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**Targeted Gene Therapy for
Canine Osteosarcoma:
Preliminary Investigations**

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**Submitted in fulfilment of the requirements for the
Degree of Doctor of Philosophy**

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Abstract

Osteosarcoma (OS) is the most common bone cancer in dogs. It is biologically aggressive and <20% survive >2 years with standard therapy. Hence, new approaches must be considered. TP53 is altered in ~50% of human and canine cancers, including OS, making it a candidate for targeted suicide gene therapy strategies. Canine OS is considered to be a good model for human OS. The aims of this study were to:

- examine the site incidence of canine OS retrieved from Glasgow University Veterinary School (GUVS) histology database;
- perform *TP53* mutational analysis in canine OS cases diagnosed at GUVS;
- investigate delivery of exogenous wild-type canine *TP53* into D17, CMT3, CMT7 and CMT8 canine OS cell lines;
- design and construct vectors for a TP53-targeted suicide gene strategy, which can selectively target canine OS cells containing accumulated TP53, and initially analyse using Dual-Luciferase® reporter assays (DLR);
- perform suicide gene/prodrug assays using nitroreductase (NTR) in combination with CB1954 or nitrofurazone (NFZ) in several canine cell lines;
- replace luciferase with NTR in vectors for TP53-targeted suicide gene strategy, and with CB1954, determine if survival of CMT7 cells possessing accumulated TP53 are reduced, in comparison to D17 cells, containing wild-type TP53.

OS were most commonly found in appendicular areas, followed by axial and extraskeletal sites; this agrees with published findings. No *TP53* mutations were found in 7 biopsies removed from 4 dogs, 5 were OS, due to analysis of a small sample number, but still fits within published data. TP53 expression did not have a significant negative effect on canine OS cell growth. Contrasting results have been shown in canine and human OS cells. Luciferase expression levels following transfection with designed constructs were higher in CMT7 cells, than in D17 cells. Similar results were shown in NTR/CB1954 assays as reductions in cell survival only occurred in CMT7 cells but not in D17 cells. NFZ was not suitable as a prodrug for NTR in canine cells as there were no differences in cell survival with cells not expressing NTR. Hence, the TP53-targeted suicide gene therapy strategy appears to selectively reduce survival of canine OS cells possessing accumulated TP53, warranting further investigation as a treatment modality for OS, in both dogs and humans.

Dedication

For the dogs in my life, past and present, who have kept me company for many an hour whilst studying and can always make me smile, come rain or shine.



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Declaration

I, Victoria Sabine, do hereby declare that the work in this dissertation is original, was carried out by myself or with due acknowledgement and has not been presented for the award of a degree at any other university.

Signed: _____

Date: 31st August 2009

Abbreviations

$\alpha\alpha$	amino acid
abs	absent
Ac	acetylation
aCGH	array CGH
ACV	acyclovir
AD	activation domain
ADP	ribosylation
Ad-p53	adenoviral-mediated p53
AML3	acute myeloid leukaemia 3
Amp	amputation
<i>Amp^r</i>	ampicillin gene
ANOVA	analysis of variance
APAF1	apoptotic peptidase activating factor 1
APC	adenomatous polyposis coli
araM	6-methoxypurine arabinonucleoside
araT	1- β -D-arabinofuranosylthymine
ARF	ADP-ribosylation factor
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	rad3 related kinase
BAC	bacterial artificial chromosome
BAX	BCL2-associated X protein
BD	binding domain
BE	bystander effect
BEA	bystander effect type A
BEB	bystander effect type B
β -gal	β -galactosidase
<i>bla</i>	β -lactamase gene
BLAST	basic local alignment search tool
bp	base pairs

BSA	bovine serum albumin
BVDU	(E)-5-(2-bromovinyl)-2'-deoxyuridine
CAR	Coxsackie Adenovirus Receptor
carbo	carboplatin
CAT/ <i>cat</i>	chloramphenicol acetyl transferase
CAT	pCMVCAT6
CAV-2	canine adenovirus type 2
CB1954	5(-aziridine-1-yl)-2, 4-dinitrobenzamide
CBFA1	core-binding factor subunit alpha 1
CDB3	cytoplasmic domain of band 3 protein
cDNA	complementary DNA
CGH	comparative genomic hybridization
CHIP	chaperone-associated ubiquitin ligase
CHEK1	CHK1 checkpoint homologue
CHEK2	CHK2 checkpoint homologue
CIAP	calf intestinal alkaline phosphatase
cm	centimetre
CMV	cytomegalovirus
CO ₂	carbon dioxide
COP1	caspase-1 dominant-negative inhibitor pseudo-ICE
COX	cyclooxygenase
CPA	cyclophosphamide
CRAd	conditionally replicative adenovirus
CSC	cancer stem cell
C-terminus/ C-ter	carboxy terminus
d	day
DAB	diaminobenzidine
DBD	DNA binding domain
DDB2	damage-specific DNA binding protein 2
ddH ₂ O	distilled water
del	deletion
DFI	Disease Free Interval
DLR	Dual-Luciferase® Reporter Assay System
<i>D. melanogaster</i>	<i>Drosophila melanogaster</i>

DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
DNA-PK	DNA-dependent protein kinase
DNA	deoxyribonucleic acid
DOB2	dietary obesity 2
DOTAP:Chol	dioleoyl trimethylammonium propane: Cholesterol
DOX	doxorubicin
E1 region	early 1 region
ECL	enhanced chemiluminescence
<i>E. coli</i>	Escherichia coli
EDTA	ethylene-diamine-tetra-acetic acid
e.g.	for example
EGFR	epidermal growth factor receptor
EHV	equine herpes virus
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1
F	forward
FASTA	FAST-All
5-FC	5-fluorocytosine
FCS	foetal calf serum
FFluc	firefly luciferase
FL	full-length
FMN	flavin mononucleotide
FOS	v-fos FBJ murine osteosarcoma viral oncogene homologue
FS	frameshift
5-FU	5-fluorouracil
GADD	growth arrest and DNA damage factor
GCG	genetics computer group
GCV	ganciclovir
GDEPT	gene directed enzyme prodrug therapy
GH	growth hormone
GLM	General Linear Model
GMCSF	granulocyte macrophage colony stimulating factor
GPX1	glutathione peroxidase 1

GUVS	Glasgow University Veterinary School
Gy	gray
HBSS	Hanks balanced salt solution
HDAC	histone deacetylase
HER-2	human epidermal growth factor-2
HGF	hepatocyte growth factor
HiFCS	heat-inactivated FCS
HIV	human immunodeficiency virus
HNSCC	head and neck squamous cell carcinoma
HPACC	Health Protection Agency Culture Collections
HPV	human papillomavirus
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homologue
HSP	heat shock protein
HSV	herpes simplex virus
IARC	International Association of Cancer Registries
IE	intermediate early enhancer/promoter
i.e.	in other words
IFO	ifosamide
IFN	interferon
IGF-1	insulin-like growth factor-1
IGF-1R	IGF-1 receptor
IHC	immunohistochemistry
IL	interleukin
ins	insertion
IPTG	isopropyl- β -D-thiogalactopyranoside
IRF	interferon regulatory factor
<i>kan^r</i>	kanamycin gene
kb	kilobase
K_{cat}	rate of catalysis
kDa	kilodalton(s)
kg	kilogram
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue
K_m	substrate concentration

LAR	luciferase assay reagent
LB	Luria-Bertani
8 LexA	8 LexA operator site sequence
<i>luc+</i>	firefly luciferase cDNA
M	Molar
Maspin	serpin peptidase inhibitor, clade B, member 5
Mb	megabase(s)
mCGH	metaphase CGH
MDCK	Madin-Darby canine kidney
MDM2	Mdm2 p53 binding protein homologue
MDM4/MDMX	Mdm4 p53 binding protein homologue
Me	methylation
MET	met proto-oncogene (HGF receptor)
mets	metastases
miR-34a	microRNA 34a
miR-34b/c	microRNA 34b/c
milk/PBS-T	low fat dried milk in phosphate buffered saline - Tween
min(s)	minute(s)
MLH1	mutL homolog 1
µg	microgram
µl	microlitre
µM	micromolar
mg	milligram
ml	millilitre
mM	millimolar
MMP	matrix metalloproteinase(s)
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethyliazol-2-yl)-2,5 diphenol tetrazolium bromide
MTX	methotrexate
MYC	v-myc myelocytomatosis viral oncogene homolog
N8	neddylation
NA	not applicable
NADH	reduced nicotinamide adenine dinucleotide

NADPH	reduced nicotinamide adenine dinucleotide phosphate
NANOG	Nanog homeobox
NCBI	National Centre for Biotechnology Information
nfsA	<i>E. coli</i> nitroreductase A
nfsB	<i>E. coli</i> nitroreductase B
NF-Y	ubiquitous heteromeric CCAAT-binding protein
NFZ	nitrofurazone
ng	nanogram(s)
nm	nanometre
NOXA	phorbol-12-myristate-13-acetate-induced protein 1
NQO1	NAD(P)H dehydrogenase, quinone 1
NR	not reported
NRH	reduced nicotinamide mononucleoside
NSCLC	non-small cell lung cancer
NTC	non-transfected cells
N-terminus/ N-ter	amino terminus
NTR	nitroreductase
nts	nucleotide positions
OC	osteocalcin
OCT4	POU class 5 homeobox 1
OD	optical density
O-Glc	glycosylation
Op	operator
ori	origin of replication
ORF	open reading frame
OS	osteosarcoma
OSU-HDAC42	S(+)-N-hydroxy-4-(3-methyl-2-phenyl-butylamino)benzamide
P	phosphorylation
p14ARF	cyclin-dependent kinase inhibitor 2A
p16INK4A	cyclin-dependent kinase inhibitor 2A
p21	cyclin-dependent kinase inhibitor 1A
p300	E1A binding protein p300
PAGE	polyacrylamide gel electrophoresis

PARC	p53-associated parkin-like cytoplasmic protein
p33ING1	inhibitor of growth family, member 1
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline - Tween
PCR	polymerase chain reaction
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PGE2	prostaglandin E2
pGL3B	pGL3-Basic vector
pGL3E	pGL3-Enhancer vector
pGL3P	pGL3-Promoter vector
pGL3B8LA	pGL3-Basic vector containing 8 LexA operator sites
pGL3P8LA	pGL3-Promoter vector containing 8 LexA operator sites
pGL3E8LA	pGL3-Enhancer vector containing 8 LexA operator sites
pGL3B8LA-NTR	pGL3-Basic vector containing 8 LexA operator sites plus nitroreductase gene
pGL3E8LA-NTR	pGL3-Enhancer vector containing 8 LexA operator sites plus nitroreductase gene
pGL3P8LA-NTR	pGL3-Promoter vector containing 8 LexA operator sites plus nitroreductase gene
PI3K	phosphatidylinositol 3-kinase
PIG3	tumour protein p53 inducible protein 3
PIRH2	ring finger and CHY zinc finger domain containing 1
pmol	picomolar
PMS2	PMS2 postmeiotic segregation increased 2
poly(A)	polyadenylation
POX	proline oxidase
pre-op	pre-operative
pres	present
post-op	post-operative
PRIMA-1	p53 reactivation and induction of massive apoptosis
p53-SP	p53 specific promoter
pSV- β -gal	pSV- β -galactosidase control vector
PTEN	phosphatase and tensin homologue

Pu	plutonium
PUMA	p53-upregulated modulator of apoptosis
PXXP	proline-rich domain containing 'PXXP' motif
R	reverse
Ra	radium
RA	rearrangement
RAD51	RAD51 homologue
RB1	retinoblastoma gene
RE	response element
RING	Really Interesting New Gene
<i>Rluc</i>	<i>Renilla luciferase</i>
RLU	relative light units
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
RUNX2	runt-related transcription factor 2
S	sumoylation
SAHA	suberoylanilide hydroxamic acid
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SCO2	SCO cytochrome oxidase deficient homolog 2
SDS	sodium dodecyl sulphate
SE	standard error
Seq	sequencing
SH3	Src Homology 3
sig. diff.	significant difference
14-3-3 sigma	stratifin
sil	silent
siRNA	short inhibitory RNA
SIS	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)
SIVA	CD27-binding protein
SN27686	3,5-dinitrobenzamide-2-bromomustard

SN-38	7-Ethyl-10-hydroxy-camptothecin
SNP	single nucleotide polymorphism
SOD2	superoxide dismutase 2
S-phase	synthesis phase
sqrt	square root
Sr	strontium
SSCP	single-strand conformation polymorphism
STAT3	signal transducer and activator of transcription 3
sub	substitution
SV40	simian virus
SV40 T antigen	simian virus transforming antigen
sx	surgery
TAE	Tris-acetate-EDTA
TAP1	transporter 1, ATP-binding cassette, sub-family B
<i>Taq</i>	<i>Thermus aquaticus</i>
TBE	Tris-borate-EDTA
TE	Tris-HCl-EDTA
TEMED	N,N,N,N,-tetramethylethylenediamine
TfR	Transferrin receptors
Th	Thorium
TIGAR	TP53-induced glycolysis and apoptosis regulator
<i>TK</i>	thymidine kinase
T_M	annealing temperature
TP53	tumour protein 53
T_{pot}	potential doubling time
TrkA	tropomyosin-related kinase
TS	transition
TSG	tumour suppressor genes
TSP1	thrombospondin 1
TV	transversion
UAS	upstream activating sequence
Ub	ubiquitin
UK	United Kingdom
USA	United States of America

USP7/HAUSP	ubiquitin specific peptidase 7
5'-UTR	5-untranslated region
UV	ultra-violet
V	voltage
VEGF	vascular endothelial growth factor
vs	versus
v/v	volume per volume
VZV	varicella-zoster virus
WT1	Wilms tumour 1
w/v	weight per volume
X	difference not able to be analysed
X-gal	5-bromo 4-chloro-3-indolyl- β -D-galactopyranoside
XPC	xeroderma pigmentosum, complementation group C

Chapter 1

Introduction

1.1 Canine osteosarcoma

1.1.1 Incidence

Osteosarcoma (OS) accounts for ~5% of all canine neoplasms and is the most frequently diagnosed and reported canine bone tumour (Merlo *et al.*, 2008; Priester & McKay 1980). OS represents 65-85% of the diagnosed primary bone sarcomas; chondrosarcoma, 8-13%; fibrosarcoma, 1-9% and haemangiosarcoma, 2-8% (Jongeward 1985). Other tumours, including lymphoma, liposarcoma and plasma cell tumours, are rarely observed and in total only account for ~5% (Jongeward 1985). OS may be more prevalent in some pure-breeds than others (Phillips *et al.*, 2007; Rosenberger *et al.*, 2007; Ru *et al.*, 1998). It is estimated that ~10000 dogs are diagnosed with OS per year in the United States (Thomas *et al.*, 2009), with reported standardized incidence rates varying between 4.6 - 57 cases/100000 dogs/year (Dobson *et al.*, 2002; Merlo *et al.*, 2008).

1.1.2 Sites

Approximately 62-77% of OS occur in the appendicular skeleton, with the remainder occurring in the axial skeleton and extraskeletal sites (Brodey & Riser 1969; Egenvall *et al.*, 2007; Loukopoulos & Robinson 2007).

The forelimbs are twice more likely to be affected than the hind limbs (Ru *et al.*, 1998; Spodnick *et al.*, 1992) but this may vary across breeds (Brodey & Riser 1969; Rosenberger *et al.*, 2007). The main sites in the foreleg are the distal radius and proximal humerus (Jongeward 1985; Spodnick *et al.*, 1992) (Figure 1.1). Tumours are fairly evenly distributed in the rear limbs between the distal femur, distal tibia and proximal tibia, with the proximal femur a slightly less common location (Jongeward 1985; Spodnick *et al.*, 1992). The major sites of axial skeletal OS are the skull, the spine and the ribs, particularly at the costochondral junction (Brodey & Riser 1969; Egenvall *et al.*, 2007; Heyman *et al.*, 1992; Jongeward 1985). Extraskeletal OS are rare malignant mesenchymal neoplasms of visceral organs and soft tissues that produce osteoid, with no

involvement of bone or periosteal tissue (Langenbach *et al.*, 1998; Patnaik 1990). The majority are found in the mammary tissue, spleen, gastrointestinal tract, liver, kidney and eye, rather than in the soft tissue of extremities (Kuntz *et al.*, 1998b; Langenbach *et al.*, 1998; Patnaik 1990).

Metastasis via the haematogenous route is most common; however, on rare occasions extension to regional lymph nodes may occur (Hillers *et al.*, 2005; Straw *et al.*, 1990). The lungs are the most commonly reported site for metastasis, but tumour spread also occurs in additional bones and/or other soft tissue sites, including spine, ribs, long bones, liver and spleen (Brodey & Riser 1969; Langenbach *et al.*, 1998; Spodnick *et al.*, 1992).

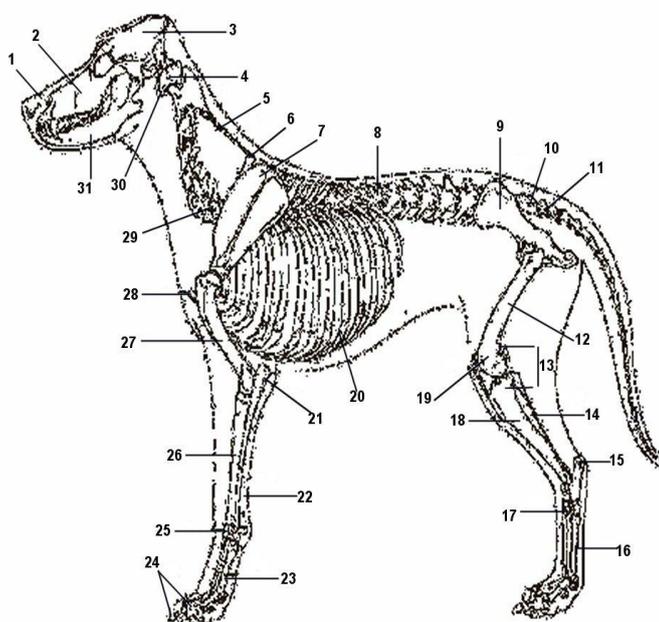


Figure 1.1: The skeleton of the dog. 1, nasal cavity & sinus; 2, maxilla*; 3, skull*; 4, spine of axis (C2)*; 5, ligamentum nuchae; 6, first thoracic vertebrae (T1)*; 7, scapula*; 8, first lumbar vertebrae (L1)*; 9, hip bone (os coxae); 10, sacrum; 11, first caudal (tail) vertebrae (Cd1); 12, femur*; 13, stifle; 14, fibula; 15, calcaneus (point of hock); 16, metatarsal bones; 17, tarsal bones; 18, tibia*; 19, patella; 20, rib*; 21, olecranon (point of elbow); 22, ulna*; 23, metacarpal bones; 24, proximal, middle and distal phalanges; 25, carpal bones; 26, radius*; 27, humerus*; 28, cranial end (manubrium) of sternum; 29, last cervical vertebrae (C7); 30, first cervical vertebrae (C1); 31, mandible*. * - sites of appendicular and axial OS typically found in dogs. Adapted from Dyce *et al.*, 1987(Dyce *et al.*, 1987).

1.1.3 Demographics

Canine OS is largely a disease of middle-aged to older dogs, with a median age of 7.5 years, although a small early peak in age incidence exists at 18 - 24 months (Egenvall *et al.*, 2007; Heyman *et al.*, 1992; Jongeward 1985; Langenbach *et al.*, 1998; Misdorp & Hart 1979). Primary rib OS tends to develop earlier with a mean age of 5.4 years (Heyman *et al.*, 1992) and mammary gland OS later with a mean age of 10.6 years (Langenbach *et al.*, 1998). OS is classically a sporadic cancer of the long bones in large to giant pure-breeds, but a tendency for familial aggregation may exist in St. Bernards, Rottweilers and Scottish Deerhound (Bech-Nielsen *et al.*, 1978; Misdorp 1980; Phillips *et al.*, 2007; Thomas *et al.*, 2009). A high prevalence of OS has also been found in Boxers, Great Danes, German Shepherds, Collies, Golden Retrievers, Doberman Pinschers, Irish Wolfhound, Greyhounds and Irish Setters (Jongeward 1985; Priester & McKay 1980; Rosenberger *et al.*, 2007; Ru *et al.*, 1998). The risk of bone sarcoma in dogs weighing >36kg is at least 60 times greater than in dogs weighing <10kg (Tjalma 1966). Increasing standard height has also been associated with an increased OS risk and has in fact been demonstrated to be a more predictive risk factor than increasing weight (Ru *et al.*, 1998). Males are often reported to be slightly more affected than females (approximate ratio of 1.5:1.0), but this finding is not consistent amongst all publications, as reports of more females than males developing OS and no sex-related differences have been published (Egenvall *et al.*, 2007; Jongeward 1985; Merlo *et al.*, 2008; Priester & McKay 1980; Ru *et al.*, 1998). In addition, conflicting evidence exists regarding neutering increasing or decreasing the risk of OS and the age at which the dog is neutered due to hormonal changes altering skeletal homeostasis (Cooley *et al.*, 2002; Priester & McKay 1980; Ru *et al.*, 1998).

1.1.4 Histopathology

OS is a malignant mesenchymal tumour of progenitor bone cells (Dernell *et al.*, 2007). These cells produce an extracellular matrix of osteoid and the presence of tumour osteoid is the basis for the histological differential diagnosis of OS versus other malignant bone sarcomas (Dahlin & Unni 1977; Dernell *et al.*, 2007)

(Table 1.1). The histological pattern may vary between tumours or even within the same tumour, hence, there are many histological subclassifications of OS based on the precursor cell type, amount of matrix and characteristics of the cells including osteoblastic, anaplastic, fibroblastic, chondroblastic, osteocytic, osteolytic, myofibroblastic, angioblastic, poorly differentiated and telangiectatic (Dahlin & Unni 1977; Kadosawa *et al.*, 1999; Misdorp & Hart 1979; Reddick *et al.*, 1980). All of these neoplasms may exhibit irregularly placed and abnormally large multi-lobed nuclei and possess mitotic figures, including aberrant ones and an unusually large quantity of heterochromatin (Kirpensteijn *et al.*, 2002a; Misdorp & Hart 1979; Reinhardt *et al.*, 2005). Mild to moderate levels of cellular necrosis and extracellular debris, adjacent to normal relatively immature cells, can be observed (Kirpensteijn *et al.*, 2002a; Reinhardt *et al.*, 2005). Alkaline phosphatase staining has been shown to aid in differentiating OS pathologically from other connective tissue tumours (Barger *et al.*, 2005; Britt *et al.*, 2007). In dogs, it has not been clearly established that there is a difference in the biological behaviour of the different histological subclassifications; however, histological grade based on microscopic features may be predictive for increased risk of metastasis (Kirpensteijn *et al.*, 2002a).

Tumour Classification	Distinguishing features
Osteosarcoma	Cells produce osteoid, not necessarily mineralized
Chondrosarcoma	Characterized by the production of neoplastic cartilage May be areas of calcification which are not areas of osteoid Bone may also develop in chondrosarcomas by endochondral ossification
Fibrosarcoma	Cells produce varying amounts of collagenous matrix
Haemangiosarcoma	Areas of undifferentiated sarcoma with areas of primitive vascular channels
Liposarcoma	Fat may be demonstrated within the tumour cells
Undifferentiated sarcoma	Cell types very primitive with insufficient organization to produce recognisable matrix
Giant cell tumour	Large numbers of giant cells Common feature of histopathology of many bony lesions including osteosarcoma and some benign tumours
Myeloma	Population of neoplastic plasma cells
Lymphoma	Population of neoplastic lymphocytes

Table 1.1: Primary malignant bone sarcomas that occur in dogs and their distinguishing features. Modified from Anderson (1996) (Anderson 1996) and Ling *et al.*, (1974) (Ling *et al.*, 1974).

1.1.5 Aetiology

Despite advances in veterinary research and molecular techniques, the precise aetiology of canine OS and the mechanisms underlying OS progression and metastasis are still to be elucidated. It is quite likely that it is multifactorial as multiple possible causes have been documented and an accepted model of multi-stage carcinogenesis is one that is underpinned by progressive genetic changes that drive malignant transformation. An alternative hypothesis has been proposed in the past few years, the cancer stem cell (CSC) theory, as cancer growth is proposed to be driven by a small population of CSCs (Pang & Argyle 2009). Recent evidence suggests that canine OS may also be a stem-cell disease (Wilson *et al.*, 2008).

1.1.5.1 Physical factors

A general theory is that OS predominate in the metaphyseal region of the major weight-bearing bones, adjacent to late-closing physes, of tall heavy dogs (Egenvall *et al.*, 2007; Ru *et al.*, 1998). There appears to be a marked correlation between the sites of predilection for OS and the growth rates of various metaphyses (Brodey & Riser 1969; Tjalma 1966). Multiple episodes of minor trauma and subsequent injury may occur to cells in these physes (Brodey 1979; Dernell *et al.*, 2007; Tjalma 1966). Hence the induction of mitogenic signals may occur that promote the development of mutant lineages that manifest themselves as OS years later (Dernell *et al.*, 2007; Tjalma 1966). This theory explains the more frequent occurrence of forelimb OS rather than rear, as heavy dogs bear more weight on their forelimbs (Brodey 1979).

OS has been associated with several fracture repair techniques including those using metallic implants (Brodey 1979; Stevenson 1991). OS has been concurrently seen in dogs with bone infarcts, but it is not clear whether there is any causal relationship, between bone infarcts and OS or, quite simply, that the stress of repair in the infarcted areas leads to a greatly increased risk of OS (Brodey 1979; Dubielzig *et al.*, 1981).

Exposure to ionising radiation may induce OS (McChesney-Gillette *et al.*, 1990; Powers *et al.*, 1989; Thurman *et al.*, 1973). The majority of radionuclides that can localize in the bone, e.g. ^{239}Pu , ^{226}Ra , ^{228}Ra , ^{244}Ra , ^{90}Sr and ^{228}Th , have the potential to cause OS in laboratory beagles (Thurman *et al.*, 1973).

However, OS is a rare late complication of radiation therapy in dogs (McChesney-Gillette *et al.*, 1990; McEntee *et al.*, 2004; Powers *et al.*, 1989).

1.1.5.2 Genetic and molecular factors

There is a body of experimental and clinical research demonstrating that several genetic and molecular factors may be involved in the pathogenesis of canine OS (Mueller *et al.*, 2007; Thomas *et al.*, 2005).

1.1.5.2.1 Chromosomal abnormalities

Cytogenetic studies have revealed that the karyotypes found in canine OS are typically complex and contain numerous numerical and structural abnormalities (Thomas *et al.*, 2009). The majority of OS cells have an aneuploid karyotype and often contain chromosomes with an abnormal number of arms and centromeres and chromosomal fragments (Thomas *et al.*, 2009). Altogether 27 of the 38 pairs of canine acrocentric autosomes and the 2 metacentric sex chromosomes have been reported to contain chromosomal aberrations (Mayr *et al.*, 1991; Thomas *et al.*, 2005).

Until recently, the canine karyotype was poorly annotated, partly due to the large number of chromosomes making identification of all 78 chromosomes by classical banding techniques difficult, but more so because of their regularly decreasing size and absence of a clear and diagnostic pattern of R bands (Breen 2008). The dog karyotype has now been described following the development over the past few years of robust genomic techniques for analysing the molecular cytogenetics of canine tumours, including OS e.g. metaphase-based comparative genomic hybridizations (mCGH) and array CGH (aCGH) (Breen 2008). A recent study by Thomas *et al.*, (2009) utilising a cytogenetically characterized, genome-anchored 10-Mb bacterial artificial chromosome (BAC) set

for aCGH revealed that canine OS possessed a vast range of DNA copy number aberrations and structural rearrangements throughout the genome, some of which were not previously identified by mCGH (Thomas *et al.*, 2009).

Furthermore, 14 loci found to contain either copy number gain or loss in >30% OS studied were located in 8 different chromosomes which included sites for 5 major cancer-related genes, *PTEN*, *MYC*, *HRAS*, *WT1* and *KIT* (Thomas *et al.*, 2009).

1.1.5.2.2 Molecular abnormalities

A number of genes and proteins are thought to be involved in the pathogenesis of canine OS, which are summarized in Table 1.2. In particular, point mutations in *TP53* have been found in 23-100% of canine OS screened (Johnson *et al.*, 1998; Kirpensteijn *et al.*, 2008; Mendoza *et al.*, 1998; Setoguchi *et al.*, 2001b; Van Leeuwen *et al.*, 1997). Abnormal levels of *TP53* mRNA have been observed in canine OS cell lines (Levine & Fleischli 2000). Immunohistochemical studies have indicated that abnormal, non-functional TP53 commonly accumulates in canine OS (Loukopoulos *et al.*, 2003; Sagartz *et al.*, 1996). Genetic evidence also indicates that *TP53* is one of the most frequently altered genes in canine OS. TP53 is a tumour suppressor, as the *TP53* gene encodes a transcription factor that regulates genes involved in cell cycle, DNA damage response and apoptosis (Riley *et al.*, 2008), which are described in further detail in 1.3. Hence the absence of a functional TP53 gene or protein may lead to the impairment of numerous pathways that TP53 participates in and consequently, contribute to unrestrained cellular proliferation and tumourigenesis.

Factor	Comments	Reference
<i>TP53</i>	Gene mutations and/or protein accumulation commonly observed in canine OS samples and cell lines	(Johnson <i>et al.</i> , 1998; Mendoza <i>et al.</i> , 1998; Sagartz <i>et al.</i> , 1996; Setoguchi <i>et al.</i> , 2001b) (Inoue & Shiramizu 1999; Kirpensteijn <i>et al.</i> , 2008; Loukopoulos <i>et al.</i> , 2003; Van Leeuwen <i>et al.</i> , 1997)
<i>MDM2</i>	Gene duplication found in canine OS	(Mendoza <i>et al.</i> , 1998)
<i>PTEN</i>	Gene mutations and/or aberrant expression of protein reported in canine OS samples and cell lines	(Levine <i>et al.</i> , 2002)
<i>ERBB2/HER-2</i>	Protein overexpression demonstrated in canine OS samples and cell lines	(Flint <i>et al.</i> , 2004)
<i>SIS/PDGF</i>	Gene amplification reported in canine OS samples and cell lines	(Kochevar <i>et al.</i> , 1990; Levine 2002)
<i>MYC</i>	Gene amplification and protein overexpression observed in canine OS samples and cell lines	(Inoue & Shiramizu 1999; Kochevar <i>et al.</i> , 1990; Levine 2002)
<i>MET/HGF</i>	Gene mutations and/or protein overexpression may contribute to metastasis in canine OS samples and cell lines	(Ferracini <i>et al.</i> , 2000; Liao <i>et al.</i> , 2006; MacEwen <i>et al.</i> , 2003) (De Maria <i>et al.</i> , 2009; Fieten <i>et al.</i> , 2009)
<i>TrkA</i>	Protein expression may enhance cell mitogenesis and survival in canine OS samples and cell lines and pulmonary metastases	(Fan <i>et al.</i> , 2008)
<i>GH</i>	Gene expression reported in canine OS	(Kirpensteijn <i>et al.</i> , 2002b)
<i>IGF-1/IGF-1R</i>	Protein expression may contribute to ability of canine OS cells to metastasize	(MacEwen <i>et al.</i> , 2004)
<i>Matrix metalloproteinases (MMP-2 & MMP-9)</i>	Elevated protein levels found in canine OS samples and cell lines	(Lana <i>et al.</i> , 2000; Loukopoulos <i>et al.</i> , 2004)
<i>Cyclooxygenase enzymes/PGE2</i>	Elevated protein levels in canine OS samples, metastatic tumours and cell lines reported	(Mohammed <i>et al.</i> , 2001; Mohammed <i>et al.</i> , 2004; Mullins <i>et al.</i> , 2004)
<i>Angiogenic factors</i>	Levels of VEGF in plasma and angiostatin in urine, both measurable in dogs with OS, may contribute to ability of canine OS to metastasize	(Pirie-Shepherd <i>et al.</i> , 2002; Thamm <i>et al.</i> , 2008; Wergin & Kaser-Hotz 2004)
<i>Telomerase reverse transcriptase</i>	Gene upregulation observed in canine OS samples and cell lines	(Yazawa <i>et al.</i> , 2003a)
<i>Ezrin</i>	Protein detected in canine OS samples and may be associated with metastasis	(Khanna <i>et al.</i> , 2004)
<i>STAT3</i>	Activation of STAT3 may contribute to cell proliferation and survival in canine OS samples and cell lines	(Fossey <i>et al.</i> , 2009; Wilson <i>et al.</i> , 2008)
<i>OCT4</i>	Gene expressed at high levels in canine OS cell lines and sarcopheres	(Wilson <i>et al.</i> , 2008)
<i>NANOG</i>	Gene expressed at high levels in canine OS cell lines and sarcopheres	(Wilson <i>et al.</i> , 2008)

Table 1.2: Molecular factors proposed to be associated with OS tumourigenesis in dogs. Adapted from Dernell *et al.*, (2007) (Dernell *et al.*, 2007).

1.1.6 Clinical presentation & diagnosis

Dogs with OS in appendicular sites generally present at clinical examination, lameness and/or swelling of the affected limb which is invariably painful (Mueller *et al.*, 2007). The history and clinical signs associated with axial and extraskeletal OS vary depending on the location of the tumour (Chun & de Lorimier 2003). The first clinical signs of OS pulmonary metastasis are rarely dogs presenting with respiratory problems, as dogs with radiographically detectable pulmonary metastasis may remain asymptomatic for many months; rather, their first signs are usually vague as most dogs develop decreased appetites and malaise within 1 month (Dernell *et al.*, 2007).

Imaging plays an important role in aiding a diagnosis and the staging of OS in dogs, in conjunction with evaluating tissue biopsies and an assessment of the patient's overall health status. A complete blood and platelet count, serum biochemical profile and urine analysis are usually obtained, as certain therapeutic decisions might be altered based on the results of such tests (Dernell *et al.*, 2007). Recently, two bone resorption markers, urine and serum N-telopeptide, were found to support a diagnosis of OS (Lucas *et al.*, 2008).

Radiographs of the affected area commonly reveal aggressive lesions (Ling *et al.*, 1974). The radiographic appearance of OS varies, as it can range from being primarily osteolytic to almost entirely osteoblastic or osteogenic, or it can be a mixture consisting of differing proportions (Wrigley 2000) (Figure 1.2).

Unfortunately a diagnosis of OS cannot be assumed solely on the basis of radiographic findings, because the radiographic appearance of OS is similar to other bone diseases, including, other primary bone tumours, multiple lymphoma of bone, and fungal and bacterial osteomyelitis (Dernell *et al.*, 2007; Ling *et al.*, 1974; Wrigley 2000). Furthermore, thoracic radiographs only reveal gross metastatic disease in less than 15% of dogs at the time of diagnosis, although it is well established, that the majority of these dogs will go on to develop pulmonary metastatic disease if treated by surgery alone (Brodey & Abt 1976; Spodnick *et al.*, 1992).

Nuclear scintigraphy (survey radiography) can be used to evaluate the degree of bone involvement from a primary bone tumour, help to predict time to

metastasis in dogs with OS, and to detect secondary skeletal sites of OS (Forrest *et al.*, 1992; Janowski *et al.*, 2003; Lamb 1987). However, the specificity of nuclear scintigraphy is relatively low for identifying sites of skeletal tumour, so any lesions identified by this technique should be verified e.g. regions of osteoblastic activity, including osteoarthritis and infection (Berg *et al.*, 1990; Chun & de Lorimier 2003; Lamb 1987; Leibman *et al.*, 2001).

Although current published treatment recommendations and prognoses are based on the results of plain radiographs for dogs with OS, the use of advanced imaging techniques, in particular, computerized tomography and magnetic resonance imaging, are becoming more commonplace for staging dogs with OS (Davis *et al.*, 2002; Wallack *et al.*, 2002).

A presumptive diagnosis of OS can be made based on symptoms, history, physical examination and radiographic findings, however, biopsy followed by histopathological evaluation remains the definitive diagnostic procedure for OS (Dernell *et al.*, 2007; Ling *et al.*, 1974). Although obtaining a bone biopsy is a painful procedure, as well as the risk of collecting a non-diagnostic biopsy and numerous post-surgical complications (Chun & de Lorimier 2003; Dernell *et al.*, 2007).

Ultrasound-guided fine-needle aspiration and cytology of lytic bone lesions are gradually being used more frequently in veterinary medicine and may provide a more rapid diagnosis and reduce complications because of the minimally invasive procedure required to obtain a sample (Barger *et al.*, 2005; Britt *et al.*, 2007). However, the cytological information obtained can be limited, in particular, OS can appear similar to chondrosarcoma, synovial cell sarcoma and fibrosarcoma (Barger *et al.*, 2005). Although, alkaline phosphatase staining of cytological samples may help to differentiate OS from other vimentin-positive tumours, including other sarcomas affecting bones (Barger *et al.*, 2005; Britt *et al.*, 2007).



Figure 1.2: The radiographic appearance of OS in a distal femur of a dog. Areas of lucency demonstrate significant bone lysis. OS is typical in the metaphyseal area (in-house photograph).

1.1.7 Prognostic factors

A number of negative prognostic indicators have been identified for canine OS, in particular the presence of metastases at the time of diagnosis (Brodey & Abt 1976; Hammer *et al.*, 1995; Ogilvie *et al.*, 1993), high histological tumour grade (Kirpensteijn *et al.*, 2002a; Straw *et al.*, 1996), high tumour microvascular density (Coomber *et al.*, 1998), and in dogs with appendicular or axial OS, elevated pre- and post-operative alkaline phosphatase levels (Ehrhart *et al.*, 1998; Fieten *et al.*, 2009; Garzotto *et al.*, 2000). Other factors which have been proposed as poor prognostic indicators are large tumour size (Forrest *et al.*, 1992; Misdorp & Hart 1979), insufficient surgical margins (Hammer *et al.*, 1995; Hendrix & Gelatt 2000; Kuntz *et al.*, 1998a), dogs younger than 5 years of age and older than 10 years (Spodnick *et al.*, 1992), OS located in the proximal

humerus (Bergman *et al.*, 1996; Mehl *et al.*, 2005; Phillips *et al.*, 2009), body weight greater than 40kg (Bergman *et al.*, 1996; Hammer *et al.*, 1995; Lascelles *et al.*, 2005), and for OS of flat or irregular bones, a telangiectatic (vascular) subtype and a rib or scapula location (Hammer *et al.*, 1995).

However, there are a few factors which are associated with possessing a lower metastatic rate and a better prognosis in dogs with OS and these include mandibular OS (Kosovsky *et al.*, 1991; Straw *et al.*, 1996), allograft infections post limb-sparing surgery (Lascelles *et al.*, 2005), >10% lymphocyte micronuclei following chemotherapy (Hahn *et al.*, 1996), and a high percentage of tumour necrosis following doxorubicin treatment (Berg *et al.*, 1995).

1.1.7.1 Molecular prognostic factors

The search continues for reliable prognostic markers in canine OS that can be determined at diagnosis. Investigations have been carried out to link the expression of various genes or their protein products with response to outcome as it is widely recognized that as various tumour suppressor genes (TSG) and oncogenes contribute to the tumourigenesis and progression of OS, they may also potentially act as negative prognostic indicators.

The presence of *TP53* mutations and protein accumulation, in addition to potentially playing a role in the aetiology of canine OS (1.1.5.2.2), have been suggested to be suitable poor prognostic indicators for canine OS (Kirpensteijn *et al.*, 2008; Loukopoulos *et al.*, 2003). Furthermore, a higher prevalence of *TP53* mutations and accumulation of TP53 are reported in appendicular rather than axial sites and multilobular tumours of bone (low-grade malignancies) (Loukopoulos *et al.*, 2003; Sagartz *et al.*, 1996). Dogs with OS originating in the appendicular skeleton have a poorer prognosis with shorter survival time and metastasis occurring earlier than dogs with OS arising in the axial skeleton or multilobular tumours of bone (Heyman *et al.*, 1992; Straw *et al.*, 1989). Hence, providing evidence for supporting TP53 as a factor in the more aggressive form of the disease, i.e. appendicular OS, and as a poor prognostic indicator.

The expression of COX2 which may also possess a role in the tumourigenesis of canine OS (Table 1.2), has also been demonstrated to be a potential prognostic indicator of poor outcome (Mullins *et al.*, 2004). Mullins *et al.*, (2004) reported that the majority of canine appendicular OS samples analysed for COX2 expression were positive and those with strong COX2 expression levels had a significantly decreased overall survival compared to those samples with negative staining (86 days versus 423 days) (Mullins *et al.*, 2004).

An increase in tumour cell proliferation rate, may be an indicator of more aggressive tumour cell kinetics (Larue *et al.*, 1994; Ohta *et al.*, 2004). The kinetic parameter, T_{pot} , has been associated with a worse prognosis in dogs with OS, in that the samples with the fastest T_{pot} were highly significant in predicting a shorter time to metastasis and reduced survival time (Larue *et al.*, 1994). The proliferation marker, Ki-67, was found to be expressed in canine OS samples at a significantly higher level than that observed in chondrosarcoma samples (Ohta *et al.*, 2004). Furthermore the survival rate of OS cases was significantly lower than that of the chondrosarcoma cases, which supports the view that in dogs, OS is clinically more aggressive than chondrosarcoma (Ohta *et al.*, 2004).

Ezrin, as well as possibly functioning in the metastatic disease process in dogs with OS (Table 1.2), has been shown to be a prognostic indicator of metastatic behaviour and survival in dogs with OS (Khanna *et al.*, 2004). The presence of high ezrin staining in the primary tumour was associated with early development of metastases and hence, a significantly shorter median disease-free interval compared to dogs with low levels of ezrin staining in the primary tumour (Khanna *et al.*, 2004).

1.1.8 Therapy

The current standard treatment for dogs with OS is amputation (Figure 1.3) or surgical resection of the primary tumour followed by either a platinum (cisplatin or carboplatin)- and/or doxorubicin- based chemotherapy protocol (summarized in Table 1.3) (Dernell *et al.*, 2007). In dogs with appendicular or axial OS, this treatment results in a median survival time of ~50 weeks and 1-year and 2-year

survival rates of ~ 45% and 20%, respectively (Berg *et al.*, 1992; Berg *et al.*, 1995; Fieten *et al.*, 2009; Kent *et al.*, 2004; Mauldin *et al.*, 1988; Phillips *et al.*, 2009). In dogs with extraskeletal OS treated with surgery and adjuvant chemotherapy, a median survival of 5 months has been reported (Kuntz *et al.*, 1998b). A systemic therapy is required because if surgery is the sole form of treatment, the median survival time in dogs with appendicular or axial OS, is ~17 weeks with 1-year and 2-year survival rates of ~ 13% and 2%, respectively (Baines *et al.*, 2002; Spodnick *et al.*, 1992; Wallace *et al.*, 1992). Dogs with extraskeletal OS have a median survival of ~1.6 months (Kuntz *et al.*, 1998b; Langenbach *et al.*, 1998). Survival rates are poor because dogs with OS are frequently euthanized due to metastatic disease, in particular pulmonary metastasis, or refractory pain in untreated dogs (Wilson *et al.*, 2008). Although pulmonary (diameter >0.5cm) or osseous metastasis is radiographically detectable in less than 15% of dogs at presentation or surgery, microscopic metastatic disease is present in ~80-90% of cases, even if there is no evidence (Langenbach *et al.*, 1998; Matthiesen *et al.*, 1992; Spodnick *et al.*, 1992; Straw *et al.*, 1991). The exception is mandibular OS, as surgery alone has been reported to provide a 1-year survival rate of >35%, and chemotherapy has not yet demonstrated any survival advantage (Kosovsky *et al.*, 1991; Straw *et al.*, 1996).

Metastasectomy may be performed in dogs with solitary bone metastases and no evidence of cancer elsewhere, although the subsequent disease-free interval is generally short (Dernell *et al.*, 2007). However, pulmonary metastasectomy may significantly increase (~50%) median survival time of dogs with appendicular OS to nearly 6 months (Brodey & Abt 1976; O'Brien *et al.*, 1993). Treatment with various combinations of therapies may increase survival times in dogs with OS diagnosed with metastases at time of presentation to that of surgery alone, albeit median survival time is only 76 days with a 1 year survival rate of 7% (Boston *et al.*, 2006). A couple of pilot studies have also observed partial responses in a small number of dogs with metastatic OS following treatment with inhalational or intravenous paclitaxel or inhalational doxorubicin for a short period of time (Hershey *et al.*, 1999; Khanna & Vail 2003; Poirier *et al.*, 2004).

Palliative treatment is provided for dogs with metastatic OS or when owners do not want to pursue more aggressive treatment options for primary OS (Mueller *et al.*, 2007). Forms of palliative treatment include radiation therapy (Coomer *et al.*, 2009), surgery alone (Chun & de Lorimier 2003), nonsteroidal anti-inflammatory drugs (e.g. piroxicam, carprofen, meloxicam) (Lester & Gaynor 2000), radiopharmaceuticals, such as samarium (Aas *et al.*, 1999; Lattimer *et al.*, 1990), and bisphosphonates (e.g. pamidronate, alendronate) (Fan *et al.*, 2009; Tomlin *et al.*, 2000).

Radiation therapy is the main modality of palliative care and in some circumstances, amputation may also be required (Dernell *et al.*, 2007). Radiotherapy has been reported to improve limb function and quality of life in over 70% of patients treated with median duration of response ~10 weeks (Knapp-Hoch *et al.*, 2009). After this time, euthanasia often occurs due to progressive local disease or the metastatic lesions becoming symptomatic. The response rate to radiotherapy may be improved by the addition of chemotherapy (Heidner *et al.*, 1991; Ramirez *et al.*, 1999), although, this has unfortunately not been found to be the case in other studies (Green *et al.*, 2002; Mueller *et al.*, 2005; Walter *et al.*, 2005a); albeit in all 5 studies, a relatively small number of dogs were treated. Recently, investigations into curative-intent radiation therapy protocols were carried out, when limb amputation was not possible in dogs with appendicular OS, which demonstrated that they can successfully control local disease, while chemotherapy was administered for metastatic disease (Coomer *et al.*, 2009). However, curative-intent radiation therapy is associated with a high rate of radiation-induced complications and their success depends on management of these complications (Coomer *et al.*, 2009). Hence, curative-intent radiation therapy protocols, as yet, cannot replace standard of care treatments for appendicular OS i.e. amputation and chemotherapy.



Figure 1.3: A cross-breed dog after forelimb amputation for OS of the distal radius (in-house photograph).

Treatment	OS Site	# chemotherapy doses	# dogs	Median DFI (days)	Median survival (days) (1-yr, 2-yr survival)	Comments	Reference
<i>Amp/limb-spare+cisplatin</i>	Appendicular	1-6	22 (17 - amp, 5 - limb-spare)	NR	325 (45.5%, 20.9%)	Increase in treatment failures possibly due to bone metastases; survival data not divided for sx types.	(Berg <i>et al.</i> , 1992)
<i>Amp+cisplatin</i>	Appendicular	2 (post-op)	17	226	262 (38%, 18%)	No sig. diff. between survival data for dogs given cisplatin before amp compared to those after amp.	(Straw <i>et al.</i> , 1991)
<i>Amp+cisplatin</i>		2 (1 pre-op;1 post-op)	19	177	282 (43%, 16%)		
<i>Surgery+cisplatin</i>	Axial (Rib)	NR	9	225	240 (NR, NR)	Age, weight, sex, number of ribs resected, tumour volume, and total cisplatin dose did not influence survival or disease-free interval.	(Pirkey-Ehrhart <i>et al.</i> , 1995)
<i>Amp+carboplatin</i>	Appendicular	1-4	48	257	321 (35.4%, NR)	Maximum tolerated cumulative dose has not been described for dogs.	(Bergman <i>et al.</i> , 1996)
<i>Amp+carboplatin</i>	Appendicular	3-4	155	256	307 (36.8%, 18.7%)	Acceptable alternative to cisplatin	(Phillips <i>et al.</i> , 2009)
<i>Amp/limb-spare+DOX</i>	Appendicular	5	35 (33 - amp, 2 - limb-spare)	NR	366 (50.5%, 9.7%)	Survival data not divided for sx types; Percentage necrosis of tumour predicted survival.	(Berg <i>et al.</i> , 1995)
<i>Surgery + cisplatin/DOX</i>	Extraskeletal	2-5	5	NR	146 (0%, 0%)	Only factor found to be prognostic for survival was use of chemotherapy.	(Kuntz <i>et al.</i> , 1998b)
<i>Amp+DOX+cisplatin</i>	Appendicular	3 (starting 2 or 10 d post-op)	102 (53 - 2d; 49 - 10d)	NR	2d post-op: 345 (48%, 28.3%) 10d post-op: 330 (46.2%, 27.5%)	12 dogs died after first cycle; 22 cases of dose reduction; no difference in post-op vs. pre-op chemotherapy.	(Berg <i>et al.</i> , 1997)
<i>Amp+DOX+cisplatin</i>	Appendicular & axial	1of each	38 (36 appendicular, 2 axial)	210	300 (37%, 26%)	No sig. diff. between survival data from this study and survival data from single agent cisplatin study.	(Mauldin <i>et al.</i> , 1988)
<i>Amp+DOX+carboplatin</i>	Appendicular	4 of each	24	195	235 (NR, NR)	Combination at these dosages was well-tolerated in most cases.	(Bailey <i>et al.</i> , 2003)
<i>Amp+DOX+carboplatin</i>	Appendicular	6 (3 of each)	50	202	258 (NR, NR)	Toxicity reported in 15 (30%) dogs. Survival times similar to single-agent protocols.	(Bacon <i>et al.</i> , 2008)
<i>Amp/limb-spare+DOX+carboplatin</i>	Appendicular	x3 cycles	32	227	320 (48%, 18%)	Minimal toxicity associated with chemotherapy.	(Kent <i>et al.</i> , 2004)
<i>Surgery+ /or chemotherapy+ /or radiotherapy</i>	Axial (mandibular)	2-3	51	285	528 (59.3%, NR)	No apparent difference between various treatment modalities.	(Straw <i>et al.</i> , 1996)
<i>Surgery+carboplatin+/- cisplatin or DOX+/- cisplatin</i>	Axial (rib+sternum)	2-8	19	149	290 (32%, NR)	Post-op chemotherapy prolongs survival time.	(Fieten <i>et al.</i> , 2009)

Table 1.3: Commonly used adjuvant chemotherapy agents and the survival outcome for dogs with OS. Abbreviations: Amp, amputation; DFI - Disease Free Interval; NA, not applicable; carbo, carboplatin; d, day; DOX, doxorubicin; post-op, post-operative; pre-op, pre-operative; sx, surgery; NR, not reported; sig. diff., significant difference. Modified from Chun and de Lorimier (2003) (Chun & de Lorimier 2003) and Dernell *et al.*, (2007) (Dernell *et al.*, 2007).

1.1.9 Comparative aspects

Animal models for the study of human diseases are important to our understanding of the mechanism and aetiology of disease and for the development and refinement of therapeutic strategies (Pang & Argyle 2009). It was not until the mid-1970's that researchers turned their attention from the development of artificial models of OS to OS found spontaneously developing in the pet population as a means of not only gaining information regarding OS in that particular species, but also into human OS (Brodey 1979).

Canine OS has many similarities to human OS and can serve well as a valuable comparative model for study (Table 1.4) (Argyle 2005; Bronden *et al.*, 2007). For example, human OS demonstrates a bimodal age distribution, frequently develops in the metaphyses of long bones, and is aggressive with distant metastases occurring in more than 80% of patients treated by surgery alone (Sandberg & Bridge 2003; Tang *et al.*, 2008). Although there is a difference in the main peak of incidence between humans and dogs, i.e. young adults vs middle-aged older dogs, this may simply be due to the fact that in young adults rapid bone growth is still occurring in the metaphyses of long bones and sufficient micro-traumas have already taken place in their earlier years, enabling the induction of mitogenic signals. In addition, children with hereditary retinoblastoma have a high risk of secondary cancers occurring, particularly in their teenage years, 50% of which are OS (Sandberg & Bridge 2003).

Standard of care treatment for human OS is also similar to that of dogs, in that neoadjuvant chemotherapy, followed by amputation or limb-sparing and adjuvant chemotherapy are employed (Tang *et al.*, 2008). Negative prognostic factors include advanced local or systemic stage, large tumour size and a lower percentage of necrosis following neo-adjuvant treatment (Tang *et al.*, 2008). Several cases of familial OS in humans have been published e.g. families with members suffering from the Li-Fraumeni syndrome possess germ-line *TP53* mutations (Tang *et al.*, 2008). In particular, OS in dogs and humans display similar types of DNA ploidy and cytogenetic abnormalities and are clinically and histopathologically virtually indistinguishable (Thomas *et al.*, 2009). Of considerable relevance to this study, is that human OS, similar to canine OS, contain a high prevalence of alterations in *TP53* and its protein which are

thought to play a significant role in the tumourigenesis of this disease (Pakos *et al.*, 2004; Toguchida & Nakayama 2009). Furthermore molecular abnormalities in MDM2, PTEN, ERBB2, SIS, MYC, MET, TrkA, GH, IGF-1, IGF-1R, MMP2, MMP9, COX1, COX2, VEGF, angiostatin, telomerase reverse transcriptase and ezrin, previously described in Table 1.2, have also been reported to be involved in the pathogenesis, aggressive tumour growth and the promotion of metastatic potential in human OS (Bakhshi *et al.*, 2009; DuBois & Demetri 2007; Freeman *et al.*, 2008; Kochevar *et al.*, 1990; Rodriguez *et al.*, 2008; Tang *et al.*, 2008).

Naturally occurring OS in dogs develops 40-50 times more frequently than in humans; therefore, case accrual is more rapid (Withrow *et al.*, 1991). Since disease progression is faster in dogs than in humans and responses to treatment are similar for the 2 species, results of treatment protocols can be reported earlier than would those of similar trials in humans (Dernell *et al.*, 2007). Research costs are less for clinical trials in dogs compared to those in humans and, from an animal welfare standpoint, no disease is induced and dogs with cancer can be helped (Argyle 2005; Mueller *et al.*, 2007). The long-term survival rate for human OS is presently 60-70% with the use of established multi-drug adjuvant protocols which contrasts to the 20% expected 5-year survival rates of the early 1980s (Tang *et al.*, 2008). However, the remaining 30-40% of patients with OS develop pulmonary metastases despite the administration of adjuvant chemotherapy and the type of surgery (amputation or limb-sparing) does not impact outcome, which together support the need for the incorporation of new forms of therapy into the adjuvant chemotherapy protocols to impact survival.

1.1.10 Summary

OS is the most commonly occurring bone cancer in the canine species and is an excellent model for human OS. A range of chromosomal aberrations and alterations in several TSG, oncogenes, proteinases, growth factors and hormone signalling systems have been documented that may be involved in the pathogenesis of OS in both species. In particular, *TP53* is the most frequently altered gene in canine and human neoplasia.

Canine OS is biologically aggressive with clinically undetectable micrometastasis occurring early on in the course of disease. If amputation of the primary OS is the sole form of treatment, the majority of dogs are dead within a year due to metastasis. Despite advances in surgical techniques and the tripling of median survival times in dogs receiving adequate primary site control, due to the use of the platinum class of chemotherapeutics, in terms of actual months, survival times have increased from a 4-month median to an 11-month median at best, furthermore, ~80% dogs still eventually die because of metastasis (Wilson *et al.*, 2008).

In summary, as OS remains a disease of high mortality in both dogs and humans, the investigation of alternative treatments is warranted, such as the use of targeted gene therapy.

Variable	Dog	Human
Total # cases diagnosed in United States/year	>8000	1000
Age (years)	Median 7.5 (early peak @ 1.5 - 2)	Peak incidence @ 15-19 (smaller peak >50)
Race/breed	Large or giant purebreds	None
Gender ratio: male: female	1.5: 1.0	1.5: 1.0
Body weight	90% > 20kg	Heavy
Site	77% long bones Metaphyseal Distal radius > proximal humerus Distal femur > tibia	90% long bones Metaphyseal Distal femur > proximal tibia Proximal humerus
Aetiology	Generally unknown	Generally unknown
Molecular & genetic alterations	Yes	Yes
% clinically confined to limb at presentation	80 - 90%	80 - 90%
% histologically high grade	95%	85 - 90%
% aneuploid	75%	75%
Prognostic indicators	Alkaline phosphatase	Alkaline phosphatase
Metastatic rate without chemotherapy	90% before 1 year	80% before 2 years
Metastatic sites	Lung > bone > soft tissue	Lung > bone > soft tissue
Improved survival with chemotherapy	Yes	Yes
Regional lymph node metastasis	<5%, negative prognosis	Poor prognosis
Radiosensitivity	Generally poor	Generally poor
Radiographic appearance	Aggressive, osteolytic and osteoblastic Codman's triangle, sunbursting.	Aggressive, osteolytic and osteoblastic Codman's triangle, sunbursting.

Table 1.4: A comparison of canine and human OS characteristics. Modified from Withrow *et al.*, (1991) (Withrow *et al.*, 1991), Dernell *et al.*, (2007) (Dernell *et al.*, 2007), and Sandberg & Bridge (2003) (Sandberg & Bridge 2003).

1.2 Gene therapy for cancer

Gene therapy can be defined as the delivery of genetic information to modify a population of target cells for the purpose of treating disease (Argyle 1999). Although gene therapy was originally envisioned as a means to treat genetic diseases, its potential to play a major role in the development of cancer therapeutic agents was quickly recognized. The goal of cancer gene therapy, similar to other treatments for cancer, is the elimination of all malignant cells whilst minimising damage to normal cells. The first approved gene transfer protocol was in human cancer patients (Rosenberg *et al.*, 1990), and the dominance of this area in gene therapy has continued, with 65% of all approved human gene therapy clinical trials being for cancer (The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide 2009). To date, nearly 1000 clinical trials for cancer gene therapy have been completed, are ongoing or approved in more than 29 countries, using over 100 genes (The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide 2009). However, very few gene therapy trials have been undertaken in patients with OS (Dass & Choong 2008).

Gene therapy has also been on trial in dogs as a method of treatment for a number of neoplastic diseases, including OS in which partial and complete responses have been observed (Dow *et al.*, 2005; Khanna *et al.*, 1997).

There is a huge amount of literature regarding cancer gene therapy and as it is beyond the scope of this chapter to review it all, that, relevant to this study will be described in the following sections.

1.2.1 Gene delivery

For gene therapy to be successful an efficient vehicle or vector for delivery is required, as the gene must be delivered to a sufficient number of target cells in the body and the gene must be expressed at an adequate level to provide a therapeutic benefit for a duration appropriate to the disease (Argyle 1999). Ensuring a sufficient supply is a priority as tumour cells, including those from canine OS, typically possess a poor and disorganized blood supply, together with a high interstitial pressure (Zachos *et al.*, 2001). The ideal delivery vehicle

would be one that could be systemically administered and would selectively target tumour cells wherever they occur in the body (Felgner 1997). Above all, the process and consequences of gene transfer should be safe. Despite intensive efforts and advances being made, the optimal universal vector does not yet exist, as many technical hurdles must still be overcome. Gene delivery techniques primarily involve viral and non-viral vectors. Viral vectors are genetically modified viruses, which are still able to transfer their genetic material to a host cell. Most commonly employed viruses include adenovirus, retrovirus, vaccinia-virus, poxvirus, adeno-associated virus and herpes simplex virus (HSV) (The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide 2009). In addition, oncolytic viruses are frequently used as they either possess an inherent tropism for tumour cells or have been engineered from viruses such as HSV, vaccinia and adenoviruses (termed conditionally replicative adenoviruses, CRAAd), to selectively replicate in and destroy cancer cells (Arendt *et al.*, 2009; Kumar *et al.*, 2008). Non-viral delivery methods frequently utilized are naked/plasmid DNA, lipofection and RNA transfer (The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide 2009). Non-viral vectors avoid the main problems associated with viral systems, in that they possess low host immunogenicity enabling repeat administrations, any size of nucleic acid can be inserted and simple large scale production can occur (Blagbrough & Zara 2009). In addition, adenoviral vectors may also be restricted by their requirement for target cells to express appropriate receptors, such as Coxsackie Adenovirus Receptor (CAR) (Yotnda *et al.*, 2002). Although the principal disadvantage of non-viral gene transfer methods is that stable long-term gene expression has, as yet, not been achieved, this may be entirely satisfactory for cancer gene therapy, in that permanent gene expression or the transmission of transferred genes to daughter cells is not necessarily required (Argyle 1999). As clinical trials progress, more data will become available for assessing the safety and efficacy of various cancer gene therapy approaches, with the vast majority of these trials employing retroviral or adenoviral vectors (The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide 2009).

Adenoviruses, including CRAAd, HSV, retroviruses, lentiviruses and liposomes have been used to deliver genes in experimental human OS investigations (Okumura *et al.*, 2007; Witlox *et al.*, 2007). In addition, adenoviruses have been employed in

Phase I/II trials in patients with refractory OS that possess lung metastases (Benjamin *et al.*, 2001). Adenoviruses, also including CRAd, have also been used to successfully introduce genes into canine OS cells *in vitro* and *in vivo* (Hemminki *et al.*, 2003; Kanaya *et al.*, 2005; Le *et al.*, 2006; Yazawa *et al.*, 2003b). Two separate Phase I studies have obtained effective gene transfer into dogs with OS lung metastases using liposome-DNA complexes via intravenous gene delivery and aerosol delivery (Dow *et al.*, 2005; Khanna *et al.*, 1997). It was also observed in the canine trials that the gene therapy treatments employed were well tolerated and produced minimal toxicity, suggesting that liposome-DNA complexes may be a relatively safe form of treatment. Furthermore, no significant signs of virus-associated toxicity were observed following the administration of a canine CRAd into normal dogs, canine adenovirus type 2 (CAV-2), which is being developed to transcriptionally target canine OS cells (Smith *et al.*, 2006). However, vector-associated toxicities have been reported following adenoviral vector-mediated gene therapy in dogs with haemophilia, although clinical benefits were observed (Brunetti-Pierri *et al.*, 2005; McCormack *et al.*, 2006). Thereby highlighting the risk of systemic delivery of adenoviral vectors and the importance of preventing immune responses against the gene delivery vector. Many publications exist pertaining to these different gene delivery systems, of which the ones utilized in OS studies are summarized in Table 1.5.

Vector	Packaging Capacity (kb)	Genetic material	Genome size (kb)	Advantage(s)	Disadvantage(s)	Comments
Adenovirus	8-9	Linear double-stranded DNA	36	Efficiently transduces wide range of non-dividing and dividing cells. Episomal gene expression	Strong immunogenicity Transient expression	These viruses have become the most popular viral delivery mechanism. Gene is packaged into a replication-incompetent adenovirus (usually E1 deleted). Conditionally replicating adenoviruses are used as oncolytic vectors.
Retrovirus	8	Linear single-stranded RNA	7-11	Long term expression Weak immunogenicity	Dividing cells only Integration into genome may induce oncogenesis.	These viruses were originally the gold standard vector. Gene is packaged into replication-defective viral particles using a packaging cell line.
Lentivirus	8	Linear double-stranded DNA	7-11	Transduces wide range of non-dividing and dividing cells. Long term expression Weak immunogenicity	Integration into genome may induce oncogenesis. Inefficient production	Subset of retrovirus group; many lentivirus vectors based on HIV-1. Become safer in recent years with many benefits of conventional retroviruses.
Herpes Simplex virus (HSV)	30	Linear double-stranded DNA	152	Transduces wide range of non-dividing and dividing cells. Large packaging capacity Long term episomal gene expression. Ability to grow to high titres	Strong immunogenicity Latent infection Difficult to manipulate due to large genome size. Transient expression	These viruses, in particular HSV-1, gained attention due to ability to infect neurons and to become latent in some neural cells. Gene is packaged into a replication-defective HSV (usually deficient for immediate-early gene 3).
Liposome/DNA conjugates	any size	Linear double-stranded DNA	any size	Delivers DNA to wide range of non-dividing and dividing cells. Episomal gene expression Safer to administer than viral vectors. Weak immunogenicity	Transfection efficiency lower than viral vectors. Rapidly inactivated in serum	Naked DNA is surrounded with liposome to improve uptake through endocytosis, enhancing efficiency of gene delivery.

Table 1.5: Summary of gene therapy vector systems employed in OS studies. Adapted from Argyle (2007) (Argyle 2007), Fang and Roth (2001) (Fang & Roth 2001), Witlox *et al.*, (2007) (Witlox *et al.*, 2007), and Okumura *et al.*, (2007) (Okumura *et al.*, 2007).

1.2.2 Cancer gene therapy strategies

A greater understanding of the TSG and oncogenes involved in carcinogenesis, along with an expanding knowledge of tumour immunology, have revealed potential molecular targets. This has enabled rationally targeted interventions at the molecular level to occur, in the development of gene therapy technologies for cancer. Essentially, 3 main strategies of cancer gene therapy, which are the subject of extensive research and clinical investigation, have been reported in OS investigations (Figure 1.4): rescue of the cancer cell through gene replacement, antisense, or related therapies which overcome the genetic abnormalities associated with the malignant phenotype; destruction of the cancer cell through delivery of ‘suicide genes’ (pro-drug activating genes i.e. gene-directed enzyme prodrug therapy, GDEPT) which result in the death of the transduced cell; and introduction of genes, e.g. cytokines, which enhance the immune response against the tumour (Witlox *et al.*, 2007).

True suicide gene therapy involves the expression of a gene whose protein product is directly toxic to the cells into which it has been introduced, rather than a prodrug activating system approach (Dr. L. Blackwood, University of Liverpool; personal communication). Cell death is achieved in GDEPT by the protein product converting a prodrug into toxic metabolites. Hence the term ‘suicide gene therapy’ is often used to describe GDEPT, however ideally it should be restricted to approaches utilising true suicide gene therapies.

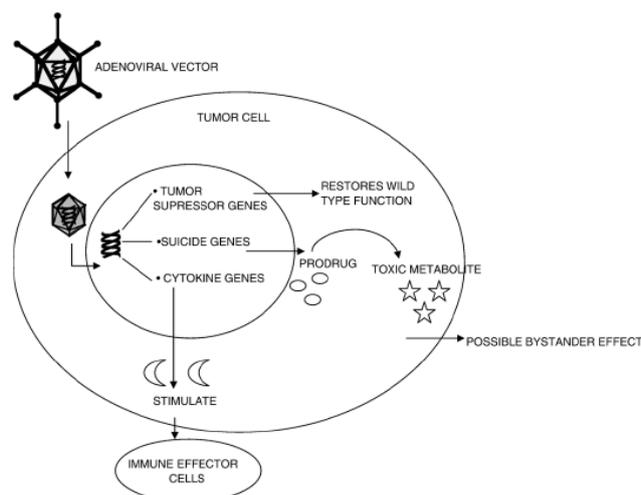


Figure 1.4: Cancer gene therapy strategies: gene replacement, suicide gene therapy and immuno-potentialiation (Witlox *et al.*, 2007).

1.2.2.1 Rescue of the cancer cell through gene replacement

TSG have been the most attractive targets for developing gene replacement strategies thus far. The loss or inactivation of both alleles of a TSG is required to eradicate the growth-suppressive function of the gene. Theoretically, then, replacement of a functional copy of the TSG in cells having homozygous loss of function could restore normal growth and proliferation pathways. Although there are concerns with this approach, especially because cancer is not a single gene defect, rather it involves multiple genetic abnormalities, and so the correction of one alteration may not significantly change the progress of the disease. Additionally, there is no technique currently available that can deliver a therapeutic gene or its product to all the tumour cells and express the gene at the required levels with acceptable levels of toxicity. However, studies undertaken by various groups have shown that it may not be necessary to reverse all the genetic changes in a cancer cell for a therapeutic effect to be observed. Several TSG have been shown to suppress tumour cell growth and proliferation and induce apoptosis following delivery into a variety of cell lines and preclinical *in vivo* models (Roth & Grammer 2005). For example, *p16INK4A*, *p14ARF*, *BAX*, *RB1*, and most notably so far, *TP53*, the most commonly altered gene in cancer, have been introduced into human OS cell lines *in vitro* and *in vivo* (Kim *et al.*, 2004; Okumura *et al.*, 2007; Toyota *et al.*, 2006; Witlox *et al.*, 2007). In addition, cell proliferation has also been inhibited in canine OS cell lines and xenografts following the expression of wild-type TP53 (Kanaya *et al.*, 2005; Yazawa *et al.*, 2003b). Furthermore, 166 clinical trials involving TSG have been approved, including 52 Phase III trials (The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide 2009). Many of these trials involve replacement of TP53 (Roth & Grammer 2005) and the replacement of TP53 is discussed in further detail in Chapter 4. Results of early clinical trials quickly delivered ‘proof of principle’ by demonstrating delivery of TSG to tumour cells, gene expression in targeted cells, minimal toxic effects, and evidence of regression or stabilization of treated tumours. Subsequent trials demonstrated that TSG replacement also rendered some tumour cells more sensitive to the effects of conventional DNA-damaging therapies, extending the potential application of TSG replacement to protocols combining TSG replacement therapy with chemotherapy and radiation e.g. introduction of *TP53* into patients with

non-small cell lung cancer (NSCLC) carcinoma, previously unresponsive to platinum treatments, enhanced their chemosensitivity to cisplatin in a Phase I trial (Smith *et al.*, 2006). Similarly in human OS cells, improvement in sensitivity to cisplatin in combination with adriamycin or doxorubicin, has also been reported after the delivery of wild-type *TP53* (Witlox *et al.*, 2007).

In vivo systems have been developed in which genes can be inserted that inhibit the production of the abnormal protein product by encoding ribozymes (transdominant sequences), short inhibitory RNAs (siRNA; RNA interference (RNAi)) or antisense (complementary) mRNA, to that produced by the TSG, oncogene or growth factor receptors. Ribozymes that can simultaneously reduce mutant *TP53* expression and restore wild-type *TP53* expression in various human cancer cell lines, including OS, have been reported (Watanabe & Sullenger 2000). The downregulation of IGF-1R and VEGF expression in human OS cells by RNAi has been reported to inhibit cell growth and induce apoptosis (Mei *et al.*, 2008; Wang *et al.*, 2009). Additionally, VEGF-siRNA has recently been reported to inhibit OS growth and pulmonary metastasis *in vivo* (Gao *et al.*, 2009). The downregulation of abnormal oncogene expression has also been shown to revert the malignant phenotype in OS e.g. antisense gene therapy using a cyclin G1 construct inhibited the growth of human OS xenografts (Chen *et al.*, 1997). Furthermore, a Phase II study for chemotherapy-resistant OS has recently been published in which this cyclin G1 construct was reported to aid in controlling tumour growth and may improve survival (Chawla *et al.*, 2009).

1.2.2.2 Destruction of cancer cells through delivery of ‘suicide genes’

The ‘suicide gene’ therapy approach has been designed to increase selectivity for cancer cells as a strategy towards improving the specificity of chemotherapy, reducing resistance and limiting the unwanted side effects of chemotherapy. This is achieved in a two-step process, in which the first step involves the delivery of a gene (‘suicide’ gene), encoding a foreign enzyme (viral, bacterial or yeast) not normally expressed by normal cells, to be expressed in cancer cells. In the second step, an appropriate non-or mildly toxic prodrug is

administered systemically and is converted locally into highly cytotoxic metabolites by the expressed foreign enzyme (GDEPT) (Altaner 2008).

Prodrugs have been used, for more than 30 years, in many areas of medicine and there is a considerable level of knowledge and understanding in their application and design for differing circumstances (Connors 1995). Prodrugs are defined as chemicals, which, even at high dose-levels are pharmacodynamically and toxicologically inert, can be converted *in vivo* to a highly pharmacologically active drug (Portsmouth *et al.*, 2007). The active drug can diffuse locally and kill even non-transfected neighbouring cells, in a so-called 'bystander' effect, by intercellular (gap junctions) and local extracellular spread (Witlox *et al.*, 2007). 'Bystander killing' of non-transfected tumour cells is actually the main reason behind the success of the GDEPT approach, as it overcomes the inability of existing gene delivery vectors to transduce all cancer cells, even if only a small fraction of the cells express the suicide gene product (Portsmouth *et al.*, 2007). The effectiveness of bystander killing also appears to be improved by additional mechanisms which include inhibition of angiogenesis, upregulation of various immune system components, and secretion of soluble pro-apoptotic proteins (Lumniczky & Safrany 2006; Nishizaki *et al.*, 1999).

More than 20 prodrug metabolising enzyme systems have been described in the literature and several have been extensively studied, some of which are summarized in Table 1.6 e.g. HSV-thymidine kinase (HSV-TK) phosphorylates ganciclovir (GCV) to produce the nucleotide toxin GCV phosphate, cytosine deaminase from *Escherichia coli* (*E. coli*) converts the antifungal drug 5'-fluorocytosine (5-FC) into the anticancer drug 5'-fluorouracil (5-FU), and nitroreductase (NTR; *nfsB*), also from *E. coli*, catalyses the reduction of CB1954, to generate a 4-hydroxylamine metabolite which reacts with thioesters, such as acetyl coenzyme A, to produce a highly cytotoxic difunctional alkylating agent that can be up to 2000-fold more cytotoxic than CB1954 itself, making it an attractive prodrug (Anlezark *et al.*, 1992; Portsmouth *et al.*, 2007; Weedon *et al.*, 2000). The majority of toxic metabolites produced by the prodrug enzyme systems are cell-cycle dependent as they inhibit cellular nucleic acid synthesis pathways. This is a disadvantage as only a small percentage of tumour cells are proliferating at any one time (Portsmouth *et al.*, 2007). However, the toxicity of

the CB1954 activated prodrug is cell-cycle independent as it can be cytotoxic to cells not in S-phase, suggesting that this prodrug may potentially be used to eliminate non-dividing neoplastic cells. Furthermore, the ideal suicide gene/prodrug combination should produce an activated drug that is at least 100-fold more cytotoxic than the prodrug; possess a sufficiently low molecular weight enabling it to diffuse easily to allow the killing of non-transfected bystander cells; possess a sufficient half-life to kill non-transfected bystander cells; and not be able to diffuse into the bloodstream and cause systemic toxicity (Portsmouth *et al.*, 2007). As yet, similar to the search for an optimal gene delivery vector, the ideal suicide gene/prodrug combination does not exist, but it is the quest of much current research.

Promising results have been obtained with numerous suicide gene therapy systems *in vitro* and *in vivo*, in various human cancer cell lines, including OS (Witlox *et al.*, 2007). On the basis of these results, over 100 suicide gene therapy clinical trials have either been approved, are ongoing or undertaken (The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide 2009). However, to the best of my knowledge no clinical trials have yet taken place in human OS patients or preclinical investigations into suicide gene therapy for canine OS. Suicide gene therapy systems in relation to this study, and in dogs, are described in further detail in Chapter 5.

Enzyme	Origin	Prodrug	Active Drug	Comments
Thymidine kinase	HSV, EHV, VZV	GCG, ACV araM, BVDU	Triphosphorylated forms Monophosphorylated forms	BEB BE present
Cytosine deaminase	<i>E. coli</i> <i>S. cerevisiae</i>	5-FC	5-FU	BEA
Cytochrome P-450	Rat, human, rabbit	CPA, IFO	4-Hydroxy forms	BEA
Carboxylesterase	Human, rabbit	Irinotecan Capecitabine Paclitaxel-2-ethylcarbonate	SN-38 5-FU Paclitaxel	
Nitroreductase	<i>E. coli</i>	CB1954	N-acetoxy derivatives	BEA
NQO1 (DT diaphorase)	Human, rat	Bioreductive agents e.g. CB1954	Reduced forms	
Carboxypeptidase A1	Human, rat	MTX- α -peptides	MTX	BEA
Deoxyribonucleoside kinase	<i>D. melanogaster</i>	BVDU, araT	Monophosphorylated forms	BE present
β -Galactosidase	<i>E. coli</i>	Self-immolative prodrugs from anthracycline antibiotics e.g. daunomycin	Anthracycline antibiotics e.g. daunorubicin	
β -Glucuronidase	<i>E. coli</i> , human, mouse	Self-immolative doxorubicin prodrugs	Doxorubicin	BEA
β -Lactamase	<i>E. coli</i>	Self-immolative prodrugs from cephem prodrugs	Alkylating agents, vinca alkaloids, anthracycline antibiotics	
Linamarase	Cassava plant	Linamarin	Cyanide	BEA

Table 1.6: Suicide gene/prodrug combinations commonly used in cancer gene therapy studies. ACV, acyclovir; araM, 6-methoxypurine arabinoside; araT, 1- β -D-arabinofuranosylthymine; BEA, bystander effect type A - does not require cell-to-cell contact; BE, bystander effect; BEB, bystander effect type B - requires cell-to-cell contact; BVDU, (E)-5-(2-bromovinyl)-2'-deoxyuridine; CB1954, 5-(Aziridine-1-yl)-2,4-dinitrobenzamide; CPA, cyclophosphamide; HSV, herpes simplex virus; EHV, equine herpes virus; GCV, ganciclovir; IFO, ifosamide; MTX, methotrexate; 5-FU, 5-fluorouracil; 5-FC, 5-fluorocytosine; VZV, varicella zoster virus; SN-38, 7-Ethyl-10-hydroxy-camptothecin. Modified from Portsmouth *et al.*, (2007) (Portsmouth *et al.*, 2007).

1.2.2.3 Gene-directed immunotherapy

Researchers have achieved some success utilising cytokines and other molecules in treating OS via augmentation of immune response (Witlox *et al.*, 2007). However, as they tend to be autocrine or paracrine in nature, significant problems are associated with the systemic use of these proteins, including short half-life, profound toxic side effects, limited dose that can be safely administered which may not even have a clinical effect and the inability to maintain potentially therapeutic levels at the desired site of action (Fang & Roth 2001). Hence, the delivery of the actual cytokine gene, e.g. IL-2, IF- γ , IL-12 or IL-18, rather than the protein is a more promising approach. This is because tumour immunogenicity is enhanced, either directly by the insertion of cytokine or costimulatory molecule encoding genes, or indirectly, by the delivery of an immunomodulatory gene, such as an immunogenic antigen or cytokine, that activates the immune system to recognize putative tumour antigens, which leads to immune-mediated cell killing (Witlox *et al.*, 2007). For example, the delivery of IL-2 gene into a primary tumour of a clinically relevant mouse OS model, displayed anti-tumoural immunity which not only inhibited primary tumour growth but also reduced metastases in distant organs (Nagano *et al.*, 2004). In addition, the introduction of the IL-12 gene significantly suppressed the growth of OS lung metastases *in vivo* and in combination with ifosfamide a greater anti-tumour effect was observed (Duan *et al.*, 2006). Furthermore, B7-1, a well-characterized co-stimulatory molecule, has also been shown *in vivo* to induce immunity against pre-established primary OS resulting in regression, systemic immunity against pre-existing pulmonary metastases and activation of T-cells (Tsujii *et al.*, 2002). In conjunction with FAS, B7-1 also induced apoptosis of OS cells and had a stronger inhibitory effect on the development of pulmonary metastasis compared to B7-1 alone (Tsujii *et al.*, 2003). This gene-directed immunotherapy approach also reduces the systemic toxicity previously observed with cytokine proteins and in addition, may increase the number of cytokines that can be used for anticancer treatment, because many proteins that are otherwise intolerable by the host may now be used (Fang & Roth 2001). In addition, the combined delivery of cytokine and suicide genes, followed by prodrug administration, have been shown to enhance the antitumour response and may improve the distant bystander effect against micrometastatic disease

(Argyle 1999). This is because the activated prodrug induces the cancer cells to undergo necrosis, allowing the exposure of anti-tumour antigens which are subsequently recognized by cytokines, thereby promoting the development of a cell-mediated immune response which enhances the antitumour response (Argyle 2007).

Gene-directed immunotherapy has been investigated in a number of small-scale veterinary studies as a potential treatment modality for canine OS. In particular, two Phase I studies have been performed in which liposome-DNA complexes containing canine IL-2 cDNA have been administered to dogs with OS lung metastases (Dow *et al.*, 2005; Khanna *et al.*, 1997). Khanna and colleagues (1997) reported that 2 of 4 dogs had complete regression of pulmonary metastases for more than 1 year following IL-2 inhalational therapy (Khanna *et al.*, 1997). Dow and co-workers (2005) observed that in 3 out of 20 dogs, partial or complete regression of lung metastases occurred and overall, survival times had significantly increased (Dow *et al.*, 2005). Both studies suggested that the tumour regressions observed were due to the activation of a substantial immune response and that IL-2 gene therapy is well tolerated as toxicity levels were minimal (Dow *et al.*, 2005; Khanna *et al.*, 1997). These investigations in the gross disease setting support further development of the gene-directed immunotherapy approach, in particular as adjuvants in the micrometastatic disease setting and in combination with currently available therapies.

1.2.3 Targeted gene therapy

In gene therapy, as in all cancer treatments, ensuring that the therapeutic agent is selectively delivered and expressed in the targeted tumour cells are not only of the highest importance, but are also the primary difficulties to be overcome when devising successful gene therapies for cancer. A major barrier to the widespread clinical use of gene therapy is the inability to give vectors systemically and to ensure that therapeutic genes are not expressed in normal cells (Argyle 2007). Whilst a number of vectors have been used for the delivery of genes into primary tumours, the larger problem in cancer is treatment of disseminated disease, requiring systemic administration of genes. A gene-based

drug that kills tumour cells *in vitro* can be relatively easily designed. However, killing only tumour cells *in vivo* and at the same time sparing normal tissue, requires an extremely selective strategy, in particular, in suicide gene therapy strategies. Numerous strategies have been attempted to target the effect of a gene-based drug, increase transfection efficiency and hence cytotoxicity. These strategies can be categorized into two main groups: transductional targeting in which gene delivery is restricted to tumour cells; and transcriptional targeting which only permits gene expression in tumour cells (Palmer *et al.*, 2006).

Although, as with other agents in cancer therapy, the heterogeneity of the malignant cell population in many tumours, including OS, makes it extremely difficult to obtain specific targeting by any one approach, hence methods of transductional and transcriptional targeting may also be combined (Fang & Roth 2001).

1.2.3.1 Transductional targeting

Transductional targeting can be achieved by the exploitation of specific properties of tumour cells by utilising the natural determinants of the virus-host cell interactions or the range of cells that the virus can infect and their proliferation rate (Fang & Roth 2001). The presence of specific receptors on the cell surface contributes to the specific uptake of an agent and its tissue distribution. In viral vector-mediated gene delivery, tissue tropisms may be used to achieve specific targeting, e.g. adenovirus quite efficiently transduces lung epithelium, retroviral vectors are integrated and expressed only in dividing cells and HSV can infect many cell types but is most efficient at infecting and then mediating prolonged gene expression in neuronal cells (Fang & Roth 2001).

Viral vectors can also be modified to redirect binding of the vector to molecules highly expressed on tumour cell surfaces rather than those it normally interacts with e.g. adenovirus fibre can be altered so that it no longer binds with its natural receptor, CAR, but instead interacts with tumour-specific receptors (Palmer *et al.*, 2006). Viral particles can be altered chemically with an inert polymer to inhibit infection or by manipulation of the genes encoding the viral capsid (coating proteins). Targeting can then be re-directed by attaching

monoclonal antibodies or specific ligands e.g. epidermal growth factor receptor (EGFR), which is overexpressed in many tumours including OS, can be targeted by adenoviral vectors containing a specifically designed antibody (Witlox *et al.*, 2007). The use of EGFR targeting has been reported to improve adenoviral gene delivery into human OS cells by 10-fold and in combination with suicide gene therapy further increased selective cell kill compared to that observed with suicide gene/prodrug alone (Witlox *et al.*, 2002). Furthermore, the infectivity of a canine conditionally replicative adenovirus has been improved following the incorporation of a polylysine peptide onto a native fibre (Le *et al.*, 2006). In addition, the use of polymer coats may reduce the immune responses observed against viral vectors (Palmer *et al.*, 2006). The identification of a single envelope protein variant, EF, screened from a random library has been reported to be highly specific for D17 canine OS cells (Bupp & Roth 2002). It was suggested that the EF envelope protein would be a useful targeting reagent in the study of suicide gene therapy for OS (Bupp & Roth 2002). Non-viral vector targeting has been accomplished primarily through the use of molecular conjugates and DNA-protein complexes.

1.2.3.2 Transcriptional targeting

Transcriptional targeting occurs once the vector has entered the cell and depends on specific regulatory sequences to direct synthesis of the protein of interest only in tissues that are capable of activating the transcriptional elements. Although every gene is represented in every cell of the body, expression of any one gene requires specific transcription factors that may be unique to a particular cell or tissue type. Certain genes have been identified that are expressed in cancer cells but not in normal cells, e.g. telomerase, or are expressed only in a specific tissue type e.g. osteocalcin (OC), which is expressed almost solely in bone tumours and mature calcified tissue (Witlox *et al.*, 2007). By using the sequences for promoters, enhancers and/or 5'-UTR that are responsive to tumour-specific transcription factors, to drive transgene expression, targeted expression can be tumour-specific, e.g. using the telomerase promoter, or tissue-specific, e.g. OC promoter in OS. Hence, control

of therapeutic gene expression in tumours may be achieved via tumour- or tissue-specific regulatory sequences (Palmer *et al.*, 2006).

The transcriptional targeting approach has been used for targeting gene expression to several tumour types including primary OS and OS pulmonary metastases, using the OC promoter which resulted in an anti-tumour effect on OS cells *in vitro* and *in vivo* (Li *et al.*, 2006). In conjunction with suicide gene therapy, the OC (Shirakawa *et al.*, 1998) and telomerase reverse transcriptase (Majumdar *et al.*, 2001) promoters enhanced the selective cell killing of OS cells *in vitro* and *in vivo*. Furthermore, in the *in vivo* experiments, normal tissues were seen to be spared (Li *et al.*, 2006; Majumdar *et al.*, 2001; Shirakawa *et al.*, 1998). The OC promoter has also been used successfully by Hemminki *et al.*, (2003) to restrict the replication of a canine oncolytic adenovirus to dog OS cells, which resulted in effective cell kill *in vitro* and yielded a therapeutic benefit *in vivo* (Hemminki *et al.*, 2003). Additionally, a Phase I/II dose study was proposed in 2001 by Benjamin and colleagues, to evaluate the OC promoter in patients with OS metastatic to the lung (Benjamin *et al.*, 2001); however, no results have yet been published. The specificity of tissue-restricted gene therapy may be further refined as a 31-amino-acid segment of the haematopoietic and osteogenic RUNX2 (CBFA1/AML3) transcription factor has been identified and utilized to direct the OC-promoter to sub-nuclear sites that support gene expression in bone cells *in vivo* (Stein *et al.*, 2000).

A combined transductional and transcriptional targeting approach has been described in which an antibody conjugate was used to redirect an adenoviral vector to EGFR and the reporter gene was placed under the control of OC (Barnett *et al.*, 2002). This dual targeting strategy resulted in a higher level of specificity for cancer cells, including OS, being demonstrated than by either approach alone (Barnett *et al.*, 2002).

Hence, the introduction of vectors containing bone tissue specific promoter-controlled transgenes provides a suitable approach for the delivery and expression of therapeutic genes in the treatment of canine and human patients, with primary OS and OS metastases. Thus TP53-targeted suicide gene therapy, may also have favourable therapeutic implications for canine and human OS.

1.2.4 Safety considerations

Although gene therapy is a highly promising strategy for the treatment of numerous diseases, including many different types of cancer, there have unfortunately been, a few incidents of serious adverse events, including death, which have had a negative impact on progress in this field (Edelstein *et al.*, 2007). These deaths were as a result of a large inflammatory response to an early generation adenoviral vector and retroviral integration near proto-oncogene promoters (Edelstein *et al.*, 2007). More recently designed viral vectors are reported to show an improved safety margin over their predecessors after administration to rodents, but their affect on large animal models is less well described. The delivery of naked DNA may also offer a safer alternative, however, all the potential safety issues with this technology, including autoimmunity and actual fate of DNA after delivery into the patient, have not yet been fully resolved (Argyle 2007). Nevertheless, in light of over 1500 clinical trials currently in place, of which nearly 1000 are Phase I (The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide 2009), there has infact been a lack of significant toxicity directly related to gene transfer in these Phase I trials. Furthermore, some patients have also been observed to benefit from the gene therapy protocol they received (Edelstein *et al.*, 2007).

Although initially in order to maximize safety, cancer gene therapy protocols only involved local, intratumoural delivery e.g. into head and neck cancers, to date several additional routes of delivery have been investigated, including intraperitoneal, intra-arterial and intravenous (Witlox *et al.*, 2007). This is because the efficacy of gene therapy is also affected by the method in which the vector is delivered as well as by the vector itself. Furthermore, in the context of pulmonary disease, e.g. pulmonary metastases, the use of aerosolized gene therapy has been regarded as an attractive route due to it being non-invasive, capable of delivering a high dose to the target site and belief that it results in fewer adverse effects compared to intravenous delivery (Witlox *et al.*, 2007). Indeed, inhalational therapy has been administered to dogs with OS pulmonary metastases resulting in clinical responses with minimal toxicity levels (Khanna *et al.*, 1997). In addition, systemic delivery may also make gene-based drugs more prone to inactivation by the immune system, considerably limiting vector

transduction efficiency and the duration of transgene expression, as well as inducing an acute inflammatory response (Witlox *et al.*, 2007).

In view of the known risks of elevated malignancy rates after conventional treatment modalities such as radiation therapy, chemotherapy and immunosuppression, gene therapy may yet prove to be an acceptable method of cancer treatment.

1.2.5 Summary

One of the most promising approaches to emerge from the improved understanding of cancer at the molecular level is the possibility of using gene therapy to selectively target and destroy tumour cells, thus improving the specificity of the therapy while reducing systemic toxicity. Intuitively, it would seem that simple eradication of cells is likely to be the best and safest. The self-renewing nature of malignant disease dictates that tumour cells should be cleared as efficiently as possible rather than genetically corrected. Although, the suicide gene therapy strategy seems no more likely to succeed than corrective gene therapy, requiring *in vivo* delivery of the therapeutic gene to every tumour cell, the bystander effect can mediate the destruction of neighbouring non-transduced tumour cells. Thus, the most frequently used genes have been designed to kill cells directly, such as suicide genes, or to induce immune-mediated destruction and are likely to provide an all-encompassing alternative to the transfer of a single gene implicated in tumourigenesis.

Many cancer gene therapy clinical trials have now taken place, with different strategies, which have confirmed that such treatments are safe and well tolerated; however, with a few exceptions, evidence of significant improvements in survival over that seen with conventional treatments, has not yet been proved. Furthermore, success rates even approaching those routinely achieved in preclinical studies, have yet to be obtained in clinical applications, including those in which real clinical benefit has been observed. It is likely that numerous obstacles are responsible for this discrepancy, including the ability to target tumour cells through systemic delivery, gene expression to occur as

required, larger size of canine and human malignancies in comparison to *in vitro* and *in vivo* models and the slower growth rate of spontaneous tumours compared to experimental xenografts. Nevertheless, gene therapy remains a highly promising strategy for the treatment of many cancers and progress in the field is increasing.

With regard to gene therapy for OS, two main problems exist for OS gene therapy to be successful, the eradication of the primary tumour and the requirement to inhibit and destroy metastases, particularly pulmonary metastases since they are the main cause of death in both canine and human OS patients. However, promising results have been obtained following the introduction of *TP53* into both canine (Kanaya *et al.*, 2005; Yazawa *et al.*, 2003b) and human (Kim *et al.*, 2004; Okumura *et al.*, 2007; Toyota *et al.*, 2006; Witlox *et al.*, 2007) OS cell lines and xenografts and in dogs with OS pulmonary metastases after gene-directed immunotherapy treatment utilising cytokine genes (Dow *et al.*, 2005; Khanna *et al.*, 1997). Although no preclinical suicide gene therapy studies have yet been published in canine OS cells, results displaying potential have been reported in *in vivo* and *in vitro* investigations of human OS cells (Witlox *et al.*, 2007). Therefore, new treatment strategies are under investigation for OS; in particular, *TP53* is a prime candidate in the development of targeted gene therapy systems.

1.3 TP53 tumour suppressor gene

TP53 was discovered in 1979 (Lane & Crawford 1979; Linzer & Levine 1979) and subsequently characterized as a tumour suppressor in 1989 (Baker *et al.*, 1989; Finlay *et al.*, 1989). Since then it has become the one of the most studied genes in cancer biology, as more than 50000 publications have been published due to it being the most commonly altered gene in human (Petitjean *et al.*, 2007b) and canine cancer (McIntyre *et al.*, 1994; Setoguchi *et al.*, 2001b; Toguchida *et al.*, 1992; Veldhoen *et al.*, 1999). TP53 has been titled “the guardian of the genome” (Lane 1992) and called a “cellular gatekeeper” (Levine 1997), as it possesses a central role in regulating the cell cycle and maintaining genomic integrity following genotoxic or cellular stress (Vogelstein *et al.*, 2000; Vousden & Lane 2007). Loss or mutation of TP53 function is highly correlated with an increased susceptibility to tumourigenesis (Olivier *et al.*, 2009). Hence, TP53 has been the subject of an extensive range of experimental approaches, extending from X-ray crystallography to clinical studies, in the pursuit of developing new and more effective therapeutic strategies for the treatment of cancer. It is beyond the scope of this chapter to describe the vast amount of literature regarding TP53, so it will be briefly reviewed and areas of relevance to this study will be discussed.

1.3.1 Structure & function of TP53

The p53 protein, consisting of 393 amino acids ($\alpha\alpha$) in humans, contains 4 well-characterized functional domains, each corresponding to specific functions that are common to several species, including dogs (Millau *et al.*, 2009; Veldhoen & Milner 1998; Zakut-Houri *et al.*, 1985) (Figure 1.5). This is because the primary amino acid sequence has been found to be highly conserved during evolution, such that five evolutionary conserved domains have been identified in cross-species comparisons (Soussi & May 1996). The amino-terminus (N-terminus), contains two transactivation domains, AD1 (1-42 $\alpha\alpha$) and AD2 (43-63 $\alpha\alpha$), and a proline-rich domain (64-97 $\alpha\alpha$) (Millau *et al.*, 2009). The transactivation domains regulate transcriptional activity due to the possession of residues which can interact with components of the basal transcriptional machinery (Ko & Prives

1996). They are also importantly the location for binding with numerous proteins that regulate the stability and activity of TP53, in particular, MDM2, and many of the post-translational modifications (Alarcon-Vargus & Ronai 2002). The proline-rich domain, containing 5 'PXXP' motifs, is reported to be essential for apoptosis as it binds with proteins containing SH3 sites (Walker & Levine 1996). The central region (102-292 $\alpha\alpha$) possesses the sequence-specific DNA-binding domain (Bargonetti *et al.*, 1993; El-Deiry *et al.*, 1992), along with four of the five evolutionary conserved domains (II-V) (Pavletich *et al.*, 1993). A 21 $\alpha\alpha$ sequence, located between 92-112 $\alpha\alpha$, in combination with the proline-rich and DNA-binding domains, are important for TP53 degradation by both MDM2 and human papillomavirus (HPV)-E6 (negative autoregulatory domain) (Gu *et al.*, 2001b; Gu *et al.*, 2001a). The carboxy-terminus (C-terminus) possesses an oligomerization domain (323-356 $\alpha\alpha$), which participates in the formation of TP53 tetramers, a nuclear export signal and ubiquitination sites for TP53 degradation (Clore *et al.*, 1994; Lohrum *et al.*, 2001). The last 30 $\alpha\alpha$ of the C-terminus (363-393), possess 3 nuclear localization signals (Shaulsky *et al.*, 1990; Stommel *et al.*, 1999), and a non-specific DNA binding domain that recognizes structural features of target DNA, including damaged DNA (Kruse & Gu 2009).

TP53 primarily functions as a tetrameric transcription factor by binding to specific DNA sequences and transactivating or repressing a large, and increasing, group of genes (El-Deiry 1998; Vogelstein *et al.*, 2000). The classical view of TP53 function is that TP53 is induced in response to a wide variety of stress signals, including DNA damage (e.g. by ultraviolet light, γ -irradiation and genotoxic chemicals), hypoxia, cytokines, DNA/RNA viral infection and activated oncogenes (Latonen & Laiho 2005; Ljungman & Lane 2004) (Figure 1.6). Following activation, TP53 is believed to initially arrest the cell cycle and based on the severity of the genomic damage, determines the fate of the cell. If the DNA is repairable, TP53 enables DNA repair to occur and once finished, allows completion of the cell cycle. However, if the damage is excessive and unreparable, TP53 irreversibly blocks cell growth and proliferation by inducing senescence, differentiation or apoptosis, thereby preventing inappropriate DNA synthesis and replication (El-Deiry 1998; Kastan *et al.*, 1991; Lane 1992; Seo & Jung 2004). Over the past few years, research suggests that TP53 may also be an active mediator of pro-survival signalling pathways due to the identification of a

multitude of TP53 targets which may actually oppose apoptosis (Janicke *et al.*, 2008; Vousden 2006). Furthermore, TP53 may also be involved in the regulation of glycolysis, autophagy, oxidative stress, invasion and motility, angiogenesis and bone remodelling (Vousden & Lane 2007). An added level of complexity to the structure and functions of TP53 now exists following the discovery in 2006 of the p53 isoform family (Bourdon *et al.*, 2005), which includes eight p53 variants truncated for either one or both of the oligomeration and transactivation domains (Murray-Zmijewski *et al.*, 2006). This indicates that the typical view of TP53 and its pathways, is based around a central TP53 rather than that of itself and its eight variants, which each possess their own characteristics. Hence, TP53 and its family is involved in many cellular activities and interacts with numerous endogenous and exogenous nuclear proteins, to produce a variety of responses (Kruse & Gu 2008; Riley *et al.*, 2008). All of these responses can be proposed to be essential for the normal development and maintenance of an organism, thus protecting the genome from accumulating mutations. Thus, if TP53 becomes defective, or the pathways of TP53 possess aberrations, cell growth and proliferation fails to be regulated, resulting in tumourigenesis.

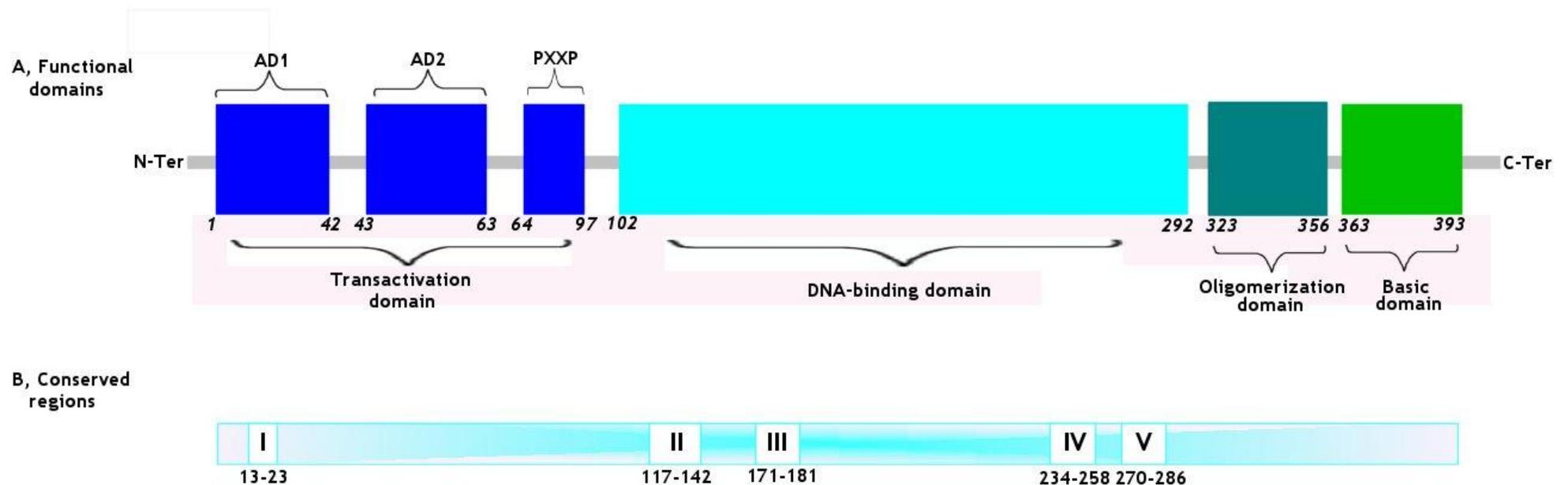
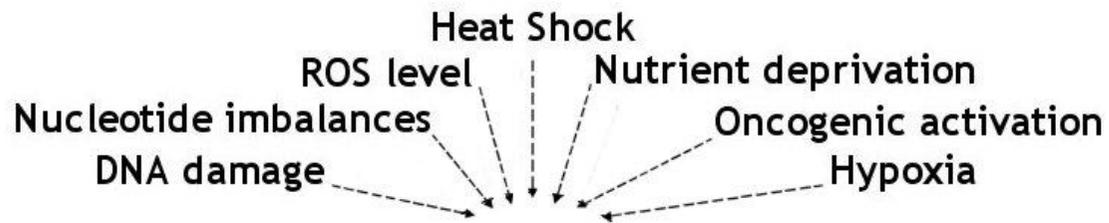


Figure 1.5: The schematic representation of the structure of the human TP53 protein in its basic form. The functional and conserved domains are shown with numbers referring to amino acid residues. Abbreviations: 'AD', activation domain; 'C-Ter', carboxy-terminus; 'N-Ter', amino-terminus; 'PXXP', proline-rich domain containing 'PXXP' motif. Adapted from Millau *et al.*, (2009) (Millau *et al.*, 2009).



p53 ↑

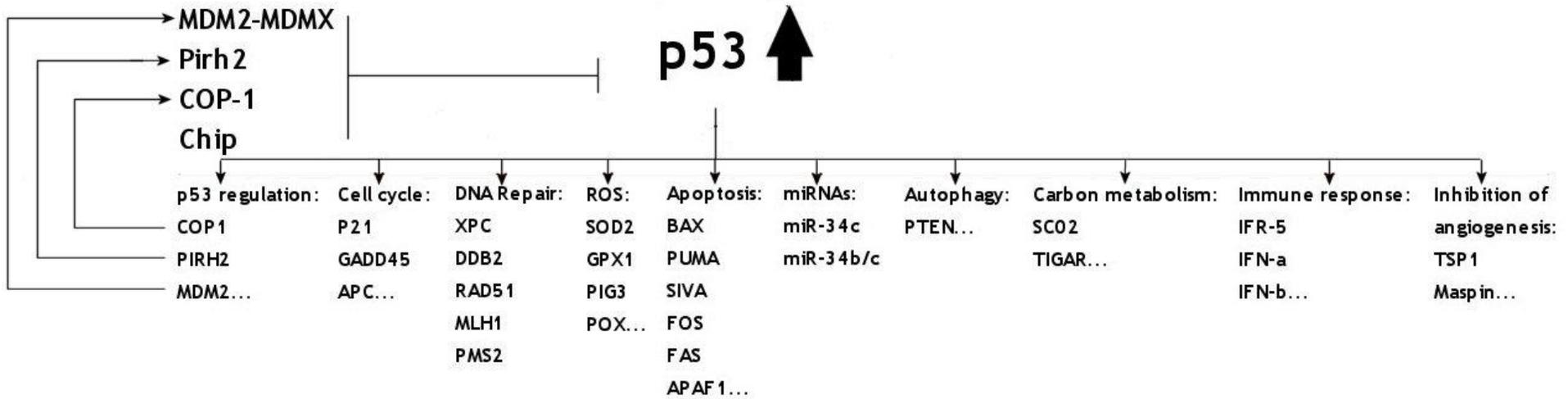


Figure 1.6: TP53 is activated in response to a wide variety of stresses and acts as a transcription factor to adapt the expression level of many target genes involved in major cellular pathways. Adapted from Millau *et al.*, (2009) (Millau *et al.*, 2009).

1.3.1.1 Transcriptional target genes

TP53 transcriptionally targets and regulates the expression of a multitude and constantly growing series of over 125 genes, all of which are the subject of extensive investigations (Laptenko & Prives 2006; Riley *et al.*, 2008) (as shown previously in Figure 1.6). This occurs via specific DNA binding of TP53 to a TP53 response element (RE) that is found either in the promoter or first introns of target genes (Millau *et al.*, 2009). Several of these genes are critically involved in the downstream propagation of growth control signals involved in cell cycle arrest, including *p21*, *GADD45* and *14-3-3-sigma*, which were some of the first TP53 target genes to be identified (Ko & Prives 1996). Canine TP53 has also shown to be capable of inducing *p21* in response to DNA damage (Zhang *et al.*, 2009). Additional TP53 transcriptional targets, which may be part of both intrinsic and extrinsic pathways of TP53-induced apoptosis, include *BAX*, and *PUMA* and *FAS*, respectively (Bossi & Sacchi 2007; Helton & Chen 2007). *TP53* also transactivates another set of genes that mediate cell survival e.g. *XPC*, *RAD51*, which sense or recognize DNA damage and initiate a repair response for the damaged DNA (Helton & Chen 2007; Janicke *et al.*, 2008; Stubbert *et al.*, 2009). A group of genes that regulate TP53 stabilization, include *MDM2* and *PIRH2*, by directly interacting with TP53 to control TP53 degradation and form negative regulatory feedback loops (Alarcon-Vargus & Ronai 2002; Leng *et al.*, 2003). These feedback loops probably act to restrain TP53 activity within cells, as well as to terminate TP53-initiated responses once the inducing signal is turned off. Further TP53 target genes play a role in anti-angiogenesis, immune response, oxidative stress, autophagy and carbon metabolism (Millau *et al.*, 2009; Vousden & Lane 2007). In addition, the eight p53 isoforms have different biochemical transcriptional activities, for example, *TP53* binds preferentially to the RE for *MDM2* and *p21* but not the RE for *BAX*, whereas p53b interacts preferentially with the *BAX* RE, as well as the *p21* RE, but not the RE for *MDM2* (Millau *et al.*, 2009).

1.3.2 Regulation of TP53 activity

The regulation of TP53 is mainly at the protein level (post-translational) by a multitude of cellular proteins that have been found to bind to various regions of

TP53 in order to regulate the specificity of its activity, including ones that it has transcriptionally activated e.g. MDM2 (Kruse & Gu 2009) (Figure 1.7). Although there are a large number, it is primarily affected by MDM2 (Jones *et al.*, 1995; Montes de Oca *et al.*, 1995).

In all normal non-stressed cells, the wild-type TP53 is expressed at low or undetectable levels, with a half-life of ~10-20 minutes. This is mainly due to the down-regulation of TP53, via the ubiquitin-mediated proteasomal degradation pathway, involving MDM2, PARC and PIRH2 (Haupt *et al.*, 1997; Leng *et al.*, 2003). These genes, as previously mentioned, are up-regulated by TP53, enabling the negative regulatory feedback loop to maintain TP53 at very low levels in normal cells.

DNA damage, caused by genotoxic or non-genotoxic stresses, is typically thought to result in the activation of TP53 through a 3-step process. First of all, TP53 is phosphorylated by a wide range of kinases, including ATM/ATR/DNA-PK and CHEK1/CHEK2, which inhibit its interaction with MDM2 and the other proteins that are involved in the negative feedback loop, resulting in the stabilization and accumulation of TP53 levels (Ashcroft & Vousden 1999; Caspari 2000; Shieh *et al.*, 1997). Phosphorylation is the most common post-translational modification and there are at least 15 phosphorylation sites, of which the majority are in the N-terminus (Appella & Anderson 2000; Meek 1998). In conjunction with this step, TP53 RNA is overtranslated to ensure the accumulation of TP53 takes place. In addition, TP53 is also stabilized by the ARF-MDM2 pathway, as a result of oncogenic activation increasing ARF levels, which enables ARF to interact with MDM2, releasing TP53 from MDM2 (Kamijo *et al.*, 1998; Lane & Lain 2002). USP7 (previously known as HAUSP), a deubiquitinating enzyme, acts as a potent TP53 stabilizer as it prevents ubiquitinated TP53 from degradation, adding further complexity to the tight regulation of TP53 stability (Li *et al.*, 2002; Salomoni & Pandolfi 2002; Wood 2002). Figure 1.8 summarizes the pathways involved in regulating TP53 stability.

Secondly, sequence-specific DNA binding of the core domain was classically thought to be negatively regulated by post-translational modifications of the C-terminus domain (Chiarugi *et al.*, 1998; Wolkowicz & Rotter 1997). However, recently this has been brought into question by studies revealing, for example,

that the C-terminus does not interact with the core domain and the core domain is already bound to DNA prior to activation of transcription (Kruse & Gu 2009). Although, sites of phosphorylation, O-linked glycosylation, ADP-ribosylation and acetylation are found in the core domain which may enhance the sequence-specific DNA binding activity of the core domain with some specific target genes (Kruse & Gu 2009).

The third step of the process is the activation of *TP53* transcription via interactions with the basal transcriptional machinery, which are aided by several post-translational modifications of the C-terminus. These modifications include ubiquitination, acetylation, phosphorylation, methylation, sumoylation and neddylation (Kruse & Gu 2009). The numerous combinations of these modifications and the timing at which they occur, enables different sets of *TP53* target genes to have different requirements for *TP53* post-translational modifications following stress-induced *TP53* activation e.g. acetylation of *TP53* is required for activation of proapoptotic genes, *BAX*, *PUMA*, *FAS* and *NOXA*, whereas no acetylation is necessary for cell survival by *MDM2* and *PIRH2* (Kruse & Gu 2009).

Although some aspects of the *TP53* post-translational regulatory network appear to be redundant, none of the stimuli can induce, for example, all of the phosphorylation pathways and the phosphorylation response of *TP53* varies to each different stimulus. However, findings from a number of studies cannot be satisfactorily explained by the classical model of *TP53* regulation which have recently led Kruse and Gu (2009) to suggest a more sophisticated model for the regulation of *TP53* activation, in which antirepression, the release of p53 from repression by *MDM2* and *MDM4*, is proposed to be central to the regulation of *TP53* (Kruse & Gu 2009).

Post-transcriptional modifications of *TP53* do exist, and these forms have been shown to differ in their response to DNA damage. In particular, an additional level in controlling the complexity of *TP53* function may occur via the eight variants of *TP53*, all of which generate altered *TP53* with either unique N- and/or C- termini (Millau *et al.*, 2009).

Hence, regardless of the actual mechanism of the regulation of TP53 activation, it is clear that the responsiveness of TP53 activity to a wide range of signals is tightly and co-ordinately controlled in response to stresses and changes in the cellular environment.

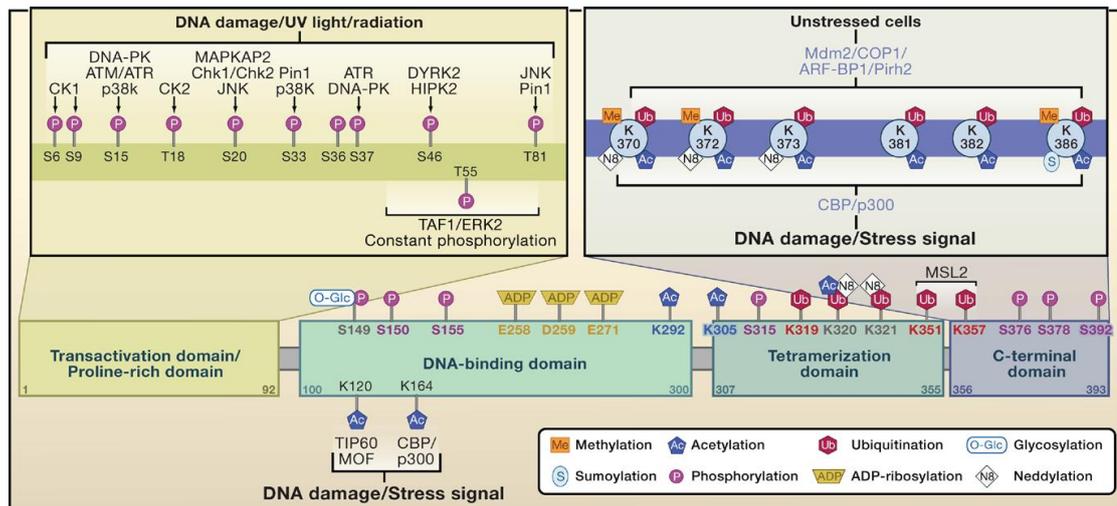


Figure 1.7: Overview of TP53 post-translational modifications. More than 36 amino acids of TP53 are reported to be modified. The major sites of TP53 phosphorylation (P), ubiquitination (Ub), and acetylation (Ac) are shown with the corresponding primary modifying enzymes and signals. Furthermore, additional phosphorylation and acetylation sites, as well as main sites of methylation (Me), sumoylation (S), neddylation (N8), glycosylation (O-Glc), and ribosylation (ADP), are indicated (Kruse & Gu 2009).

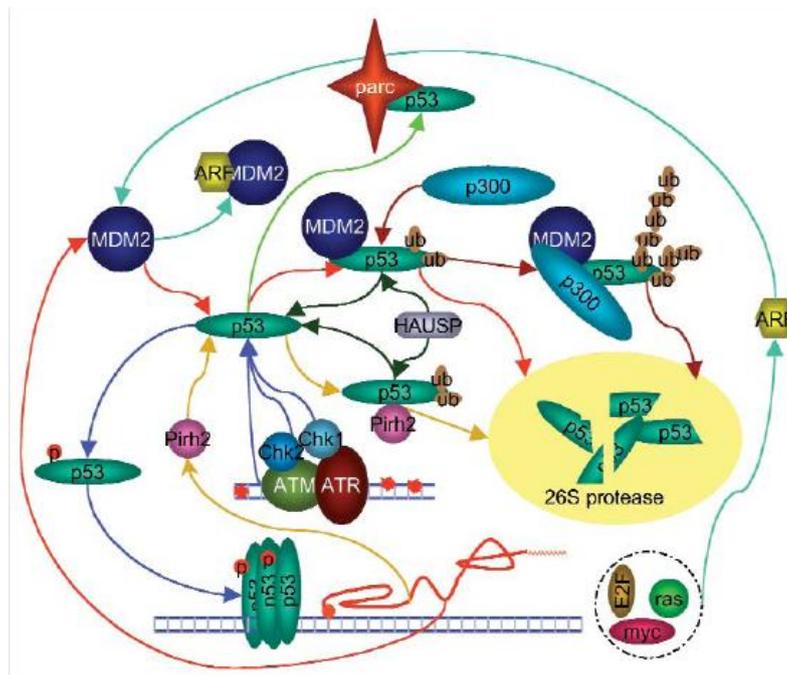


Figure 1.8: Pathways of TP53 stabilization and degradation. MDM2 binds to TP53 and ubiquitinates TP53 through its RING domain that possesses E3 ligase activity. The ubiquitinated TP53 is then degraded by 26S proteasomes or further polyubiquitinated by p300 for more efficient degradation. DNA-damage stabilizes TP53 by inducing TP53 phosphorylation, which prevents the binding of MDM2. The stabilized TP53 in the form of a tetramer transactivates target genes, which include MDM2. MDM2 therefore forms a negative feedback loop with TP53 and regulates TP53 stabilization. Another feedback loop regulating TP53 stabilization is the PIRH2 pathway. Activation of oncogenes stabilizes TP53 by the ARF pathway, in which ARF binds to MDM2 and releases TP53 from MDM2-mediated degradation. HAUSP (USP7), which contains deubiquitinating enzyme activity, acts as a potent TP53 stabilizer, and can rescue TP53 from degradation. PARC, a large molecular weight cytosolic protein, also participates in regulating TP53 by anchoring TP53 in the cytoplasm. In case of DNA damage, TP53 is heavily phosphorylated by ATM/CHEK2 or ATR/CHEK1. Phosphorylation of TP53 at the amino-terminus prevents MDM2 binding and TP53 is stabilized (Wang *et al.*, 2003).

1.3.3 Inactivation of TP53

TP53 fails to function correctly in the majority of human cancers, including OS (Petitjean *et al.*, 2007b; Tang *et al.*, 2008). In ~50% of these tumours, the TP53 is directly inactivated due to mutations in the *TP53* and in the remainder, the wild-type TP53 can be indirectly inactivated through interactions with viral proteins or aberrations in genes whose products are either upstream mediators

or downstream events of *TP53* (Vogelstein *et al.*, 2000) (Table 1.7). It is acknowledged that different forms of mutant *TP53* possess different functional and biological effects to that of wild-type *TP53*. Disruption of the *TP53* response pathway strongly correlates with tumourigenesis. This is clearly demonstrated by the predisposition to a wide range of early-onset tumours, including OS, in patients who suffer from Li-Fraumeni and related syndromes due to the inheritance of *TP53* mutations (Kansara & Thomas 2007), and *TP53* null mice which develop normally, but are prone to the spontaneous development of a variety of neoplasms at an early age, thereby mimicking Li-Fraumeni syndrome (Donehower *et al.*, 1992). Direct and indirect inactivation of *TP53* is also a common event in canine malignancies, including OS (Johnson *et al.*, 1998; Setoguchi *et al.*, 2001a; Thomas *et al.*, 2009; Van Leeuwen *et al.*, 1997).

Mechanism of inactivating <i>TP53</i>	Typical tumours	Effect of inactivation
Amino-acid-changing mutation in the DNA-binding domain	Colon, breast, lung, bladder, brain, pancreas, stomach, oesophagus and many others	Prevents <i>TP53</i> from binding to specific DNA sequences and activating the adjacent genes
Deletion of the carboxy-terminal domain	Occasional tumours at many different sites	Prevents the formation of tetramers of <i>TP53</i>
Multiplication of the <i>MDM2</i> gene in the genome	Sarcomas, brain	Extra <i>MDM2</i> stimulates the degradation of <i>TP53</i>
Viral infection e.g. SV40 large T antigen, HPV16/18, adenovirus E1B	Cervix, liver, lymphomas	Products of viral oncogenes bind to and inactivate <i>TP53</i> in the cell, in some cases stimulating <i>TP53</i> degradation
Mutations in pathways upstream/ downstream of <i>TP53</i> e.g. deletion of p14ARF or ATM genes; PI3K/ AKT/PTEN	Breast, brain, lung and others, especially when <i>TP53</i> itself is not mutated	Failure to inhibit <i>MDM2</i> and keep <i>TP53</i> degradation under control
Mislocalization of <i>TP53</i> to the cytoplasm, outside the nucleus	Breast, neuroblastomas	Lack of <i>TP53</i> function (<i>TP53</i> functions only in the nucleus)

Table 1.7: Mechanisms of *TP53* inactivation in cancer (Vogelstein *et al.*, 2000).

1.3.3.1 Mutational spectra of *TP53*

Over 2000 original publications (published between 1989 and December 2006) have described alterations to *TP53* in human tumours, totalling 24785 somatic and 423 germ-line mutations, of which there at least 1700 different mutations in over 230 of the 393 residues (International Association of Cancer Registries (IARC) *TP53* Mutation Database, R13 release, November 2008, www-p53.iarc.fr) (Petitjean *et al.*, 2007b). Several residues have sustained multiple alterations,

implying that diverse structural alterations in TP53 can promote carcinogenesis (Greenblatt *et al.*, 1994; Petitjean *et al.*, 2007b). It is believed that the main reason why *TP53* is so frequently mutated is due to the ability of TP53 to respond to the many different types of stress stimuli (Appella & Anderson 2000; Pluquet & Hainaut 2001).

Mutations in *TP53* include any one or a combination of the following aberrations: somatic or germline missense (point), frameshift, nonsense, silent, insertion, deletion and splice site mutations (Petitjean *et al.*, 2007b); gene rearrangements (Miller *et al.*, 1990; Toguchida *et al.*, 1992); and allelic gains and losses (Kansara & Thomas 2007; Kaur *et al.*, 2007; Ozaki *et al.*, 2002; Yamaguchi *et al.*, 1992). The most commonly occurring mutation is missense, as missense mutations account for 73.4% and 77.3% of the somatic and germline mutations in human cancers, respectively (IARC TP53 Mutation Database R13 release, November 2008) (Petitjean *et al.*, 2007b). Although missense mutations are found throughout the open reading frame (ORF), the nature of the mutations generally depends on the domain as the mutations mainly observed in the N- and C-termini are nonsense (truncation) and frameshift mutations (Hainaut & Hollstein 2000).

Whilst mutations are found throughout the ORF and at splice junctions, over 98% are clustered in four of the five evolutionarily conserved domains, located in the central region of the *TP53*, and less than 1% occur in the oligomerization domain of the C-terminal domain (Hainaut & Hollstein 2000; Petitjean *et al.*, 2007b). The 4 evolutionarily conserved domains lie between codons 110 and 310 and cover exons 5 to 8. Within this stretch of amino acids, the majority of mutations are missense (84.1%) and the most commonly mutated codons ('hotspots'), are located at amino acid residues 175, 245, 248, 249, 273 and 282, which account for 50% of mutations within this domain (Petitjean *et al.*, 2007b).

The most frequently occurring *TP53* mutation found in all tumour types is G:C to A:T transitions at CpG dinucleotide sites, accounting for 24.7% of the 24785 somatic mutations analysed in the IARC TP53 Mutation Database R13 release, November 2008) (Petitjean *et al.*, 2007b). Furthermore, five of the six mutational 'hotspots' in *TP53* are CpG dinucleotide sites (175, 245, 248, 273 and 282) (Tornaletti & Pfeifer 1995). CpG dinucleotides are estimated to have at

least a 10-fold higher mutation rate than that of other dinucleotides, due to their preferential methylation to 5-methylcytosine which has the ability to spontaneously deaminate (Holliday & Grigg 1993; Rideout, III *et al.*, 1990). It is recognized that G:C→A:T transitions at CpG sites typically represent endogenous DNA damage, whereas G:C→T:A transversions are generally associated with carcinogen exposure (Greenblatt *et al.*, 1994; Walker *et al.*, 1999).

The mutational spectra of *TP53* in human OS, similar to that for all tumour types, indicates that the majority of alterations occur between exons 5 and 8 and are missense, with mutations being reported at all six 'hotspot' codons, of which 175, 245, 248, 273 and 282, are also found to be frequently mutated (IARC TP53 Database R13 release, November 2008), (Figure 1.9) (Petitjean *et al.*, 2007b). The most commonly occurring mutation in human OS is also G:C to A:T transitions at CpG dinucleotide sites (26.7%, n=172) (IARC TP53 Mutation Database R13 release, November 2008) (Petitjean *et al.*, 2007b). Thus, from the current knowledge of exogenous and endogenous mutation events in *TP53*, it seems likely that mutations in human OS tumours are as a result of endogenous factors.

A similar pattern may exist for *TP53* in canine tumours, including OS, as many of the aberrations detected, so far, are located in *TP53* DNA binding domain and to a lesser extent in the transactivation and oligomerization domains (Kirpensteijn *et al.*, 2008; Setoguchi *et al.*, 2001b). The majority of mutations in exons 5-8 are missense along with the occurrence of a few frameshifts, deletions and insertions (McIntyre *et al.*, 1994; Setoguchi *et al.*, 2001b; Toguchida *et al.*, 1992; Veldhoen *et al.*, 1999). In particular, three studies also reported that between 25-50% of the mutations found in canine OS were transitions at CpG sites (Johnson *et al.*, 1998; Mendoza *et al.*, 1998; Van Leeuwen *et al.*, 1997).

These findings therefore indicate the involvement of *TP53* in the genesis of canine tumours in a way comparable to that of human tumours and mutations in the conserved domains of *TP53* appear to play a significant role in OS, in both species.

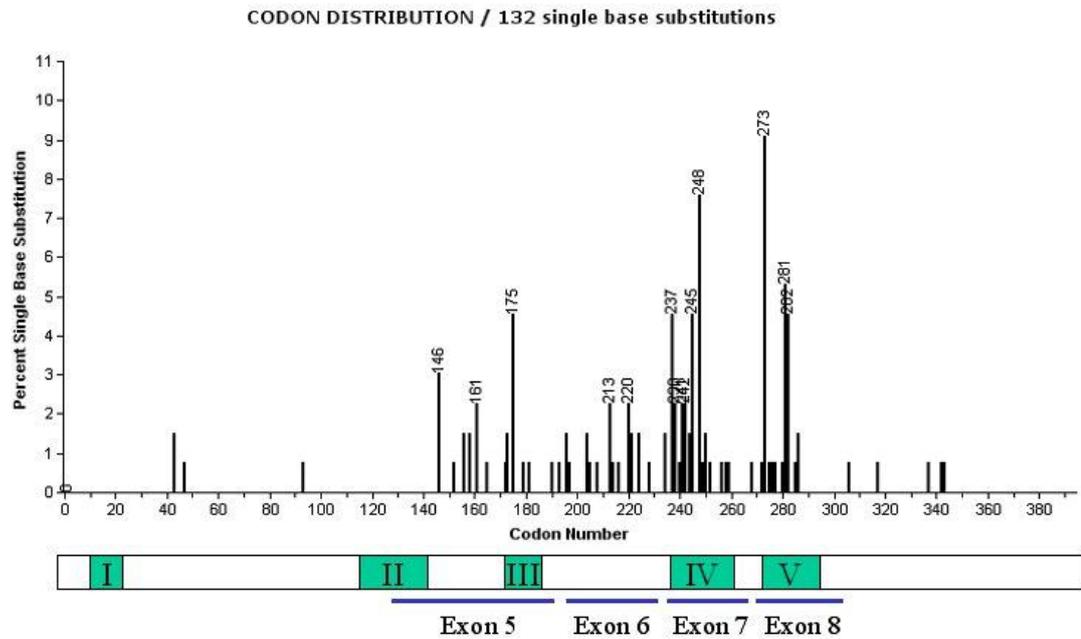


Figure 1.9: *TP53* mutations found in human OS. The green boxes represent evolutionarily conserved regions. The length of the black vertical lines above the main box represent the frequency at which mutations are found at each particular residue and are clustered in conserved regions II-V. Most commonly mutated codons are highlighted, of which 175, 245, 248, 273 and 282 are also ‘hotspots’. The horizontal blue lines represent the locations of exons 5-8 within *TP53*, which contain 4 of the 5 evolutionary conserved domains. Modified from IARC *TP53* Mutation Database, R13, Release November 2009 (Petitjean *et al.*, 2007b).

1.3.3.2 Effect on *TP53* function

Cells with *TP53* functional deficiencies are unable to respond properly to stresses and accumulate DNA damage, while the ability to initiate cell cycle arrest and apoptosis are usually lost. These conditions act synergistically to promote the transformation of normal cells into malignant cells. Deficiency of *TP53* function also confers a growth advantage under hypoxic conditions, which contributes to tumour progression (Pluquet & Hainaut 2001). In addition, conventional cancer treatments are based on genotoxic stresses inducing cell death through different pathways e.g. DNA damage, cell cycle inhibition, hence the loss of *TP53*-mediated apoptosis considerably reduces the response to these therapies (Lowe *et al.*, 1993).

Structurally, *TP53* mutations can be divided into two classes: class one contains alterations in residues regulating sequence-specific binding e.g. arginines 248, 273 and 282; and class two, aberrations in residues for the global conformation

of TP53 structure e.g. arginines 175 and 249 (Cho *et al.*, 1994; Hainaut *et al.*, 1998). In both cases, mutated TP53 is typically characterized by a prolonged half-life and by altered or abolished wild-type RE binding activity (Hainaut & Hollstein 2000). Furthermore, *TP53* mutations contribute to a broad spectrum of *TP53* transcriptional activities, which may result in either a loss of function or a gain of function (Lin *et al.*, 1995).

1.3.3.2.1 TP53 loss of function

Many *TP53* mutations produce a loss in transcriptional function. A total loss of function may occur when mutated TP53 exerts a dominant negative effect within TP53 tetramers. This is because mutated TP53 is more abundant in the cell due to the possession of a prolonged half-life, resulting in its over-representation in tetramers. Hence, even if the remaining wild-type p53 allele is present, *TP53* transcriptional activities are lost (Chene & Bechter 1999). For example, transgenic mice expressing p53^{+/-} or p53^{-/-} are very prone to both spontaneous and induced tumours (Hann & Lane 1995; Harvey *et al.*, 1995). A dominant negative effect has been reported to be important for switching off *TP53* transcriptional activity, since 80% of the most common mutants are able to exert a dominant negative effect (Petitjean *et al.*, 2007b). However, mutated TP53 may retain some transcriptional activity, with each one possessing a different amount of activity, thereby having a different effect on different target genes e.g. p53-175P mutants are correlated with a loss of apoptotic properties but retain the ability to induce cell cycle arrest (Rowan *et al.*, 1996). The function and structure of all 2314 possible amino acid substitutions caused by a point mutation in *TP53* showed that 635 mutants exhibited decreased transcriptional activities for specific target genes and 361 demonstrated a total loss of transcriptional activities (Petitjean *et al.*, 2007b). Furthermore, several TP53 mutants have been shown to possess some ability to regulate target genes similar to that of wild-type TP53, as well as varying their response to different stresses. For example, three mutated p53 proteins, each containing a different mutation in the DNA-binding domain (p53-143A, p53-273H and p53-175H), were exposed to γ -irradiation, and whilst p53-143A induced G1/S arrest, in the same manner as the wild-type protein, p53-273H and p53-175H did not (Pocard *et al.*,

1996). Whereas, following treatment with a mitotic spindle inhibitor, p53-273H induced G1/S arrest, but p53-143A and p53-175H did not (Dridi *et al.*, 2003). From these findings, it was suggested that mutations in the DNA-binding domain may only prevent DNA-binding of the TP53 tetramer to certain target gene RE in a stress-dependent manner (Dridi *et al.*, 2003). This could have important consequences during cancer treatments since different genotoxic stresses may be used, thereby influencing the TP53 response.

1.3.3.2.2 TP53 gain of function

The discovery that TP53 mutants can regulate new target genes, which are normally not regulated by wild-type TP53 e.g. the multi-drug resistance gene, led to the TP53 gain of function concept (Dittmer *et al.*, 1993). Gain of function switches TP53 from acting as a tumour suppressor to one of an oncogene. It is proposed that mutations can alter the sequence-specific DNA-binding activity of TP53 to recognize and regulate different target genes, while the activity of the target genes normally regulated by wild-type is decreased (O'Farrell *et al.*, 2004). This provides an explanation as to why TP53 in tumour cells contains primarily missense mutations rather than truncation or frameshift mutations, which induce total disruption. Also, it has been hypothesized that mutant TP53 requires the functional integrity of the N- and C-termini to enable it to acquire gain of function status (Levine *et al.*, 1995). Hence, in addition to losing tumour-suppressor activity, mutant TP53 has the potential to be oncogenic, providing a proliferative advantage to tumour cells. It was initially thought that RE of new target genes recruited TP53 mutants (O'Farrell *et al.*, 2004), however, despite several studies, neither TP53 RE, nor any consensus sequences bound by mutated TP53 were found in the promoter regions of these genes (Kim & Deppert 2004). It has since been shown that most TP53 mutants bind to non-linear DNA structures in a highly structure selective manner, but are unable to bind the same sequence when presented in linear DNA (Gohler *et al.*, 2005). Hence, instead of TP53 mutants recognising new target genes via new RE, they bind to DNA secondary structure motifs located in the promoter of these genes (Gohler *et al.*, 2005; Walter *et al.*, 2005b). In agreement with this idea of structure-based binding, another explanation to elucidate the lack of TP53 mutant RE, is

that TP53 regulation of new target genes may occur indirectly through other DNA-binding factors e.g. involving sequence-specific transactivators *ETS1* and *NF-Y* (Gu *et al.*, 2004; Strano *et al.*, 2007). *ETS1* plays an important role in cell differentiation, cell proliferation, apoptosis and tissue remodelling and *NF-Y* in cell cycle regulation. The transcriptional activities of *ETS1* and *NF-Y* are inhibited following binding to wild-type TP53, but are increased by mutant TP53, resulting in the up-regulation of the transcription levels of their respective target genes (Gu *et al.*, 2004; Strano *et al.*, 2007).

1.3.3.3 Detection of TP53 alterations

A number of methods have been typically used to detect aberrations in *TP53* in human OS, including single-strand conformation polymorphism (SSCP) of polymerase chain reaction (PCR) products and PCR in conjunction with direct DNA sequence analysis (Overholtzer *et al.*, 2003; Toguchida *et al.*, 1992). SSCP has also been used to identify *TP53* aberrations in several canine tumours, including OS (Mendoza *et al.*, 1998; Setoguchi *et al.*, 2001b; Van Leeuwen *et al.*, 1997). In particular, PCR and direct DNA sequence analysis is one of the most precise methods for determining *TP53* mutation status (Cheah & Looi 2001; Osuna & de Alava 2009; Petitjean *et al.*, 2007b), and has been employed to determine the presence of *TP53* alterations in various canine tumours (Muto *et al.*, 2000; Nasir *et al.*, 2001). More recent methods of detecting *TP53* mutations, including high-throughput screening techniques, are described in 3.4.2.2.2.

Mutant TP53 is typically metabolically stable, and possess a 10- to 20-fold longer half-life than that of wild-type TP53 (Bartek *et al.*, 1991; Gannon *et al.*, 1990). Consequently, transformed cells in culture and in human and canine tumours often possess far higher levels of TP53 (5-100 fold) due to the accumulation of TP53 in the nucleus (Reich *et al.*, 1983). This enables detection, using antibodies specific for TP53 and visualization by immunohistochemistry (IHC) (Bartek *et al.*, 1991; Gannon *et al.*, 1990; Legros *et al.*, 1994) (Figure 1.10). Since wild-type TP53 is rapidly degraded, possesses a short half-life and is present at a level in normal cells usually below the amount required for detection using this technique (Esrig *et al.*, 1993; Imamura *et al.*, 1994). Many authors have

interpreted the detection of nuclear immunopositivity as evidence for mutations in *TP53*, due to the presence of mutant or stabilized forms of TP53, rather than the up-regulation of wild-type TP53 due to an increase in the steady-state level of *TP53* mRNA (Gamblin *et al.*, 1997; Haga *et al.*, 2001; Hall & Lane 1994). Although this can be controversial, due to the occurrence of false-positive and false-negative results (Wynford-Thomas 1992). Nonetheless, IHC has been extensively performed on many human and canine tumours, including OS, and has clearly demonstrated that the accumulation of TP53 is a common occurrence (Loukopoulos & Robinson 2007; Pakos *et al.*, 2004; Sagartz *et al.*, 1996).

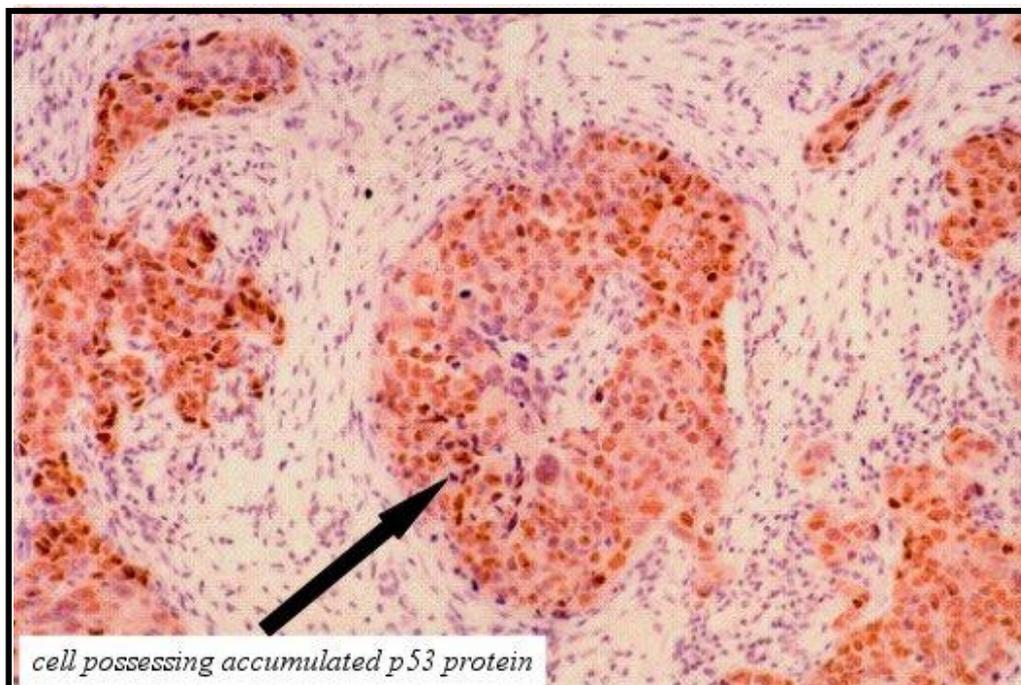


Figure 1.10: Immunocytochemical detection of accumulated TP53 using CM-1 polyclonal anti-p53 antibody (Novocastra Laboratories, Newcastle Upon Tyne, UK; diluted 1:1000 in 0.1% bovine serum albumin (BSA)) in a canine OS (x100). Regions of cells containing accumulated TP53 are stained dark brown, e.g. arrow pointing to a cell demonstrates nuclear TP53 expression (in-house photograph). Section immunostained using a streptavidin-biotin complex (DakoCytomation, UK), and immunoreactivity visualised using diaminobenzidine (DAB) (Sigma, UK) and counterstained with haematoxylin.

1.3.3.4 Effect of TP53 mutations on prognosis

The identification of molecular markers for diagnosis, prognosis and prediction of therapy response is of paramount importance in improving the treatment of

cancer. Several studies have investigated the possibility of the existence of a link between specific TP53 mutations and the prognosis and response to treatment. Tumour cells containing wild-type TP53 are usually more sensitive to current treatments than those bearing mutant TP53. For a variety of human cancers, including breast, colorectal, gastric, poor prognosis and/or poor response to treatments have been correlated with TP53 mutational status (Petitjean *et al.*, 2007b; Petitjean *et al.*, 2007a). Although TP53 status was reported not to be a predictive factor for a histological response to chemotherapy in human OS patients, TP53 alterations may be a prognostic factor as they were associated with decreased survival (Pakos *et al.*, 2004). Importantly, the presence of TP53 mutations and accumulation of TP53 may also be suitable poor prognostic indicators for canine OS (Kirpensteijn *et al.*, 2008; Loukopoulos *et al.*, 2003), as previously discussed in 1.1.7.1. Additionally, different types of TP53 mutations have been related to different outcomes, e.g. missense mutations were associated with the poorest outcome in several cohorts (Petitjean *et al.*, 2007a; Taubert *et al.*, 1996). Evidence also suggests that expression of specific p53 isoforms are associated with the prognostic value of TP53 mutations (Olivier *et al.*, 2009). Furthermore, recent findings based on computational approaches are able to predict the structural and functional effect of TP53 mutations and directly correlate with experimentally validated data (Carlsson *et al.*, 2009). This research opens the possibility of classifying mutations according to the extent to which they disrupt TP53 structure/function and to determine categories of ‘aggressive’ or ‘low-risk’ mutations. Structure/function predictions might therefore be useful when TP53 status is used as a molecular prognosis marker and could be an important tool for clinicians. However, penetrance of TP53 mutants, may be influenced by various factors, such as genetic background, genetic mutations due to carcinogenesis and tissue specificity (Soussi 2007). Numerous other studies have been reported, including some in human OS, which were unable to demonstrate any correlations between either TP53 gene or protein mutational status and poor prognosis (Pakos *et al.*, 2004; Petitjean *et al.*, 2007a). In addition, in some human tumours, e.g. bladder cancer, TP53 mutations were found to correlate with increased drug sensitivity as tumours positive for TP53 responded better to chemotherapy than tumours with undetectable TP53 levels (Cote *et al.*, 1997).

Similarly, investigations of breast cancer cells possessing wild-type TP53 were protected from some forms of cytotoxic chemotherapy and associated with a poor response to treatment (Bertheau *et al.*, 2008). It has been suggested that these contradictory results could be due to one or a combination of methodological and clinical differences, or as TP53 mediates a wide range of cellular responses, from cell death to cell survival, the consequences of wild-type or mutated TP53 activity could thus have a similar spectrum of effects (Pakos *et al.*, 2004; Petitjean *et al.*, 2007a).

1.3.4 Summary

TP53 acts as a tumour suppressor and controls the transcription of many different genes in response to a wide variety of stress signals (Riley *et al.*, 2008). TP53 truly fulfils in its role as the ‘guardian of the genome’, by acting as the main co-ordinator of the repair of damaged DNA. The TP53 pathway has been divided into five parts: (i), the stress signals that activate the pathway; (ii), the upstream mediators that detect and interpret the upstream signals; (iii), the core regulation of TP53 through its interaction with several proteins that modulate its stability; (iv), the downstream events, mainly transcriptional activation or protein-protein interactions; and (v), the final outcome, growth arrest, apoptosis or DNA repair (Levine *et al.*, 2006). Post-translational modifications of TP53 and specific interactions with distinct cellular proteins, as well as the recent discovery of eight p53 isoforms, are the main mechanisms responsible for the molecular versatility that enables a single gene to address the multiple functions attributed to TP53.

Inactivation of *TP53* is primarily due to either small mutations (missense and nonsense mutations or insertions/deletions of several nucleotides), which lead to either expression of a mutant protein (90% of cases) or absence of protein (10% of cases), or indirectly, for example, by interactions with viral proteins or aberrations in genes whose products are either upstream mediators or downstream events of TP53 (Vogelstein *et al.*, 2000). Epidemiological data reveals that *TP53* is the most frequently altered gene in human and canine neoplasia, including OS. No inactivation of *TP53* expression by hypermethylation

of transcription promoters has been demonstrated at the present time, which supports the hypothesis of a function for TP53 mutants. Two main problems are posed by the loss of gene regulation by TP53, tumourigenesis and insensitivity and resistance to cancer treatments.

The determination of TP53 status is clinically important, since the functional status of TP53 has been related to prognosis, progression and therapeutic response in a variety of human tumour types (Petitjean *et al.*, 2007a).

Furthermore, in human OS patients, *TP53* alterations may be associated with decreased survival (Pakos *et al.*, 2004), and in dogs with OS, the presence of *TP53* mutations and accumulation of TP53 may be classed as poor prognostic factors (Kirpensteijn *et al.*, 2008; Loukopoulos *et al.*, 2003).

Hence, all these characteristics make TP53 an ideal molecular target for cancer therapy, including canine OS.

1.4 Summary & overall aim

OS is the most commonly occurring bone cancer in the canine species. It is an excellent model for human OS due to the fact that molecular characteristics between the two species are similar, including alterations in *TP53*. In recent years, it has become increasingly clear that the immunocytochemical subclassification of OS, as with all cancers, significantly enhances the accuracy of pathological diagnosis (Ueda *et al.*, 1993). The determination of *TP53* status of OS is of the utmost importance, since an accumulation of mutant *TP53* in the nucleus of neoplastically transformed cells may strongly correlate with the genetic instability, proliferative potential and ultimately, survival of the patient.

Although improvements have been made in the surgical modalities for OS, conventional therapies are at best only palliative as survival rates are still poor and so OS remains a disease of high mortality in both human and veterinary medicine (Ling *et al.*, 1974). In general, patients with OS ultimately die of metastatic disease distant to the site of their primary tumour, in that they do not die from a lack of adequate surgical therapy for the primary site; rather, they die as a result of inadequate adjuvant medical therapy for micro- and macro- metastatic disease. However, overall improvements in survival await advances in systemic therapy. Hence, in recent years, attention has turned to more innovative ideas of combating the problem of metastasis in OS, in both dogs and humans. New approaches, based on gene therapy strategies, currently showing promise as future methods of adjuvant treatment are the subject of intense research. These include modulation of the immune response, novel chemotherapeutics incorporating suicide gene/prodrug combinations and targeted molecular strategies, in particular, the use of *TP53*. The latter two approaches are of considerable relevance to this study.

The overall aim of this study, was to perform preliminary investigations into *TP53*-targeted suicide gene therapy as a treatment modality for canine OS by constructing and analysing a *TP53*-targeted suicide gene therapy strategy, which could selectively target and initiate the destruction of canine OS cells possessing accumulated *TP53*. This approach could also be easily adapted to target human

OS cells accumulating TP53 and furthermore, potentially be modified and analysed in any cancer possessing accumulated TP53.

Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 Maintenance of canine cell lines

Six adherent canine cell lines, with known p53 status (Table 2.1), were used in this study. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Solutions were pre-warmed (37°C) before use. D17, MDCK and CML-10 cell lines, were maintained in Dulbecco's modified eagle medium (DMEM) with GLUTAMAX-I™ (L-Ananyl-L-Glutamine), supplemented with 10% (v/v) heat-inactivated foetal calf serum (HiFCS; Appendix A), penicillin and streptomycin (both 100 units/ml), fungizone (250µg/ml; all GIBCO BRL) and ciprofloxacin hydrochloride (10µg/ml; Bayer Diagnostics). CMT3, CMT7 and CMT8 cell lines, were maintained in RPMI 1640 medium (GIBCO BRL), containing 10% HiFCS, L-glutamine (0.292mg/ml; GIBCO BRL), penicillin (100 units/ml), streptomycin (100 units/ml) and fungizone (250 µg/ml). Cell culture plastics were purchased from Greiner.

2.1.2 Cell passaging

Medium was replaced every 3 days and cells routinely passaged when confluent. Medium was removed, cells were washed twice with Hanks balanced salt solution (HBSS) and incubated in trypsin-ethylene-diamine-tetra-acetic acid (EDTA; both GIBCO BRL) at 37°C/ 5% CO₂ until completely detached. To block trypsin, detached cells were washed with medium containing 10% HiFCS and pelleted by centrifugation at 254 x g for 10 minutes. The cell pellet was resuspended in medium and an appropriate volume transferred to a fresh culture vessel and placed in an incubator. All experiments were performed within a window of 15 passages throughout this study.

2.1.2.1 Cell counting

The cell suspension was introduced to a Neubauer haemocytometer chamber and cells counted under an inverted microscope with 10x objective; cells lying on the

top and right hand perimeter of each large (1mm) square were included, those on the bottom or left hand perimeter were excluded. Cell concentration (cells/ml) was calculated by multiplying the mean number of cells per large square by 10^4 and correcting for the dilution factor. Live cell numbers were estimated by adding 0.4% trypan blue (Sigma) to the cell suspension (1:1), incubating at room temperature (RT) for 5 minutes prior to counting and excluding dead (blue) cells from the count.

2.1.3 Cryopreservation of cells: freezing and recovery

Cells at 70-80% confluence were detached as above (2.1.2) and cell pellets resuspended in freezing medium (Appendix A), at a concentration of $\sim 2 \times 10^6$ cells/ml, and transferred into cryovials in 1ml aliquots. Cryovials were cooled at $1^\circ\text{C}/\text{min}$ to -70°C and transferred to a liquid nitrogen tank for permanent storage.

Cells recovered from liquid nitrogen were thawed in a water bath at 37°C . When almost thawed, cells were slowly added to tubes containing 10ml of medium with 10% HiFCS, pelleted by centrifugation at $254 \times g$ for 5 minutes, washed with 10ml of medium containing 10% HiFCS and recentrifuged. The cell pellet was resuspended in medium, transferred to a culture flask and placed in an incubator at $37^\circ\text{C}/5\% \text{CO}_2$. The following day, cells were either passaged if confluent or medium was replaced.

Canine cell line	Cell type	Derived from	TP53 status	References
CML-10	melanoma	primary melanoma	wildtype (exons 5-8)	(Helfand <i>et al.</i> , 1996; Sabine <i>et al.</i> , 1999; Wolfe <i>et al.</i> , 1987)
CMT3	atypical benign mixed tumour	primary mammary	mutated (exon 5: Cys176Phe)	(Hellmen 1992;Van Leeuwen <i>et al.</i> , 1996)
CMT7	osteosarcoma	primary mammary osteosarcoma	mutated (exon 7: Gly245Ala)	(Hellmen 1992;Van Leeuwen <i>et al.</i> , 1996)
CMT8	osteosarcoma	primary mammary osteosarcoma	mutated (exon 7: Tyr236Asn)	(Hellmen 1992;Van Leeuwen <i>et al.</i> , 1996)
D17	osteosarcoma	primary osteosarcoma (metastatic to lung)	wildtype (exons 5-8)	(Riggs <i>et al.</i> , 1974;Sabine <i>et al.</i> , 1999)
MDCK	kidney	normal kidney	wildtype (exons 5-8)	(Gaush <i>et al.</i> , 1966;Sabine <i>et al.</i> , 1999)

Table 2.1: TP53 status of canine cell lines. CMT3, CMT7 and CMT8 cell lines were kindly donated by Dr. E. Hellman, Swedish University of Agricultural Sciences, Uppsala, Sweden. D17 and MDCK cell lines were purchased from Health Protection Agency Culture Collections (HPACC; Reference numbers, 89090403 and 84121903, respectively). CML-10 cell line was kindly donated by Dr. L. Wolfe, Auburn University, Alabama, US.

2.2 General nucleic acid manipulation & cloning

2.2.1 Polymerase chain reaction

PCR was used to amplify *TP53* exons 5-8 and produce DNA fragments for cloning. Both *Taq* DNA Polymerase kit (QIAGEN) and Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech) were used according to the manufacturer's instructions.

Standard PCR conditions in this study were, depending on the template, 50-200ng of DNA in a 50µl or 25µl reaction mixture containing 1.5 units of *Taq* DNA polymerase (0.75µl), 10x PCR buffer (5µl or 2.5µl; including 22.5mM MgCl₂ and detergents, pH 9.2), 200µM of each nucleotide (0.5µl), 0.2µM of each primer (1µl) and distilled water to the final volume. A negative control was prepared which included all components, but replacing template DNA with distilled water. The reaction was gently mixed and aliquoted into 0.2ml thin-walled PCR reaction tubes. PCR was performed using a GeneAmp PCR System 2400 (PerkinElmer Applied Biosystems).

PCR involved an initial denaturation step at 95°C for 5 minutes followed by 30 cycles of: denaturation at 95°C, annealing and elongation 72°C each for 1 minute; after which a final extension step at 72°C for 30 minutes was performed. Annealing temperatures varied depending on the primers used (MWG-Biotech) and were optimized for each set of primers used.

PCR products (5 or 10µl) were analysed by agarose gel electrophoresis (2.2.2). If a single DNA fragment was present, the PCR product was purified using QIAquick PCR Purification Kits (QIAGEN), following the manufacturer's guidelines and sequenced (2.2.8). If non-specific fragments were present, the product of interest was excised and purified (2.2.3).

2.2.2 Gel electrophoresis

Gels containing 0.8-2% agarose (w/v) in 1xTBE (Appendix A) or 1xTAE (Appendix A) were used to separate DNA (Sambrook *et al.*, 1989), and to verify the purity of PCR fragments of a certain size. 50, 150 or 300 ml gels were poured into perspex trays and wells were cast using appropriate combs. Gel loading buffer (Appendix A) was added to samples at a final concentration of 1x, samples were then loaded into the wells and electrophoresed in 1xTBE or 1xTAE until sufficient separation of the DNA had occurred (typically 100V for 30-45 minutes). When TAE gels were used, the running buffer was changed frequently to prevent buffer exhaustion. Appropriate DNA size markers (Appendix A; GIBCO BRL) were included to gauge product size. Gels were stained by adding 0.2 µg/ml of ethidium bromide (Sigma) to the molten agarose prior to casting, viewed on a T2201 UV transilluminator (Sigma) and photographed with a UP-890 CE Videographic Printer (Sony).

2.2.3 Purification of DNA from agarose gels

Following electrophoresis, DNA fragments were excised from gels using a clean scalpel. DNA was purified using either QIAEX II Agarose Gel Extraction kit (QIAGEN) or JETSORB Gel Extraction kit (GENOMED), as described by the manufacturer's instructions, quantified (2.2.4) and stored at -20°C.

2.2.4 DNA quantification

DNA samples were usually quantified by spectrophotometry (2.2.4.1), however, low DNA concentrations were analysed by gel electrophoresis (2.2.4.2).

2.2.4.1 Determination by spectrophotometry

DNA samples were diluted (usually 1:20) in distilled water. The optical densities (OD) of the diluted samples were measured at 260nm and 280nm, in comparison

to a blank of ddH₂O, in a Smartspec 3000 UV spectrophotometer (Bio-Rad). Concentrations were calculated, assuming double-stranded DNA has an OD of 1.0 at 260nm, at a concentration of ~50µg/ml. Purity was determined by OD₂₆₀/OD₂₈₀ ratio, with acceptable ratios being between 1.6 - 2.1; lower values suggest possible protein or phenol contamination.

2.2.4.2 Determination by gel electrophoresis

Following DNA electrophoresis alongside low DNA mass ladder (Appendix A), DNA concentrations were estimated by comparing the intensity of fluorescence of DNA in the sample to that of the DNA in the low DNA mass ladder.

2.2.5 DNA sequence analysis

Cycle sequencing reactions were carried out using the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer Applied Biosystems), according to the manufacturer's recommendations, with appropriate primers. Briefly, 200-500ng of plasmid DNA or 20-90ng of PCR product, 3.2pmol of primer (MWG-Biotech) and 4µl of Terminator Ready reaction mix were mixed in a 0.2ml PCR tube and made up to 20µl with ddH₂O. The reaction mixture was then passed through 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Data analysis was performed using an ABI PRISM 310 DNA Sequencer (PerkinElmer Applied Biosystems). Typically, 400-500 bp of sequence were obtained from one run. Mrs Elizabeth Gault, Department of Veterinary Clinical Science, University of Glasgow, carried out the preparation of the sequencing reactions.

DNA sequences were analysed using Genetics Computer Group (GCG) package (Wisconsin Package Version 10.0, Madison, Wisconsin). Database searches were carried out by FAST-All (FASTA) searches in GCG or Basic Local Alignment Search Tool (BLAST) searches on the National Centre for Biotechnology Information's (NCBI) website (<http://www.ncbi.nlm.nih.gov/BLAST/>).

2.2.6 Digestion of DNA with restriction endonucleases

Restriction endonucleases (Promega) were used to linearize plasmids, obtain DNA fragments for cloning and for integration of inserts, in accordance with the manufacturer's guidelines. DNA was mixed with an appropriate amount of enzyme and incubated at the recommended temperature, typically 37°C, for 2-4 hours.

2.2.7 Cloning of DNA fragments

2.2.7.1 Ligation reactions

Vector DNA (typically 1 µg) was linearized using an appropriate restriction enzyme. Re-circularization was prevented by the hydrolysis of 5'-phosphate groups, at 37°C for 30 minutes, with 1 unit of calf intestinal alkaline phosphatase (CIAP; Promega). This was followed by the addition of another 1 unit of CIAP and the incubation repeated.

DNA fragments generated by:

(i), restriction digestion were ligated into a linearized vector overnight at both 14°C and 4°C, using T4 DNA ligase (Promega) at 1:1, 1:3 and 3:1 molar ratios of vector:insert DNA using the following formula:

$$\text{ng insert required} = \frac{\text{ng vector} \times \text{insert size (kb)}}{\text{vector size (kb)}} \times \text{molar ratio}$$

(ii), PCR products were ligated, following the manufacturer's recommendations, into either pCR[®]2.1-TOPO[®] using the TOPO TA Cloning[®] Kit (Invitrogen; Appendix C, Figure C1) or pTarget[™] Mammalian Expression Vector (Promega; Appendix C, Figure C2) via TA cloning.

2.2.7.2 Growth of competent cells

DH5α (GIBCO BRL; Appendix B) and TOP10 (Invitrogen; Appendix B) cells were streaked onto Luria-Bertani (LB) agar (Agar Bacteriological, Oxoid) plates and incubated overnight at 37°C. JM109 cells were maintained on LB agar plates

supplemented with 1ml/litre of 1M thiamine hydrochloride (GIBCO BRL). 5ml of Luria Broth base (LB broth; GIBCO BRL) were inoculated and placed in an orbital incubator (200 rpm) at 37°C overnight. Next morning, a 1:50 dilution was prepared in LB broth and grown at 37°C for 2-4 hours until the OD 650 reached approximately 0.6. The culture was then centrifuged at 671 x g for 10 minutes at RT. The supernatant was discarded and the cell pellet gently resuspended in 2.5ml of 0.1M CaCl₂ (pre-cooled) and incubated on ice for 20 minutes with occasional gentle shaking. The suspension was centrifuged as before and cells resuspended in 500µl of 0.1M CaCl₂. The cells, if immediately required were dispensed into 50µl aliquots and kept on ice, or, if for later use, 5ml of 50% sterile glycerol were added, dispensed into 100µl aliquots and frozen at -70°C.

2.2.7.3 Transformation of bacteria

DH5α, JM109 (Promega; Appendix B) or TOP10 high efficiency competent cells, were used for blue/white colour screening and standard ampicillin selection. Aliquots containing 25µl of competent bacterial cells were removed from -70°C and placed on ice until just thawed. Cells were mixed by gently flicking the tubes and ligation mix (~2µl) containing 5-25ng of total DNA was added and incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 30 seconds and immediately returned to ice for 2 minutes. 250µl of SOC medium (GIBCO BRL) were added to each tube and cells were incubated with shaking at 200 rpm, for 1 hour at 37°C. 50µl and 200µl of each transformation culture were spread evenly onto LB agar plates containing 100µg/ml of ampicillin (filter sterilized; GIBCO BRL). For *lacZ* complementation selection, plates were also treated with 5-bromo 4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Sigma; Appendix B; DH5α and TOP10 cells), plus isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma; Appendix B; JM109 cells). Plates were incubated overnight at 37°C. Positive and negative controls in the form of pUC18 DNA (Invitrogen) and ligation mix including or excluding insert DNA were used, respectively.

2.2.7.4 Identification of recombinants

Positive (white) colonies were picked, used to inoculate 3-5ml of LB broth containing ampicillin (100 μ g/ml) and cultured overnight at 37°C in an orbital shaker (200 rpm), Colonies with appropriate inserts were initially identified by small-scale plasmid purification (2.2.8.1) and restriction digestion (2.2.6) and later on in the study, by PCR analysis (2.2.1). For PCR analysis, after inoculated broth had been shaken for 5 hours, 50 μ l of culture were removed, heated to 95°C for 10 minutes, followed by centrifugation at 17900 x g for 5 minutes. 5 μ l of supernatant were used in PCR with primers specific to the DNA of interest. The PCR product was electrophoresed on agarose stained with ethidium bromide (2.2.2) and colonies containing the insert identified.

2.2.7.4.1 Storage of recombinants

15% glycerol stocks were prepared by the addition of 90 μ l of 50% glycerol to 210 μ l of culture and stored at -70°C. For subsequent preparations of DNA, stocks were thawed, 5 μ l streaked onto a LB agar plate and incubated overnight at 37°C. Next morning, colonies were picked, used to inoculate 3-5ml of LB broth plus ampicillin before DNA isolation (2.2.8).

2.2.8 Isolation of plasmid DNA

Recombinant plasmid DNA was prepared using 3 different procedures, outlined below, depending on the intended use. Unless otherwise stated, a single colony was inoculated in LB broth with ampicillin and shaken overnight at 200-300 rpm at 37°C.

2.2.8.1 Small-scale isolation of plasmid DNA

For sequencing, plasmid DNA was prepared from 3-5ml of an overnight culture, using the QIAGEN QIAprep Spin Miniprep kit as described by the manufacturer's instructions.

2.2.8.2 Large-scale isolation of plasmid DNA

Large quantities of high quality plasmid DNA were produced from 100ml of an exponentially growing overnight culture of recombinant bacteria, using the QIAGEN Plasmid Purification Maxi kit in accordance with the manufacturer's guidelines.

2.2.8.3 Large-scale isolation of transfection grade plasmid DNA

For transfection, plasmid DNA were purified from 100ml of an exponentially growing overnight culture of recombinant bacteria, using the QIAGEN Endofree Plasmid Purification Maxi kit as described by the manufacturer's recommendations.

2.2.9 Transfection

Transfections into cells were carried out using Transfast™ reagent (Promega), following the manufacturer's instructions.

2.2.10 Cell survival assay

Cell survival was measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer's guidelines. Assays were incubated at 37°C, for 1.5 hours, in an atmosphere of 5% CO₂. Using a 96 well plate reader (Dynex technologies MRX) all assays were read at both 450nm and 540nm.

2.3 General manipulation of protein

2.3.1 Preparation of cell lysates

Cells for protein lysates were prepared by first replacing media with 2ml of fresh medium. A sterile pasteur pipette was used to scrape cells from the base of each

well/flask and each 2ml sample was divided into 1.5ml and 0.5ml aliquots, centrifuged for 6 minutes at 17900 x g and the supernatant from each sample discarded. 50µl of protein treatment sample buffer (2x; Appendix A) were added to cell pellets from the 1.5ml samples, heated in a boiling water bath for 10-20 minutes and allowed to cool to RT, before storing at -20°C. 50µl of PBS (Sigma; Appendix A) were added to the cell pellets from the 0.5ml samples and sonicated for 3 seconds before storage.

2.3.2 Protein quantification (Bradford assay)

The Bradford reagent (Sigma) was used to determine the total protein concentration for each cell lysate (Bradford 1976). A stock solution of 2 mg/ml BSA (Sigma) was serially diluted in ddH₂O, 1:1, to provide 7 protein standards (1000, 500, 250, 125, 62.5, 31.3 and 15.6 µg/ml). An appropriate volume of each sonicated cell lysate was adjusted to 100µl with ddH₂O. 900µl of Bradford reagent were added to each of the blanks, standards and samples, followed by incubation for 5 minutes at RT to allow the colour to develop. The absorbance at 595nm was then measured using a spectrophotometer. The absorbance of each BSA standard was plotted against its respective protein concentration on a calibration curve, from which the concentration of protein in each of the samples was determined.

2.3.3 Western blotting

2.3.3.1 Protein SDS-polyacrylamide gel electrophoresis

A 7.5% separating gel was prepared by mixing 3ml of 40% (w/v) acrylamide/bis-acrylamide (Severn Biotech Ltd.), 4.4ml of dH₂O, 2.5ml of 1.5M Tris-HCl (pH 8.8), 100µl of 10% SDS, 75µl of 10% Ammonium persulphate (BIO-RAD) and 6µl of TEMED (Sigma). The gel was poured into the casting apparatus using the Mini-PROTEAN II system (Bio-Rad), the appropriate comb inserted and the gel allowed to polymerize. Denatured protein samples were sonicated for 30 seconds - 2 minutes, centrifuged for 10 minutes at 17900 x g and the supernatant from each sample transferred to a fresh tube. An appropriate amount was loaded onto the

gel and electrophoresed in Laemmli Running Buffer (Appendix A), alongside a protein ladder (Bio-Rad; Appendix A), at 60V for 4-5 hours.

2.3.3.2 Protein Transfer

Proteins were transferred from the gel to Trans-Blot Transfer Medium membrane (0.45 μ M; Bio-Rad) using the Mini-PROTEAN II Trans-Blot Cell system (Bio-Rad). During the last hour of electrophoresis, nitrocellulose membrane, 2 pieces of filter paper and 2 cassettes sponges were equilibrated in transfer buffer (Appendix B) for at least 30 minutes. Following SDS-polyacrylamide gel electrophoresis (PAGE), a cassette was opened onto which a cassette sponge was placed, followed by a piece of filter paper and any bubbles present were removed by using a plastic 'rolling pin'. The gel was then lifted from the glass plates and placed on top of the filter paper, followed by the nitrocellulose membrane, the second piece of filter paper and another sponge. The cassette was closed, placed in the Mini-PROTEAN II Trans-Blot Cell system and run in transfer buffer at 60V for 2 hours.

2.3.3.3 Blocking, antibody incubations and washes

The membrane was rinsed in 0.1% (v/v) Tween-20 in PBS (PBS-T) and blocked by immersion in 5% (w/v) low fat dried milk (Safeway) in PBS-T (milk/PBS-T) at RT on a shaker for 1 hour and then 4°C overnight.

Next morning the membrane was rinsed twice with PBS-T, washed in PBS-T for 15 minutes and finally washed twice in PBS-T for 5 minutes; all washes were carried out at RT with shaking. The primary antibody was diluted in 1% milk/PBS-T (Table 2.2), the membrane placed inside a plastic bag, the diluted antibody added and the bag sealed. The bag was incubated on a rotary mixer for 1 hour at RT. The membrane was removed from the bag, washed as before in PBS-T and placed in a new bag. The secondary donkey anti-rabbit horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech) diluted 1:3000 with 1% milk/PBS-T, was added to the membrane, the bag sealed and incubated 1 hour

at RT as before. The membrane was then removed from the bag, rinsed twice, washed once for 15 minutes and 4x for 5 minutes in PBS-T.

Primary antibody	Dilution	Source
Anti-LexA	1:5000	Invitrogen
CM-1 (anti-p53)	1:500	Novocastra Laboratories
<i>E.coli</i> NTR	1:500	Donated by Dr. G. Anlezark (Anlezark <i>et al.</i> , 1992).

Table 2.2: Primary antibodies (rabbit polyclonal) used in western blotting.

2.3.3.4 Signal detection

Signals were detected using the ECLTM system (Amersham Pharmacia Biotech) following the manufacturer's recommendations. The membrane covered with ECLTM reagent was transferred protein side up to an X-ray film cassette. In a dark room, a sheet of HyperfilmTM ECLTM (Amersham Pharmacia Biotech) was placed over the membrane for between 10 seconds and 5 minutes before developing in a Curix 60 developer (Agfa).

2.4 Statistical analyses

Statistical analyses were carried out in association with Dr. Mohammed Reza Baneshi, Medical Statistics Unit, University of Edinburgh. Background absorbance counts (wells containing no cells, only cell culture medium) were removed from the data in experiments run over more than 1 time point and where appropriate, data were $\log(x+1)$, \log , \sqrt{x} and $\sqrt{x}/(x+1)$ transformed. Significant differences between the treatment groups were identified by analysis of variance (ANOVA) General Linear Model (GLM), followed by Scheffe's post hoc pairwise comparison of means with Bonferroni's adjustment for multiple comparisons. Mean significant difference was set at the 0.05 level.

Chapter 3

Role of *TP53* in Canine Osteosarcoma

3.1 Introduction

3.1.1 Canine osteosarcoma

Over 90% of primary neoplasms found in canine bones are malignant sarcomas, of which 65-85% are OS (Jongeward 1985). Approximately 75% of OS occur in the appendicular skeleton (Brodey & Riser 1969), with the forelimbs twice as likely to be affected as the hind limbs (Ru *et al.*, 1998; Spodnick *et al.*, 1992). Most remaining OS arise in the axial skeleton (Brodey & Riser 1969; Loukopoulos & Robinson 2007). The most common sites of axial skeleton OS in the dog are skull, spine and ribs, particularly at the costochondral junction (Brodey & Riser 1969; Egenvall *et al.*, 2007; Heyman *et al.*, 1992; Jongeward 1985). Extraskeletal OS is rare (<7% of canine OS) but mostly confined (86%) to visceral organs (Kuntz *et al.*, 1998b; Langenbach *et al.*, 1998; Patnaik 1990). Metastases are commonly found in the lungs and may also be found in additional bones and/or other soft tissue sites e.g. ribs, liver, spleen (Brodey & Riser 1969; Langenbach *et al.*, 1998; Spodnick *et al.*, 1992).

3.1.2 The canine TP53 gene

The development of canine and human OS is linked to alterations in a number of genes (previously described in 1.1.3.1), with a high prevalence of aberrations in TP53 (Johnson *et al.*, 1998; Kirpensteijn *et al.*, 2008; Tang *et al.*, 2008). Canine TP53, assigned to chromosome 5 consists of 11 exons and 10 introns, similar to human TP53 (Guevara-Fujita *et al.*, 1996; Kraegel *et al.*, 1995; Veldhoen & Milner 1998). Several cytogenetic aberrations at the TP53 locus (17p13.1) have been reported in human OS samples (Kansara & Thomas 2007). Recently, Thomas *et al.*, (2009) observed a range of frequently occurring chromosomal copy number imbalances in canine OS, with both gains and losses observed in TP53 (Thomas *et al.*, 2009).

The first exon of canine TP53 is untranslated and the first intron is possibly large, while exons 2-11 are coding exons. The gene transcript is spliced to produce a 2.2-2.5kb mRNA. The full length canine TP53 is 381 $\alpha\alpha$ in length (Veldhoen & Milner 1998) and displays strong sequence homology with TP53

identified in other vertebrates, e.g. 78.4% human TP53 (393 $\alpha\alpha$) (Zakut-Houri *et al.*, 1985) and 86.3% feline p53 (386 $\alpha\alpha$) (Okuda *et al.*, 1993). Veldhoen and Milner (1998) proposed that the difference in length between canine and human TP53 might be due to the deletion, during evolution, of amino acid residues 61-79, located within the proline rich region of canine TP53 (Veldhoen & Milner 1998). However, they were unable to identify the precise deletion (Veldhoen & Milner 1998). Between exons 5 and 8 of TP53, the canine sequence approaches 100% homology with the human sequence, this region encompassing 4 of the 5 evolutionary conserved domains II to V (Kraegel *et al.*, 1995; Soussi & May 1996; Veldhoen & Milner 1998). This region, includes the majority of mutations identified in human (IARC TP53 Mutation Database R13 release, November 2008 (Petitjean *et al.*, 2007b) and canine neoplasms, including OS (McIntyre *et al.*, 1994; Setoguchi *et al.*, 2001b; Toguchida *et al.*, 1992; Veldhoen *et al.*, 1999). PCR and DNA sequence analysis, is regarded as a robust and precise method for mutation detection (Cheah & Looi 2001; Osuna & de Alava 2009; Petitjean *et al.*, 2007b), and has been employed to determine the presence of TP53 alterations in human OS (Overholtzer *et al.*, 2003) and various canine tumours (Muto *et al.*, 2000; Nasir *et al.*, 2001).

3.1.3 Summary

The development of OS, in both humans and dogs, is thought to be linked to alterations in a number of genes, in particular, TP53. The majority of TP53 mutations identified in OS samples from human and canine patients are located in exons 5-8. Samples, where possible, were taken from the tumour, metastases (if detected) and normal tissues of the dogs diagnosed with OS at Glasgow University Veterinary School (GUVS), and exons 5-8 of TP53 were analysed by PCR and direct DNA sequence analysis. The main aims of this study were to analyse the site incidence of canine OS diagnosed at GUVS and to extend data on TP53 mutations in canine OS.

3.2 Materials & methods

3.2.1 Canine osteosarcoma at Glasgow University Veterinary School

The numbers and locations of canine OS, at GUVS either by case referral and/or by the histopathology diagnostic service and recorded from January 1986-September 2001, were retrieved from GUVS histology report database. Tumour biopsies were performed on 4 of 8 canine OS cases presented at GUVS from September 1998-September 2001, with normal tissue samples also being removed from 2 of the 4 dogs. A biopsy of normal lung tissue was obtained from a healthy dog as a control. Tissue was cut into approximately 1 cm³ pieces, placed into labelled 5ml bijoux tubes, snap frozen in liquid nitrogen and stored at -70°C. Clinical case records were available for 3/4 dogs with OS (Table 3.1). These 3 dogs, 2 female and 1 male, were all large breed dogs (Vizsla Hungarian, Rottweiler and German Short-haired Pointer) and weighed over 18kg. The mean age of the dogs was 2.7 years (range: 1-6 years). All 3 dogs had primary OS in appendicular sites, in 2 the distal radius and the third, the proximal tibia and in 2 dogs, metastases were found.

3.2.2 Extraction of DNA from frozen tissue samples

Approximately 50mg of frozen tissue sample were finely minced and 2ml lysis buffer (Appendix A) added and mixed using a vortex. The mixture was incubated at 55°C for 2 hours and if sufficient digestion had not occurred, at 37°C overnight. Following incubation, 1ml of each of phenol, chloroform and isoamylalcohol were added to the digested material. The mixture was then vortexed and centrifuged for 10 minutes at 489 x g. The aqueous layer was removed, transferred into a clean tube and 2ml chloroform added. The mixture was then vortexed and centrifugation repeated as before. The top aqueous layer was again removed, transferred into a fresh tube and 200µl 3M sodium acetate (pH 5.2) and 4ml cold 100% ethanol added. The mixture was stored at -20°C for at least 1 hour, or overnight, to precipitate DNA. DNA was pelleted by centrifugation at 4°C for 10 minutes at 17900 x g. The supernatant was removed, the pellet washed with 1ml 70% ethanol and centrifuged as before. The ethanol was then decanted and the DNA air-dried for 10 minutes before dissolving in 50µl

TE buffer (additional TE buffer was used if the DNA did not dissolve after 5 minutes) at RT for at least 15-20 minutes. The DNA was quantified and its purity assessed by spectrophotometry (2.2.4.1) and quality determined by gel electrophoresis (2.2.2). DNA was stored at -20°C and thawed on ice when required.

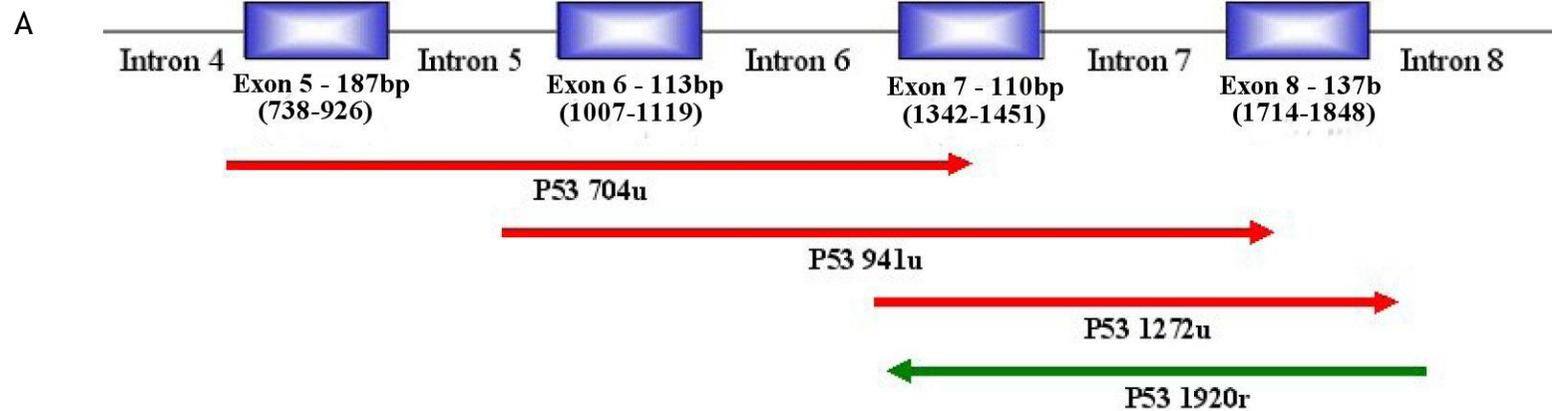
3.2.3 DNA sequence analysis of TP53 in canine osteosarcoma

PCR (2.2.1) was carried out to enable the DNA sequence analysis of exons 5-8 of TP53 from DNA samples prepared from each of the 7 tissue biopsies removed from the 4 dogs with OS and the normal tissue biopsy extracted from the healthy dog. The PCR products were purified if required (2.2.3), quantified by gel electrophoresis (2.2.4.2) and sequenced (2.2.5).

The oligonucleotide primers used in the PCR and sequencing reactions (Figure 3.1) were designed from a partial DNA sequence of the *canis familiaris* TP53 (GenBank Accession number U62133). Each PCR and sequencing reaction was repeated in triplicate. The nucleotide sequences of each PCR product were compared, manually and with the GCG package, to 10 complete or partial canine wild-type TP53 nucleotide sequences from GenBank for exons 5-8 (Accession numbers U62133, U51857, L27630, NC_006587, S77819, L37107, AF159062, DN444655, AF060514 and AB020761). Sequence differences between the 7 DNA samples, control DNA sample and exons 5-8 in the 10 published wild-type TP53 sequences were recorded.

Dog	Case no.	Clinical details	Date first presented @ GUVS	Treatment	Outcome	Biopsy	Sample no.
1	136964	18.5kg 1 year old female Vizsla Hungarian Primary appendicular OS affecting proximal tibia of right hindlimb. Radiological evidence of pulmonary and lymph node metastases.	01/05/1999	Amputation and cisplatin therapy	Euthanized 2 weeks after limb amputation	Lung metastasis Lymph node metastasis	1 2
2	Unknown	Unknown - Primary OS	07/08/2000	Unknown	Unknown	Primary OS	3
3	138398	56kg 6 year old female neutered Rottweiler Primary appendicular OS affecting distal radius of left forelimb. No radiological evidence of metastasis, however post-mortem report indicated metastases in lungs, nodular hyperplasia of liver and spleen and a hyperplastic draining lymph node.	04/11/1999	None	Euthanized	Primary OS Normal kidney	4 5
4	141572	28.3kg 1 year old male German Short-haired Pointer Primary appendicular OS affecting distal radius of right forelimb (25.5kg after amputation). No radiological evidence of pulmonary metastasis.	17/01/2001	Amputation and cisplatin therapy	Still alive 1 yr later	Primary OS Normal muscle	6 7

Table 3.1: Clinical case details of dogs with OS presented at Glasgow University Veterinary School (GUVS), from which biopsies were obtained, DNA extracted and *TP53* sequence analysed.



B

Primer	Primer Sequence (5' --> 3' orientation)	T _m	Location (nts)	Reaction
P53 704u	tga cct gtc cat ctg tcc tt	57	714-733	PCR & Seq
P53 941u	gct aca gat ggg aca agg ct	59	941-960	Seq
P53 1272u	act gag gct gat aga cta ca	55	1272-1291	Seq
P53 1920r	atc atg cct gat gct caa cc	57	1901-1920	PCR & Seq

Figure 3.1: Details of the oligonucleotide primers used in the PCR and Sequencing (Seq) reactions designed from a partial canine *TP53* DNA sequence (GenBank Accession number U62133): A, region of canine *TP53* DNA sequence able to be analysed with each of the 4 sequencing primers. Red arrows indicate forward primers and green arrow indicates a reverse primer. Size, in base pairs (bp), and location of exons 5,6 7 and 8 within the nucleotide sequence of GenBank Accession number U62133 are displayed (location in parentheses); B, Sequence of each primer, annealing temperature (T_m) and nucleotide (nts) location within the sequence of GenBank Accession number U62133 are tabulated. Forward primers are indicated by 'u' at end of primer name and reverse primer by 'r'.

3.3 Results

3.3.1 Incidence of canine osteosarcoma by site

132 OS cases, obtained from the GUVS histology report database, were grouped into 26 defined and 8 non-defined sites. Defined sites were separated into 3 main locations, appendicular, axial and extraskeletal (Table 3.2); unfortunately sites of metastatic OS could not be identified in this database. The number of OS situated in the appendicular locations (n=51, 38.7%) is slightly greater than those in the axial sites (n=48, 36.4%). The extraskeletal locations (n=25, 19.15%) represent the lowest proportion of OS cases. The non-defined sites account for 8 (6.1%) of the 132 cases.

Of the 51 appendicular tumours, approximately twice as many OS were diagnosed in the forelimbs (n=27) than the hindlimbs (n=13). The 4 major weight-bearing bones, radius (plus ulna), humerus, tibia and femur, accounted for 51% (n=26) of the OS originating in the appendicular sites (19.7% of the 132 cases). Of the 48 OS diagnosed in 12 axial sites, 52.1% (n=25) were located in the maxilla & mandible. The remaining 23 OS were distributed between 11 other sites. Of the 25 OS diagnosed in 11 extraskeletal sites, 56% (n=14) were located in the mammary glands. Overall, the site in dogs most commonly diagnosed with OS at GUVS was the maxilla & mandible (n=25, 18.9%), followed by the mammary glands (n=14, 10.6%), radius & ulna (n=11, 8.3%) and the scapula (n=7, 5.3%).

3.3.2 DNA sequence analysis of TP53 in canine osteosarcoma

All samples generated PCR products of the expected size, ~1140 bp, revealing the absence of major gene deletions/insertions. DNA sequence analysis indicated that there were no disparities in the nucleotide sequences for TP53 exons 5-8 between each of the 5 tumour DNA samples and the 3 normal tissue DNA samples.

The alignment of the control DNA sequence with exons 5-8 of the 10 nucleotide sequences accessible in Genbank are summarized in Table 3.3 and displayed in Appendix D. The control DNA sequence is most similar to the sequences in GenBank Accession numbers U51857 and NC_006587, as it only had 1 sequence

difference with each of these Genbank sequences. In contrast, the control DNA sequence was most different to GenBank Accession number U62133 as 6 sequence differences were found. A total of 11 nucleotide sequence differences were observed between exons 5-8 of the published sequences, 5 in exons and 6 in intronic sequences. 6 of the 11 differences, (located in exon 5, intron 7 and exon 8 (canine codons 294-295)), are between the sequence of GenBank Accession number U62133 and the sequences of the remaining 9 GenBank Accession numbers. No differences were identified between the sequences of exons 5-8 in GenBank Accession numbers NC_006587, S77819, L37107, AF159062, AF060514 and AB020761.

Appendicular Site	No. cases	% Appendicular cases	% Total cases
Scapula	7	13.73	5.3
Humerus	6	11.76	4.55
Radius & Ulna	11	21.57	8.33
Carpus	3	5.88	2.27
Hock	3	5.88	2.27
Femur	4	7.84	3.03
Stifle	1	1.96	0.78
Tibia	5	9.8	3.79
Foot	4	7.84	3.03
Leg (not defined)	7	13.73	5.3
Sub-total	51	99.99	38.65
Axial Site	No. cases	% Axial cases	% Total cases
Sternum	3	6.25	2.27
Ribs	5	10.42	3.79
Spine	6	12.5	4.55
Pelvis	1	2.08	0.78
Skull	4	8.33	3.03
Maxilla & Mandible	25	52.08	18.94
Nasal Cavity & Sinus	4	8.33	3.03
Sub-total	48	99.99	36.39
Extraskeletal Site	No. cases	% Extraskeletal cases	% Total cases
Trachea	1	4	0.78
Axilla	2	8	1.52
Lung	1	4	0.78
Lymph node	1	4	0.78
Pancreas	1	4	0.78
Mammary gland	14	56	10.61
Gastric	1	4	0.78
Soft tissue (subcutaneous, perianal)	3	4	2.27
Muscle	1	4	0.78
Sub-total	25	100	19.15
Non-defined site	8	-	6.06
Total	132	-	100.25

Table 3.2: Incidence of OS cases at Glasgow University Veterinary School, from January 1986 - September 2001, categorized into appendicular, axial and extraskeletal sites. N.B. Sites of metastatic OS could not be identified.

Nucleotide Sequence Differences												
Location	Exon 5	Intron 5	Exon 6	Intron 6	Exon 7	Intron 7	Intron 7	Intron 7	Intron 7	Exon 8	Exon 8	
Canine Codon	113-114	-	198	-	215	-	-	-	-	259	294-295	
Equivalent to Human Codon	126-127	-	210	-	227	-	-	-	-	271	306-307	
Nucleotide alteration	3bp del <u>ACT</u>	1bp del <u>G</u> pres/abs	<u>AAC</u> --> <u>TAC</u> A:T --> T:A	1bp sub <u>A/G</u>	<u>TCT</u> --> <u>TIT</u> G:C --> A:T	1bp del <u>A</u>	1bp ins <u>G</u>	1bp ins <u>G</u>	2bp RA <u>GA</u> --> <u>AG</u>	<u>GAG</u> --> <u>GAA</u> G:C --> A:T	2bp del <u>AG</u>	
Amino acid substitution	FS	-	Asn --> Tyr TV	-	Ser --> Phe TS	-	-	-	-	Glu --> Glu (sil) TS	FS	
Control DNA Sequence	X	<u>G</u> pres 229bp	A 332bp	<u>A</u> 464bp	C 606bp	No del 749bp	No ins 814-815bp	No ins 824-825bp	No RA 846-847bp	G 1002bp	<u>AG</u> del 1106-1107bp	
GenBank Accession Number	U62133	<u>ACT</u> del 743-744bp	<u>G</u> abs 972-973bp	A 1075bp	<u>G</u> 1207bp	C 1349bp	<u>A</u> del 1491-1492bp	<u>G</u> ins 1555bp	<u>G</u> ins 1566bp	<u>AG</u> 1588-1589bp	G 1744bp	<u>AG</u> del 1848-1849bp
	U51857	X	<u>G</u> pres 204bp	A 307bp	<u>A</u> 439bp	C 581bp	No del 724bp	No ins 787-788bp	No ins 797-798bp	No RA 819-820bp	<u>A</u> 975bp	X
	L27630	X	<u>G</u> abs 117-118bp	<u>I</u> 220bp	<u>G</u> 352bp	<u>I</u> 494bp	X	X	X	X	X	X
	NC_006587	No del 1099-1101bp	<u>G</u> pres 1331bp	A 1434bp	<u>A</u> 1566bp	C 1708bp	No del 1851bp	No ins 1914-1915bp	No ins 1924-1925bp	No RA 1946-1947bp	G 2102bp	No del 2207-2208bp
	S77819	No del 266-268bp	X	A 520bp	X	C 572bp	X	X	X	X	G 705bp	No del 810-811bp
	L37107	No del 54-56bp	X	A 308bp	X	C 360bp	X	X	X	X	G 493bp	No del 598-599bp
	AF159062	No del 24-26bp	X	A 278bp	X	C 330bp	X	X	X	X	G 463bp	No del 568-569bp
	DN444655	No del 472-474bp	X	X	X	X	X	X	X	X	X	X
	AF060514	No del 354-356bp	X	A 608bp	X	C 660bp	X	X	X	X	G 793bp	No del 898-899bp
	AB020761	No del 384-386bp	X	A 638bp	X	C 690bp	X	X	X	X	G 823bp	No del 928-929bp

Table 3.3: Summary of the nucleotide sequence differences between the control *TP53* DNA sequence (normal canine lung tissue) with exons 5-8 in the 10 canine wild-type *TP53* sequences accessible in GenBank (U62133, U51857, L27630, NC_006587, S77819, L37107, AF159062, DN444655, AF060514, AB020761). 'Equivalent to Human Codon', according to GenBank Accession number X54156; 'X', difference is not able to be analysed; 'del', deletion; 'ins', insertion; 'sub', substitution; 'pres', present; 'abs', absent; 'sil', silent; 'RA', rearrangement; 'FS', frameshift; 'TS', transition; 'TV', transversion.

3.4 Discussion

3.4.1 Locations of canine osteosarcoma

The locations of 132 cases of canine OS, retrieved from the GUVS histology report database, were categorized into appendicular, axial and extraskeletal regions. Overall, it was determined that the most commonly affected region was the appendicular area, of which, the majority originated in the forelimbs followed by similar numbers occurring in the hindlimbs and non-defined regions of the leg or foot, then axial sites and the least affected, extraskeletal locations. In general, these findings are in agreement with those previously published (Brodey & Riser 1969; Langenbach *et al.*, 1998; Loukopoulos & Robinson 2007; Ru *et al.*, 1998; Spodnick *et al.*, 1992). It was observed that approximately twice as many OS were diagnosed in radius/ulna and humerus of the forelimbs to the femur and tibia of the hindlimbs. It is generally accepted that OS occur most frequently in these forelimb and hindlimb bones as they are the 4 major weight-bearing bones, with forelimbs being twice as affected as hindlimbs (Brodey 1979). Hence, multiple episodes of minor trauma and injury to these bones whilst they are growing may result in DNA damage, which could subsequently lead to the development of OS (Dernell *et al.*, 2007; Tjalma 1966).

It was also seen that the axial sites most often diagnosed with OS were the maxilla & mandible followed by ribs and in the extraskeletal sites, OS were primarily found in the mammary glands. This concords with the locations previously described in the literature (Brodey & Riser 1969; Heyman *et al.*, 1992; Jongeward 1985; Langenbach *et al.*, 1998).

One main distinction found between this study and those previously published was that the total percentages of OS diagnosed in the appendicular, axial and extraskeletal regions were different. In this study, the OS located in appendicular regions are ~50% lower, whereas the incidence of axial and extraskeletal OS are ~2 and 3-fold higher. This is likely to be due to GUVS possessing a biased population as a result of its key role as a referral/diagnostic centre. Veterinarians at local practices are more likely to biopsy a tumour at an axial/extraskeletal site than one at an appendicular location due to the greater uncertainty of the clinical diagnosis. In addition, the GUVS histology report

database grouped together all diagnosed OS, so that sites of primary OS could not be distinguished from metastatic sites. For example, metastases to the lungs are more frequently reported than primary extraskkeletal OS in the lung (Brodey & Riser 1969; Kuntz *et al.*, 1998b; Langenbach *et al.*, 1998).

To the best of my knowledge, the only publication reporting the site incidence of canine OS in the UK across all breeds is by Dobson *et al.*, 2002 (Dobson *et al.*, 2002) at 57 cases/100000 dogs/year (confidence interval range: 44-70). The majority of studies described in the literature were carried out in the U.S.A., Netherlands, Australia and New Zealand. However, the study by Dobson and colleagues, utilizes incidence rates from an insured dog population (Dobson *et al.*, 2002). Therefore, to gain a more accurate representation of the site incidence of OS in the UK canine population as a whole, attempts should be made in future studies to obtain information from numerous veterinary practices and the remaining 5 university veterinary hospitals in the UK.

3.4.2 Analysis of TP53 in canine osteosarcoma

3.4.2.1 Case studies

Only 8 of the 132 cases of canine OS diagnosed at GUVS between January 1986 - September 2001, were case referrals, as the majority were diagnosed by the histopathology diagnostic service. Of these 8, samples were only obtained from 4 dogs, due to weekend admissions and owner permission. Unfortunately, the complete case records (including age, sex, breed, weight, site of primary OS and presence and site of metastases) for the remaining 124 cases were not accessible from the histology report database used to retrieve the OS sites of these cases. Thus, comparisons to previously published reports are limited due to the small sample size in this study. Any future investigations would require the complete case details for each dog to be recorded to enable more detailed comparisons to occur.

However, a number of observations can be made between this study and those previously published. Clinical case records were available for 3 of 4 dogs from which samples were taken. All 3 were large-breed dogs (Rottweiler, German Short-haired Pointer and Vizsla Hungarian) which agree with the findings that

large/giant breeds are more likely to be affected by OS than small breed dogs (Ru *et al.*, 1998; Tjalma 1966). The sites of OS diagnosed in the 3 dogs were in the radius and tibia, 2 of the 4 major-weight bearing bones, which is in agreement with previous studies, as described in 3.4.1. Metastases were found in lungs and lymph nodes, which have also been previously reported (Brodey & Riser 1969; Hillers *et al.*, 2005; Langenbach *et al.*, 1998; Spodnick *et al.*, 1992). Appendicular OS have also been previously reported in Rottweilers and Pointers (Jongeward 1985; Rosenberger *et al.*, 2007). In addition, these 3 dogs each weighed over 18kg which correlates with the observations that the risk of OS increases as weight increases (Ru *et al.*, 1998; Tjalma 1966). In this study, 2 dogs were females and 1 was male. There is disagreement between published literature as to whether males are more affected than females or vice versa or no sex-related differences exist (Egenvall *et al.*, 2007; Jongeward 1985; Merlo *et al.*, 2008; Priester & McKay 1980; Ru *et al.*, 1998). In addition, conflicting evidence exists regarding neutering increasing or decreasing the risk of OS and the age at which the dog is neutered due to hormonal changes altering skeletal homeostasis (Cooley *et al.*, 2002; Priester & McKay 1980; Ru *et al.*, 1998). Thus in future studies, information should be recorded regarding neutering status and if so, at what age the dog was neutered. In this study, 1 dog is 6 years old and 2 dogs are 1 year old, and although in the literature, generally middle-aged to older dogs are reported to be afflicted (Langenbach *et al.*, 1998), there is a small peak in age incidence at 12-24 months (Jongeward 1985). Owen and Steel (1969) have also reported that the significance of age statistics is doubtful in a series in which there are many breeds of dogs with widely differing life expectancies and so age incidence figures may also be probably more significant when each breed is considered separately (Owen & Steel 1969).

3.4.2.2 DNA sequence analysis of TP53

DNA sequence analysis revealed that there were no differences in the nucleotide sequences for TP53 exons 5-8 between the 7 DNA samples (3 primary OS, 2 metastases and 2 normal tissues) with the control sample. This is probably as a result of a small sample number. However, several studies in the literature have reported the detection of TP53 mutations in exons 5-8 in 23-100% canine OS

studied (Johnson *et al.*, 1998; Mendoza *et al.*, 1998; Setoguchi *et al.*, 2001b; Van Leeuwen *et al.*, 1997). Hence, the absence of TP53 mutations in the 5 OS samples analysed in this study is well within statistical probability. The majority of the reported mutations are not only missense mutations, the most commonly occurring type of mutation in human TP53, but also located in evolutionary conserved domains (II, IV, and V) (Petitjean *et al.*, 2007b). In particular, 4 of the codons affected (248*, 249, 273* and 282*), of which 3 contain CpG dinucleotide sites (*) belong to the 6 most commonly mutated codons in human TP53 ('hotspots') for somatic single base substitutions (IARC TP53 Database, R13 release, November 2008 (Petitjean *et al.*, 2007b)). The effects of TP53 mutations on p53 protein function p53 are previously described in 1.3.3.2.

The frequency of TP53 mutations detected in the published canine OS studies, is comparable to 18-100% found in human OS and some of the mutations reported in canine OS have also been reported to be altered in human OS e.g. 'hotspot' codons 273 and 282 (Iavarone *et al.*, 1992; McIntyre *et al.*, 1994; Miller *et al.*, 1996; Overholtzer *et al.*, 2003; Toguchida *et al.*, 1992).

11 nucleotide sequence differences were noted between the 7 GenBank sequences, NC_006587, S77819, L37107, AF159062, DN444655, AF060514 and AB020761, with GenBank sequences, U62133, U51857 and L27630. These differences are probably due to the replication error rate of *Taq* DNA polymerase, although some may be polymorphisms or even mutations (Chu *et al.*, 1998). Indeed, the codons involved in 3 of these differences, canine 113-114, 215 and 259 (equivalent to codon's 126-127, 227 and 271 in human TP53, respectively (GenBank Accession number X54156)), have been found to be altered in canine and/or human OS samples (McIntyre *et al.*, 1994; Setoguchi *et al.*, 2001b; Toguchida *et al.*, 1992). In particular, canine codon's 113 and 259 are located in evolutionary conserved domains, II and V, respectively.

These findings indicate the involvement of TP53 in the tumourigenesis of canine OS in a manner comparable to that of human OS, as there are many sites within TP53 that are susceptible to alterations and that in both species, mutations in the conserved domains of TP53 appear to play a significant role in OS. Although no TP53 mutations were detected in the canine OS investigated in this study,

this study fits within the range of published data given only 5 samples were analysed from 4 dogs.

3.4.2.2.1 Mutations outside of TP53 exons 5-8

Although the small sample size is likely to be the main reason for the lack of TP53 aberrations detected in this study, another potential cause could be that mutations were present but located outside of exons 5-8. Even though it is generally accepted that very few alterations in TP53 occur outside of these 4 exons, over 80% of TP53 mutational studies have limited their investigations to the analysis of only exons 5-8 (Soussi & Beroud 2001). In addition, the small number of studies published regarding TP53 mutations in canine OS, also possessed small sample sizes. In particular, two studies which have examined the entire TP53 coding region found that of the mutations identified, 42-56% were located in exons 5-8, whilst the remaining were located outside this region (Kirpensteijn *et al.*, 2008; Setoguchi *et al.*, 2001b). Additionally, numerous research groups have reported that between 4-33% of mutations in TP53 they found in human OS were situated outside of exons 5-9 (Toguchida *et al.*, 1992; Overholtzer *et al.*, 2003). Thus a future improvement to this study, as the canine genome sequence has now been published (GenBank Accession Number NC_006587; (Lindblad-Toh *et al.*, 2005)), would be to screen, at minimum, exons 4-10, including the splice junctions, as recommended by the IARC TP53 Mutation Database R13 release November 2008, to reduce the number of missed mutations to an acceptable level (Petitjean *et al.*, 2007b).

3.4.2.2.2 Methods of mutational analysis

Another explanation for the difference in the frequency of TP53 mutations observed in this study to that of previous studies might be due to the use of different methodologies.

Wedge sections of tissue were removed from the OS analysed in this study by a pathologist, from which, DNA was extracted from the wedge tip. False negative

results may still have arisen from tumour heterogeneity and/or sampling error, e.g. normal stromal contamination (Thomas *et al.*, 2006).

The PCR and sequencing primers employed in this study were designed using the GenBank sequence U62133. This partial canine *TP53* DNA sequence has also been used for the design of primers in several published *TP53* mutational investigations (Chu *et al.*, 1998; Muto *et al.*, 2000; Nasir & Argyle 1999; Stoica *et al.*, 2004). It has also been used as the reference *TP53* sequence in the study by Stoica *et al.*, (2004), for comparison of sequences from tumour samples.

PCR and DNA sequence analysis is the gold standard for the detection of mutations in genes (Osuna & de Alava 2009), especially when performed in triplicate as in this study, and has been used to successfully identify *TP53* mutations in human OS (Overholtzer *et al.*, 2003) and various canine tumours (Muto *et al.*, 2000; Nasir *et al.*, 2001). Unfortunately its main limitation is that it can only detect mutations when the fraction of mutated alleles is greater than ~20% (Collins *et al.*, 1989; van Ommen *et al.*, 1999). Hence, in future *TP53* mutational studies, microscopic assessment of each tumour sample should be performed by a pathologist and if necessary, manual/laser microdissection carried out prior to PCR and DNA sequence analysis (Takahashi *et al.*, 1995). IHC should also be carried out as post-translational aberrations may occur to *TP53*, resulting in TP53 accumulation in the absence of sequence mutations (Sjogren *et al.*, 1996). IHC is the most extensively reported method for determining the presence of TP53 accumulation in a wide variety of canine and human tumour samples, including OS (Loukopoulos & Robinson 2007; Pakos *et al.*, 2004; Sagartz *et al.*, 1996).

High through-put *TP53* mutational screening including large population-based studies, can now be performed using DNA microarrays and single-nucleotide polymorphism (SNP) assays, and more recently high-resolution melting assays (Garritano *et al.*, 2009; Tennis *et al.*, 2006). Kits are also commercially available designed specifically to examine the entire *TP53* coding sequence in human tumours. For example, the 'GeneChip p53 Assay' (Affymetrix), consists of more than 50000 unique probes to analyse *TP53* exons 2-11, includes more than 400 mutations (Affymetrix 2001), and has been successfully used to analyse human tumours from a variety of tissue types e.g. breast (Tennis *et al.*, 2006), bladder

(Wikman *et al.*, 2000), and OS (Chen *et al.*, 2004). Wikman *et al.*, (2000) also found that the 'GeneChip p53 assay' could detect as little as 1% mutated DNA (Wikman *et al.*, 2000). Hence, similar studies in canine OS warrant investigation.

3.4.2.2.3 Non-TP53 alterations in osteosarcoma

Another putative reason for the lack of mutations detected in *TP53* in the OS analysed in this study may be due to the presence of non-*TP53* aberrations. Alternative mechanisms for the inactivation of *TP53* are described in 1.3.3, which were identified after a number of tumours were reported to no longer possess wild-type *TP53* function, even though no mutations in *TP53* could be observed (Leach *et al.*, 1993; Thomas *et al.*, 1999). Frequent chromosomal aberrations have been observed on numerous loci, besides *TP53* locus, in canine and human OS samples (Tang *et al.*, 2008; Thomas *et al.*, 2009), thereby indicating that more than 1 gene is altered in OS. Indeed, 14 loci found to contain either copy number gain or loss in >30% canine OS studied were located in 8 different chromosomes which included sites for 5 major cancer-related genes, *PTEN*, *MYC*, *HRAS*, *WT1* and *KIT* (Thomas *et al.*, 2009). Alterations in several TSG, oncogenes, proteinases, growth factors and hormone signalling systems have also been documented, as previously described in 1.1.5.2, in addition to *TP53* that may also be involved in the pathogenesis of canine OS. In particular, amplification of the *MDM2* gene or overexpression of the *MDM2* protein can be detected, whilst *TP53* remains wild-type. It is generally accepted that *MDM2* and *TP53* mutations are mutually exclusive, suggesting that either of these 2 mechanisms may inactivate *TP53* (Tang *et al.*, 2008).

It would therefore be worthwhile in any future canine OS mutational studies, to analyse, in addition to *TP53*, chromosomal aberrations and expression of these other genes. This could be achieved using aCGH, cDNA microarrays and next-generation sequencing. For example, canine gene expression microarrays are now commercially available (Affymetrix 2005; Agilent Technologies 2006; Holzwarth *et al.*, 2005) and integrated molecular profiling using whole-genome analysis of DNA copy number and gene expression analysis of human OS (Sadikovic *et al.*, 2008) has been reported. Thomas *et al.*, (2008) have recently

published the development of a comprehensive 1-Mb resolution BAC microarray which also enables the direct translation of dog tumour-associated copy numbers into DNA sequence, revealing the consequences of genomic aneuploidy on gene dosage (Thomas *et al.*, 2008). This array has yet to be used to analyse canine OS and will no doubt provide valuable information once this has been performed.

Breed agencies and support organizations are presently involved in tissue archiving with a goal of genetic screening for susceptibility prediction to OS as a tendency for familial predisposition may exist in some pure-breeds (Lord *et al.*, 2007; Phillips *et al.*, 2007; Urfer *et al.*, 2007). Whether these are true 'cancer families' due to a genetic defect, for example, the Li-Fraumeni syndrome in humans which has a known aberration in *TP53* (Kansara & Thomas 2007), has still to be determined.

Hence, molecular events involved in canine OS tumourigenesis can be elucidated more quickly with the increasing widespread use of molecular-based technologies and the publication of the entire canine genome sequence. The frequency and distribution of such molecular changes are essential to establish not only the molecular aetiology of the disease, but also improve understanding of the basic biology of the tumour, such that management and treatment may be improved.

Chapter 4
TP53 as a Target for Canine
Osteosarcoma Gene Therapy

4.1 Introduction

TP53 is functionally involved in tumour suppression, cell cycle control and apoptosis and is mutated in 40-80% of human and canine cancers, making it the most frequently altered and studied gene in canine and human neoplasia. In particular, aberrations in *TP53* are a common occurrence in canine OS (Johnson *et al.*, 1998; Mendoza *et al.*, 1998; Setoguchi *et al.*, 2001b; Van Leeuwen *et al.*, 1997). It is anticipated that a better understanding of the biology of TP53 can be exploited to develop new and more effective therapeutic strategies for treating cancer, as well as better diagnostic and prognostic tools. Hence, *TP53* is an attractive candidate in the development of a targeted suicide gene therapy strategy for OS.

4.1.1 *TP53* gene therapy strategies

A number of *TP53* gene therapy approaches have addressed the problem of overcoming the vast number of *TP53* mutations or inactivation of wild-type TP53 present in cancer. These approaches include, introducing an exogenous wild-type *TP53* to replace the defective *TP53*, which can also be carried out in combination with conventional DNA-damaging agents or other gene therapy strategies e.g. expression of p14ARF, p33ING1, p16, IL-2 and GM-CSF or inhibition of telomerase, cyclin D1, PCNA and clusterin genes (Almazov *et al.*, 2007); *TP53* antisense, ribozymes and RNAi that reduce mutant TP53 expression and restore wild-type TP53 expression (Watanabe & Sullenger 2000); enhancing or reactivating transcriptional function to mutant TP53 by small synthetic molecules e.g. PRIMA-1, C-terminal peptides, antibodies against the C-terminus, and single-chain Fv antibody fragments corresponding to defined TP53 domains e.g. CDB3 (Bassett *et al.*, 2008); activation of wild-type TP53 by the inhibition of MDM2 with Nutlin-3 (Vassilev *et al.*, 2004); and selective killing of TP53 mutant tumours, which includes suicide gene therapy strategies and a TP53-targeting oncolytic adenovirus, ONYX-015 (Bischoff *et al.*, 1996; Nemunaitis *et al.*, 2001). Although several of these approaches e.g. ONYX-015, are not classed as a gene therapy strategy per se, they do utilize the genetic aberrations of tumour cells. It is beyond the scope of this introduction to discuss all these approaches, as

there is an extensive amount of literature available, however the aim for all of them, is to eliminate tumour cells through the induction of apoptosis. Hence, the strategies that will be discussed here are the approaches that have undergone the most investigation in canine or human OS, being the delivery of wild-type *TP53* and its delivery in combination with conventional therapies, and those that are also of particular relevance to this study i.e. suicide gene therapy systems utilising TP53.

4.1.1.1 Introduction of exogenous wild-type TP53

The restoration of TP53 function in tumours has been pursued as a promising strategy for cancer therapy as its inactivation is advantageous for tumour survival. One of the main approaches employed to achieve this purpose, is the delivery and expression of exogenous wild-type *TP53* in cancer cells and studies on more than 100 cell lines derived from many different tumour types have been published, using viral and non-viral vectors. The common theme throughout the literature is that expression of wild-type TP53 effectively restores suppression of growth and proliferation and induction of apoptosis in most cancer cells *in vitro* and *in vivo*, albeit with reduced efficacy in tumour cells containing wild-type TP53 (Tang *et al.*, 2004; Tsuchiya *et al.*, 2000). Additionally, minimal levels of toxicity are observed in animal models (Kastan 2007). In particular, cell proliferation has been inhibited and apoptosis induced in canine OS cell lines and xenografts (Kanaya *et al.*, 2005; Yazawa *et al.*, 2003b). Similar observations were also reported in human OS cells *in vitro* and *in vivo* (Densmore *et al.*, 2001; Hellwinkel *et al.*, 2005; Nakase *et al.*, 2005a).

Several pre-clinical studies have also importantly shown that normal non-transformed cells from different tissue types with functional endogenous TP53, e.g. fibroblasts, bone marrow cells, and astrocytes, may tolerate the presence and expression of low to moderate levels of exogenous wild-type TP53 as their levels of proliferation were at most, only minimally affected (Harris *et al.*, 1996; Seth *et al.*, 1996).

Numerous *TP53* gene therapy clinical trials have been completed, are ongoing or approved in humans with a variety of different cancers, although none as yet

with patients suffering from OS (US National Institutes of Health 2009). Encouraging results have been reported in several Phase I and Phase II clinical trials carried out in patients with unresectable NSCLC and head and neck squamous cell carcinoma (HNSCC), which had failed to respond to conventional treatments (Clayman *et al.*, 1998; Swisher *et al.*, 1999). As successful gene transfer, low toxicity (<5% incidence of serious adverse events), evidence of apoptosis, reduction in tumour size, disease stabilization and partial and complete responses were observed to have occurred in some patients (Clayman *et al.*, 1998; Swisher *et al.*, 1999). Furthermore, Phase III clinical trials are currently underway with replication-deficient recombinant adenoviral-mediated p53 (Ad-p53) gene therapy products, Advexin and SCH 585500, and under more intensive investigation in China, is Gendicine (Vazquez *et al.*, 2008) (Table 4.1). However, the efficacy of TP53 therapy has been brought into question, as clinical studies carried out in lung, bladder, breast and ovarian carcinoma resulted in the absence of additional beneficial effects in comparison to conventional treatments (Vecil & Lang 2003; Zeimet & Marth 2003). In addition, the therapeutic effect of TP53 delivery may be limited due to the majority of cancers containing mutant TP53, which possess a dominant negative activity that can inactivate the function of exogenous wild-type TP53 (Watanabe & Sullenger 2000).

Various methods of TP53 delivery have been used in both experimental cancer models and clinical trials. Among them, the use of Ad-p53 is a common approach, due to its ability to generate a large viral titre and target a broad spectrum of cells (Fujiwara *et al.*, 2000; Wilson 2002). Although, the efficient induction of apoptosis in OS cells, following delivery of wild-type TP53, using an Ad5-based adenoviral vector, can only be achieved if OS cells express high levels of Ad5 receptors (Hellwinkel *et al.*, 2005). Intratumoural and intraperitoneal administrations of high titre Ad-p53 have generally proved to be safe and efficient with no significant toxicity observed even in repeated subsequent injections (Merritt *et al.*, 2001; Wadler *et al.*, 2002; Wen *et al.*, 2003). Induction of immune responses by the virus infection may also contribute to the tumour shrinkage by Ad-p53 delivery (Carroll *et al.*, 2001). In addition, exogenous TP53 expression and TP53 target gene activation were detected in clinical trials even in the presence of a high titre of anti-adenovirus antibodies in the circulation.

However, the endogenous TP53 status, whether wild-type or mutant, appears to be critically decisive to the response of Ad-p53 therapy, where endogenous wild-type TP53 is associated with reversible cell growth arrest and mutant TP53 with apoptosis. Thus ironically, a poorer response and greater resistance to Ad-p53 therapy is observed in the wild-type TP53 context, due to DNA repair and recovery from cell cycle arrest.

Agent	Stage of development	Types of cancers in trials
Gendicine	Approval in China	Head and neck
Advexin	Phase I-III	Head and neck, NSCLC, breast, oesophageal, prostate, ovarian, bladder, bronchoalveolar and glioblastoma
SCH 58500	Phase I-III	Ovarian, lung, bladder and liver

Table 4.1: Clinical trials of recombinant adenoviral-mediated p53 gene therapy agents that restore wild-type TP53 function. NSCLC, non-small-cell lung cancer (Vazquez *et al.*, 2008).

4.1.1.2 Introduction of exogenous TP53 in combination with standard therapeutic modalities

Inactivation of TP53 is not only involved in tumourigenesis but also in conferring resistance to chemo- and radiotherapy and consequently a poor prognosis as most of these treatments elicit DNA damage and induce TP53-dependent apoptosis in cells (Lowe *et al.*, 1993; Petitjean *et al.*, 2007a). For example, the presence of TP53 mutations and accumulation of TP53 may be poor prognostic indicators for canine OS (Kirpensteijn *et al.*, 2008; Loukopoulos *et al.*, 2003), as previously discussed in 1.1.7.1 and 1.3.3.4. In addition, TP53 gene alterations may be associated with decreased survival in human OS patients (Pakos *et al.*, 2004). Hence, the therapeutic replacement of the wild-type TP53 in combination with these conventional modalities has been investigated.

Preclinical studies have demonstrated that delivery of wild-type TP53 significantly increases the therapeutic effects of chemotherapy and radiotherapy in several different tumour types (Fujiwara *et al.*, 1994; Gallardo *et al.*, 1996; Spitz *et al.*, 1996; Xu *et al.*, 2001a). In particular, restoring wild-type TP53 has

been shown to increase sensitivity of human OS cells (Saos-2, p53 null) to chemotherapeutic agents, cisplatin (Tsuchiya *et al.*, 2000) and adriamycin (Song & Boyce 2001). In addition, after delivery of Ad-p53, the viability of 4 different human OS cell lines (3 p53 mutants, 1 p53 null) was reported to decrease with an increase in sensitivity to cisplatin and doxorubicin (Ganjavi *et al.*, 2006). Furthermore, it has recently been published that upregulating the expression of wild-type TP53 in canine OS cells, already possessing wild-type TP53, enhanced their sensitivity to radiation at clinically relevant doses and significantly decreased their survival in comparison to cells in which TP53 was not upregulated (Shiomitsu *et al.*, 2008). However, results reported by Hellwinkel *et al.*, (2005), who found that Ad-p53 treatment with cisplatin or doxorubicin, increased sensitivity in 2 human OS cell lines, U2OS and Saos-2 cells but not in 3 other human cell lines studied, K-HOS, MG-63 and SJSA (Hellwinkel *et al.*, 2005).

In clinical trials, similar to those of wild-type TP53 administered alone, conflicting results have been observed with the delivery of wild-type TP53 in conjunction with conventional treatments. For example, treatment of Ad-p53 plus chemotherapy in patients with ovarian cancer and NSCLC was found to provide no additional survival benefit over that of chemotherapy alone, although NSCLC tumours had significantly decreased in size (Buller *et al.*, 2002; Schuler *et al.*, 2001; Wen *et al.*, 2003). In comparison, randomized Phase II trials of intratumoural Ad-p53 followed by radiotherapy in patients with HNSCC, nasopharyngeal carcinoma and NSCLC, reported significantly improved response rates compared to radiotherapy alone (Chen *et al.*, 2003; Swisher *et al.*, 2003; Zhang *et al.*, 2003). Phase II and III clinical trials on 135 patients with late-stage HNSCC, treated with Gendicine and radiotherapy, showed complete regression in 64% patients and partial regression in 32%, with no serious side effects observed (unknown 2007). Clinical benefit has also been observed in NSCLC patients, previously unresponsive to conventional treatments, after Ad-p53 administration in combination with cisplatin (Fujiwara *et al.*, 2006; Nemunaitis *et al.*, 2000). Advexin, as well as demonstrating a consistent safety profile and clinical efficacy as a monotherapy, has also shown additive or synergistic effects in a variety of tumour types in combined modality regimens with chemotherapy and/or radiotherapy (Senzer & Nemunaitis 2009; unknown 2007).

Hence, findings from studies in which *TP53* therapy has been combined with DNA-damaging agents appear encouraging as additional efficacy has been observed over *TP53* therapy alone and together may interact in synergistic or additive ways. Furthermore, no observations of antagonistic interactions between *TP53* therapy and standard cancer treatments have been reported.

4.1.1.3 Selective killing of TP53 mutant tumours

Although the restoration of TP53 function has shown promise, there are inherent problems, in that TP53 plays a pivotal role in tumour progression and tumourigenesis is a multi-step process. Most tumour cells already contain a broad spectrum of genetic abnormalities before they become clinically apparent; thus it seems likely that corrective gene therapy will not realistically be able to produce the required clinical benefit in all tumour types. Hence, gene therapy protocols for cancer using non-corrective genes to enhance tumour cell killing have occurred, including many suicide gene therapy preclinical and clinical studies.

Surprisingly though, at present, only 2 preclinical studies have been published that involve TP53 directly in a suicide gene therapy system. Firstly, Da Costa *et al.*, (1996) devised a novel strategy, in which the human *TP53* C-terminus, employed as a TP53 binding domain, can bind to intact TP53, recruiting the N-terminus transactivation domain (Da Costa *et al.*, 1996). This can occur, even in accumulated TP53, resulting in the transcriptional activation of a toxic gene, because in most TP53 mutants, as previously described in 1.3.3.1, only a small percentage of mutations are located in the N- and C-termini as the majority are found in the central DNA-binding domain (Petitjean *et al.*, 2007b; Zambetti & Levine 1993). Thereby, the transactivation domain is generally preserved in TP53 mutants, enabling the wild-type *TP53* C-terminus to be utilized in a gene therapy approach that can specifically kill tumour cells possessing accumulated TP53. Secondly, Mizumoto and colleagues (2002) published a TP53-mutated cancer cell-specific suicide gene therapy strategy using *TP53* as a transcriptional activating factor (Mizumoto *et al.*, 2002). This strategy used the HSV-TK expression system containing the wild-type *TP53*-specific promoter (p53-SP) and

the Cre/loxP exchange system to specifically induce expression of the HSV-TK gene in TP53-mutated cells, whilst in the cells containing wild-type TP53, to reduce HSV-TK expression. On addition of GCV, growth inhibition occurred in the TP53-mutated cells but not in the wild-type TP53 cells. Thus, enabling the selective growth inhibition of cells possessing mutated TP53.

Two other suicide gene therapy studies have been published, which indirectly target TP53 to inhibit the growth of tumour cells possessing mutant TP53 (Lipinski *et al.*, 2001; Zhu *et al.*, 2000). This was achieved through the expression of a suicide gene being placed under the control of heat shock protein (HSP) 70 promoter and similar to the study by Mizumoto *et al.*, (2002), suicide gene expression was able to be minimized in cells containing wild-type TP53, as wild-type TP53 is able to repress HSP70.

It is the study by Da Costa and colleagues (1996) (Da Costa *et al.*, 1996), which is of specific relevance to this study, in that a similar strategy was to be investigated as a treatment modality for canine OS, as the canine TP53 C-terminus was to be utilized instead of the human TP53 C-terminus. Importantly, Veldhoen and Milner (1998) had previously shown that the canine TP53 produced *in vitro* recognizes and binds TP53-specific DNA targets derived from *p21* and *GADD45* promoters and to a consensus TP53 binding site (Veldhoen & Milner 1998). They also reported that canine TP53 associates with oligonucleotides representing damaged DNA sites and undergoes proteolytic cleavage similar to that described for murine and human TP53; as well as demonstrating that the canine TP53 is able to transcriptionally activate a TP53-dependent reporter gene *in vivo*. These results add further evidence to the likelihood that canine TP53 is similar, in function as well as structure, to human TP53 and that canine cancer may provide a useful model for human TP53 therapy approaches.

4.1.2 Summary

Since TP53 mutation, as well as wild-type TP53 impairment, is observed in most canine and human malignancies, restoring TP53 function by TP53 delivery in tumours was the initial approach pursued as a strategy for cancer therapy. TP53

is also an attractive candidate for gene therapy protocols because it can directly elicit apoptosis in cancer cells, possessing both wild-type and mutant TP53, without apparent toxicity to normal cells and enhances the therapeutic efficacy of conventional therapies. Previously published findings in canine and human OS cells suggest that their proliferative ability can be suppressed *in vitro* and *in vivo* by the restoration of normal TP53 function (Densmore *et al.*, 2001; Kanaya *et al.*, 2005; Nakase *et al.*, 2005a; Oshima *et al.*, 2007; Yazawa *et al.*, 2003b). However the usefulness of this approach continues to be debated in light of the wide range of aberrations found in cancer cells, in that restoring just one gene, is unlikely to produce the desired clinical effect. This led to the development of several alternative TP53 therapy strategies, including the use of TP53 in a suicide gene therapy approach to selectively target and destroy tumour cells. However, to the best of my knowledge, none of the clinical trials reported so far, involve TP53 in a suicide gene therapy system or have studied the effects of TP53 delivery in canine or human OS patients. In addition, none of the cell lines utilized in the preclinical TP53 suicide gene therapy studies by Da Costa *et al.*, (1996) and Mizumoto *et al.*, (2002), in which growth was inhibited in TP53-mutated cancer cells, but not in wild-type TP53-producing, were OS (Da Costa *et al.*, 1996; Mizumoto *et al.*, 2002).

The aims of this chapter were two-fold. Firstly, to investigate the effects of introducing exogenous wild-type TP53 into 4 canine OS cell lines, D17, CMT3, CMT7 and CMT8. This was carried out because the delivery of wild-type TP53 has not been previously reported in the CMT3, CMT7 and CMT8 cell lines, only one publication to date has reported TP53 gene delivery in D17 cells (Shiomitsu *et al.*, 2008), and only 3 other canine OS cell lines have been investigated to date (Kanaya *et al.*, 2005; Yazawa *et al.*, 2003b). Thereby, extending data on TP53 restoration gene therapy in canine OS cells.

Secondly, and more importantly, to design, construct and analyse the vectors required for a TP53-targeted suicide gene therapy strategy, in which the canine TP53 C-terminus domain (294-382 $\alpha\alpha$) was to be utilized. This was performed to determine if the transactivation domain of accumulated canine TP53 can be recruited to drive, the expression of a luciferase reporter gene in this study and ultimately a suicide gene (as described in Chapter 5), via the use of a Lex-DNA

binding domain interacting with its operator site acting as an upstream activating sequence, as highlighted in Figure 4.1. Hence, enabling the selective targeting of canine OS cells possessing accumulated TP53. The analysis of this transcriptional targeting approach was the primary aim of the entire thesis.

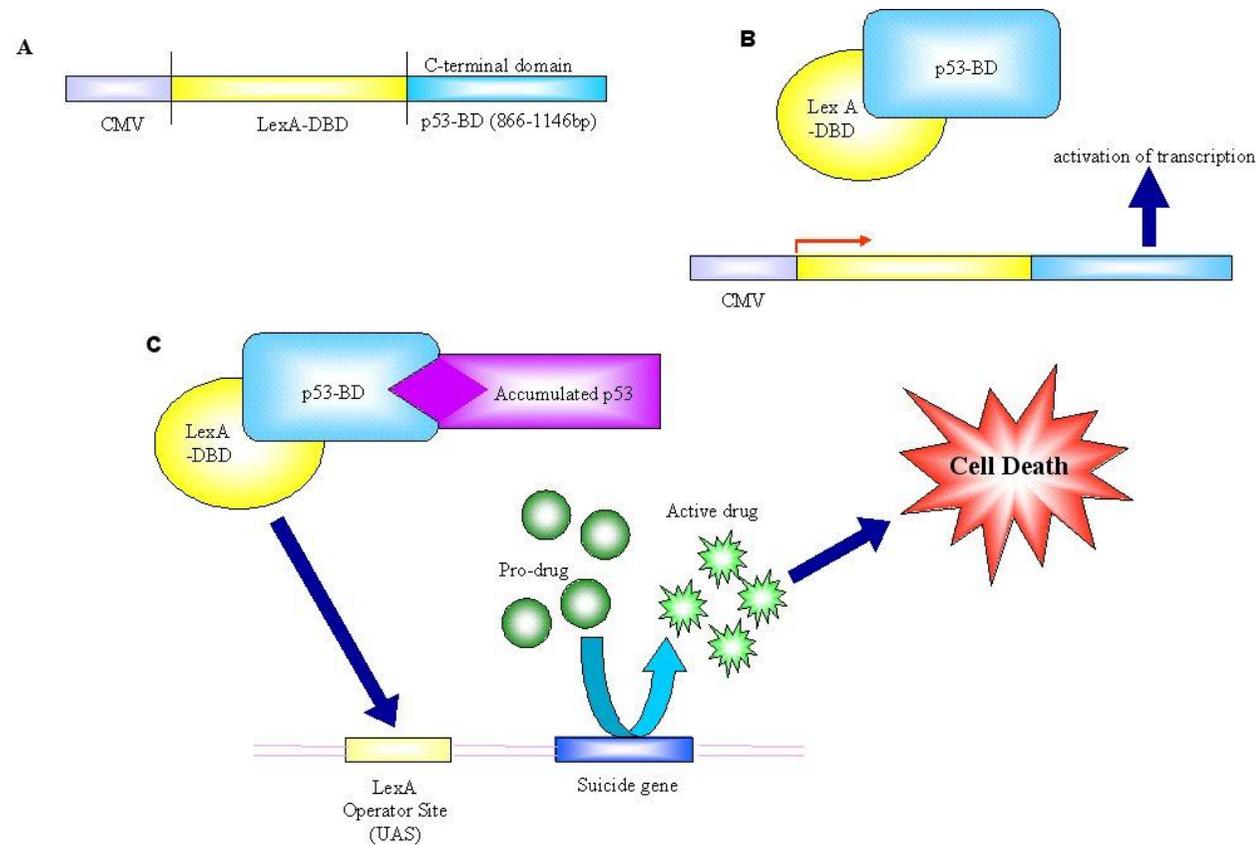


Figure 4.1: Transcriptional targeting using canine TP53 to initiate tumour cell destruction: (A), LexA DNA Binding Domain (DBD) is fused in frame to p53-binding domain (BD) of canine TP53 (C-terminal domain); (B), Production of LexA-p53 protein; (C), LexA-p53 protein binds to both accumulated TP53 to recruit its transactivation domain and the LexA operator site (acting as an upstream activating sequence, UAS) to be able to drive the expression of the suicide gene. The suicide gene converts the inactive prodrug to the active drug, thereby causing cell death.

4.2 Materials & methods

4.2.1 Optimization Assays

4.2.1.1 Cell number

Cells were approximately 80% confluent on the day of transfection (2.2.9), as recommended by the manufacturer's instructions. For all of the transfections performed in this study, the cells were transfected 16-24 hours after placement into 96-well plates or 25cm² flasks. The canine OS cell lines utilized in this study were D17, CMT3, CMT7 and CMT8 and were each cultured and maintained as described in 2.1.1.

The concentration of cells required to achieve 80% confluence for each of the four cell lines, was determined by plating out a range of cell concentrations into 96-well plates in quadruplicate per cell concentration (200 µl/well):

- (i), D17: 35000-100000 cells/ml at increments of 5000 cells/ml (35000, 40000, 45000, 50000, 55000, 60000, 65000, 70000, 75000, 80000, 85000, 90000, 95000 and 100000);
- (ii), CMT 3: 25000-80000 cells/ml at increments of 5000 cells/ml (25000, 30000, 35000, 40000, 45000, 50000, 55000, 60000, 65000, 70000, 75000 and 80000);
- (iii), CMT7: 15000-65000 cells/ml at increments of 10000 cells/ml (15000, 25000, 35000, 45000, 55000, 65000);
- (iv), CMT8: 30000-85000 cells/ml at increments of 5000 cells/ml (30000, 35000, 40000, 45000, 50000, 55000, 60000, 65000, 70000, 75000, 80000 and 85000).

The percentage of cells confluent at each cell concentration was estimated by eye after 24 hours, enabling the concentration of cells required to achieve 80% confluence for the D17, CMT3, CMT7 and CMT8 cell lines to be determined.

4.2.1.2 Transfection efficiency

The '*In situ* Staining of Cells for β-galactosidase (β-gal) Activity' of the 'β-Galactosidase Enzyme Assay System' (Promega) protocol was used to determine the: optimal amount of plasmid DNA to be transfected; and charge ratio of

transfection reagent (Transfast™, Promega) to DNA, for the subsequent experimental assays utilising D17, CMT3, CMT7 and CMT8 cells.

The four cell lines were each cultured and maintained (2.1.1), and 16-24 hours prior to transfection occurring, seeded at the appropriate density (as determined in 4.3.1.1) per well of a 96-well plate. 50, 100, 150 and 200 ng of pSV-β-Galactosidase control vector (pSV-β-gal) DNA were each transfected into D17, CMT3, CMT7 and CMT8 cells, in quadruplicate. Two charge ratios of Transfast™ to DNA were used: 1:1; and 2:1. 48 hours later the cells were fixed, as directed by the manufacturer's instructions, using a 0.25% glutaraldehyde solution and incubated with the substrate X-gal, at 37°C for 2-4 hours, until the cells were visibly stained blue. The cells were viewed with a light microscope (10x magnification) and if the cells retained a normal visual appearance, the number of blue stained (transfected) and total number of cells were recorded per well. This enabled the transfection efficiency (%) to be calculated, hence the amounts of plasmid DNA to be transfected and the charge ratio of Transfast™ to DNA in the subsequent experimental assays, employing D17, CMT3, CMT7 and CMT8 cells, to be determined.

4.2.2 Construction of expression vectors

The methods involved in constructing the vectors in this study are previously described in Chapter 2. Methods included DNA purification (2.2.3), restriction digestion (2.2.6), PCR (2.2.1), cloning (2.2.7) and DNA sequence analysis (2.2.5). PCR conditions are as previously described in 2.2.1 and details of primers used for PCR and DNA sequence analysis are displayed in Table 4.2.

4.2.2.1 pTarget-p53-full-length construct

The construction of the pTarget-p53-full-length expression vector (pTarget-p53FL) was carried out by designing 2 PCR primers, 'p53FLF' and 'p53FLR', which allowed the amplification of the full-length (FL) canine *TP53* cDNA sequence from the pK9 vector (kindly donated by Dr. N. Veldhoen (Veldhoen & Milner 1998); Figure 4.2). The p53FL fragment was directly ligated, at 4°C for 20

hours, into the pTarget mammalian expression vector system of TA cloning (Promega), and cloned using JM109 competent cells (Promega), to produce the pTarget-p53FL expression vector. DNA sequence analysis, using the sequencing primers 'T7 Promoter', 'p531006F' and 'pTarget1358R', was carried out to confirm that the p53FL fragment had been correctly ligated into the pTarget™ Mammalian Expression Vector (Figure 4.3).

Expression Vector(s)	Primer	Forward (F) / Reverse (R)	Primer Sequence (5' --> 3' orientation)	T _m	Location (nts)	Vector(s)
A, Construction						
pTarget-p53FL	p53FLF	F	cgt aat acg act cac tag tgg gta	61	2447-2470	pK9
	p53FLR	R	gga att cct gca gga tat cgt gga	62.7	3620-3643	pK9
pTarget-LexDBD	LexF358	F	gcc gct gca caa tat ttc aag cta t	61.3	358-378	pHybLex/Zeo
	LexR1052	R	ctg aga tct gag ctc aag ctt	57.9	1032-1052	pHybLex/Zeo
pTarget-Lexp53	Xholp53F	F	gcc tcg agc tcg aga att tcc aca aga ag	68.1	3297-3311	pK9
	p53XhoIR	R	gcc tcg agc tcg aga tac gac tca ctt aa	68.1	3697-3710	pK9
	pRcLexXbaIF	F	gcc tct aga tct aga ata cgg cct tcc ttc	68.1	221-233	pHybLex/Zeo-p53
	pRcp53XbaIR	R	gcc tct aga tct aga gaa att cgc ccg ga	68.1	1515-1528	pHybLex/Zeo-p53
pGL3B8LA, pGL3E8LA & pGL3P8LA	2KpnIpGL3F	F	ggt acc ggt acc ggc att acc acc ata tac	69.5	227-244	pSH18-34
	NhelpGL3FR	R	gct agc gct agc tac ccc aga aat aag gct	69.6	463-480	pSH18-34
B, DNA Sequence Analysis						
pTarget-p53FL, pTarget-LexDBD & pTarget-Lexp53	T7 Promoter	R	taa tac gac tca cta tag ggc ga	58.9	1229-1252	pTarget
	pTarget1358R	R	aga tat gac cat gat tac gcc aag	59.3	1358-1381	pTarget
pTarget-p53FL & pTarget-Lexp53	p531006F	F	ttg gag ctg aag gat gcc cag agt	64.4	3477-3500	pK9
pTarget-LexDBD & pTarget-Lexp53	Lex735F	F	aag ccg aat gct gat ttc ctg ctg	62.7	735-758	pHybLex/Zeo
pGL3B8LA, pGL3E8LA & pGL3P8LA	RV3primer	F	cta gca aaa tag gct gtc cc	57.3	4760-4779; 5006-5025; 4952-4971	pGL3B, pGL3E & pGL3P
	GL2primer	R	gga aga cgc caa aaa cat aaa g	57.1	111-89; 111-89; 303-281	pGL3B, pGL3E & pGL3P

Table 4.2: Details of the oligonucleotide primers used in the overall process of constructing each expression vector by: (A), PCR; and (B), DNA sequence analysis. For each primer the following information is provided: the sequence, acting as a forward (F) or a reverse (R) primer, annealing temperature (T_m) and nucleotide (nts) location on the particular vector undergoing PCR/DNA sequence analysis. (pGL3B, pGL3-Basic; pGL3E, pGL3-Enhancer; pGL3P, pGL3-Promoter; 8LA, 8 LexA operator).

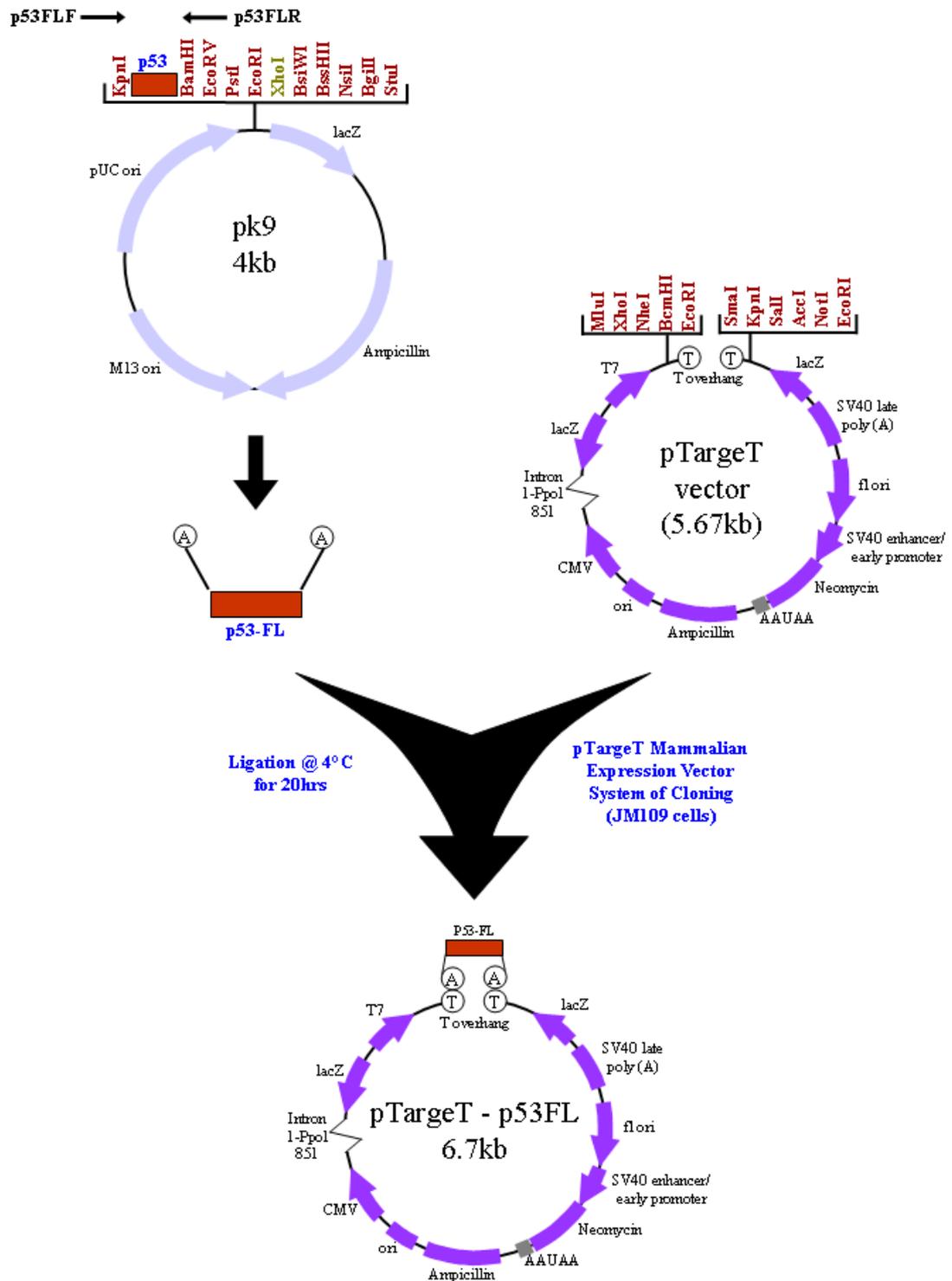


Figure 4.2: Flow-diagram indicating the processes involved in the construction of the pTargetT-p53FL expression vector. The PCR primers, 'p53FLF' & 'p53FLR,' were used to amplify the full-length (FL) canine TP53 cDNA sequence from the pk9 vector.

```

                pTarget vector                p53FLF
1252  ATTCGGATCC TTGCTAGCCT CGAGACGCGT GATTCGTAAT ACGACTCACT

                pK9                full length p53
1302  AGTGGGTACC GGTGACTGCA ATG GAG GAG TCG CAG TCA GAG CTC

1346  AAT ATC GAC CCC CCT CTG AGC... (1077bp)...AGA GAA GGG CTT

                pK9                p53FLR
2456  GAC TCA GAC TGA GGTTAAGGAT CCACGATATC CTGCAGGAAT

                pTarget vector
2498  TCAATCTTTC CCGGGGGTAC CGTCGACTGC GGCCGCGAAT TCCAAGCTTG

```

Figure 4.3: Confirmation by DNA sequence analysis that the pTarget-p53FL expression vector contains the full-length (FL) canine *TP53* cDNA sequence from the pK9 vector. The sequences of the PCR primers, 'p53FLF' and 'p53FLR', are indicated. (XXX, represents start and stop codons).

4.2.2.2 pTarget-LexA DNA Binding Domain construct

The pTarget-LexA DNA Binding Domain expression vector (pTarget-LexDBD) was constructed by designing the 2 PCR primers, 'LexF358' and 'LexR1052', which enabled the amplification of the LexDBD ORF from pHybLex/Zeo (Invitrogen; Appendix C, Figure C3) (Figure 4.4). The LexDBD was directly ligated, at 4°C for 20 hours, into the pTarget™ Mammalian Expression Vector (Promega) and cloned using JM109 competent cells (Promega), to produce the pTarget-LexDBD expression vector. DNA sequence analysis was employed to confirm that the LexDBD fragment had been correctly ligated into the pTarget™ Mammalian Expression Vector, using the sequencing primers, 'T7 Promoter', 'Lex735F' and 'pTarget1358R' (Figure 4.5).

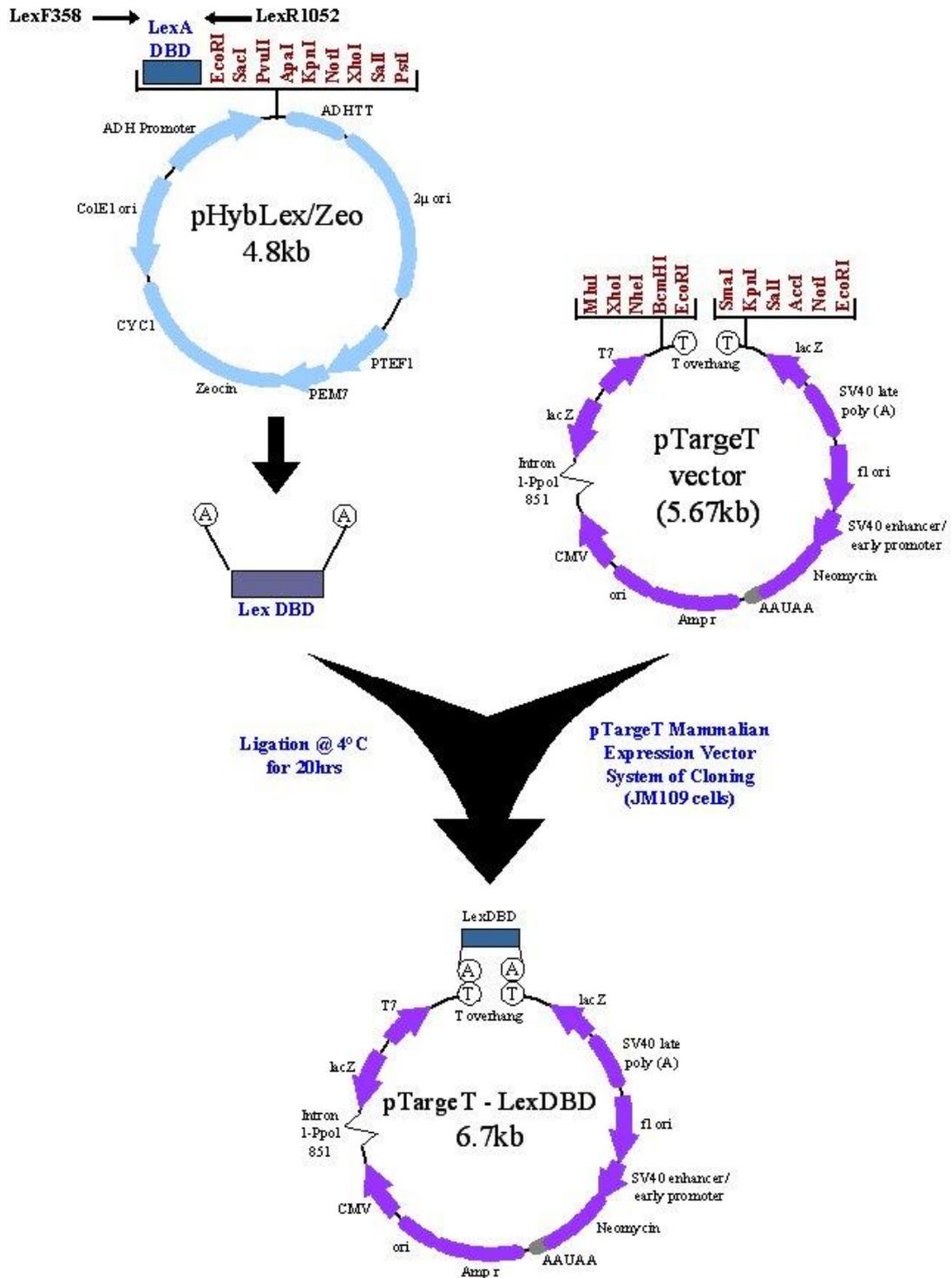


Figure 4.4: Flow-diagram indicating the processes involved in the construction of the pTargetT-LexDBD expression vector. The PCR primers, ‘LexF358’ and ‘LexR1052’, were used to amplify the LexA DNA-binding domain (LexDBD) from the pHybLex/Zeo vector.

```

                pTarget vector                                LexF358
1252  CTTGCTAGCC TCGAGACGCG TGATTGCCGC TGCACAATAT TTCAAGCTAT

                pHybLex/Zeo
1302  ACCAAGCATA CAATCAACTC CAAGCTTTAA TTCCGGGCGG A ATG AAA

                LexA ORF
1349  GCG TTA ACG GCC AGG CAA CAA GAG GTG ... (558bp)...AAC GGC

                pHybLex/Zeo LexR1052
1940  GAC TGG CTG GAATTCAAGC TTGAGCTCAG ATCTCAGAAT CTTTCCCGGG

                pTarget vector
1989  GGTACCGTCG ACTGCGGCCG CGAATTCCAA GCTTGAGTAT

```

Figure 4.5: Confirmation by DNA sequence analysis that the pTarget-LexDBD expression vector contains the LexA DNA-binding domain (LexDBD) open reading frame (ORF) from the pHybLex/Zeo vector. The sequences of the PCR primers, 'LexF358' and 'LexR1052', are shown. (XXX, represents start and stop codons).

4.2.2.3 pTarget-Lexp53 construct

The pTarget-Lexp53 expression vector, containing the LexDBD and canine *TP53* C-terminus, was constructed in 3 main stages (Figure 4.6). The first stage involved the design of 2 PCR primers, 'Xholp53F' and 'p53XhoIR', enabling the amplification of the C-terminus of canine *TP53* cDNA (826-1146bp) from the pK9 vector. These 2 PCR primers had been constructed with *XhoI* restriction enzyme sites to allow the incorporation of *XhoI* sites at the 5' and 3' -ends of the *TP53* C-terminus PCR products. The *TP53* C-terminus PCR products were electrophoresed on an agarose gel, extracted, purified and ligated into the pCR[®]2.1-TOPO[®] cloning vector (Invitrogen) using the TOPO TA Cloning[®] Kit. The pCR[®]2.1-TOPO[®] vector was subsequently digested with the *XhoI* restriction enzyme to create *TP53* C-terminus fragments with *XhoI* digested 5' and 3' -ends.

The second stage focussed on the preparation of the 'Lexp53' fragment. The LexDBD in the pHybLex/Zeo vector (Invitrogen) is located upstream of a multiple cloning site, containing a *XhoI* site. pHybLex/Zeo was digested using the *XhoI* restriction enzyme, dephosphorylated and purified. The *XhoI* digested *TP53* C-terminal fragments were ligated into the purified *XhoI* digested pHybLex/Zeo

vector in frame with the LexA ORF, at 4°C for 20 hours, and cloned using Max Efficiency DH5 α competent cells (GIBCO BRL). The fused in frame LexA DBD and *TP53* C-terminus sequence was amplified by PCR, in which the primers ‘pRcLexXbaIF’ and ‘pRcp53XbaIR’ were utilized, to produce ‘Lexp53’ PCR products.

The third and final stage involved the direct ligation of these ‘Lexp53’ PCR products into the pTargetTM Mammalian Expression Vector System of TA cloning (Promega), at 4°C for 20 hours, and cloned using JM109 competent cells (Promega), to produce the pTarget-Lexp53 expression vector. DNA sequence analysis was subsequently performed, using the ‘T7 Promoter’, ‘Lex735F’, ‘p531006F’ and ‘pTarget1358R’ sequencing primers, to confirm that the LexA DBD and *TP53* C-terminus sequence had been correctly ligated into the pTargetTM Mammalian Expression Vector (Figure 4.7).

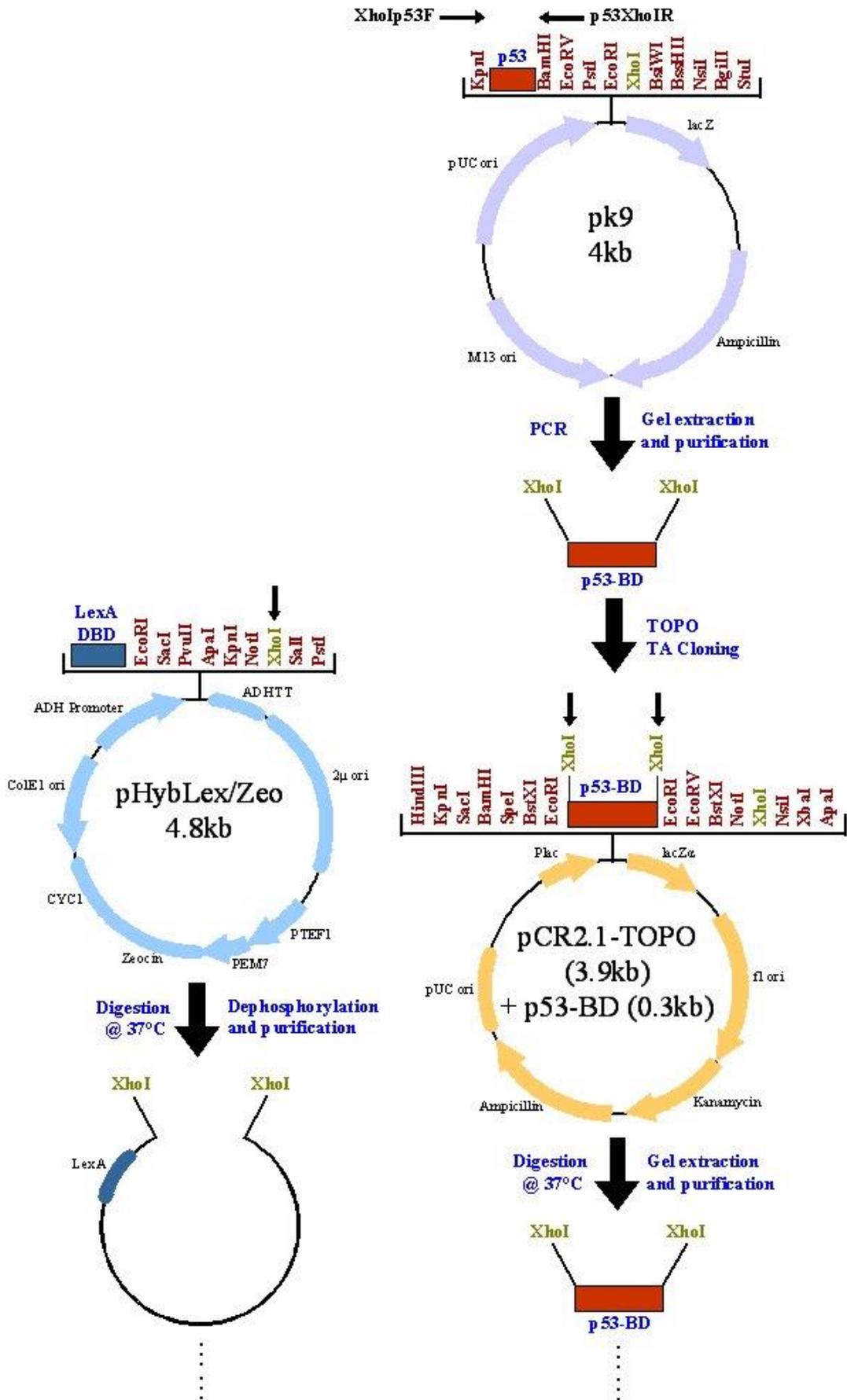


Figure 4.6: continued on next page.

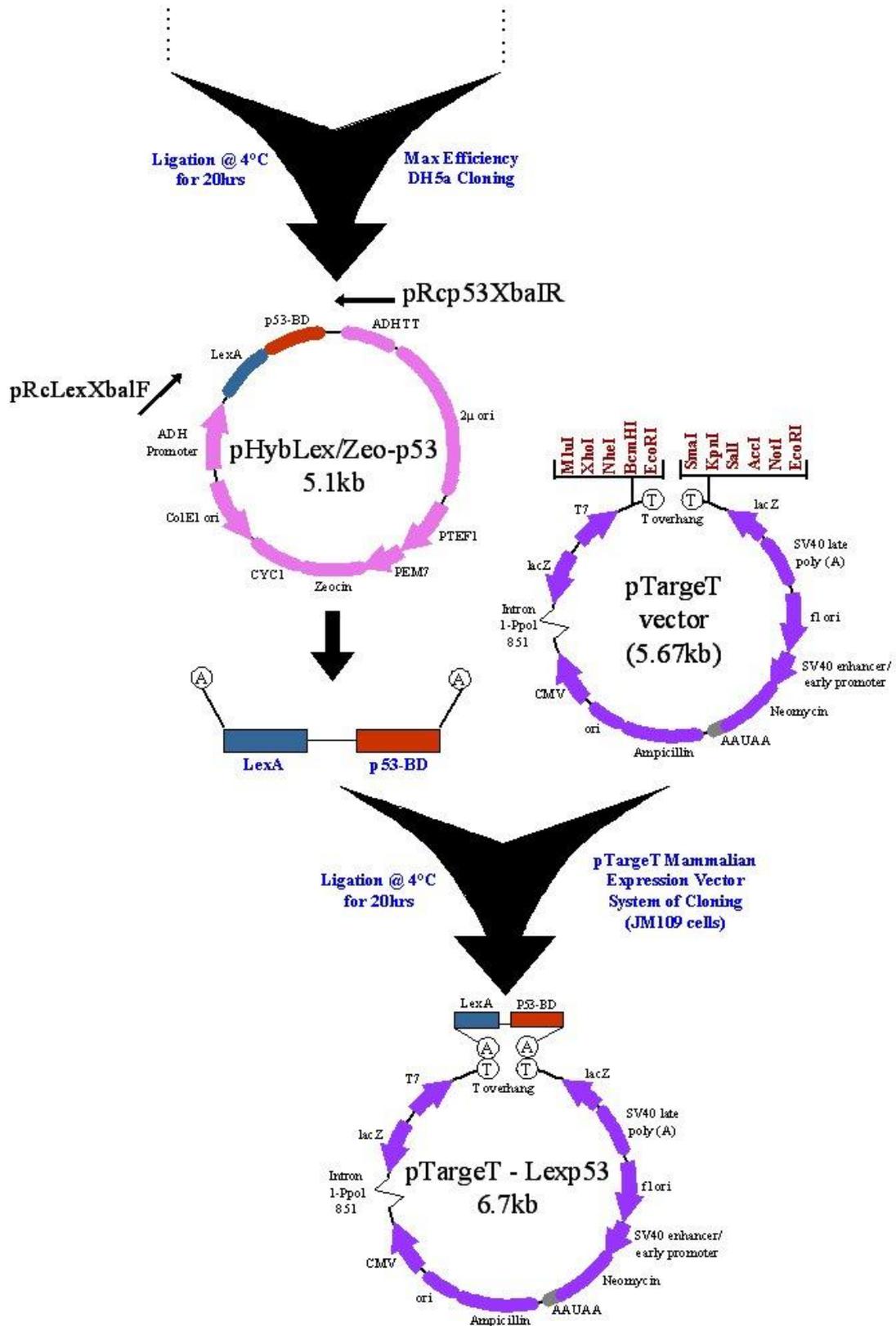


Figure 4.6: Flow-diagram indicating the processes involved in the construction of the pTargetT-Lexp53 expression vector. The PCR primers, 'XhoI p53F' & 'p53XhoIR' and 'pRcLexXbaIF' and 'pRcp53XbaIR', were used to amplify the canine TP53 C-terminus from the pK9 vector and the LexA-p53 fragment from the pHybLex/Zeo-p53 vector, respectively.

```

                pTarget vector                                pRcLexXbaIF
1252  ATTCGGATCC TTGCTAGCCT CGAGACGCGT GATTGCCTCT AGATCTAGAA

                pHybLex/Zeo
1302  TACGGCCTTC CTTCAGTTA CTTGAATTTG A...(160bp)...CCGGGCGGA

                LexA ORF
1502  ATG AAA GCG TTA ACG GCC AGG CAA CAA GAG GTG

1536  ... (558bp) ... AAC GGC GAC TGG CTG GAA TTC AAG

2119  CTT GAG CTC AGA TCT CAG CTG GGC CCG GTA CCH

                XhoI                                p53 C-terminus (826-1146bp)
2152  CGG CCG CTC GAG AAT TTC CAC AAG AAG GGG GAG CCT TGT

2191  GAG CCA CCC CCC GGG AGT ACC AAG...(243bp)...AGA GAA GGG CTT

                pK9
2470  CCT GAC TCA GAC TGA GGTTAAGGAT CCACGATATC CTGCAGGAAT

                XhoI                                pHybLex/Zeo                                pRcp53XbaIR
2515  TCCTCGAGTC GACCTGCAGC CAAGCTAATT CCGGGCGAAT TTCTCTAGAT

                pTarget vector
2565  CTAGAGGC AA TCTTTCCCGG GGGTACCGTC GACTGCGGCC GCGAATTCCA

```

Figure 4.7: Confirmation by DNA sequence analysis that the pTarget-Lexp53 expression vector contains the canine TP53 C-terminus (826-1146 bp), from the pK9 vector, fused in frame with the LexA open reading frame (ORF), from the pHybLex/Zeo vector. The sequences of the PCR primers, 'pRcLexXbaIF' and 'pRcp53XbaIR', and the XhoI restriction enzyme site, are shown. (XXX, represents start and stop codons).

4.2.2.4 pGL3-Basic 8LexA Operator, pGL3- Enhancer 8LexA Operator & pGL3-Promoter 8LexA Operator constructs

The construction of the pGL3-Basic 8LexA Operator (pGL3B8LA), pGL3-Enhancer 8LexA Operator (pGL3E8LA) and pGL3-Promoter 8LexA Operator (pGL3P8LA) vectors involved the insertion of the 8 LexA operator site from the pSH18-34 vector (Invitrogen; Appendix C, Figure C4), into the pGL3-Basic (pGL3B), pGL3-Enhancer (pGL3E) and pGL3-Promoter (pGL3P) firefly luciferase reporter vectors (all Promega; Appendix C, Figure C5), respectively (Figure 4.8). This was carried out in 2 stages.

The first stage involved the use of the PCR primers, '2KpnlpGL3F' and 'NhelpGL3R', to amplify the 8LexA operator site from pSH18-34. The '2KpnlpGL3F' and 'NhelpGL3R' primers had been designed with *KpnI* and *NheI* restriction sites, respectively, to enable the incorporation of *KpnI* and *NheI* sites at the 5'- and 3' -ends of the 8LexA operator site PCR products, respectively. The 8LexA operator site PCR products were electrophoresed on an agarose gel, extracted and purified.

The second stage consisted of the ligation of the purified 8LexA operator site PCR products into the pGL3B, pGL3E and pGL3P firefly luciferase reporter vectors, to create pGL3B8LA, pGL3E8LA and pGL3P8LA. This was achieved by digesting the 3 pGL3 vectors, each possessing *KpnI* and *NheI* restriction enzyme sites in their multiple cloning sites, with the *KpnI* and *NheI* restriction enzymes. The digested pGL3 vectors were subsequently electrophoresed on an agarose gel, extracted and purified. The purified 8LexA operator site PCR products were ligated into each of the purified pGL3 vectors, at 4°C for 20 hours, and cloned using JM109 competent cells (Promega). DNA sequence analysis was performed, using the 'RV3primer' and 'GL2primer' sequencing primers, to confirm that the 8LexA operator site sequence had been correctly ligated into the pGL3B, pGL3E and pGL3P firefly luciferase reporter vectors (Figure 4.9).

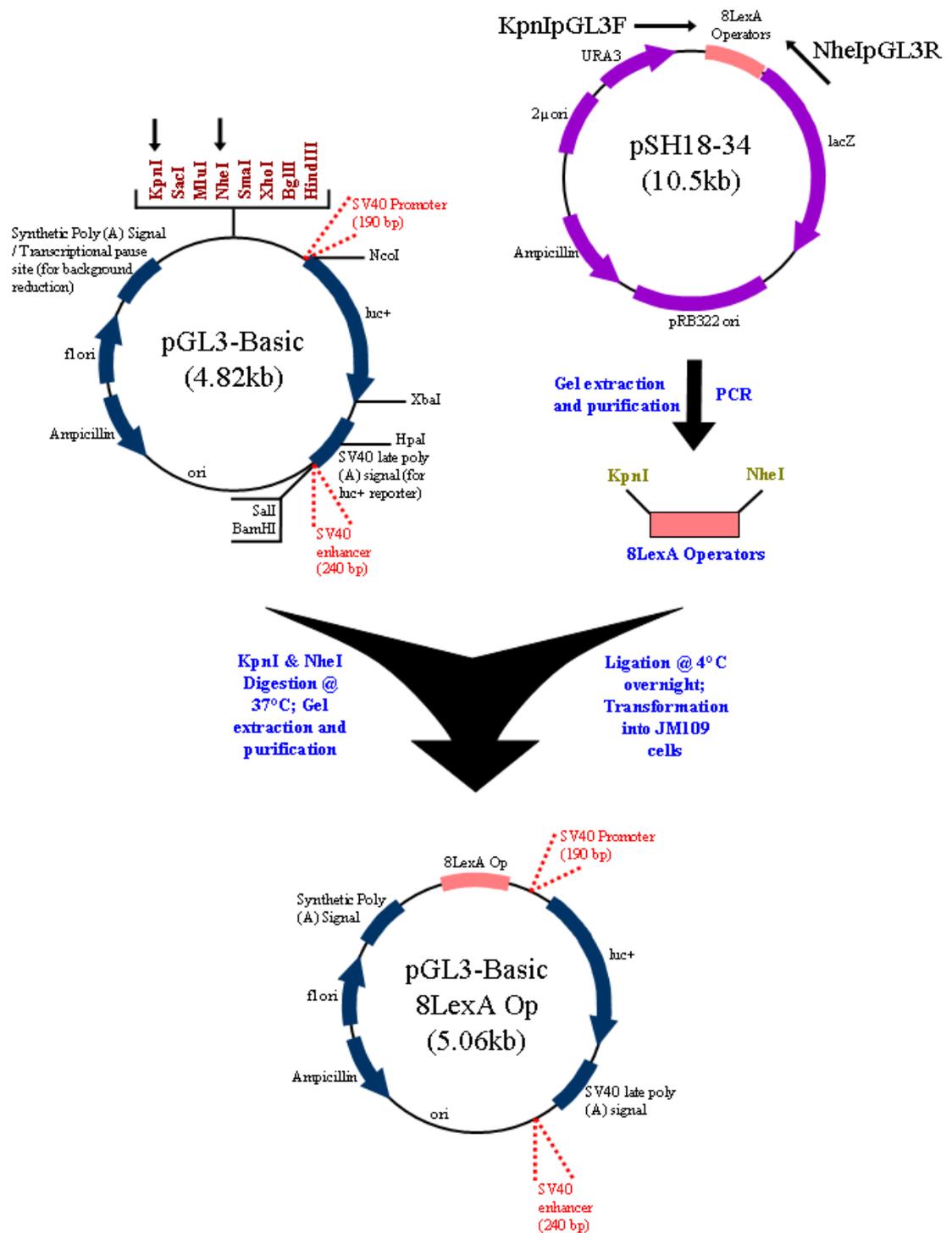


Figure 4.8: Flow-diagram indicating the processes involved in the construction of the pGL3-8LexA Operator (Op) firefly luciferase reporter vectors, pGL3-Basic 8LexA Op (lacks the SV40 Promoter and Enhancer sequences), pGL3-Enhancer 8LexA Op (pGL3-Basic 8LexA Op plus the SV40 Enhancer sequence) and pGL3-Promoter 8LexA Op (pGL3-Basic 8LexA Op plus the SV40 Promoter sequence). The PCR primers '2KpnIpGL3F' and 'NheIpGL3R' were used to amplify the 8LexA Op site from the pSH18-34 vector.

```

B5060 }
E5310 }      pGL3 vector          1 KpnI
P5250 }      TGCAAGTGCA GGTGCCAGAA CATTCTCTA TCGATAGGTA CCCATGGCAT

      KpnIPGL3F
51      TACCACCATA TACATATCCA TATACATATC CATATCTAAT CTTACCTCGA

101     CTGCTGTATA TAAAACCAGT GGTTATATGT ACAGTACTGC TGTATATAAA

      8 Lex A Operator
151     ACCAGTGGTT ATATGTACAG TACGTCGACT GCTGTATATA AAACCAGTGG

201     TTATATGTAC AGTACTGCTG TATATAAAAC CAGTGGTTAT ATGTACAGTA

      NheIPGL3R
251     CGTCGAGGGA TGATAATGCG ATTAGTTTTT TAGCCTTATT TCTGGGGTAG

      NheI                               SV40 promoter →
301     CTAGCCCGGG CTCGAGATCT GCGATCTAAG TAAGCTTGGC ATTCCGGTAC

      NcoI                               1UC
351 (P546) TGTGGTAAA GCCACCATG G AAGACGCCA AAAACATAAA

401 (P596) GAAAGGCCCG GCGCCATTCT ATCCGCTGGA AGATGGAACC

451 (P646) GCTGGAGAGC AACTGCATAA...(1490bp)... GTCTTACCGG

1971 (P2166) AAAACTCGAC GCAAGAAAAA TCAGAGAGAT CCTCATAAAG

      XbaI
2011 (P2206) GCCAAGAAGG GCGGAAAGAT CGCCGTGTAA TCTAGA GTCG

      pGL3 vector
2051 (P2246) GGGCGGCCGG CCGCTTCGAG CAGACATGAT AAGATACATT GATGAGTTTG

2101 (P2296) GACAAACCAC AACTAGAATG CAGTGAAAAA AATGCTTTAT TTGTGAAATT

2151 (P2346) TGTGATGCTA TTGCTTTATT TGTAACCATT ATAAGCTGCA ATAAACAAGT

2201 (P2396) TAACAACAAC AATTGCATTC ATTTTATGTT TCAGGTTTCTAG GGGGAGGTGT

2251 (P2446) GGGAGGTTTT TTAAAGCAAG TAAAACCTCT ACAAATGTGG TAAAATCGAT

      SV40 enhancer
2301 (P2496) AAGGATCCGT CGACCGATGC CCTTGAGAGC CTTCAACCCA GTCAGCTCCT

```

Figure 4.9: Confirmation by DNA sequence analysis that the pGL3-8LA Operator firefly luciferase (LUC) reporter vectors, pGL3-Basic 8LexA Operator (B), pGL3-Enhancer 8LexA Operator (E) and pGL3-Promoter 8LexA Operator (P) contain the 8LexA operator site. The sequences of the PCR primers '2KpnIPGL3F' and 'NheIPGL3R' and the *KpnI*, *NheI*, *NcoI* and *XbaI* restriction enzyme sites, are shown. The locations at which the SV40 promoter and enhancer sequences are positioned in the pGL3-Promoter 8LexA Operator (P) and pGL3-Enhancer 8LexA Operator (E) vectors, respectively, are also indicated. (ATG represents start codon).

4.2.3 Detection of endogenous TP53 and proteins expressed by pTarget-Lexp53, pTarget-p53FL & pTarget-LexDBD

4.2.3.1 Transfection of DNA & preparation of cell lysates for western blotting

The pTarget-Lexp53, pTarget-p53FL and pTarget-LexDBD expression vectors were prepared using the QIAGEN EndoFree Plasmid Purification Maxi kit (2.2.8.3). Each vector was transfected, in duplicate, into each of the D17, CMT3, CMT7 and CMT8 cell lines using the Transfast™ reagent (Promega) (2.2.9).

These four cell lines were each cultured and maintained (2.1.1) and 16-24 hours prior to transfection occurring, seeded at the appropriate density (as determined in 4.3.1.1) per 25cm² flask. 3.25µg and 6.5µg of construct were transfected per flask (as determined in 4.3.1.2) of D17 cells and CMT3, CMT7 and CMT8 cells, respectively. One pair of flasks for each cell line was not transfected to act as negative controls and also enable the endogenous levels of TP53 to be determined. 48 hours after transfection, the cells in each pair of flasks were harvested in protein treatment sample buffer (2.3.1) and the protein concentration determined (2.3.2).

4.2.3.2 SDS-polyacrylamide gel electrophoresis & western blotting

Following protein concentration determination, SDS-PAGE was performed, in which 100µg of each protein sample were loaded per lane (2.3.3.1). Western blotting was subsequently carried out, using primary antibodies of rabbit antiserum raised against TP53 on the pTarget-Lexp53, pTarget-p53FL and non-transfected control samples and LexA on the pTarget-Lexp53, pTarget-LexDBD and non-transfected control samples (2.3.3).

4.2.4 Analysis of exogenous wild-type TP53 delivery

The pTarget-p53FL and pTarget-LexDBD expression vectors were used to investigate the effects of delivering the exogenous wild-type *TP53* into the 4 canine OS cell lines, D17, CMT3, CMT7 and CMT8. The 2 vectors were prepared using the QIAGEN EndoFree Plasmid Purification Maxi kit (2.2.8.3). Each vector

was transfected into the D17, CMT3, CMT7 and CMT8 cell lines using the Transfast™ reagent (Promega), in quadruplicate per time point (2.2.9). The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega; 2.2.10) was employed to determine cell numbers immediately before transfection (0 hour time point), and 12, 24, 48, 96 hours post-transfection for all four cell lines and at 144 hours for only D17 and CMT7 cells. This experiment was carried out in triplicate.

The four cell lines were each cultured and maintained (2.1.1) and 16-24 hours prior to transfection occurring, seeded at the appropriate density (as determined in 4.3.1.1) per well of a 96-well plate. 50ng and 100ng of construct were transfected per well (as determined in 4.3.1.2) of D17 cells and CMT3, CMT7 and CMT8 cells, respectively. 4 wells remained untransfected for each cell line (NTC - non-transfected cells) and 4 wells containing no cells for each cell culture medium (background) were used at each time point to act as negative controls. The cell culture medium was replaced after 72 hours with fresh medium.

The mean absorbance reading for each set of 4 wells was determined. The background readings were removed and the percentages of cell survival (+/- standard error (SE)), compared to the 0 hour time point for the NTC (100%), calculated.

4.2.5 Analysis of vectors required for TP53-targeted suicide gene therapy strategy using Dual-Luciferase® Reporter Assay System

The vectors used in the following Dual-Luciferase® Reporter Assay System (DLR) experiments were prepared using the QIAGEN EndoFree Plasmid Purification Maxi kit (2.2.8.3). They were transfected, in quadruplicate, into the D17, CMT3, CMT7 and CMT8 cell lines using the Transfast™ reagent (Promega; 2.2.9). The DLR experiments were performed 48 hours post-transfection, as directed by the manufacturer's instructions, except for the use of 50µl of each of the Luciferase Assay Reagent (LAR) II and Stop & Glo Reagent, instead of the indicated 100µl.

This was because no difference was observed in the production of luciferase activity by these 2 volumes, thereby increasing the cost efficiency of a DLR kit.

The four cell lines were each cultured and maintained (2.1.1) and 16-24 hours prior to transfection occurring, seeded at the appropriate density (as determined in 4.3.1.1) per well of a 96-well plate. 4 wells remained untransfected for each cell line (NTC - non-transfected cells) at each time point to act as negative controls.

The firefly luciferase (FFluc) and *Renilla* luciferase (*Rluc*) activities for each well were separately measured on a Dynex plate-reading luminometer. This enabled the mean FFluc activity (+/-SE), normalized for *Rluc* activity, to be determined for each set of 4 wells.

4.2.5.1 Optimization of Dual-Luciferase® Reporter Assay System

Preliminary experiments were performed in D17, CMT3, CMT7 and CMT8 cell lines, using the pGL3-Control vector (Promega; Appendix C, Figure C5) to assess which of the: two *Rluc* co-reporter vectors (both Promega; Appendix C, Figure C6), pRL-*TK* or pRL-cytomegalovirus (*CMV*); and at which of the 3 charge ratios, 5:1, 10:1 and 25:1, of pGL3-Control to co-reporter vector, were optimal for use in the DLR experiments, to enable transfection efficiency to be accounted for, allowing normalization of FFluc activity. The amounts of DNA of each vector transfected, per ratio, are displayed in Table 4.3A. The DLR optimization experiment was performed once.

4.2.5.2 Dual-Luciferase® Reporter Assay System experiments

Three DLR experiments were carried out, and each in duplicate, to analyse the vectors required for the TP53-targeted suicide gene therapy strategy:

- (i), pTarget-Lexp53 and pGL3-P8LA with pRL-*CMV* as co-reporter vector (ratio 5:1);
- (ii), pTarget-Lexp53 and pGL3-P8LA with pRL-*TK* as co-reporter vector (ratio 25:1);

(iii), pTarget-Lexp53 and pGL3-B8LA, pGL3-E8LA and pGL3-P8LA with pRL-*TK* as co-reporter vector (ratio 25:1).

The following vectors were required in some or all of the 3 DLR experiments: pRL-*TK*, pRL-*CMV*; pGL3-Control; pGL3-B8LA, pGL3-E8LA pGL3-P8LA; pTarget-Lexp53; pTarget-p53FL (as a means for producing a cellular environment possessing increased levels of TP53 - positive control); and pBLCAT6 (contains chloramphenicol acetyl transferase (*CAT*) gene and utilized as 'carrier DNA' due to its lack of promoter sequences, ensuring all wells were transfected with similar amounts of DNA, regardless of number of vectors transfected; Appendix C, Figure C7).

The vectors transfected into each set of wells for each DLR experiment were labelled as:

- (i), Carrier DNA - comprising pBLCAT6 and pRL-*CMV* or pRL-*TK*;
- (ii), Control - containing pGL3-Control, pBLCAT6 and pRL-*CMV* or pRL-*TK*;
- (iii), 8LA - comprising pBLCAT6, pGL3-8LA (B, E, or P) and pRL-*CMV* or pRL-*TK* (carried out due to the expectation of background FFluc activity as a result of basal promoter activity by pGL3-P8LA);
- (iv), p53-FL - containing pTarget-Lex-p53, pTarget-p53FL, pGL3-P8LA and pRL-*CMV* or pRL-*TK*
- (v), Lex-p53 - comprising pTarget-Lex-p53, pGL3-8LA (B, E or P) and pRL-*CMV* or pRL-*TK*.

The amounts of DNA transfected at a ratio of experimental DNA: co-reporter vector of 25:1, per combination of vectors, are displayed in Table 4.3B.

A, Optimization of DLR co-reporter vectors		Positive Control		Ratio					
				5:1		10:1		25:1	
Cell Line		D17	CMT3,7 & 8	D17	CMT3,7 & 8	D17	CMT3,7 & 8	D17	CMT3,7 & 8
Vector DNA (ng)	pGL3-Control	50	100	45	83	50	91	50	96
	pRL-CMV or pRL-TK	-	-	9	17	5	9	2	4
Total amount of DNA transfected (ng)		50	100	54	100	55	100	52	100

B, DLR experiments		Carrier		Control		8LA		p53-FL		Lex-p53	
Cell Line		D17	CMT3,7 & 8	D17	CMT3,7 & 8	D17	CMT3,7 & 8	D17	CMT3,7 & 8	D17	CMT3,7 & 8
Vector DNA (ng)	pBLCAT6	50	100	40	80	40	80	-	-	-	-
	pTarget-Lex-p53	-	-	-	-	-	-	20	40	40	80
	pTarget-p53FL	-	-	-	-	-	-	20	40	-	-
	pGL3-8LA (B, E or P)	-	-	-	-	10	20	10	20	10	20
	pGL3-Control	-	-	10	20	-	-	-	-	-	-
pRL-CMV or pRL-TK		2	4	2	4	2	4	2	4	2	4
Total amount of DNA transfected (ng)		52	104	52	104	52	104	52	104	52	104

Table 4.3: The amounts of DNA (in ng) and combinations of vectors transfected into D17, CMT3, CMT7 and CMT8 cells to enable the analysis of vectors required for TP53-targeted suicide gene therapy strategy using the Dual-Luciferase® Reporter Assay System (DLR): (A), Optimization of DLR co-reporter vectors, pRL-TK and pRL-CMV, in combination with pGL3-Control; (B), DLR experiments, in which sets of wells were labelled as ‘Carrier’, ‘Control’, ‘8LA’, ‘p53-FL’ and ‘Lex-p53’, and ratio of experimental DNA: co-reporter vector of 25:1, per combination of vectors, was utilized.

4.3 Results

4.3.1. Optimization assays

4.3.1.1 Cell number

The concentrations of cells required for the D17, CMT3, CMT7 and CMT8 cell lines to obtain 80% confluence were: D17 - 90000 cells/ml; CMT3 - 75000 cells/ml; CMT7 - 35000 cells/ml; and CMT8 - 75000 cells/ml. This enabled the same number of cells to be transfected for each cell line, in all the subsequent assays performed in this study, without any toxicity occurring due to the transfection of low cell densities (200 μ l/well of a 96-well plate; 13ml per 25cm² flask).

4.3.1.2 Transfection efficiency

The D17, CMT3, CMT7 and CMT8 cell lines appeared normal following the transfection of 50ng pSV- β -gal DNA, at the charge ratio of 1:1, for which the transfection efficiencies were calculated as: 18.1% D17 cells; 2.75% CMT3 cells; 1% CMT7 cells; and 0.5% CMT8 cells (Table 4.4). A greater percentage of cells were transfected using 100ng pSV- β -gal DNA at the ratio of 1:1: D17 cells, 27.9%; CMT3 cells, 19.7%; CMT7 cells, 10.0%; and CMT8 cells, 4.75%. However, a small number of the D17 cells were observed to have an abnormal visual appearance, in contrast all the CMT3, CMT7 and CMT8 cells appeared normal. Following the transfection of 150 and 200ng pSV- β -gal DNA into the four cell lines, at the charge ratio of 1:1, all the cells possessed an abnormal visual appearance. 100% cytotoxicity was observed in the D17, CMT3, CMT7 and CMT8 cells after the transfection of 50, 100, 150 and 200ng pSV- β -gal DNA at the ratio of 2:1, TransfastTM reagent to DNA.

Therefore, the amounts of plasmid DNA to be transfected and the ratio of TransfastTM to DNA in the subsequent experimental assays employing these four cell lines, were determined to be 50ng DNA per well (or 3.25 μ g per 25cm² flask) of D17 cells and 100ng DNA per well (or 6.5 μ g per 25cm² flask) of CMT3, CMT7 and CMT8 cells, at a ratio of 1:1 TransfastTM: DNA. These amounts enabled an acceptable level of transfection efficiency to be achieved for each cell line,

without appearing to compromise the normal visual appearance of these cells. Hence, any cytotoxicity induced in the experimental assays could be deemed to be as a result of the active construct/prodrug and not the components involved in the transfection protocol.

		% Cells Transfected									
		1:1				2:1					
Ratio of Transfast™: pSV-β-gal DNA											
Amount of pSV-β-gal DNA transfected (ng)		50	100	150	200	50	100	150	200		
Cell line	D17	18.1	27.9*	100% abnormal visual appearance				100% cytotoxicity			
	CMT3	2.75	19.7	100% abnormal visual appearance				100% cytotoxicity			
	CMT7	1	10	100% abnormal visual appearance				100% cytotoxicity			
	CMT8	0.5	4.75	100% abnormal visual appearance				100% cytotoxicity			

Table 4.4: The transfection efficiency (%) and the effect on the normal visual appearance of D17, CMT3, CMT7 and CMT8 cells following the transfection of 50, 100, 150 and 200 ng pSV-β-galactosidase control vector (pSV-β-gal) DNA at the charge ratios of transfection reagent (Transfast™) to DNA, 1:1 and 2:1 (* - small number of D17 cells possessed an abnormal visual appearance).

4.3.2 Detection of endogenous TP53 and expression of proteins by pTarget-Lexp53, pTarget-p53FL & pTarget-LexDBD

Endogenous TP53 was detected, using the CM-1 antibody (anti-p53), in the NTC lysates of CMT7 and CMT8 cells and possibly in CMT3 cells but not in D17 cells (Figure 4.10A). LexA immunopositivity was not detected, using the anti-LexA antibody, in the NTC lysates of all four cell lines (Figure 4.10B).

Immunopositivities of the full-length p53 and LexA DNA binding domain proteins employing the CM-1 (Figure 4.10C) and anti-LexA (Figure 4.10D) antibodies, respectively, were detected in the D17, CMT3, CMT7 and CMT8 cell lysates, after transfection of the pTarget-p53FL and pTarget-LexDBD constructs, respectively. Immunopositivity of the Lexp53 protein, following the transfection of the pTarget-Lexp53 construct, was detected with both antibodies in the cell lysates from all four cell lines (Figure 4.10 E & F). Faint bands indicated the possible detection of immunopositivity of the Lexp53 protein bound to endogenous TP53 in CMT3, CMT7 and CMT8 cell lysates, using the anti-LexA antibody (Figure 4.10E); however, no bands were visible employing the CM-1 antibody (Figure

4.10F). The Lexp53 protein was not detected bound to endogenous TP53, using either antibody, in the lysate from D17 cells (Figure 4.10 E & F).

These results demonstrate that the pTargetT-Lexp53, pTargetT-p53FL and pTargetT-LexDBD constructs can be successfully transfected into the D17, CMT3, CMT7 and CMT8 cell lines and the Lexp53, full-length TP53 and LexA DNA binding domain proteins expressed, respectively. The NTC lysates of CMT7 and CMT8 cells, and possibly CMT3 cells, are shown to possess endogenous TP53, whereas no endogenous TP53 is evident in the D17 cell lysate. There is also evidence that the Lexp53 protein can bind to the endogenous TP53 in the CMT3, CMT7 and CMT8 cell lines.

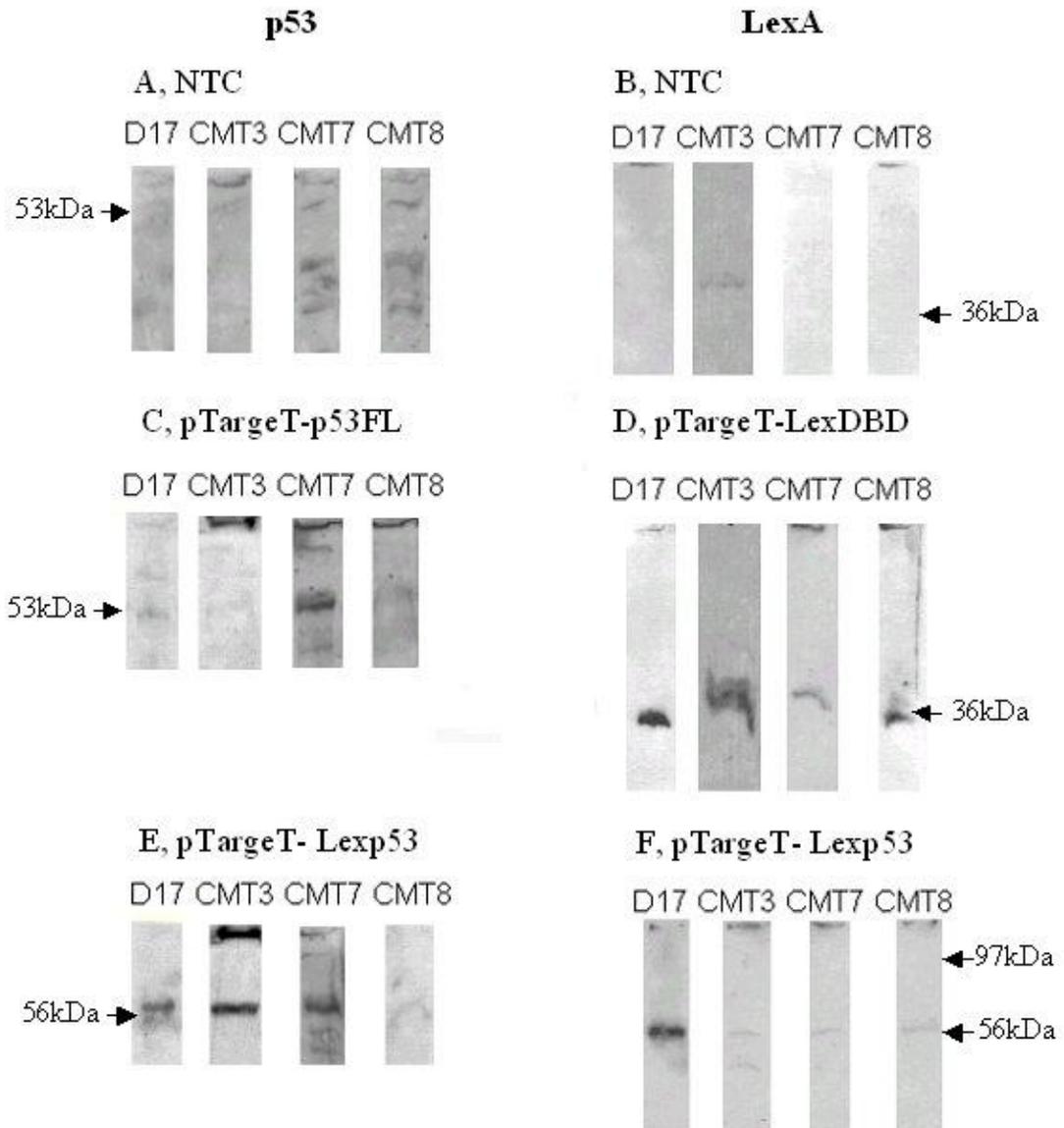


Figure 4.10: The chemiluminescent immunoblots obtained using anti-p53 (CM1) and anti-LexA primary antibodies, in cell lysates prepared from D17, CMT3, CMT7 and CMT8 cells, to detect the expression of: endogenous levels of TP53 (A) and LexA DNA binding domain (B) proteins in non-transfected cells (NTC); full-length p53 (53kDa) and LexA DNA binding domain (36kDa) proteins following the transfection of the pTargetT-p53FL construct (C) and the pTargetT-LexDBD construct (D), respectively; Lexp53 protein (56kDa) following the transfection of the pTargetT-Lexp53 construct (E & F; 97kDa indicates the possible detection of the Lexp53 protein bound to endogenous TP53). (100 μ g protein loaded per lane; black arrows indicate the position of the relevant protein).

4.3.3 Analysis of exogenous wild-type TP53 delivery

The survival curves of the pTarget-p53FL and pTarget-LexDBD transfected and NTC for each of the four cell lines are displayed in Figure 4.11. It can be observed that the expression of the full-length TP53 and the negative control protein, LexA DNA binding domain by pTarget-p53FL and pTarget-LexDBD, respectively, did not appear to reduce cell survival. Indeed, there are no statistically significant differences between the survival curves for the pTarget-p53FL and pTarget-LexDBD transfected cells and NTC for each cell line (Table 4.5).

Increases in cell survival seen at the 96-hour time point, were probably due to the cell culture medium being replaced with fresh medium at 72 hours.

Thus as the expression of the full-length TP53 by pTarget-p53FL was not observed to decrease the survival of D17, CMT3, CMT7 and CMT8 cells, a requirement was demonstrated for the investigation of a TP53-targeted suicide gene therapy strategy in these canine OS cell lines.

Treatment Comparison		D17	CMT3	CMT7	CMT8
NTC vs.	LexDBD	0.998	0.161	0.82	0.713
	p53FL	0.954	0.98	0.954	0.064
LexDBD vs.	p53FL	0.918	0.11	0.948	0.296

Table 4.5: p-values of post hoc pairwise comparisons of treatment groups in Figure 4.11, which were not significantly different, for the D17, CMT3, CMT7 and CMT8 cell lines (significance was set at $p = 0.05$).

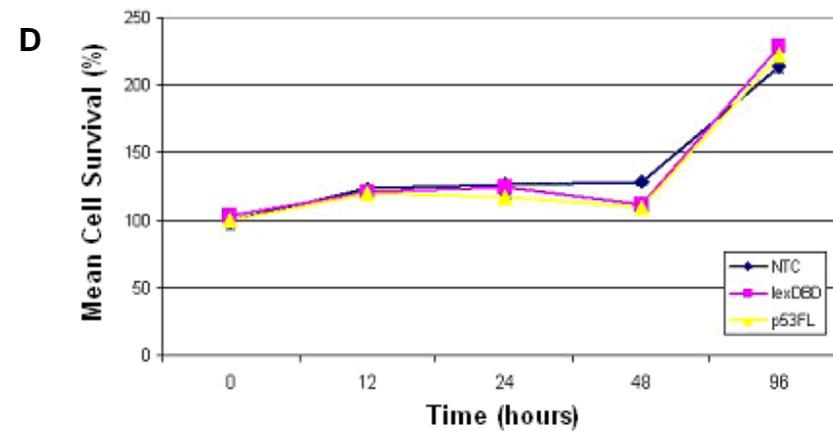
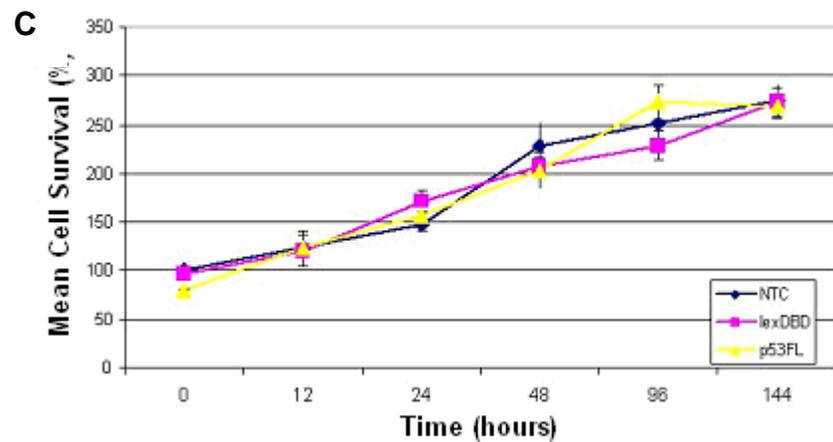
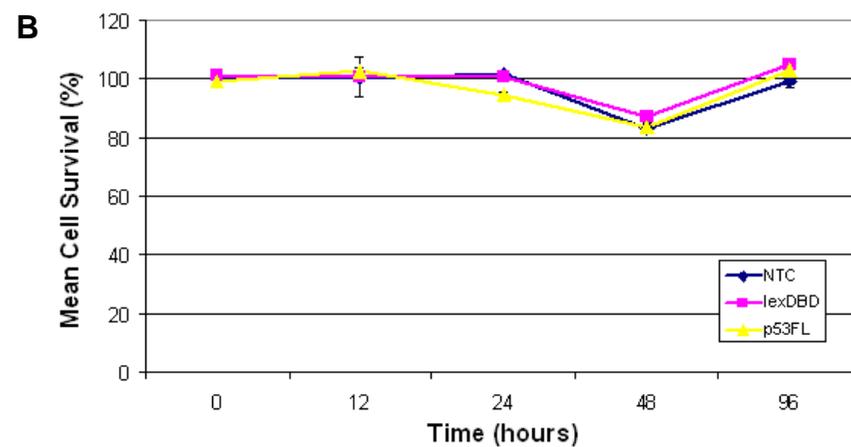
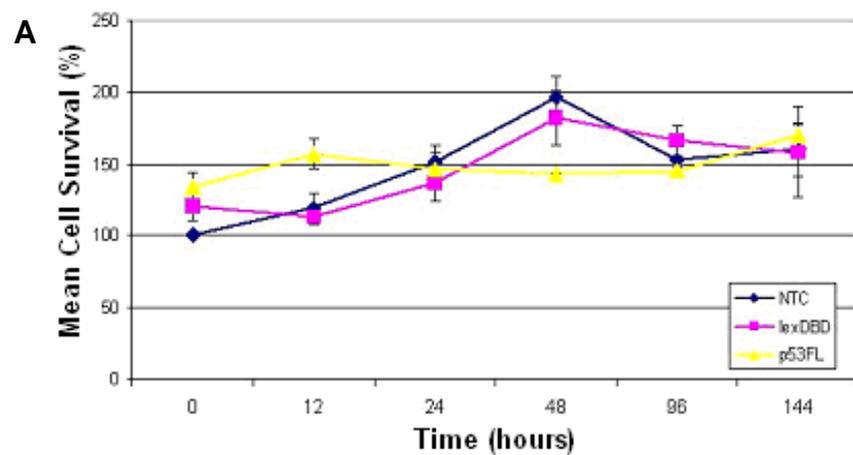


Figure 4.11: The effect of the expression of the full-length p53 and LexA DNA binding domain proteins following the transfection of the pTarget-p53FL (p53FL) and the pTarget-LexDBD (lexDBD) constructs, respectively, over a 96/144 hour time course on the D17 (A), CMT3 (B), CMT7 (C) and CMT8 (D) cell lines. ('NTC', non-transfected cells; each assay was repeated in triplicate and each time point in quadruplicate; bars represent +/-SE). Please note different scales on Y-axes.

4.3.4 Analysis of vectors required for TP53-targeted suicide gene therapy strategy using Dual-Luciferase® Reporter Assay System

4.3.4.1 Optimization of Dual-Luciferase® Reporter Assay System

The data from the D17, CMT3, CMT7 and CMT8 optimization experiments are summarized in Figure 4.12 A, B, C and D, respectively. The post hoc pairwise comparison results of *FFluc* values are displayed in Table 4.6. There were, unexpectedly, no statistically significant differences between the *FFluc* activities of the NTC and pGL3-Control transfected cells in any of the cell lines. However, as expected, the background *Rluc* activities of the NTC and pGL3-Control transfected cells were similar to each other. Although, there does appear to be two anomalous results in CMT3 cells: firstly, the *FFluc* activities of the NTC and cells transfected with pGL3-Control are greater than those cells transfected with pGL3-Control in combination with pRL-*TK* or pRL-*CMV*; and secondly, the *Rluc* activities of the NTC and cells transfected with pGL3-Control are higher than the activities produced by the cells transfected with pRL-*TK*, even though no co-reporter vector was transfected. It was realized at a later date that 50ng of pGL3-Control, instead of 100ng, and half the required amount of co-reporter vector, had accidentally been transfected into each of the six charge ratio experiments. In comparison, 100ng of pGL3-Control had been transfected into the cells with no co-reporter vector. Even though the incorrect amounts of pGL3-Control plus pRL-*TK* or pRL-*CMV* had been transfected, the charge ratios of pGL3-Control to pRL-*TK*/ pRL-*CMV* were correct. Hence, these anomalies can probably be explained, but the experiment should have been repeated, to confirm these explanations. In addition, the background *FFluc* and *Rluc* activities of CMT3 cells appear to be much higher than those of D17, CMT7 and CMT8 cells, and this should be investigated further.

The observed *Rluc* activities are greater, as expected, following transfection of pRL-*CMV* than pRL-*TK* in all four cell lines. This is due to pRL-*CMV* containing the *CMV* enhancer and early promoter elements, which are stronger than the *TK* promoter in pRL-*TK* and able to provide a higher-level expression of *Rluc* in co-transfected cells. However, anomalous results were observed in the CMT7 and CMT8 cells, as similar *Rluc* readings were observed for each pRL-*TK* ratio, suggesting that comparable amounts of pRL-*TK* had been transfected into these

cells. Furthermore, the activities of *Rluc* in the CMT8 cells appear to be lower than the *Rluc* reading for NTC indicating that pRL-*TK* might not have actually been transfected into these cells. These two experiments should have been repeated, to validate the *Rluc* activities obtained.

It can also be seen that, as expected, the activity of *Rluc* decreases as the ratio of pGL3-Control to pRL-*TK*/ pRL-*CMV* increases i.e. reduction in amount of co-reporter vector transfected resulted in a lower *Rluc* measurement, in the D17, CMT3, CMT7 and CMT8 cells, except for CMT7 and CMT8 cells transfected with pRL-*TK* (as previously described).

There was a statistically significant difference between *Ffluc* activities of D17 cells transfected with pGL3-Control on their own to those co-transfected with pRL-*TK* or pRL-*CMV* at 5:1, 10:1 and 25:1, even though similar amounts of pGL3-Control vector had been transfected in all cells. Although theoretically this should not occur, one of the primary objectives for carrying out this optimization assay was to ensure that independent genetic expression of the experimental and control reporter genes occurs, due to the reported possibility of a *trans* effect occurring with the use of a strong promoter, such as *CMV* (Farr & Roman 1992). Significant differences were also found in the CMT7 cells, between those cells transfected with pGL3-Control and those co-transfected with pRL-*CMV* at 5:1 and 10:1. In addition, a significant difference was observed between the CMT8 cells transfected with pGL3-Control and those co-transfected with pRL-*CMV* at 5:1. The likely reason for significant differences not being found between CMT7 and CMT8 cells transfected with pGL3-Control on their own to those co-transfected with pRL-*CMV* at 25:1 and 10:1 and 25:1, respectively, is also due to the error in which only 50ng of pGL3-Control, instead of 100ng, and half the required amount of co-reporter vector, albeit at the correct ratio, had been transfected. In addition, statistically significant differences cannot be assessed in the CMT3 cells and CMT7 and CMT8 cells between the cells only transfected with pGL3-Control to those co-transfected with pRL-*TK*, due to the discrepancies previously described.

It was observed that within the six charge ratio experiments of the D17 cells, the *Ffluc* activity of the cells transfected with pGL3-Control in combination with pRL-*TK* at 5:1 ratio were higher and statistically significantly different from all

the other ratios of pRL-*TK* and pRL-*CMV*, except pRL-*CMV* at 10:1. This was not expected as *TK* is a weaker promoter than *CMV* and theoretically, a stronger *trans* effect is more likely to occur with pRL-*CMV* than pRL-*TK*, due to *CMV* being the stronger promoter. The FFluc activity of the cells transfected with pGL3-Control in combination with pRL-*CMV* at 10:1 was also higher and significantly different to those transfected with pRL-*CMV* at 5:1 and 25:1. The higher FFluc activity of cells transfected with pRL-*CMV* at 10:1 to those at 5:1 is also unexpected, as theoretically lower readings should be obtained with weaker ratios in comparison to stronger ratios. In the CMT3 cells, there were no statistically significant differences between the CMT3 cells transfected with pGL3-Control in combination with pRL-*TK* or pRL-*CMV* at 5:1, 10:1 or 25:1. This shows that in the CMT3 cells, the FFluc activity produced by pGL3-Control is affected equally by pRL-*TK* and pRL-*CMV* and the ratio at which they are utilized. In the CMT7 cells transfected with pGL3-Control in combination with pRL-*CMV*, there was a statistically significant difference between FFluc activities of the cells transfected with pRL-*CMV* at 10:1 to those transfected with pRL-*CMV* at 25:1. This indicates that in the CMT7 cells, higher levels of *CMV*, may interact with the pGL3-Control vector to increase FFluc expression, due to the reported *trans* effect of the *CMV* reporter. In the CMT 8 cells transfected with pGL3-Control in combination with pRL-*CMV*, there were also no significant differences at 5:1, 10:1 or 25:1. Showing that the ratios of pRL-*CMV* in combination with pGL3-Control possess a similar effect on FFluc activity.

A second primary objective of this optimization assay was to determine relative light units produced by each co-reporter to enable reliable quantification. Although, it can be observed that the readings of FFluc activities in CMT3, CMT7 and CMT8 transfected with pGL3-Control in combination with pRL-*CMV* or pRL-*TK* are much lower than the equivalent D17 transfected cells, this is likely to be due to the transfection of 50ng of pGL3-Control instead of 100ng. It could be hypothesized that as ~ 7-, 10- and 9.5- fold increases in transfection efficiency were observed between 50ng and 100ng in CMT3, CMT7 and CMT8 cells, respectively, in 4.4.1, similar increases in FFluc activity would also be observed.

Overall, these results show, that even in light of the identified discrepancies, similar FFluc activities were generally observed between the cells transfected

with pGL3-Control in combination with pRL-CMV or pRL-TK at 5:1, 10:1 and 25:1. Hence, pRL-CMV could be used initially in the subsequent assay for normalising FFluc activity, and at a ratio of 5:1, as CMV is the stronger promoter and to ensure adequate levels of *Rluc* expression occur in all four cell lines.

Group	Treatment	D17	CMT3	CMT7	CMT8
1	NTC	All except 2	All except 2	6, 7	6
2	pGL3-Control	All except 1	All except 1	6, 7	6
3	5:1 TK	All except 7	1, 2		6
4	10:1 TK	1, 2, 3	1, 2		
5	25:1 TK	1, 2, 3	1, 2		
6	5:1 CMV	1, 2, 3, 7	1, 2	1, 2	1, 2, 3
7	10:1 CMV	1, 2, 6, 8	1, 2	1, 2, 8	
8	25:1 CMV	1, 2, 3, 7	1, 2	7	

Table 4.6: Post hoc pairwise comparisons of FFluc activities for the treatment groups in Figure 4.12, listing the treatment groups which were significantly different from each group for the D17, CMT3, CMT7 and CMT8 cell lines (significance was set at $p = 0.05$).

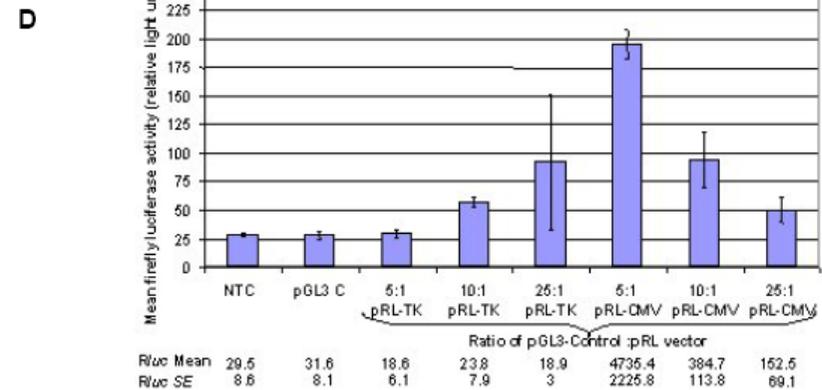
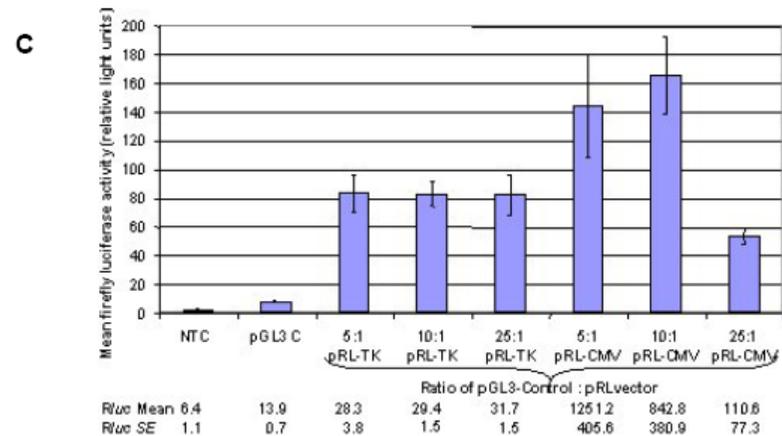
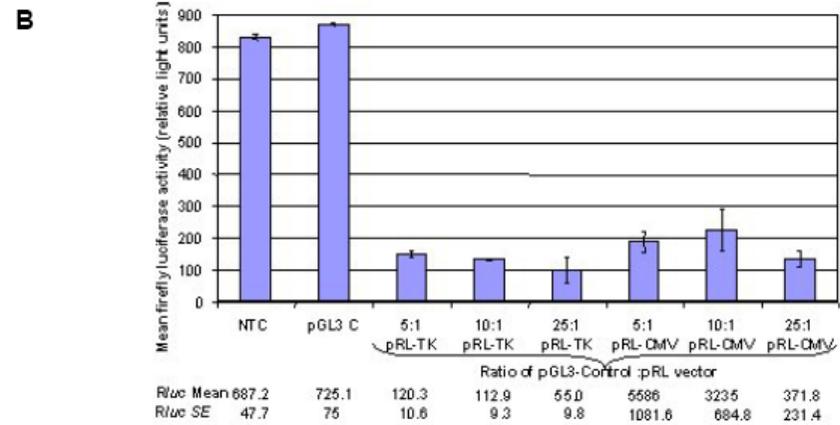
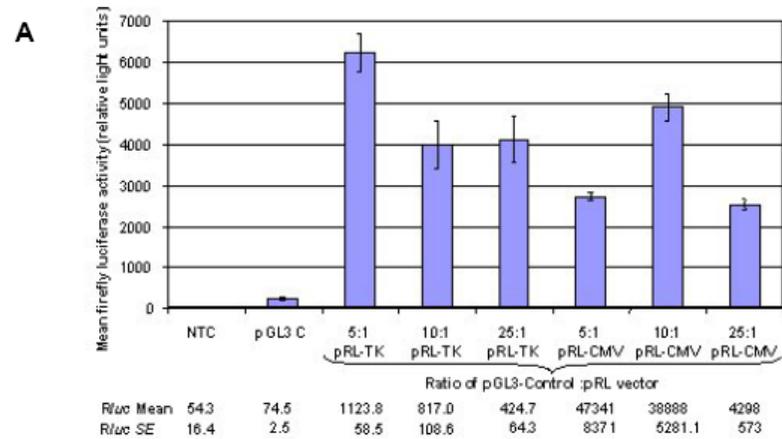


Figure 4.12: Optimization of Dual-Luciferase® Reporter Assay System to determine which of the two *Renilla* luciferase co-reporter vectors, pRL-*TK* or pRL-*CMV*, and ratio of experimental vector (in this assay pGL3-Control) to *Renilla* vector, 5:1, 10:1 and 25:1, were to be used in the Dual-Luciferase® Reporter Assay System experiments for the D17 (A), CMT3 (B), CMT7 (C) and CMT8 (D) cell lines. ‘NTC’ - non-transfected cells in which cells were only treated with cell culture medium. Each transfection was repeated in quadruplicate; bars represent +/-SE (Rluc mean and SE values are displayed at the bottom of each graph). Please note different scales on Y-axes.

4.3.4.2 Dual-Luciferase® Reporter Assay System experiments

4.3.4.2.1 Analysis of pTarget-Lexp53 and pGL3-Promoter 8LexA Operator with pRL-CMV as co-reporter vector

In this DLR assay, pTarget-Lexp53 in combination with pGL3-Promoter 8LexA Op (pGL3-P8LA) was assessed, using pRL-CMV as the co-reporter vector, in the D17, CMT3, CMT7 and CMT8 cell lines. The data from this experiment are summarized in Figure 4.13 and the post hoc pairwise comparison results of *FFluc* values are displayed in Table 4.7. It was observed in all four cell lines that the NTC had very low *FFluc* and *Rluc* (D17, 6.6 +/- 2.2 relative light units (RLU); CMT3, 3.0 +/- 0.2 RLU; CMT7, 3.9 +/- 0.4 RLU; CMT8, 5.6 +/- 0.5 RLU (+/-SE)) activities; of which the *FFluc* values were statistically significantly different to all the other treatments except for the D17 and CMT3 cells in the 'Carrier' experiment. *FFluc* values were measured in all the cells transfected with carrier DNA, although only pRL-CMV and pBLCAT6 had been transfected into them, however the *FFluc* readings were statistically significantly different to all the other treatments except for the D17 cells in 'Lex-p53' experiment. Variable *Rluc* activity was observed in all four cell lines (D17, 69693 +/- 7263 RLU; CMT3, 6620 +/- 1285 RLU; CMT7, 24974 +/- 3353 RLU; CMT8, 25744 +/- 2990 RLU (+/-SE)), demonstrating that normalization of *FFluc* activity was required. The cells treated in the 'Control' experiment produced relatively high *FFluc* readings, indicating that an acceptable percentage of the cells had been transfected and the assay was functioning correctly. The cells treated in the 'P8LA' experiment produced *FFluc* readings not statistically significantly different to those cells treated with 'Control', except for CMT7 cells, which is generally as anticipated due to pGL3-P8LA also possessing the strong SV40 promoter (only difference being that pGL3-Control also contains the SV40 enhancer). Finally, the 'p53FL' cells which had been transfected with pGL3-P8LA, pTarget-p53FL and pTarget-Lexp53, produced the highest *FFluc* readings, statistically significant different to all other treatments except for 'Lex-p53' in CMT7 cells, indicating that pTarget-p53FL was acting as required, as a positive control to show that the designed system could function correctly in the presence of increased levels of TP53 i.e. exogenous wild-type TP53 expressed by pTarget-p53FL was able to bind to Lexp53 protein expressed by pTarget-Lexp53 and in turn, the Lexp53 protein was able to bind to 8LexA operator site in pGL3-P8LA, thereby driving the

expression of *FFluc*. Hence, cells treated with 'p53FL' were suitable as a model for cells possessing accumulated TP53.

The cells treated with 'Lex-p53', to produce *FFluc* activity from pGL3-P8LA, would have to utilize the endogenous TP53 present in each cell line. It can be seen in D17, CMT3 and CMT8 cells that 'Lex-p53' *FFluc* activities in these cell lines are statistically significantly lower than those of 'p53-FL' treated cells. In addition the *FFluc* values of the 'Lex-p53' treated cells in the D17, CMT3 and CMT8 cells were not statistically significantly different to the cells treated with '8LA'. It could be proposed that as pGL3-P8LA is a component of the 'Lex-p53' treatment, the *FFluc* activities measured were mainly due to pGL3-P8LA. In contrast in the CMT7 cells, there is no statistically significant difference between the cells treated with 'Lex-p53' and 'p53FL' but there is a difference between 'Lexp53' and 'P8LA'. These results indicate that generally the endogenous TP53 levels within each cell line are able to control the amount of *FFluc* expressed by this TP53-targeted gene therapy system. This is because D17 cells possess wild-type *TP53* and CMT3 cells although containing mutated *TP53* (Table 2.1), both display none or very low levels of endogenous TP53, as previously shown in Figure 4.10. In comparison, CMT7 cells, contain mutated *TP53* and express higher levels of endogenous TP53. However, the CMT8 cells although possessing mutated *TP53* and demonstrating equivalent endogenous TP53 levels as that of CMT7 cells in Figure 4.10, did not appear to upregulate *FFluc* expression. Hence, the low levels of TP53 present in the D17, and CMT3 cells, provide little or nothing for the Lex-p53 protein to bind with, resulting in lower expression levels of *FFluc*. In comparison, the higher levels of endogenous TP53 in the CMT7 cells interact with the Lexp53 protein, producing increased levels of *FFluc* expression from pGL3-P8LA.

Thus, as pRL-CMV produced high levels of *Rluc* expression in this experiment, the possibility of reducing the occurrence of any *trans* effect, particularly in the CMT7 cells, was able to be investigated in the next experiment, in which pRL-TK was employed instead of pRL-CMV, and at a ratio of 25:1 (vector DNA:pRL-TK).

Group	Treatment	D17	CMT3	CMT7	CMT8
1	NTC	All except 2	All except 2	All other treatments	All other treatments
2	Carrier	1, 3, 4, 5	All except 1	All other treatments	All other treatments
3	Control	1, 2, 5	1, 2, 5	All except 4	All except 4
4	P8LA	1, 2, 5	1, 2, 5	All except 3	1, 2, 5
5	p53FL	All other treatments	All other treatments	All except 6	All other treatments
6	Lexp53	1, 5	1, 2, 5	All except 5	All except 4

Table 4.7: Post hoc pairwise comparisons of treatment groups in Figure 4.13, listing the treatment groups which were significantly different from each group for the D17, CMT3, CMT7 and CMT8 cell lines (significance was set at $p = 0.05$).

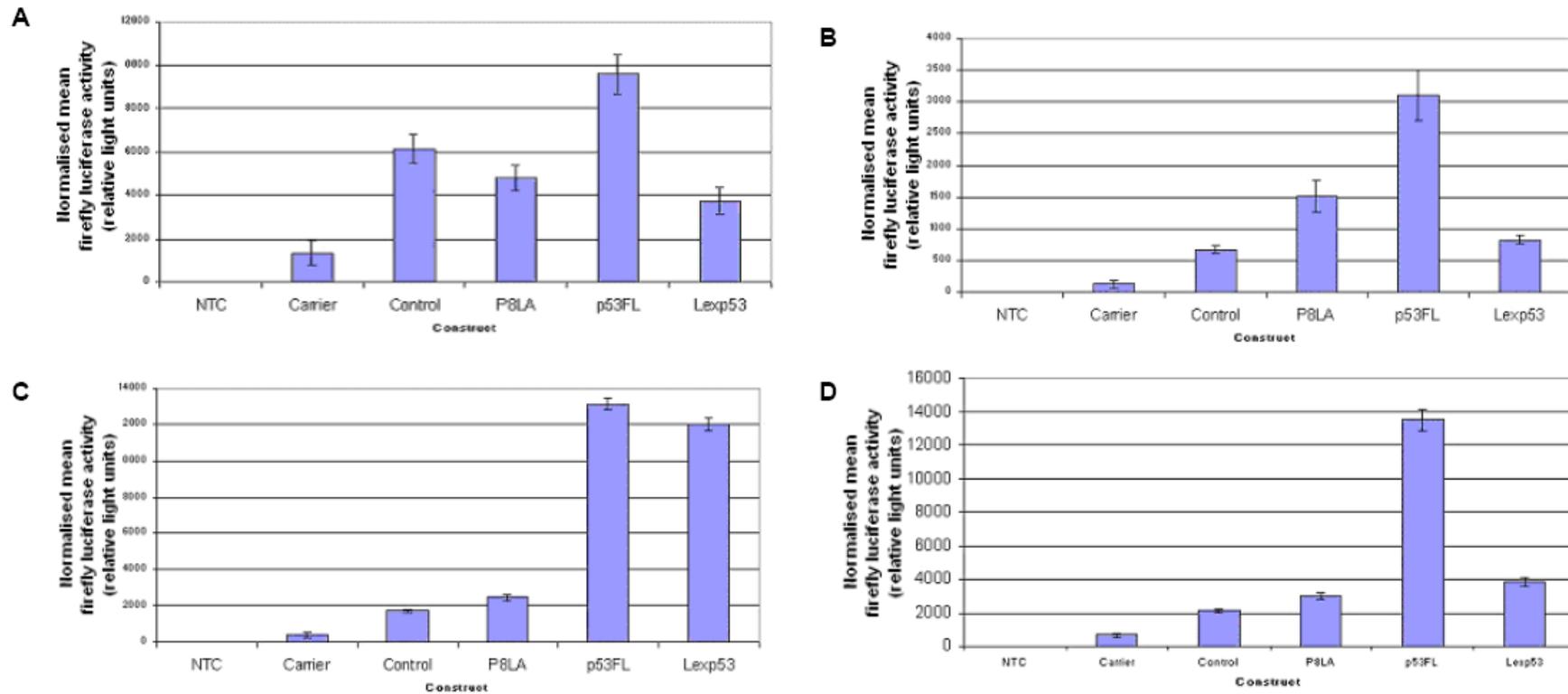


Figure 4.13: Dual-Luciferase® Reporter analysis of pTargetT-Lexp53 (Lexp53) and pGL3-Promoter 8LexA Op (P8LA) with pRL-CMV as co-reporter vector in the D17 (A), CMT3 (B), CMT7 (C) and CMT8 (D) cell lines. ('NTC' - non-transfected cells; 'Carrier DNA' - pBLCAT6 and pRL-CMV; 'Control' - pGL3-Control, pBLCAT6 and pRL-CMV; 'P8LA' - pBLCAT6, pGL3-P8LA and pRL-CMV; 'p53-FL' - pTargetT-Lex-p53, pTargetT-p53FL, pGL3-P8LA and pRL-CMV; 'Lex-p53' - pTargetT-Lex-p53, pGL3-P8LA and pRL-CMV). Firefly luciferase activity was normalized using pRL-CMV. Each assay (4 replicates/transfection) was repeated in duplicate; bars represent +/-SE. Please note different scales on Y-axes.

4.3.4.2.2 Analysis of pTargetT-Lexp53 and pGL3-Promoter 8LexA Op with pRL-TK as co-reporter vector

In this DLR assay, pTargetT-Lexp53 in combination with pGL3-Promoter 8LexA Op (pGL3-P8LA) was assessed, using pRL-*TK* as the co-reporter vector, in the D17, CMT3, CMT7 and CMT8 cell lines. The data from this experiment are summarized in Figure 4.14 and the post hoc pairwise comparison results of *FFluc* values are displayed in Table 4.8. It was observed in all four cell lines that the NTC had extremely low *FFluc* and *Rluc* (D17, 2.5 +/- 0.1 RLU; CMT3, 2.8 +/- 0.1 RLU; CMT7, 2.6 +/- 0.1 RLU; CMT8, 3.0 +/- 0.2 RLU (+/-SE)) activities; of which the *FFluc* values were statistically significantly different to all the other treatments except for the cells treated with 'Carrier'. *FFluc* was measured in all the cells transfected with 'Carrier', although only *Rluc* had been transfected into them, however the *FFluc* readings were statistically significantly different to all the other treatments except for the CMT3 cells in 'p53FL' and 'Lex-p53' experiments. The cells treated in the 'Control' experiment had high *FFluc* values, indicating that an acceptable percentage of the cells had been transfected and the assay was functioning correctly. Finally, in comparison to the previous DLR assay (4.3.4.2.1) in which the 'p53FL' cells had produced the highest *FFluc* readings, in this assay the 'p53FL' transfected cells produced *FFluc* values statistically significantly lower than cells treated with 'P8LA' in D17, CMT7 and CMT8 experiments. As pGL3-P8LA is a component of the 'p53FL' treatment it could be suggested that the *FFluc* activities measured were mainly due to pGL3-P8LA. This implies that pTargetT-p53FL may not have been expressing TP53 as required to increase levels of TP53 present or the use of pRL-*TK* affected the expression of *FFluc*. It was also observed that the expression of *Rluc* was, as expected, much lower from pRL-*TK* (D17, 314 +/- 21 RLU; CMT3, 116 +/- 18 RLU; CMT7, 134 +/- 18 RLU; CMT8, 137 +/- 11 RLU (+/-SE)), than the expression previously observed using pRL-*CMV*, as *TK* is a weaker promoter than *CMV*. In addition, *TK* was likely to have a reduced effect on the promoters in the other transfected constructs, in comparison to *CMV*, including those in the 'pTargetT-p53FL' vector, which contains both the *CMV* and SV40 enhancers and promoters.

Similar to the previous DLR assay (4.3.4.2.1), the cells treated with ‘Lex-p53’ would have to utilize the endogenous TP53 levels present in each cell line to produce FFluc activity from pGL3-P8LA. It can be seen in D17 cells that ‘Lex-p53’ FFluc activity is not statistically significantly different than ‘p53FL’ treated cells but is significantly lower than ‘P8LA’ treated cells. In the CMT3 and CMT8 cells, the ‘Lex-p53’ FFluc activities are not statistically significantly different to those of ‘p53-FL’ and ‘P8LA’ treated cells. In comparison, in the CMT7 cells, the ‘Lex-p53’ FFluc activities are statistically significantly higher to those of ‘p53-FL’ and ‘P8LA’ treated cells. The lower FFluc expression levels observed in the D17 and CMT3 cells are as a result of these cells possessing low levels of endogenous TP53 and the higher FFluc expression levels in the CMT7 cells, due to the possession of higher levels of endogenous TP53. Again, the CMT8 cells did not appear to increase FFluc expression levels. These results are generally in agreement with the findings made in the previous assay using pRL-CMV (4.3.4.2.1.2).

The aim of decreasing the expression of *Rluc* to lower levels was achieved using the pRL-*TK* vector and, it was hoped that any *trans* effect would also be reduced. It was also noted that the FFluc expression levels obtained in each cell line were much lower than that observed in the previous assay, however similar overall findings were obtained. Hence, the pRL-*TK* co-reporter could be used in the subsequent DLR experiment, in which the possibility of reducing the background FFluc expression by ‘P8LA’ was investigated using the pGL3-Enhancer 8LexA Op and pGL3-Basic 8LexA Op constructs.

Group	Treatment	D17	CMT3	CMT7	CMT8
1	NTC	All except 2	All except 2	All except 2	All except 2
2	Carrier	All except 1	3, 4	All except 1	All except 1
3	Control	All other treatments	1, 2, 5	All except 5	1, 2
4	P8LA	All other treatments	1, 2	All other treatments	1, 2, 5
5	p53FL	All except 6	1, 3	All except 3	1, 2, 4
6	Lexp53	All except 5	1	All other treatments	1, 2

Table 4.8: Post hoc pairwise comparisons of treatment groups in Figure 4.14, listing the treatment groups which were significantly different from each group for the D17, CMT3, CMT7 and CMT8 cell lines (significance was set at $p = 0.05$).

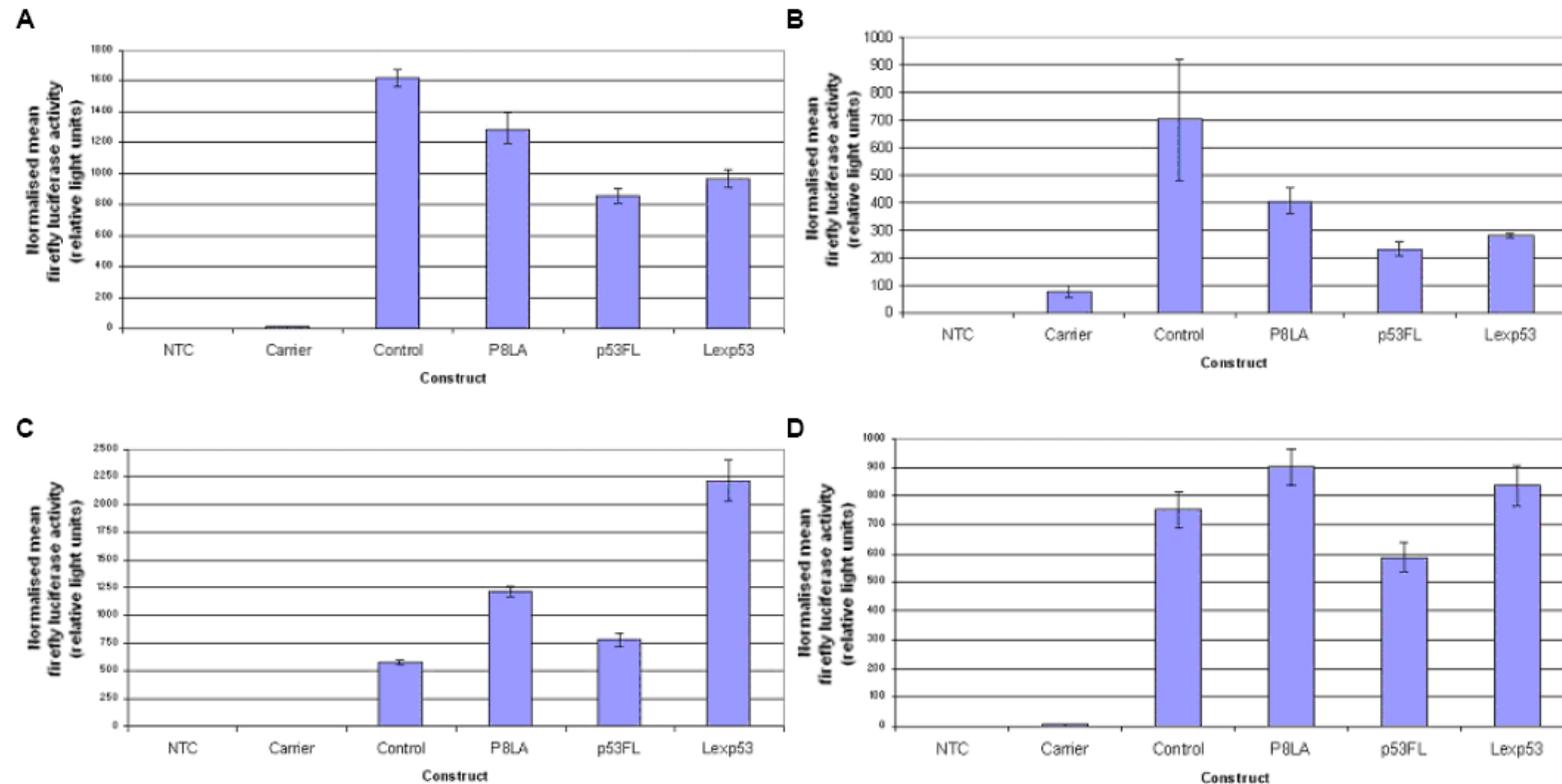


Figure 4.14: Dual-Luciferase® Reporter analysis of pTargetT-Lexp53 (Lexp53) and pGL3-Promoter 8LexA Op (P8LA) with pRL-*TK* as co-reporter vector in the D17 (A), CMT3 (B), CMT7 (C) and CMT8 (D) cell lines. ('NTC' - non-transfected cells; 'Carrier DNA' - pBLCAT6 and pRL-*TK*; 'Control' - pGL3-Control, pBLCAT6 and pRL-*TK*; 'P8LA' - pBLCAT6, pGL3-P8LA and pRL-*TK*; 'p53-FL' - pTargetT-Lex-p53, pTargetT-p53FL, pGL3-P8LA and pRL-*TK*; 'Lex-p53' - pTargetT-Lex-p53, pGL3-P8LA and pRL-*TK*). Firefly luciferase activity was normalized using pRL-*TK*. Each assay (4 replicates/transfection) repeated in duplicate; bars represent +/-SE. Please note different scales on Y-axes.

4.3.4.2.3 Analysis of pTarget-Lexp53 and pGL3-Basic 8LexA Op, pGL3-Enhancer 8LexA Op and pGL3-Promoter 8LexA Op with pRL-*TK* as co-reporter vector

The data from this experiment are summarized in Figure 4.15 and the post hoc pairwise comparison results of *FFluc* values are displayed in Table 4.9. It was observed in the D17, CMT3, CMT7 and CMT8 cell lines that the NTC had minimal *FFluc* and *Rluc* (D17, 6.5 +/- 0.5 RLU; CMT3, 6.5 +/- 0.2 RLU; CMT7, 10.0 +/- 2.4 RLU; CMT8, 8.8 +/- 1.3 RLU (+/-SE)); activities; of which the *FFluc* values were statistically significantly different to all the other treatments except for the CMT7 cells treated with 'Carrier'. *FFluc* was measured in all the cells transfected with 'Carrier', although only *Rluc* had been transfected into them, however the *FFluc* readings were statistically significantly different to all the other treatments except for the D17 cells treated with 'B8LA'. It was also observed that the expression of *Rluc* from pRL-*TK* in this experiment (D17, 349 +/- 62 RLU; CMT3, 268 +/- 47 RLU; CMT7, 190 +/- 34 RLU; CMT8, 180 +/- 32 RLU (+/-SE)), was similar to that in the previous assay using pRL-*TK* as the co-reporter. High *FFluc* values were obtained in the cells treated in the 'Control' experiment, indicating that an acceptable percentage of the cells had been transfected and the assay was functioning correctly. The cells treated with 'B8LA' produced statistically significantly lower *FFluc* values than cells treated with 'E8LA' or 'P8LA' except for 'E8LA' treated cells in CMT3 cells. Similarly 'E8LA' treated cells gave statistically significantly lower *FFluc* readings than 'P8LA' treated cells. Thereby demonstrating that the background *FFluc* activity of pGL3-P8LA can be reduced, although it is at the expense of an overall reduction in the level of *FFluc* expressed. There were no statistically significant differences between the cells treated with 'B8LA+Lexp53' and 'E8LA+Lexp53', showing that in the 'Lexp53 setting' pGL3-B8LA and pGL3-E8LA constructs behave similarly. However there are statistically significant differences between the cells treated with 'P8LA+Lexp53' and those with 'B8LA+Lexp53' and 'E8LA+Lexp53', except in CMT3 cells between 'P8LA+Lexp53' and 'E8LA+Lexp53', showing that greater activity is achieved with a construct containing a promoter sequence.

The D17 and CMT3 cells treated with 'B8LA+Lexp53', 'E8LA+Lexp53' or 'P8LA+Lexp53' did not produce statistically significantly higher *FFluc* readings

than their respective counterparts treated without 'Lexp53', except in the 'B8LA' treated D17 cells. Whereas there is a statistically significant difference between the CMT7 cells treated with 'B8LA+Lexp53', 'E8LA+Lexp53' or 'P8LA+Lexp53' and those without 'Lexp53'. In the CMT8 cells, there is also a statistically significant difference between the cells treated with 'B8LA+Lexp53' or 'E8LA+Lexp53' and those without 'Lexp53' but not between those treated with 'P8LA+Lexp53'. This indicates that in the D17 and CMT3 cell lines, as they possess low levels of TP53, FFluc expression is unable to be increased, whereas the CMT7 cells containing high levels of accumulated TP53 are able to increase FFluc expression. These findings are in agreement with the previous DLR experiments. However, there is evidence in this experiment that, in the absence of the SV40 promoter in the pGL3-B8LA and pGL3-E8LA constructs, the levels of TP53 present in CMT8 cells may be able to significantly increase the expression of FFluc. But in the presence of SV40 promoter, this significance is lost in the CMT8 cells regardless of endogenous TP53 levels, probably due to the inherent strength of the SV40 promoter in pGL3-P8LA to drive FFluc expression.

Group	Treatment	D17	CMT3	CMT7	CMT8
1	NTC	All other treatments	All other treatments	All except 2	All other treatments
2	Carrier	All except 4	All other treatments	All except 1	All other treatments
3	Control	1, 2, 4, 5, 7, 8	1, 2, 4, 5, 7, 8	All except 6	1, 2, 4, 5, 7, 8
4	B8LA	All except 2	1, 2, 3, 6, 8, 9	All other treatments	All other treatments
5	E8LA	1, 2, 3, 4, 6, 9	1, 2, 3, 6, 9	All other treatments	All except 7
6	P8LA	1, 2, 4, 5, 7, 8	1, 2, 4, 5, 7, 8	All except 3	1, 2, 4, 5, 7, 8
7	B8LA+Lexp53	1, 2, 3, 4, 6, 9	1, 2, 3, 6, 9	All except 8	1, 2, 3, 4, 6, 9
8	E8LA+Lexp53	1, 2, 3, 4, 6, 9	1, 2, 3, 4, 6	All except 7	All except 7
9	P8LA+Lexp53	1, 2, 4, 5, 7, 8	1, 2, 4, 5, 7	All other treatments	1, 2, 4, 5, 7, 8

Table 4.9: Post hoc pairwise comparisons of treatment groups in Figure 4.15, listing the treatment groups which were significantly different from each group for each of the D17, CMT3, CMT7 and CMT8 cell lines (significance was set at $p = 0.05$).

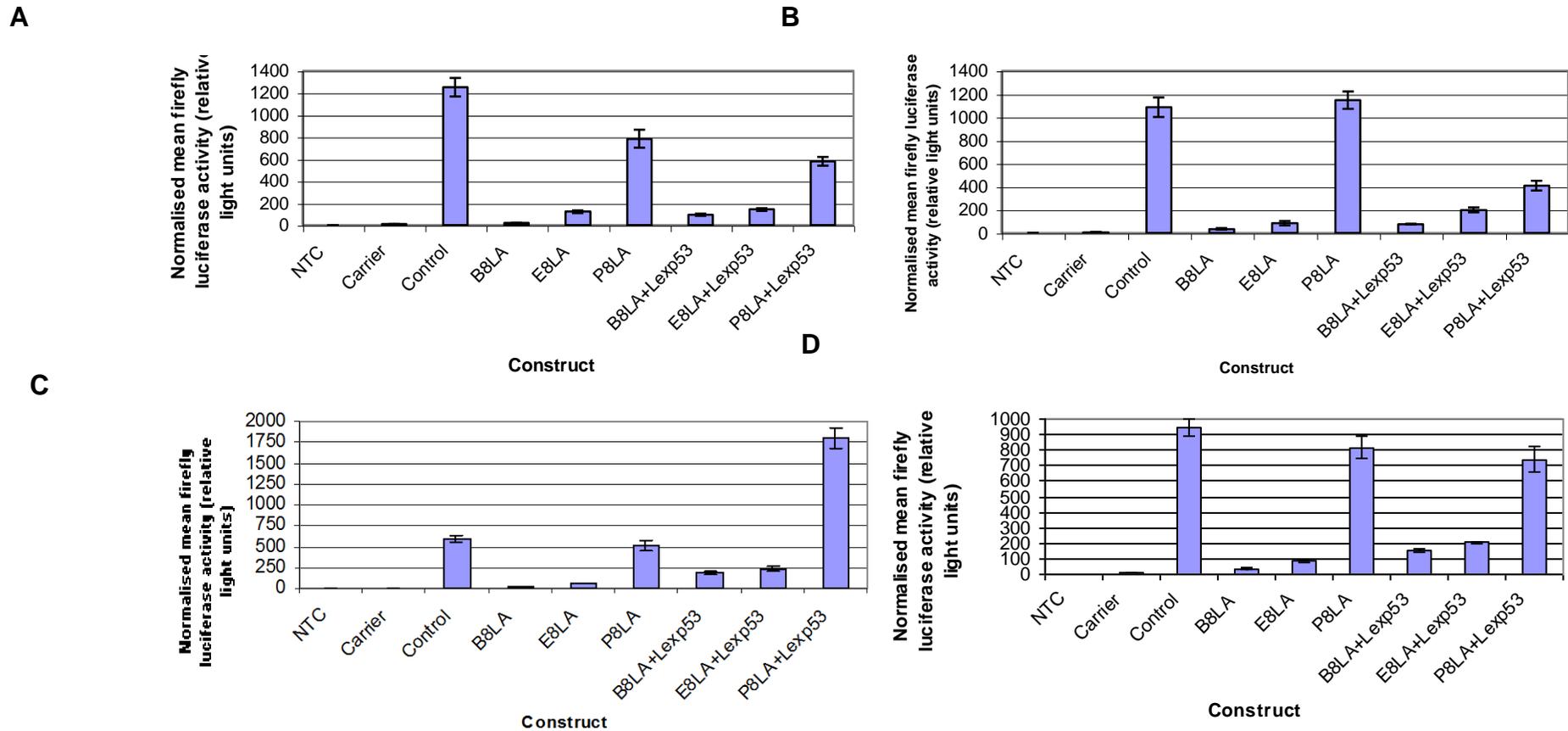


Figure 4.15: Dual-Luciferase® Reporter analysis of pTargetT-Lexp53 (Lexp53) in combination with either pGL3-Basic 8LexA Op (B8LA), pGL3-Enhancer 8LexA Op (E8LA), or pGL3-Promoter 8LexA Op (P8LA), and pRL-*TK* as the co-reporter in the D17 (A), CMT3 (B), CMT7 (C) and CMT8 (D) cell lines. ('NTC' - non-transfected cells; 'Carrier DNA' - pBLCAT6 and pRL-*TK*; 'Control' - pGL3-Control, pBLCAT6 and pRL-*TK*; 'B8LA' - pBLCAT6, pGL3-B8LA and pRL-*TK*; 'E8LA' - pBLCAT6, pGL3-E8LA and pRL-*TK*; 'P8LA' - pBLCAT6, pGL3-P8LA and pRL-*TK*; 'B8LA+Lex-p53' - pGL3-B8LA, pTargetT-Lex-p53 and pRL-*TK*; 'E8LA+Lex-p53' - pGL3-E8LA, pTargetT-Lex-p53 and pRL-*TK*; 'P8LA+Lex-p53' - pGL3-P8LA, pTargetT-Lex-p53 and pRL-*TK*). Firefly luciferase activity was normalized using pRL-*TK*. Each assay (4 replicates/transfection) repeated in duplicate; bars represent +/-SE. Please note different scales on Y-axes.

4.4 Discussion

4.4.1 Detection of endogenous TP53 and expression of proteins by pTargetT-Lexp53, pTargetT-p53FL & pTargetT-LexDBD

The CM-1 rabbit-origin polyclonal antibody is raised against recombinant human wild-type TP53 produced in a bacterial expression system (Bartek *et al.*, 1991). CM-1 recognizes, in humans, a range of TP53 epitopes, including those present in wild-type and several forms of mutant TP53 (Aprikian *et al.*, 1994; Auvinen *et al.*, 1994). The CM-1 antibody has been used previously to successfully detect both wild-type and accumulated canine TP53 in a number of different types of neoplasms, including OS (Gamblin *et al.*, 1997; Sagartz *et al.*, 1996). It was demonstrated in this study that it is also able to recognize the canine wild-type TP53 expressed by pTargetT-p53FL and detect the presence of endogenous TP53 in the NTC lysates of CMT7 cells, CMT8 cells and possibly in CMT3 cells; endogenous TP53 was not detected in D17 cells. Since the half-life of mutant TP53 is long (up to 12 hours) and wild-type TP53 half-life is comparatively short (30 minutes), it is assumed that the amount of wild-type TP53 stained by CM-1 is negligible (Jaffe *et al.*, 2000). Immunopositivity is often accepted as evidence of an underlying TP53 abnormality, because there is a strong positive correlation between immunopositivity and the possession of missense base substitutions in TP53 as previously discussed in 1.3.3.3 (Gamblin *et al.*, 1997; Haga *et al.*, 2001; Hall & Lane 1994). Hence, it can be proposed that CMT7 and CMT8 cells, and maybe CMT3 cells, possess accumulated TP53, and D17 cells do not. These results are in agreement with a number of studies. DNA sequence analyses of TP53 from each of the cell lines CMT7, CMT8 and CMT3 have been shown to contain missense mutations (Hellmen 1992; Van Leeuwen *et al.*, 1996), and the sequence in D17 cells found to be that of wild-type TP53 (Sabine *et al.*, 1999).

The transfected pTargetT-Lexp53 construct was detected, with both CM-1 and anti-LexA antibodies, in the cell lysates from the four cell lines, which indicates that CM-1 is able to bind to the C-terminal domain of TP53 present in pTargetT-Lexp53. Although no bands were visible using the CM-1 antibody to detect pTargetT-Lexp53 binding to accumulated endogenous TP53 in CMT3, CMT7 and CMT8 cells. There is possible evidence that this interaction can be detected as faint bands were detected in the immunoblot, in the expected region of 97kDa,

using the anti-LexA antibody. This result may be due to the length of time the endogenous TP53 is bound to the C-terminal domain of TP53 in pTarget-Lexp53 as the on/off rate of TP53 DNA binding is in the order of seconds (Bargonetti *et al.*, 1992). At any one point in time only a percentage will be bound and the level of detection will depend on the number bound. Hence, the number of bound molecules was probably below the detection limit of the ECL™ western blotting analysis system (Amersham Pharmacia Biotech). No binding was detected in the D17 cell lysates, as expected, as these cells contain wild-type TP53, which has a short half-life.

An improvement in further studies would be to determine if binding could be detected by using a more sensitive chemiluminescent method. Although, the ECL™ western blotting analysis system is well-publicized and can detect down to low-picogram (10^{-12}) quantities, kits are commercially available that possess detection limits of zeptomole amounts (10^{-20}) e.g. ECL Advance Western Blotting Detection Kit (Amersham Biosciences), and SuperSignal® West Femto Maximum Sensitivity Substrate Kit (PIERCE). In addition, the cell lysates in this study were prepared using a standard protocol, which may be suitable for enabling the detection of stable protein expression but not necessarily for transient binding. Thus, this method should be optimized for the detection of p53-p53 protein interactions. Furthermore, the expression of Lexp53, full-length TP53, and LexA DNA binding domain proteins were determined after a single time point (48 hours post-transfection), hence, it would be worthwhile investigating the expression levels of these proteins over a time course.

4.4.2 Analysis of exogenous wild-type TP53 delivery

The expression of full-length p53 by pTarget-p53FL did not reduce the survival of the D17, CMT3, CMT7 and CMT8 cells employed in this study. Acceptable levels of transfection efficiency were deemed to be achieved for each cell line (D17 cells, 18.1%; CMT3 cells, 19.7%; CMT7 cells, 10.0%; and CMT8 cells, 4.75%). These percentages are comparable to those previously reported using cationic liposomes to deliver β -gal expression plasmid into human OS cells (11.5-17%) (Nakase *et al.*, 2005b), and wild-type TP53 into human HNSCC (5-20%) (Xu *et al.*,

1997). These 4 cell lines were used in this study as they were the only canine OS cell lines available in-house at the time of the study. To the best of my knowledge, the introduction of exogenous wild-type TP53 has only been previously reported in D17 cells, in one publication, in which D17 cells were stably transfected with wild-type *TP53* and decreases in cell survival were achieved above radiation doses of 1 Gy (Shiomitsu *et al.*, 2008). Hence, it appears that in this study by Shiomitsu *et al.*, (2008), the upregulation of TP53 expression also had no inhibitory effect, by itself, on the growth of D17 cells. The results obtained in this study and by Shiomitsu *et al.*, (2008), are in contrast to two other studies investigating the delivery of wild-type *TP53* in canine OS cells, in which cell proliferation was found to be inhibited and apoptosis induced *in vitro* (Yazawa *et al.*, 2003b) and *in vivo* (Kanaya *et al.*, 2005). However, a total of 3 different canine OS cell lines were utilized in these 2 studies and the 2 cell lines in the *in vitro* studies were TP53 null (*in vivo* study contained a mutant TP53 cell line). Contrasting results have also been demonstrated in human OS *in vitro* and *in vivo* studies, in which the restoration of wild-type TP53 has proved to be both ineffective in some cells lines, possessing wild-type and mutant TP53, but in others, either TP53 null or containing wild-type or mutant TP53, cell proliferation was reduced and apoptosis induced (Densmore *et al.*, 2001; Endo *et al.*, 2001; Nakase *et al.*, 2005a; Oshima *et al.*, 2007). Hence, showing that the differences in the results obtained in canine OS cell lines, including those in this study, are in agreement with those previously published in human OS *in vitro* studies. Furthermore, the delivery of wild-type *TP53* into human OS cells only inhibited cell proliferation and induced apoptosis in some studies, if it was introduced in combination with chemotherapy (Endo *et al.*, 2001; Ganjavi *et al.*, 2006).

Several explanations have been proposed for these varying results. Firstly, differences observed may be due to the small number of OS cell lines studied, particularly canine cell lines. Secondly, variations in experimental conditions employed. For example, previous studies using adenoviral constructs have reported dose-dependent, p53-specific effects as higher levels of *TP53* transfer and expression correlated with a greater efficiency in inhibiting DNA synthesis of infected cells (Harris *et al.*, 1996; Hellwinkel *et al.*, 2005; Wills *et al.*, 1994). Additionally, the cellular level of transduced wild-type *TP53* dictates the

response of the cell because *in vitro*, p53-induced apoptosis seems to occur only above a certain percentage of transfected cells (Gomez-Manzano *et al.*, 1996). Thus, the amount of pTarget-p53FL introduced and/or wild-type TP53 expressed, following delivery of pTarget-p53FL into D17, CMT3, CMT7 and CMT8 cells, may not have been sufficient to have an inhibitory effect on cell proliferation. In addition, the half-life of full-length TP53 expressed by pTarget-p53FL was unknown, thereby requires investigation. Although the optimal amount of pTarget-p53FL was delivered into the four cell lines using the Transfast™ transfection reagent, a cationic liposomal based method, improvements in further studies would be to investigate alternative methods of gene delivery or increase the transfection efficiency gained using this method. This could be achieved with regard to the next explanation. The presence of specific cell surface receptors, can increase the uptake of gene delivery vectors e.g. targeting of EGFR improves adenoviral gene delivery into human OS cells by 10-fold (previously described in 1.2.3.1) (Witlox *et al.*, 2002), and efficient induction of apoptosis in OS cells, following delivery of wild-type TP53, using an Ad5-based adenoviral vector, can only be achieved if OS cells express high levels of Ad5 receptors (Hellwinkel *et al.*, 2005). Furthermore, the transfection efficiency of cationic liposomes in human OS cells has been improved with the use of magnetic cationic liposomes, which subsequently increased the level of apoptosis (Hirao *et al.*, 2003), and the presence of transferrin receptor ligand on the liposomes, enabling receptor-mediated endocytosis to occur (Nakase *et al.*, 2005a; Pirollo *et al.*, 2000). Transferrin receptors (TfR) levels are elevated in various types of cancer cells, including OS and correlate with the aggressive or proliferative ability of tumour cells (Cheng 1996; Thorstensen & Romslo 1993). Finally, generally only the TP53 status of the cell lines are known, while knowledge of the abnormalities in the TP53 signalling networks are limited, differences in individual molecular pathologies of different cancer lines do vary, each having a different impact on TP53 function, even if TP53 is wild-type (Momand *et al.*, 1998; Ternovoi *et al.*, 2006; Trotman & Pandolfi 2003). For example, elevated MDM2 protein levels may enhance the degradation of TP53, particularly in tumour cells, including OS, possessing endogenous wild-type TP53 (Ternovoi *et al.*, 2006), and other genetic alterations may impair the ability of TP53 to trigger an apoptotic response (Atencio *et al.*, 2001; Fulci *et al.*, 2000;

Ternovoi *et al.*, 2006). Although D17 cells possess endogenous wild-type TP53 (Sabine *et al.*, 1999; Shiomitsu *et al.*, 2008), and it is accepted that the efficacy of exogenous wild-type TP53 in human tumour cells, containing wild-type TP53, including OS, is likely to be less than that observed in tumour cells possessing null or mutant TP53 (Harris *et al.*, 1996; Tang *et al.*, 2004; Tsuchiya *et al.*, 2000). Mutant TP53 whilst retaining certain functions of the wild-type molecule, react very differently to the introduction of TP53 than a straightforward TP53 inactivation (TP53 somatic deletion and knockout cells) (Cimoli *et al.*, 2004). In addition, different mutant TP53 may vary in their individual mechanism of response to the introduction of wild-type TP53, because of the diverse mechanism of action of different TP53 mutants (Cimoli *et al.*, 2004). Hence, the effects of the introduction of wild-type TP53 on cell growth, including D17, CMT3, CMT7 and CMT8 cells, are likely to be different than that hypothesized or demonstrated in other cell lines, regardless of the known endogenous status of TP53. Furthermore, the transcriptional regulation of expression of exogenous TP53 in different cell lines may be affected differently by the same promoter-enhancer (Lu *et al.*, 2002).

These explanations, particularly the latter two, bear important potential consequences for the clinical use of the restoration of TP53 approach. The extrapolation of these findings on cell lines to that of a patient's tumour, indicate that knowledge of the TP53 status alone will be highly unlikely to succeed in trying to determine the response of a patient to this strategy. Indeed, the therapeutic benefit observed in clinical trials, carried out following preclinical studies that reported successful results, were lower than expected.

Therefore, even though suggestions have been made to modify and improve the methodology employed in this study, in an attempt to determine if cell survival can be reduced following expression of wild-type TP53 in D17, CMT3, CMT7 and CMT8 cells, it is doubtful if these results would be translated positively in an *in vivo* setting. Hence, investigations into the effects of the TP53-targeted suicide gene therapy strategy are warranted on the D17, CMT3, CMT7 and CMT8 cell lines.

4.4.3 Analysis of vectors required for TP53-targeted suicide gene therapy strategy using Dual-Luciferase® Reporter Assay System

4.4.3.1 Optimization of Dual-Luciferase® Reporter Assay System co-reporter vectors

pGL3-Control was utilized in this experiment, due to the information provided by the manufacturer protocol, stating that pGL3-Control provides an indicator to the efficacy of the assay itself as it can produce strong expression of *FFluc* in many mammalian cell types due to the possession of SV40 promoter and enhancer sequences. Although, to the best of my knowledge, the use of pGL3-Control has not been previously reported in the D17, CMT3, CMT7 and CMT8 canine cell lines, it has been used in MDCK cells (Campbell *et al.*, 2001; Yoshida *et al.*, 2002). Hence, the expression of *FFluc* from pGL3-Control observed following transfection in the cell lines in this study, appears to be remarkably low for that expected from a positive control, as a statistically significant difference was expected with the NTC. As significant differences were not observed, this entire assay should have been repeated twice more, as is standard practice, and because of the anomalous results observed in the CMT3, CMT7 and CMT8 experiments.

It was observed in this experiment that generally low levels of background firefly and *Renilla* luminescence were recorded in the NTC. In addition, reduced levels of background *Renilla* luminescence were usually determined in the cells only transfected with pGL3-Control. It has been reported that as the *FFluc* and *Rluc* enzymes are not endogenously expressed in mammalian cells, all background measurements, typically arise from the instrumentation and/or the sample plates (Promega Corporation 1999). In addition, some of the background *Renilla* luminescence activity can be attributed to *Rluc* substrate, coelenterazine. It has been documented that the primary limitation of *Rluc* assay chemistry is the emission of a low level non-enzymatic background luminescence, termed 'auto-luminescence' as a result of coelenterazine rapidly undergoing non-enzymatic oxidation as a result of being unstable in cell culture media (Hawkins *et al.*, 2005; Promega Corporation 2003; Wood 1998). Furthermore, the level of auto-luminescence can be increased by the presence of lipids and proteins found in cell lysates and detergents used in cell lysis buffers to protect the luminescent

excited state from quenching by water (Promega Corporation 2003; Wood 1998). Although the reagents in the DLR Assay System are formulated to minimize the level of coelenterazine auto-oxidation, they are not 100% effective because the presence of small amounts of detergents are still required to enable cell lysis to occur (Sherf *et al.*, 1996).

Similar FFluc activities were generally observed between the cells transfected with pGL3-Control in combination with pRL-CMV or pRL-TK at 5:1, 10:1 and 25:1. The only probable indication of a *trans* effect by the CMV promoter was observed in the CMT7 cells, as the FFluc activities of the cells transfected with pGL3-Control in combination with pRL-CMV at 10:1 were statistically significant higher than those cells transfected with pGL3-Control in combination with pRL:CMV at 25:1.

It was apparent however, that statistically significant differences occurred between the FFluc activities produced in the cells only transfected with the pGL3-Control vector to those co-transfected with either of the pRL-reporter vectors. One of the main requirements of the experimental and control reporters is that they should both be devoid of undesirable regulatory sites in the gene sequence so that their expression should not interfere significantly with the expression of the other. However, these findings suggest that a regulatory sequence(s) may exist within the backbone of the pRL- and/or pGL3- vector, which is interacting with the presence of an unknown transcription factor, to artificially increase the activities of FFluc and Rluc. This would result in the normalization of FFluc activity, in the subsequent DLR experiments, to be inaccurately calculated.

Indeed, since the DLR experiments were performed in this study, the presence of ~300 consensus sequences for binding mammalian transcription factors have been identified in Rluc (Zhuang *et al.*, 2001), and also a large number in both the FFluc gene, *luc+* and the pGL3 Vector backbone (Bert *et al.*, 2000; Paguio *et al.*, 2005). These may yield high backgrounds and anomalous transcriptional behaviour in mammalian cells under certain experimental conditions, thereby compromising the gene's reliability as a reporter molecule (Bert *et al.*, 2000; Thirunavukkarasu *et al.*, 2000). For example, several of the consensus binding sites are for the GATA transcription factors and Ho and colleagues (2004), have

reported that *Rluc* expression, by the pRL-*TK* and pRL-SV40 vectors, increased 2 - 8 -fold following the co-transfection of plasmids expressing members of the GATA transcription factor family (Ho & Strauss 2004). Furthermore, several other research groups have also reported experimental evidence in which the co-transfection of different pRL plasmids influenced the expression of the experimental vector and as a consequence, limited the evaluation of their results (Matuszyk *et al.*, 2002; Mulholland *et al.*, 2004). The DLR manual now recommends a charge ratio of experimental vector to pRL-vector from 10:1 to 50:1; the optimization assay employed ratios of 5:1 - 25:1, the first DLR experiment, a ratio of 5:1, and the subsequent two experiments, 25:1.

These studies highlight the need to be aware of potential artefacts when using reporter gene plasmids and assessing the results obtained, as well as ensuring suitable controls are included, as it is apparent that there may be major contributions to reporter gene expression from the vectors themselves. Thus, the presence of an unidentified transcription factor(s) in the solutions used in the co-reporter optimization assays was likely to have interacted with its respective binding site either in *Rluc*, FFluc gene, *luc+* and/or pGL3 Vector backbone to result in the overall increased expression of FFluc.

Zhuang and co-workers (2001) and Paguio and colleagues (2005), as a result of identifying the transcription factor binding sites in the native *Rluc* gene and *luc+*, developed synthetic *Rluc*, *hRluc*, and FFluc, *luc2*, genes and reporter gene vectors, the pGL4 Luciferase Reporter Vectors, respectively (Paguio *et al.*, 2005; Zhuang *et al.*, 2001). The number of mammalian transcription factor binding sites were reduced to an absolute minimum, thereby decreasing the risk of anomalous transcription behaviour and improving expression levels, sensitivity and reliability over the native *Rluc* and *luc+* genes and pGL3 vector backbone in mammalian cells (Bhaumik *et al.*, 2004; Paguio *et al.*, 2005; Zhuang *et al.*, 2001). Additionally, the resultant increase in *Rluc* expression enables the risk of *trans* effect to be reduced because instead of strong promoters being used in the control reporters, weaker ones can be employed (Zhuang *et al.*, 2001). Furthermore, both *hRluc* and *luc2* were cloned into the pGL4 Luciferase Reporter Vectors, obviously creating a greater structural similarity between a pGL4 control reporter and a pGL4 experimental reporter than that of a pGL3 Vector to

a pRL Vector. This structural similarity may enable the pGL4 Vectors to respond similarly to any biochemical changes that might occur, thereby resulting in the accuracy of normalization potentially being greater than that previously obtained with a pGL3 experimental reporter and a pRL control reporter.

Thus it would certainly be worthwhile analysing pGL4.13[*luc2/SV40*] instead of pGL3-Control as the experimental reporter and pGL4.74[*hRluc/TK*] and pGL4.75[*hRluc/CMV*] instead of pRL-*TK* and pRL-*CMV*, respectively, as the control reporters, in future DLR assays, as a means for improving independent genetic expression from the reporter vectors. This would also hopefully reduce the difference in FFluc expression observed between pGL4.13[*luc2/SV40*] alone with that of pGL4.13[*luc2/SV40*] in combination with either pGL4.74[*hRluc/TK*] or pGL4.75[*hRluc/CMV*]. In addition, a further control that could be employed in future studies, is the transfection of pGL4.74[*hRluc/TK*] and pGL4.75[*hRluc/CMV*] alone as the transfection of the pRL vectors alone was not carried out in the co-reporter optimization assays. This would be used to confirm that pGL4.74[*hRluc/TK*] and pGL4.75[*hRluc/CMV*] are themselves not contributing directly to FFluc2 activity expressed from pGL4.13[*luc2/SV40*]. Any abnormal expression that did occur was again possibly due to the presence of the unknown transcription factors being able to bind to regulatory sequences. These control transfections could also be used to determine the level of *hRluc* expression from pGL4.74[*hRluc/TK*] and pGL4.75[*hRluc/CMV*], in the absence of an experimental reporter, as a means for comparing to the *hRluc* activity gained following co-transfection with pGL4.13[*luc2/SV40*].

4.4.3.2 Dual-Luciferase® Reporter Assay System experiments

In the first DLR experiment, although pRL-*CMV* was used and at a ratio that would have permitted a *trans* effect to occur, this experiment did demonstrate promising initial results, as the p53-targeted gene therapy system appeared to function as designed. In that not only were high levels of FFluc expression observed with the introduction of pTarget-p53FL (positive control), in combination with pTarget-Lexp53 and pGL3-P8LA, into D17, CMT3, CMT7 and CMT8 cells, but also, after the transfection of pTarget-Lexp53 in combination

with pGL3-P8LA in the CMT7 cells. These observations were generally confirmed in the subsequent two DLR experiments. In addition, in the third DLR experiment, a significant difference was also observed between the CMT7 cells treated with 'B8LA+Lexp53' and 'E8LA+Lexp53' to those treated without 'Lexp53'. These results indicate that the canine TP53 C-terminus of the Lexp53 protein expressed by pTarget-Lexp53 is able to recruit the transactivation domain of the accumulated TP53 present in the CMT7 cells and bind to the 8 LexA Op in pGL3-8LA, to significantly increase FFluc expression. In comparison, in the D17 and CMT3 cells, possessing none and low levels of accumulated TP53, non-significant levels of FFluc expression were found. Thereby, indicating that the presence of accumulated TP53 is able to drive the expression of FFluc, which is in agreement with the results previously published by Da Costa *et al.*, (1996) (Da Costa *et al.*, 1996). Also providing further evidence that canine TP53 C-terminus is similar in function, as well as structure, to human TP53 C-terminus. It was observed however, that although CMT8 cells were proposed to contain accumulated TP53 (as previously described in 4.4.1), no significant increase in FFluc expression occurred in the first two DLR experiments. This could possibly be due to either the mutation already known to be present in CMT8 TP53 inhibiting the interaction between the transactivation domain and Lexp53 protein, or, an unknown mutation is located in the transactivation domain as TP53 mutational analysis of the first 108 codons has not yet been reported in the CMT8 cell line (Van Leeuwen *et al.*, 1996). Although both explanations are not commonly reported (as previously described in 1.3.3.1 and 4.1.1.3), they are still likely to occur. In addition, as mentioned in 4.4.2, the differences in individual molecular pathologies and differentiations of different cancer lines, does not enable knowledge of the endogenous TP53 status to be entirely predictive for a response to a TP53-based gene therapy. However, in the third DLR experiment utilising pGL3-B8LA and pGL3-E8LA in addition to pGL3-P8LA, there is evidence that the lack of a significant increase in FFluc expression may be due to the ability of SV40 promoter in pGL3-P8LA to drive FFluc expression to such an extent that it 'swamps' the effect of the endogenous TP53 present, as a significant difference is observed in the absence of the SV40 promoter. Hence, further investigations are warranted to study these different explanations. Additionally, the reduction in the number of LexA operator sites upstream of the

luciferase gene should be analysed. This is because the greater the number of LexA sites, the weaker the interaction that can be detected, however, the number of false positives measured may increase. In contrast, using a smaller number of upstream sites would reduce the number of false positives and increase the stringency of the protein-protein interactions detected. Furthermore, optimizations into the amounts of different constructs transfected into the cells treated with 'Control', '8LA', and 'Lex-p53' should be performed, as this was not done.

Although a *trans* effect was only apparent in the optimization assay employing CMT7 cells, there did appear to be one between the DLR experiments as there was an overall reduction in FFluc expression between the first DLR experiment using pRL-CMV as the co-reporter, at a ratio of 5:1, and the second and third DLR experiment, using pRL-TK at a ratio of 25:1. In addition to this *trans* effect, it has also been reported that pRL-TK is unresponsive to human TP53 (Thavathiru & Das 2001). In the first DLR experiment, the D17, CMT3, CMT7 and CMT8 cells treated with 'p53FL' all produced FFluc expression levels significantly greater than the cells treated with 'P8LA'. In comparison, in the second experiment, significantly lower FFluc expression levels were observed (except for CMT3 cells). Hence, not only indicating that pRL-TK may also be unresponsive to canine TP53, but also that pRL-CMV is affected by TP53. Although the possibility exists that pTarget-p53FL may not have been expressing TP53 as required to increase levels of TP53 present. However, the overall indication that pTarget-Lexp53 in combination with pGL3-P8LA was able to utilize accumulated TP53 present in canine OS cells to drive FFluc expression did not appear to be affected by the use of either co-reporter, as similar results were obtained in all 3 DLR experiments.

Therefore, these DLR assays demonstrated the TP53-targeted suicide gene therapy strategy shows promise, enabling further investigations to take place, which are described in the next chapter. Briefly, the luciferase gene was replaced with a suicide gene to determine if, in combination with a prodrug, the significant differences observed in FFluc activity with pTarget-Lexp53 in combination with pGL3-P8LA, pGL3-E8LA and pGL3-B8LA resulted in significant reductions in survival of canine OS cells possessing accumulated TP53.

Chapter 5

Suicide Gene Therapy for Canine Osteosarcoma

5.1 Introduction

5.1.1 Suicide gene therapy in dogs

A small number of suicide gene therapy studies, using HSV-TK/GCV or ACV and linamarase/linamarin, have been carried out in canine cell lines and Beagles, to investigate their potential as treatments for cancer (Garcia-Escudero *et al.*, 2008; Matsukura *et al.*, 1999; Park *et al.*, 2003; Rainov *et al.*, 2000). Additionally, two Phase I/II canine clinical trials have been reported, one a clinical protocol for dogs with mammary tumours using cytochrome P450/cyclophosphamide (Winiarczyk *et al.*, 2002), and the second, in dogs with melanoma using HSV-TK/GCV (Finocchiaro *et al.*, 2008). In the study by Finocchiaro *et al.*, (2008), survival was demonstrated to be significantly extended and toxicity levels observed were minimal (Finocchiaro *et al.*, 2008). GCV treatment has also been reported to be well tolerated in healthy dogs with no significant toxicities occurring (Appelbaum *et al.*, 1988; Link Jr *et al.*, 2000). The canine herpesvirus TK has been cloned and characterized, but as yet has not been utilized in a suicide gene therapy study (Solaroli *et al.*, 2006). As far as I am aware, there is no literature evaluating the use of suicide gene therapy in canine OS cell lines; hence, this study is likely to be the first. Studies have been performed investigating the use of suicide gene therapy in human OS *in vitro* and *in vivo*, including HSV-TK/GCV (Charissoux *et al.*, 1999; Ketola *et al.*, 2004), varicella-zoster virus (VZV)-TK/(E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) (Degreve *et al.*, 1997), carboxylesterase-2/CPT-11 (Oosterhoff *et al.*, 2003), and cytosine deaminase/5-FC (Ramnaraine *et al.*, 2003). These studies demonstrated inhibition of tumour growth and induction of cytotoxicity and a proximal bystander effect was also observed following treatment with HSV-TK/GCV (Charissoux *et al.*, 1999; Ketola *et al.*, 2004) and cytosine deaminase/5-FC (Ramnaraine *et al.*, 2003). Furthermore, Charissoux *et al.*, (1999) reported that the HSV-TK/GCV prodrug system may have the potential to suppress pulmonary metastasis (Charissoux *et al.*, 1999). As yet, no suicide gene therapy clinical trials have been performed in human OS patients. Although, a Phase II clinical study reported that after chemotherapy with 5-FU (active drug) in human OS patients, in addition to no objective responses, numerous grade 3/4 toxicities were observed (Pratt *et al.*, 1994). Pratt and colleagues (1994) concluded no

further evaluation of 5-FU would occur in patients with malignant solid tumours, including OS (Pratt *et al.*, 1994). In addition, the use of 5-FU in the treatment of dogs with carcinomas of the mammary gland, gastro-intestinal tract, liver and lung, is at best palliative (Bishop 1996; Karayannopoulou *et al.*, 2001; Yamashita *et al.*, 2001). 5-FU is regarded not to be suitable as a prodrug in dogs, particularly those with cancer, as serious side-effects of 5-FU are well-documented and which can be fatal after exposure to high levels (Davis *et al.*, 1994; Dorman *et al.*, 1990; Hammer *et al.*, 1994). However, S-1, a relatively new oral formulation of 5-FU, has been reported to have a low level of toxicity in Beagles (Kato *et al.*, 2001), and human Phase II clinical trials (Saif *et al.*, 2009). Furthermore, as therapeutic benefits were observed in patients with several different types of cancer, S-1 is currently being tested in a Phase III trial (Saif *et al.*, 2009).

Hence, investigations into additional suicide gene/prodrug systems, including *E.coli* NTR/CB1954, are warranted for canine cancers, in particular OS, to extend the publications currently available. In addition, all of the original toxicological work on CB1954 was performed in dogs (Workman *et al.*, 1986b), and other aromatic nitrogen mustard alkylating agents, such as cyclophosphamide and chlorambucil are used therapeutically to treat a variety of different tumours in dogs (Ahrens, 1996).

5.1.2 *E.coli* NTR/CB1954 system

The *E.coli* NTR/CB1954 system is now established as a successful pre-clinical prodrug activating system. NTR is an enzyme (24kDa), encoded by the *nfsB* gene of *E. coli*, which contains flavin mononucleotide (FMN) cofactors and was originally identified through its involvement in bacterial sensitivity to nitrofurans e.g. nitrofurazone (NFZ, 5-nitro-2-furaldehyde semicarbazone) (Searle *et al.*, 2004). It is also oxygen-insensitive, enabling it to function even within hypoxic cells present in large solid tumours (Lemmon *et al.*, 1997), and can be successfully introduced into a broad range of mammalian species (Blackwood *et al.*, 2001; Denny 2003). NTR functions as a monomer, thus minimising the requirement for post-translational modifications and can reduce

a variety of quinone and nitroaromatic substrates including non-toxic prodrugs, such as CB1954 (5(-aziridine-1-yl)-2,4-dinitrobenzamide)) into toxic metabolites. Its use however, is limited to the activation of intracellular prodrugs as it acts via a substituted enzyme ('ping-pong') mechanism, which requires either reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) cofactors to first reduce the tightly bound FMN molecule (Searle *et al.*, 2004). In the second step, the substrates are then able to bind to NTR, which enables their reduction by FMN (Friedlos *et al.*, 1992a). Four broad classes of prodrugs for NTR have been described: dinitroaziridinylbenzamides, which includes CB1954; dinitrobenzamide mustards; 4-nitrobenzylcarbamates; and nitroindolines; but the majority of current research to date has focussed primarily on CB1954 (Denny 2003).

Although CB1954 is now regarded as a non-toxic prodrug, it was initially developed as a chemotherapeutic agent, which was found to be extremely selective and toxic against the rat Walker carcinoma 256 *in vitro* and *in vivo* (Cobb *et al.*, 1969). Based on these results, in early 1970's CB1954 was entered into human clinical trials, but no anti-tumour effects were observed (Knox *et al.*, 1993). In addition, CB1954 was found to possess no significant toxicity on several other animal or human tumours *in vitro* or *in vivo* (Connors & Melzack 1971; Workman *et al.*, 1986a). The sensitivity of the Walker tumour towards CB1954 was subsequently attributed to the rat NQO1 (DT diaphorase) enzyme, and that the human equivalent of this enzyme was far less efficient at reducing CB1954 to its cytotoxic metabolite (4-(N-acetoxy)-5-(aziridin-1-yl)-2-nitrobenzamide) (Boland *et al.*, 1991; Knox *et al.*, 1991). The addition of this enzyme alone to cells normally insensitive to CB1954 resulted in a large increase in their sensitivity towards CB1954 (Knox *et al.*, 1988). It was later determined that *E. coli* NTR could also reduce CB1954, increase sensitivity to CB1954 by 770-fold compared to untransfected controls and is ~90-fold more efficient than rat NQO1 (Anlezark *et al.*, 1992; Bailey *et al.*, 1996; Knox *et al.*, 1992). NTR converts CB1954 from a weak, monofunctional alkylating agent into a toxic bifunctional alkylating agent that can be upto 2000-fold more cytotoxic than CB1954 itself in human cancer cell lines (Green *et al.*, 1997; Knox *et al.*, 1988; Weedon *et al.*, 2000). This is because activated CB1954 is capable of reacting with DNA to form a large number of interstrand DNA crosslinks, which are poorly

repaired by the cell inducing p53-independent apoptosis (Cui *et al.*, 1999; Friedlos *et al.*, 1992c). Thus, activated CB1954 is independent of ongoing cellular DNA replication for its cytotoxicity and can kill both proliferating and non-proliferating cells (Bridgewater *et al.*, 1995; Weedon *et al.*, 2000). This is an advantage over, for example, activated GCV or 5-FU, as they are cell-cycle dependent and their toxicity is mainly restricted to cells in S-phase, which is further limited by there only being a small percentage of tumour cells proliferating at any one time (Bridgewater *et al.*, 1995). As with the other enzyme/prodrug combinations, bystander cell killing has been observed with NTR/CB1954 due to local spread of the activated prodrug (Bridgewater *et al.*, 1997; Green *et al.*, 1997; McNeish *et al.*, 1998b). This enabled an antitumour effect to be achieved *in vivo* even when a minimal number of cells expressed NTR (Djeha *et al.*, 2000). Furthermore, as the conversion of a prodrug by NTR to its active species relies on the presence of NADH, an intracellular co-factor, an element of safety is introduced, since no extracellular activation can occur (Palmer *et al.*, 2004). Additionally, NADH is not a cofactor for human NQO1, hence it does not potentiate any unwanted CB1954 cytotoxicity (Friedlos *et al.*, 1992b). CB1954 is also toxic to tumours, which have acquired resistance to other chemotherapeutic agents e.g. cisplatin (McNeish *et al.*, 1998b; Weedon *et al.*, 2000).

The *E. coli* NTR/CB1954 system has shown high levels of selectivity (100-2000 fold) in several pre-clinical studies, using a wide variety of human cancer cell lines *in vitro* (Bridgewater *et al.*, 1995; Green *et al.*, 1997; McNeish *et al.*, 1998a), and in tumour xenografts (Djeha *et al.*, 2000; McNeish *et al.*, 1998a; Weedon *et al.*, 2000). In addition, this system has been used to indirectly inhibit the growth of tumour cells possessing mutant TP53, in which NTR expression was placed under the control of the HSP70 promoter (Lipinski *et al.*, 2001). Following systematic treatment with CB1954, toxicity was achieved in the required cells/tissue, reducing tumour volume, whilst healthy cells containing wild-type TP53 remained unaffected. Furthermore, clinical trials have been carried out separately with NTR and CB1954 in human cancer patients, which demonstrated that they were well tolerated at doses required for use in combination with each other (Chung-Faye *et al.*, 2001; Palmer *et al.*, 2004). Recently, results from the first Phase I/II clinical trial of NTR in combination with CB1954, carried out in

prostate cancer patients, were reported (Patel *et al.*, 2009). Minimal levels of toxicity were observed and there was evidence of a time-delay in progression of prostate specific antigen.

5.1.3 Nitrofurazone

Several alternative prodrugs have been investigated as to their efficacy with NTR, in the hope of revealing more potent and selective killing of cells and/or superior bystander effect than that obtained with CB1954. In particular, Bailey *et al.*, (1996) reported that NTR reduced NFZ more actively than CB1954 (Bailey *et al.*, 1996). NFZ has a wide range of antimicrobial effects, and is used in both human and veterinary medicine (Hiraku *et al.*, 2004; Kobierska-Szeliga & Czeczot 1994). Although, cytotoxic analyses of Chinese hamster lung V79 cells revealed that CB1954 was actually more cytotoxic than NFZ to NTR-expressing cells than non-expressing cells by ~8-fold, NFZ still exhibited 97-fold differential cytotoxicity between NTR- and non- expressing cells (Bailey *et al.*, 1996).

5.1.4 Summary

The *E.coli* NTR/CB1954 system possesses several intrinsic advantages that are of potential value for its use as a treatment for canine OS. Firstly, NTR is oxygen-insensitive, enabling it to function even within hypoxic cells present in large solid tumours (Lemmon *et al.*, 1997), and it can be introduced into a wide range of mammalian species (Blackwood *et al.*, 2001; Denny 2003). Secondly, CB1954 is not dependent on a cell cycle phase (Bailey & Hart 1997), and all the original toxicological work on CB1954 was performed in dogs (Workman *et al.*, 1986b). Finally, NTR-mediated cell death by CB1954 is very rapid and of particular importance to this study is that it is by a TP53-independent apoptotic pathway (Cui *et al.*, 1999). In addition, NFZ is a well-known veterinary antimicrobial drug accepted for use in dogs (Hiraku *et al.*, 2004).

Hence, the aims of this chapter were two-fold. Firstly, to perform preliminary *in vitro* suicide gene/prodrug assays, using NTR in combination with CB1954 or NFZ,

to assess the suitability of CB1954 and NFZ as prodrugs for use with NTR in several canine cell lines, primarily OS; thereby extending *in vitro* data on the effects of suicide gene therapy in canine cancer cells.

Secondly, to replace the luciferase gene in the constructed vectors, belonging to the TP53-targeted suicide gene therapy strategy, with the NTR gene, to determine if the TP53-targeted suicide gene therapy strategy in combination with CB1954, translates the promising results obtained in the DLR experiments (Chapter 4), into significant reductions in survival of canine OS cells possessing accumulated TP53.

5.2 Materials & methods

5.2.1 Optimization assays

5.2.1.1 Cell number

Cells were approximately 80% confluent on the day of transfection (2.2.9), as recommended by the manufacturer's instructions. For all of the transfections performed in this study, the day of transfection was 24 hours after the cells had been placed into the 96-well plates or 25cm² flasks. The canine cell lines employed in the NFZ (Sigma; Appendix A) assays were D17, CMT3, CMT7, CMT8, MDCK and CML-10 and in the CB1954 assays, D17 and CMT7 were used.

The concentration of cells required to obtain 80% confluence for the D17, CMT3, CMT7 and CMT8 cells had been previously determined in 4.3.1.1. The MDCK and CML-10 cells were each cultured and maintained as described in 2.1.1. The determination of the concentration of cells required to obtain 80% confluence for the MDCK and CML-10 cell lines was carried out by plating the following cell concentrations into a 96-well plate, in quadruplicate per cell concentration (200 µl/well): MDCK - 50000 cells/ml (E. Gault, Department of Veterinary Clinical Studies, University of Glasgow; personal communication); and CML-10 - 75000 cells/ml, due to the observation that these cells showed a similar growth rate to CMT3 and CMT8 cells.

The percentage of cells confluent at each cell concentration was estimated by eye after 24 hours, enabling the concentration of cells required to achieve 80% confluence for the MDCK and CML-10 cell lines to be confirmed.

5.2.1.2 DMSO

Dimethyl sulphoxide (DMSO; Sigma) was used as the solvent for NFZ and CB1954 (kindly donated by Dr. N. Keith, University of Glasgow; Appendix A). DMSO was applied at 0, 0.125, 0.25, 0.5, 1 and 2% to D17, CMT3, CMT7 and CMT8 cells, in quadruplicate, to determine the optimal percentage of DMSO that could be used in the final volume of prodrug for the subsequent assays.

The D17, CMT3, CMT7 and CMT8 cell lines were each cultured and maintained (2.1.1), and 16-24 hours prior to DMSO treatment, seeded at the appropriate

density (as determined in 4.3.1.1), per well of a 96-well plate. MDCK and CML-10 cell lines were not available at the time of this optimization assay.

Following 24 hours of DMSO treatment, the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega; 2.2.10) was employed to determine cell numbers.

The mean absorbance reading for each set of 4 wells was determined and the percentages of cell survival (+/-SE), compared to the cells treated with 0% DMSO (100%), were calculated.

5.2.2 Construction of nitroreductase expression vectors

The methods involved in constructing the NTR expression vectors are previously described in Chapter 2. Methods included DNA purification (2.2.3), restriction digestion (2.2.6), PCR (2.2.1), cloning (2.2.7) and DNA sequence analysis (2.2.5). PCR conditions are as previously described in 2.2.1 and details of primers used for PCR and DNA sequence analysis are displayed in Table 5.1.

The construction of the NTR expression vectors, pGL3-Basic 8LexA Operator NTR (pGL3B8LA-NTR), pGL3-Enhancer 8LexA Operator NTR (pGL3E8LA-NTR) and pGL3-Promoter 8LexA Operator NTR (pGL3P8LA-NTR) involved the replacement of the luciferase gene in pGL3-B8LA, pGL3-E8LA and pGL3-P8LA vectors, respectively, with the NTR gene (Figure 5.1). This was carried out in 2 stages.

The first stage involved the use of the PCR primers, '2NcolpNTRF' and 'XbalpNTRR', to amplify the full-length NTR gene from the pCMVNTR expression vector (kindly donated by Dr. L. Blackwood, (Blackwood *et al.*, 2001); Appendix C, Figure C8). The '2NcolpNTRF' and 'XbalpNTRR' primers had been designed with *NcoI* and *XbaI* restriction enzyme sites, respectively, to enable the incorporation of *NcoI* and *XbaI* sites at the 5' and 3' -ends of the NTR PCR product, respectively. The NTR PCR product was electrophoresed on an agarose gel, extracted and purified.

The second stage consisted of the excision of the luciferase gene and the ligation of the purified NTR PCR product into the pGL3-B8LA, pGL3-E8LA and

pGL3-P8LA vectors, to create the NTR expression vectors, pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR. This was achieved by digesting the pGL3-B8LA, pGL3-E8LA and pGL3-P8LA vectors, each possessing *NcoI* and *XbaI* restriction enzyme sites at the 5' and 3' -ends of the luciferase gene, respectively, with the *NcoI* and *XbaI* restriction enzymes. The digested pGL3-8LA vectors were subsequently electrophoresed on an agarose gel, extracted and purified. The purified NTR PCR product was ligated into each of the purified pGL3-8LA vectors, at 4°C for 20 hours, and cloned using JM109 competent cells (Promega). DNA sequence analysis was performed, using the 'RV3primer' and 'RV4primer' sequencing primers, to confirm that the NTR gene sequence had been correctly ligated into pGL3-B8LA, pGL3-E8LA and pGL3-P8LA (Figure 5.2).

Primer	Reaction	Forward (F) / Reverse (R)	Primer Sequence (5' --> 3' orientation)	T _m	Location (nts)	Vector(s)
2NcolpNTRF	PCR	F	cca tgg cca tgg ata tca ttt ctg tcg	65	820-838	pCMVNTR
XbaI pNTRR	PCR	R	tct aga tct aga tta cac ttc ggt taa gg	62.4	1458-1474	pCMVNTR
RV3primer	Sequencing	F	cta gca aaa tag gct gtc cc	57.3	4760-4779; 5006-5025; 4952-4971	pGL3-B8LA; pGL3-E8LA; pGL3-P8LA
RV4primer	Sequencing	R	gcg cgg ggc atg act atc gtc	61	2080-2061; 2326-2307; 2272-2253	pGL3-B8LA; pGL3-E8LA; pGL3-P8LA

Table 5.1: Details of the oligonucleotide primers involved in the construction and DNA sequence analysis of the nitroreductase expression vectors, pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR, from pGL3-B8LA, pGL3-E8LA and pGL3P8LA firefly luciferase reporter vectors, respectively, and pCMVNTR expression vector. For each primer the following information is provided: the sequence, acting as a forward (F) or a reverse (R) primer, annealing temperature (T_m) and nucleotide (nts) location on the particular vector undergoing PCR/DNA sequence analysis.

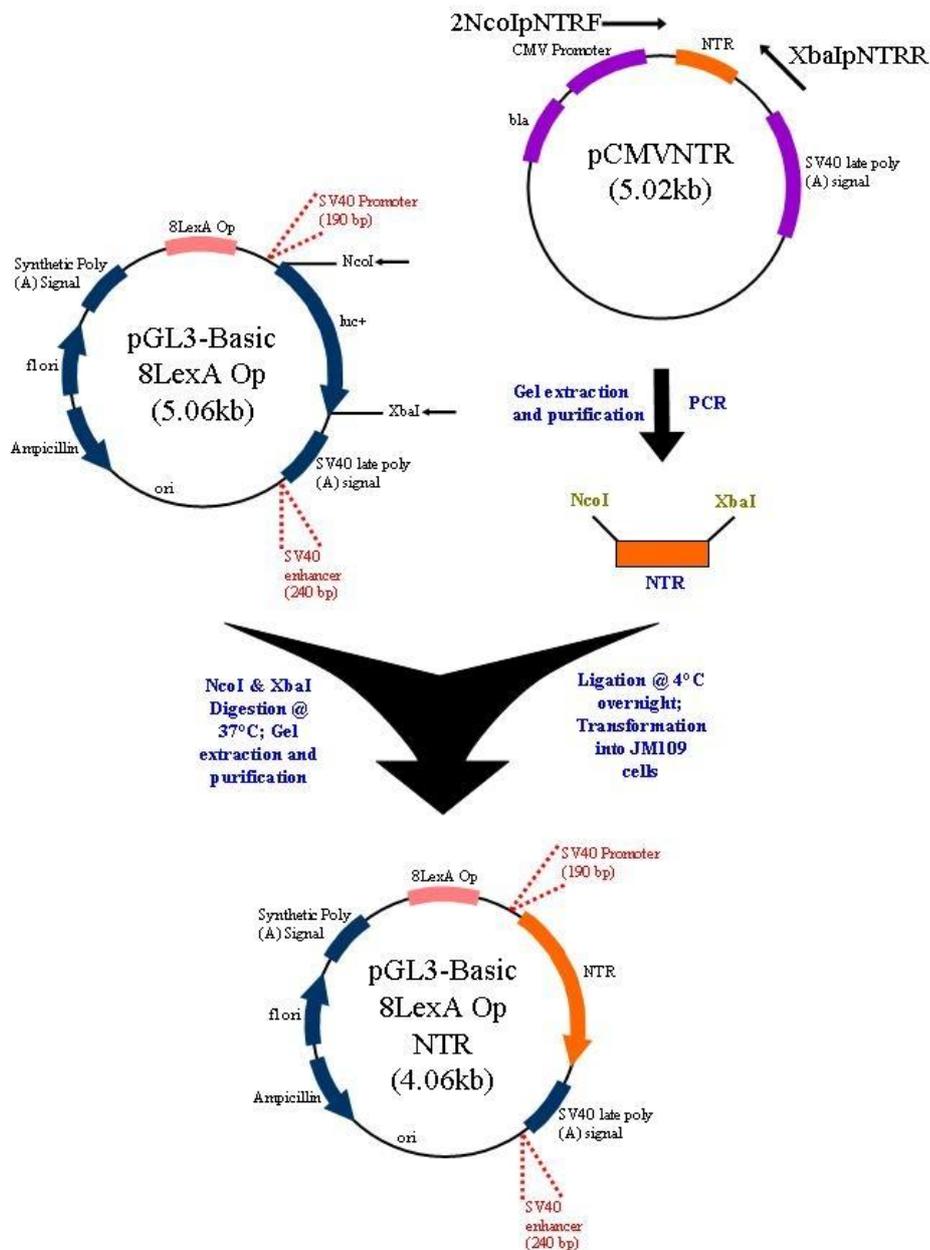


Figure 5.1: Flow-diagram indicating the processes involved in the construction of the pGL3-8LexA Operator NTR expression vectors, pGL3-Basic 8Lex A Op NTR (lacks the SV40 Promoter and Enhancer sequences), pGL3-Enhancer 8LexA Op NTR (pGL3-Basic 8LexA Op NTR plus the SV40 Enhancer sequence) and pGL3-Promoter 8LexA Op NTR (pGL3-Basic 8Lex A Op NTR plus the SV40 Promoter). The PCR primers, '2NcolpNTRF' and 'XbalpNTRR' were used to amplify the NTR gene from the pCMVNTR vector.

B5678 } E5922 } P6117 }		pGL3 vector	1 <i>KpnI</i>	
51		TGCAAGTGCA GGTGCCAGAA CATTCTCTA TCGATAGGTA CC CATGGC		
		2KpnIPGL3F		
		TACCACCATA TACATATCCA TATACATATC CATATCTAAT CTTACCTCGA		
101		CTGCTGTATA TAAAACCAGT GGTTATATGT ACAGTACTGC TGTATATAAA		
		8 Lex A Operator		
151		ACCAGTGGTT ATATGTACAG TACGTGCACT GCTGTATATA AAACCAGTGG		
201		TTATATGTAC AGTACTGCTG TATATAAAAC CAGTGGTTAT ATGTACAGTA		
		NheIPGL3R		
251		CGTCGAGGGA TGATAATGCG ATTAGTTTTT TAGCCTTATT TCTGGGGTAA		
		<i>NheI</i>	SV40 promoter →	
301		CTAGCCCGGG CTCGAGATCT GCGATCT TAAG TAAGCTTGGC ATTCCGGT		
		<i>NcoI</i>	2NcoIpNTRF	
351 (P546)		TGTTGGTAAA GCCACC <u>ATGG</u> ATATCATTTC TGTCGCCTTA AAGCGTCA		
401 (P596)		CCACTAAGGC ATTTGATGCC AGCAAAAAAC TTACCCCGGA ACAGGCCGAG		
451 (P646)		CAGATCAAAA CGCTACTGCA ATACAGCCCA TCCAGCACCA ACTCCCAGCC		
501 (P696)		GTGGCATTTC ATTGTTGCCA GCACGGAAGA AGGTAAGCG CGTGTGCCA		
551 (P746)		AATCCGCTGC CGGTAATTAC GTGTTCAACG AGCGTAAAAAT GCTTGATGCC		
601 (P796)		TCGCACGTCG TGGTGTCTG TGCAAAAAACC GCGATGGACG ATGTCTGGCT		
		NTR		
651 (P846)		GAAGCTGGTT GTTGACCAGG AAGATGCCGA TGGCCGCTTT GCCACGCCG		
701 (P896)		AAGCGAAAAG CGCGAACGAT AAAGGTCGCA AGTTCCTCGC TGATATGCAC		
751 (P946)		CGTAAAGATC TGCATGATGA TGCAGAGTGG ATGGCAAAAC AGGTTTATCT		
801 (P996)		CAACGTCGGT AACTTCTGTC TCGGCGTGGC GGCTCTGGGT CTGGACGCCG		
851 (P1046)		TACCCATCGA AGGTTTGAC GCCGCCATCC TCGATGCAGA ATTTGGTCTG		
901 (P1096)		AAAAGAGAAAG GCTACACCAG TCTGGTGGTT GTTCCGGTAG GTCATCACAG		
951 (P1146)		CGTTGAAGAT TTTAACGCTA CGCTGCCGAA ATCTCGTCTG CCGCAAAACA		
		XbaIpNTRR	<i>XbaI</i>	
1001 (P1196)		TCACCTTAAC CGAAGTGTA TCTAGAGTCG GGGCGGCCG CCGCTTCG		
1051 (P1246)		CAGACATGAT AAGATACATT GATGAGTTTG GACAAACCAC AACTAGAATG		
1101 (P1296)		CAGTGAAAAA AATGCTTTAT TTGTGAAATT TGTGATGCTA TTGCTTTATT		
		pGL3 vector		
1151 (P1346)		TGTAACCATT ATAAAGCTGCA ATAAACAAGT TAACAACAAC AATTGCATTC		
1201 (P1396)		ATTTTATGTT TCAGGTTCCAG GGGGAGGTGT GGGAGGTTTT TTAAGCAAG		
			SV40 enhancer	
1251 (P1446)		TAAAACCTCT ACAAATGTGG TAAAATCGAT AAGGATCCGT CGACCGATGC		

Figure 5.2: Confirmation by DNA sequence analysis that the pGL3-8LexA Operator NTR expression vectors, pGL3-Basic 8LexA Op NTR (B), pGL3-Enhancer 8LexA Op NTR (E) and pGL3-Promoter 8LexA Op NTR (P), contain the NTR gene. The sequences of the PCR primers '2KpnIpGL3F', 'NheIpGL3R', '2NcoIpNTRF' and 'XbaIpNTRR' and the *KpnI*, *NheI*, *NcoI* and *XbaI* restriction enzyme sites are shown. The locations at which the SV40 promoter and enhancer sequences are positioned in the pGL3-Promoter 8LexA Op NTR (P) and pGL3-Enhancer 8LexA Operator (E) vectors, respectively, are also indicated.

5.2.3 Detection of nitroreductase protein expression by pCMVNTR and pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR, in combination with pTarget-Lexp53 or pCMVCAT6

5.2.3.1 Transfection of DNA & preparation of cell lysates for western blotting

The 3 NTR constructs, pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR, and the pTarget-Lexp53, pCMVNTR and pCMVCAT6 (kindly donated by Dr. L. Blackwood; Appendix C, Figure C9) vectors were prepared using the QIAGEN EndoFree Plasmid Purification Maxi kit (2.2.8.3). All transfections were carried out, in duplicate, using the Transfast™ reagent (Promega; 2.2.9). The pCMVNTR construct (positive control) was transfected into the D17, CMT3, CMT7, CMT8 and MDCK cell lines and pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR, with and without pTarget-Lexp53, were transfected into D17 and CMT7 cells. The pCMVCAT6 vector was used as the carrier DNA to ensure all wells were transfected with similar amounts of DNA. The amounts of DNA of each vector transfected per combination per cell line are displayed in Table 5.2. CML-10 cells were not used in this experiment as they had previously been shown to be difficult to transfect (E. Gault, Department of Veterinary Clinical Studies, University of Glasgow; personal communication).

These 5 cell lines were each cultured and maintained (2.1.1) and 16-24 hours prior to transfection occurring, seeded at the appropriate density (as determined in 4.3.1.1 and 5.3.1.1) per 25cm² flask. A total of 3.25µg and 6.5µg of vector DNA were transfected per flask (as determined in 4.3.1.2) of D17 cells and CMT3, CMT7 and CMT8 cells, respectively. 6.5µg of vector DNA were transfected per flask of MDCK cells (E. Gault, Department of Veterinary Clinical Studies, University of Glasgow; personal communication). One pair of flasks remained untransfected for each cell line to act as negative controls and enable the endogenous levels of NTR protein to be determined. 48 hours after transfection, the cells in each pair of flasks were harvested in protein treatment sample buffer (2.3.3.1) and the protein concentration determined (2.3.3).

5.2.3.2 SDS-PAGE and western blotting

Following protein concentration determination, SDS-PAGE (2.3.3.1) was performed, in which 3 separate gels were used. The first gel was loaded, per lane, with 50µg protein sample from each of the D17, CMT3, CMT7, CMT8 and MDCK cell lines transfected with pCMVNTR in combination with pCMVCAT6 and NTC lysates. 5ng purified *E. coli* NTR enzyme were also loaded as a positive control. In the second and third gels, 25µg protein sample from each of the D17 and CMT7 cells, respectively, transfected with pCMVCAT6 alone, pCMVNTR in combination with pCMVCAT6, pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR in combination with pCMVCAT6 or pTarget-Lexp53 and NTC lysates, were loaded per lane. Western blotting was subsequently carried out using a primary antibody of rabbit antiserum raised against *E.coli* NTR, kindly provided by Dr. G. Anlezark (Centre for Applied Microbiology and Research, Porton Down, Salisbury) (Anlezark *et al.*, 1995) (2.3.3). The primary antibody was detected by a secondary anti-rabbit horseradish peroxidase conjugated antibody.

<i>Cell Line</i>		NTR		CAT		B/E/P		B/E/P+Lp	
		<i>D17</i>	<i>CMT3, CMT7, CMT8 & MDCK</i>	<i>D17</i>	<i>CMT 7</i>	<i>D17</i>	<i>CMT7</i>	<i>D17</i>	<i>CMT7</i>
Vector DNA (μg)	pCMVCAT6	2.6	5.2	3.25	6.5	2.6	5.2	-	-
	pTargetLex-p53	-	-	-	-	-	-	2.6	5.2
	PGL3-8LANTR (B, E or P)	-	-	-	-	0.65	1.3	0.65	1.3
	pCMVNTR	0.65	1.3	-	-	-	-	-	-
Total amount of DNA transfected (μg)		3.25	6.5	3.25	6.5	3.25	6.5	3.25	6.5

Table 5.2: The amounts of DNA (in μ g) and combinations of vectors transfected, to enable the detection of NTR expression by: pCMVNTR in combination with pCMVCAT6 (NTR) in D17, CMT3, CMT7, CMT8 and MDCK cells; and pCMVCAT6 (CAT), and pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR, in combination with pCMVCAT6 (B/E/P, respectively), or pTarget-Lexp53 (B/E/P+Lp, respectively), in D17 and CMT7 cells.

5.2.4 Analysis of pCMVNTR in combination with nitrofurazone

The pCMVNTR and pCMVCAT6 vectors were used to investigate the effects of NFZ, following the transfection of pCMVNTR, on the survival of D17, CMT3, CMT7, CMT8, MDCK and CML-10 cells. 3 main assays were performed:

- (i), D17 assays consisting of 2 preliminary experiments and 1 ‘proper’ experiment;
- (ii), CMT3, CMT7 and CMT8 assays composed of 4 preliminary experiments and 1 ‘proper’ experiment;
- (iii), non-OS cell line assays in which experiments on MDCK and CML-10 cells were performed.

All the experiments assessed the transfection of pCMVNTR in combination with pCMVCAT6 (acting as carrier DNA) and NTC, except for the CML-10 cells in which only NTC were studied. The D17 ‘proper’ experiment also examined the transfection of pCMVCAT6 alone. The preliminary experiments were each performed once and the ‘proper’ experiments in triplicate, unless otherwise stated.

The pCMVNTR and pCMVCAT6 vectors were prepared using the QIAGEN EndoFree Plasmid Purification Maxi kit (2.2.8.3). Each vector was transfected into the cell lines using the Transfast™ reagent (Promega; 2.2.9), in quadruplicate per time point. The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega; 2.2.10) was employed to determine cell numbers immediately before the addition of NFZ (0 hour time point) and after cells had been treated for the required amount of time, typically 12, 24, 48, 96 and 144 hours (2.2.10), unless otherwise stated.

The 6 cell lines were each cultured and maintained (2.1.1), and 16-24 hours prior to transfection occurring, seeded at the appropriate density (as determined in 4.3.1.1 and 5.3.1.1) per well of a 96-well plate. A total of 50ng and 100ng of vector DNA were transfected per well (as determined in 4.3.1.2 and 5.2.3.1) of D17 cells and CMT3, CMT7, CMT8 and MDCK cells, respectively. The amounts of DNA of each vector transfected per cell line are displayed in Table 5.3. 4 wells remained untransfected for each cell line (NTC) and 4 wells containing no cells for each cell culture medium (background) were used at each time point to act as negative controls. 24 hours after transfection, NFZ was

added to the cells. The appropriate amount of freshly made NFZ stock solution in DMSO was added to each cell culture medium and DMSO added, to adjust the final DMSO concentration to 0.5% (5.3.1.2). The cell culture medium was replaced after 72 hours with fresh medium.

The mean absorbance reading for each set of 4 wells was determined. The background readings were removed and the percentages of cell survival (+/-SE), compared to the 0 hour time point for the NTC (100%), calculated.

Cell Line		NTR		CAT	
		D17	CMT3, CMT7, CMT8 & MDCK	D17	CMT3, CMT7, CMT8 & MDCK
Vector DNA (ng)	pCMVCAT6	40	80	50	100
	pCMVNTR	10	20	-	-
Total amount of DNA transfected (ng)		50	100	50	100

Table 5.3: The amounts of pCMVNTR and pBLCAT6 (in ng) transfected into D17, CMT3, CMT7, CMT8 and MDCK cell lines to enable the analysis of the effects of NFZ, following transfection of pCMVNTR, on cell survival.

5.2.4.1 D17 assays

In the first preliminary experiment, D17 cells were treated with 1 μ M and 5 μ M NFZ and cell numbers determined after 12 and 24 hours. The second preliminary experiment was carried out to determine the concentration at which NFZ reduces the survival of pCMVNTR-transfected cells by ~50%, but NTC remain unaffected. A range of concentrations from 0.002 - 1 μ M (0.002, 0.004, 0.008, 0.016, 0.031, 0.063, 0.125, 0.25, 0.5 and 1 μ M) NFZ were applied to D17 cells for 144 hours.

The 'proper' experiment consisted of treating D17 cells with 0.125 μ M and 0.25 μ M NFZ for 96 hours and cell numbers determined at 0, 12, 24, 48 and 96 hours. This experiment was repeated 4 times.

5.2.4.2 CMT3, CMT7 and CMT8 assays

Concentrations below 0.125 μ M NFZ were observed to not reduce cell survival in the second D17 preliminary experiment. The first two preliminary experiments were carried out on CMT3 cells, using a range of concentrations from 0.0625 - 0.5 μ M (0.0625, 0.125, 0.25 and 0.5 μ M) NFZ in the first and 0.5 - 4 μ M (0.5, 1, 2

and 4 μ M) in the second, to obtain 2 concentrations at which NFZ reduces the survival of pCMVNTR-transfected cells by ~ 50% and 100%. The CMT3 cells in both experiments were treated with the various concentrations of NFZ for 96 hours and cell numbers determined at 0, 12, 24, 48 and 96 hours.

The next preliminary experiment involved the treatment of CMT7 cells with 0.0625, 0.125, 0.25 and 0.5 μ M NFZ, over 144 hours. The fourth consisted of treating CMT8 cells with 0.0625, 0.125, 0.25 and 0.5 μ M NFZ over 96 hours and cell numbers determined at 0, 12, 24, 48 and 96 hours.

In the 'proper' experiment, CMT3 cells were treated with 0.5 μ M and 1 μ M NFZ and CMT7 and CMT8 cells with 0.25 μ M and 0.5 μ M NFZ, over 144 hour time period.

5.2.4.3 Non-osteosarcoma cell line assays

MDCK and CML-10 cells were treated with 0.25 μ M and 0.5 μ M NFZ, over 144 hour time period.

5.2.5 Analysis of pCMVNTR and TP53-targeted suicide gene therapy strategy in combination with CB1954

The pCMVNTR, pCMVCAT6, pTarget-Lexp53, pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR vectors were used to investigate the effects of CB1954 on the survival of D17 and CMT7 cells. 3 experiments were carried out, each only performed once, 1 preliminary experiment repeated in quadruplicate per time point, followed by 2 'proper' experiments, in triplicate per time point. All the experiments assessed the transfection of pCMVNTR in combination with pCMVCAT6 and NTC. The 2 'proper' experiments also examined the transfection of pCMVCAT6 alone, and pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR in combination with pCMVCAT6 or pTargetLex-p53. The vectors were prepared using the QIAGEN EndoFree Plasmid Purification Maxi kit (2.2.8.3). Each vector was transfected into the cell lines using the TransfastTM reagent (Promega; 2.2.9). The CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega;

2.2.10) was employed to determine cell numbers immediately before the addition of CB1954 (0 hour time point) and after cells had been treated for the required amount of time.

In the preliminary experiment, D17 and CMT7 cells were treated with 0.15, 0.3, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 μ M CB1954 for 48 hours and cell numbers determined at 0, 12, 24 and 48 hours. This was performed to obtain 2 concentrations at which CB1954 reduces the survival of pCMVNTR-transfected cells, particularly CMT7 cells, by ~ 50 and 100%.

The 2 'proper' experiments were similar in that D17 and CMT7 cells were treated with 5 μ M and 20 μ M CB1954, and cell numbers determined at 0, 12, 24, 48, 96 and 144 hours. The cell culture medium was replaced after 72 hours with fresh medium. The exception was that the first was carried out one in the absence of NADH and the second in the presence of NADH (Sigma; Appendix A). In this second experiment, NADH was applied at the same time as CB1954, at a final concentration of 100 μ M.

The 2 cell lines were each cultured and maintained (2.1.1) and 16-24 hours prior to transfection occurring, seeded at the appropriate density (as determined in 4.3.1.1) per well of a 96-well plate. A total of 50ng and 100ng of vector DNA were transfected per well (as determined in 4.3.1.2 and 5.2.3.1) of D17 and CMT7 cells, respectively. The amounts of DNA of each vector transfected per cell line are displayed in Table 5.4. 4 wells remained untransfected for each cell line (NTC) and 4 wells containing no cells for each cell culture medium (background) were used at each time point to act as negative controls. 24 hours after transfection, CB1954 was added to the cells. The appropriate amount of freshly made CB1954 stock solution in DMSO was added to each cell culture medium and DMSO added, to adjust the final DMSO concentration to 0.5% (5.3.1.2).

The mean absorbance reading for each set of 4 wells was determined. The background readings were removed and the percentages of cell survival (+/-SE), compared to the 0 hour time point for the NTC (100%), calculated.

<i>Cell Line</i>		NTR		CAT		B/E/P -8LA		B/E/P -8LA+p53	
		D17	CMT7	D17	CMT7	D17	CMT7	D17	CMT7
Vector DNA (ng)	pCMVCA76	40	80	50	100	40	80	-	-
	pTargetLex-p53	-	-	-	-	-	-	40	80
	PGL3-8LANTR (B, E or P)	-	-	-	-	10	20	10	20
	pCMVNTR	10	20	-	-	-	-	-	-
Total amount of DNA transfected (ng)		50	100	50	100	50	100	50	100

Table 5.4: The amounts of DNA (in ng) and combinations of vectors transfected into D17 and CMT7 cell lines to enable the analysis of pCMVNTR and TP53-targeted suicide gene therapy strategy, in combination with CB1954, on cell survival. Abbreviations: ‘NTR’, pCMVNTR in combination with pCMVCA76; ‘CAT’, pCMVCA76; ‘B/E/P -8LA’, pGL3B8LA-NTR, pGL3E8LA-NTR or pGL3P8LA-NTR, in combination with pCMVCA76, respectively; ‘B/E/P -8LA+p53’, pGL3B8LA-NTR, pGL3E8LA-NTR or pGL3P8LA-NTR, in combination with pTargetT-Lexp53, respectively. .

5.3 Results

5.3.1 Optimization assays

5.3.1.1 Cell number

The concentrations of MDCK and CML-10 cells proposed to obtain 80% confluence, 50000 cells/ml and 75000 cells/ml, respectively, were correct. This enabled the same number of cells to be transfected for each cell line, in all the subsequent assays performed in this study, without any toxicity incurring due to the transfection of low cell densities (200 μ l/well of a 96-well plate; 13ml per 25cm² flask).

5.3.1.2 DMSO

The survival curves for the D17, CMT3, CMT7 and CMT8 cell lines treated with 0, 0.125, 0.25, 0.5, 1 and 2% of DMSO for 24 hours are displayed in Figure 5.3. The post hoc pairwise comparison results are shown in Table 5.5. There were no statistically significant differences between the untreated (0%) cells to those treated with 0.125, 0.25, 0.5, 1 and 2% DMSO. However, the appearance of the cells treated with 1% DMSO for all four cell lines was not normal and in the presence of 2% DMSO clear signs of cytotoxicity were apparent. Therefore it was determined that DMSO would contribute 0.5% to the final volume of NFZ and CB1954 in solution.

The maximum percentage of DMSO that could be used, in the final volume, to dissolve NFZ and CB1954 without having a cytotoxic effect on D17, CMT3, CMT7 and CMT8 cells was determined to be 0.5%. In all NFZ and CB1954 experiments, the NTC were cultured in medium containing 0.5% DMSO and appeared to grow normally.

Group	Treatment (% DMSO)	D17	CMT3	CMT7	CMT8
1	0				
2	0.125			6	
3	0.25	6		6	
4	0.5	6		6	
5	1				
6	2	3, 4		2, 3, 4	

Table 5.5: Post hoc pairwise comparisons of treatment groups in Figure 5.3, listing the treatment groups which were significantly different from each group for the D17, CMT3, CMT7 and CMT8 cell lines (significance was set at $p = 0.05$).

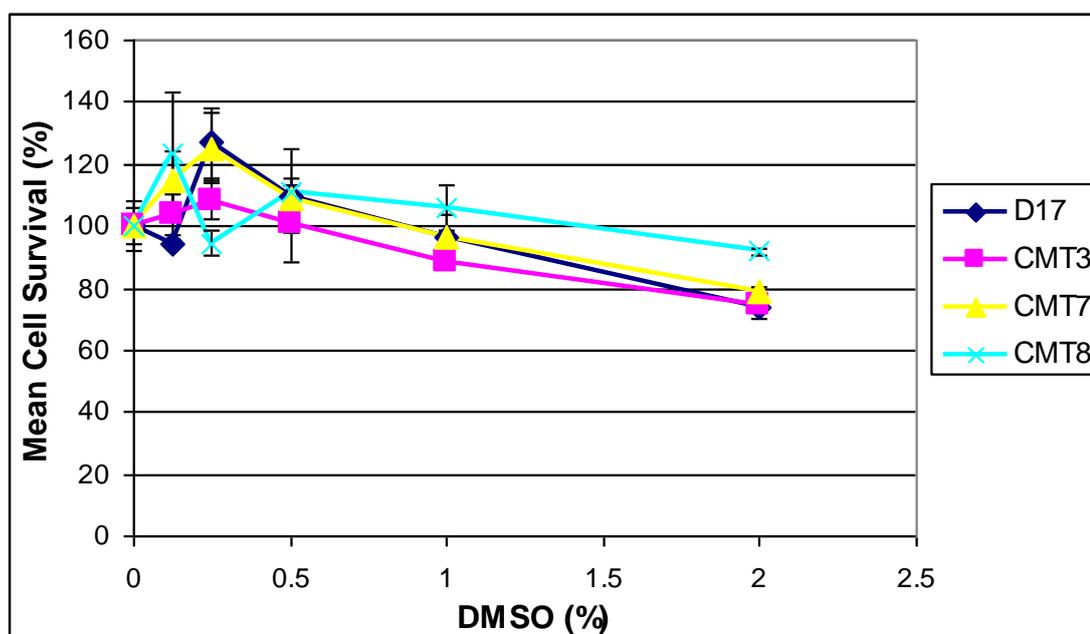


Figure 5.3: The effect of DMSO, at 0, 0.5, 1 and 2%, for 24 hours on the survival of D17, CMT3, CMT7 and CMT8 cells. (Each data point represents the mean of 4 values; bars represent \pm SE).

5.3.2 Detection of nitroreductase protein expression by pCMVNTR and pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR, in combination with pTarget-Lexp53 or pCMVCAT6

NTR immunopositivity was detected, using the anti-NTR antibody, in the positive control, purified *E. coli* NTR enzyme and the cell lysates of D17, CMT3, CMT7,

CMT8 and MDCK cells transfected with pCMVNTR, in combination with pCMVCAT6 (Figure 5.4). NTR was not detected in the NTC lysates of any of the 5 cell lines. There was no evidence of immunopositivity in the: D17 and CMT7 cell lysates only transfected with pCMVCAT6; D17 cells transfected with pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR, in combination with pTargetT-Lexp53 or pCMVCAT6 (Figure 5.4B); and CMT7 cell lysates transfected with pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR, in combination with pCMVCAT6 (Figure 5.4C). However, NTR is detected in the CMT7 cell lysates transfected with pGL3P8LA-NTR in combination with pTargetT-Lex53; although expression was not as strong as that detected in the pCMVNTR transfected cell lysates. Unfortunately no evidence was provided of NTR immunopositivity following transfection of pGL3B8LA-NTR and pGL3E8LA-NTR in combination with pTargetT-Lexp53.

These results indicate that the D17, CMT3, CMT7, CMT8, and MDCK NTC lysates do not appear to endogenously express NTR. pCMVNTR can be successfully transfected into these 5 cell lines and expression of NTR detected. NTR expression was only detected in the CMT7 cells transfected with pGL3P8LA-NTR in combination with pTargetT-Lexp53. These results are in agreement with those obtained using the DLR, previously described in 4.3.4. Hence, the TP53-targeted suicide gene therapy system was then able to be tested in cell survival assays.

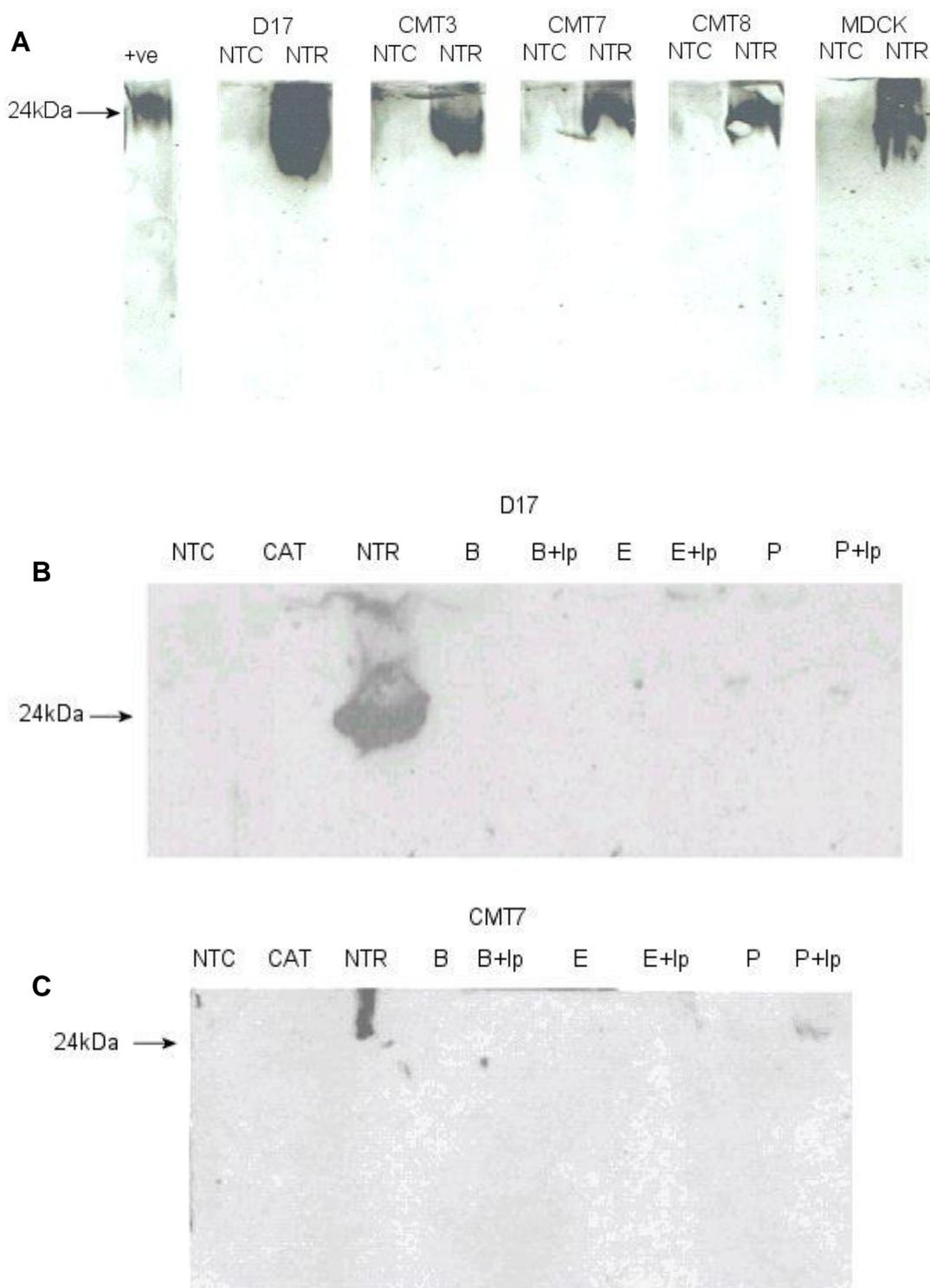


Figure 5.4: Chemiluminescent immunoblots of NTR (24kDa) detected using anti-NTR primary antibody in lysates prepared from: (A) D17, CMT3, CMT7, CMT8 and MDCK cells transfected with pCMVNTR (NTR), in combination with pCMVCAT6 (50 μ g protein loaded per lane; +ve - positive control, 5ng purified *E. coli* nitroreductase enzyme); D17 (B) and (C) CMT7 cells, transfected with pCMVCAT6 (CAT), pCMVNTR in combination with pCMVCAT6 (NTR), and pGL3B8LA-NTR pGL3E8LA-NTR and pGL3B8LA-NTR in combination with pCMVCAT6 (B, E and P, respectively) or pTargetLex-p53 (B+lp, E+lp and P+lp, respectively) (25 μ g protein loaded per lane). (NTC - non-transfected cell lysates; arrow indicates position of NTR protein).

5.3.3 Analysis of pCMVNTR in combination with nitrofurazone

5.3.3.1. D17 assays

5.3.3.1.1 Preliminary experiment 1

The data from this experiment are summarized in Figure 5.5 and the post hoc pairwise comparison results are displayed in Table 5.6. In the absence of NFZ treatment, it was observed that the NTC, appeared to grow normally during the 24 hour treatment period, whereas, the cells transfected with pCMVNTR suffered a statistically significant decrease in cell survival, probably due to toxicity from transfection. In the presence of 1 μ M and 5 μ M NFZ treatment, the survival of NTC was statistically significantly reduced in comparison to those not treated and after 24 hours ~10% cells remained in both sets of treated cells. There was also a statistically significant difference between the cells transfected with pCMVNTR and those transfected with pCMVNTR and treated with 1 μ M and 5 μ M NFZ. After 12 hours of 5 μ M NFZ treatment, none of the pCMVNTR transfected cells were alive and with 1 μ M NFZ treatment, only ~10% had survived. After 24 hours, none of the NTR-transfected cells treated with 1 μ M NFZ were alive. There were no statistically significant differences between NTC and pCMVNTR transfected cells treated with 1 μ M or 5 μ M NFZ.

Hence, 1 μ M and 5 μ M NFZ results in statistically significant decreases in survival of both NTC and pCMVNTR transfected D17 cells.

Group	Treatment	D17
1	NTC	All other treatments
2	NTC + 1 μ M NFZ	1, 4
3	NTC + 5 μ M NFZ	1, 4
4	NTR	All other treatments
5	NTR + 1 μ M NFZ	1, 4
6	NTR + 5 μ M NFZ	1, 4

Table 5.6: Post hoc pairwise comparisons of treatment groups in Figure 5.5, listing the treatment groups which were significantly different from each group for the D17 cell line over the 24-hour treatment period (significance was set at $p = 0.05$).

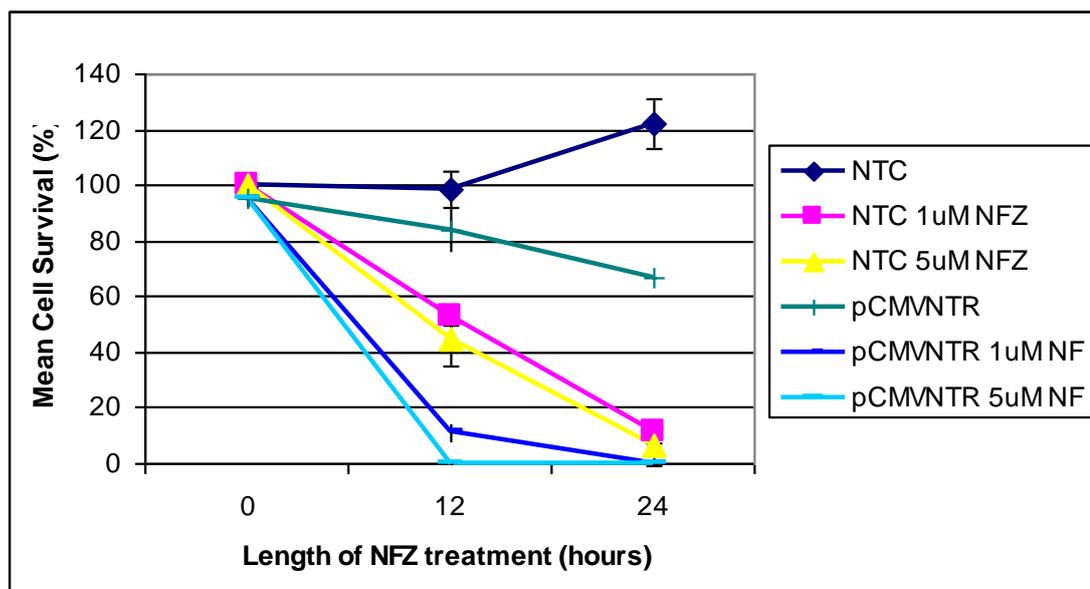


Figure 5.5: The effect of $1\mu\text{M}$ and $5\mu\text{M}$ nitrofurazone (NFZ) on survival of D17 cells, following the transfection of pCMVNTR (NTR), over 24 hours. ('NTC', non-transfected cells; each time point was repeated in quadruplicate; bars represent \pm SE).

5.3.3.1.2 Preliminary experiment 2

The data from this experiment are summarized in Figure 5.6 and the post hoc pairwise comparison results are displayed in Table 5.7. There were no statistically significant differences in mean cell survival percentages after 144 hours between the NTC, cells transfected with pCMVNTR and the NTC and pCMVNTR-transfected cells treated with $0.002 - 0.0625\mu\text{M}$ NFZ. There were also no statistically significant decreases between the NTC and cells transfected with pCMVNTR, with NTC treated with $0.125\mu\text{M}$ and $0.5\mu\text{M}$ NFZ. Statistically significant reductions in cell survival were observed between the NTC and cells transfected with pCMVNTR, with NTC treated with $0.5\mu\text{M}$ and $1\mu\text{M}$ NFZ and pCMVNTR-transfected cells treated with $0.125 - 1\mu\text{M}$ NFZ. The NTC treated with $0.5\mu\text{M}$ and $1\mu\text{M}$ NFZ were not statistically different to each other and nor were the pCMVNTR-transfected cells treated with $0.125 - 1\mu\text{M}$ NFZ. The pCMVNTR-transfected cells treated with $0.25 - 1\mu\text{M}$ NFZ were significantly different to NTC treated with $0.5\mu\text{M}$ NFZ but not to NTC treated with $1\mu\text{M}$ NFZ.

Hence, it appears that 0.125 μ M NFZ was the only concentration to cause a large decrease in survival of pCMVNTR-transfected cells and a minimal amount in NTC after 144 hours treatment. Treatment of pCMVNTR-transfected cells with 0.25, 0.5 and 1 μ M NFZ induced >85% reduction in cell survival after 12 hours and ~100% by 24 hours. In comparison, the NTC treated with 0.25 μ M NFZ remained largely viable for 48 hours and by 144 hours, ~60% were still alive. The NTC treated with 0.5 μ M and 1 μ M NFZ after 12 hours were reduced to ~80% and 50% after 12 hours, and ~15% and 0% after 144 hours, respectively. Therefore, 0.125 μ M and 0.25 μ M NFZ concentrations were to be used in the subsequent D17 NFZ experiments.

Group	Treatment	D17
1	NTC	10, 11, 19, 20, 21, 22
2	NTC + 0.002 μ M NFZ	10, 11, 19, 20, 21, 22
3	NTC + 0.004 μ M NFZ	10, 11, 19, 20, 21, 22
4	NTC + 0.008 μ M NFZ	10, 11, 19, 20, 21, 22
5	NTC + 0.016 μ M NFZ	10, 11, 19, 20, 21, 22
6	NTC + 0.031 μ M NFZ	9, 10, 11, 19, 20, 21, 22
7	NTC + 0.0625 μ M NFZ	10, 11, 19, 20, 21, 22
8	NTC + 0.125 μ M NFZ	10, 11, 19, 20, 21, 22
9	NTC + 0.25 μ M NFZ	6, 11, 19, 20, 21, 22
10	NTC + 0.5 μ M NFZ	1, 2, 3, 4, 5, 6, 7, 8, 12, 13, 14, 15, 16, 17, 20, 21, 22
11	NTC + 1 μ M NFZ	1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, 15, 16, 17, 18
12	NTR	10, 11, 19, 20, 21, 22
13	NTR + 0.002 μ M NFZ	10, 11, 19, 20, 21, 22
14	NTR + 0.004 μ M NFZ	10, 11, 19, 20, 21, 22
15	NTR + 0.008 μ M NFZ	10, 11, 19, 20, 21, 22
16	NTR + 0.016 μ M NFZ	10, 11, 19, 20, 21, 22
17	NTR + 0.031 μ M NFZ	10, 11, 19, 20, 21, 22
18	NTR + 0.0625 μ M NFZ	11, 19, 20, 21, 22
19	NTR + 0.125 μ M NFZ	1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, 15, 16, 17, 18
20	NTR + 0.25 μ M NFZ	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18
21	NTR + 0.5 μ M NFZ	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18
22	NTR + 1 μ M NFZ	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18

Table 5.7: Post hoc pairwise comparisons of treatment groups in Figure 5.6 (A&B), listing the treatment groups which were significantly different from each group over the 144hour treatment period (significance was set at $p = 0.05$).

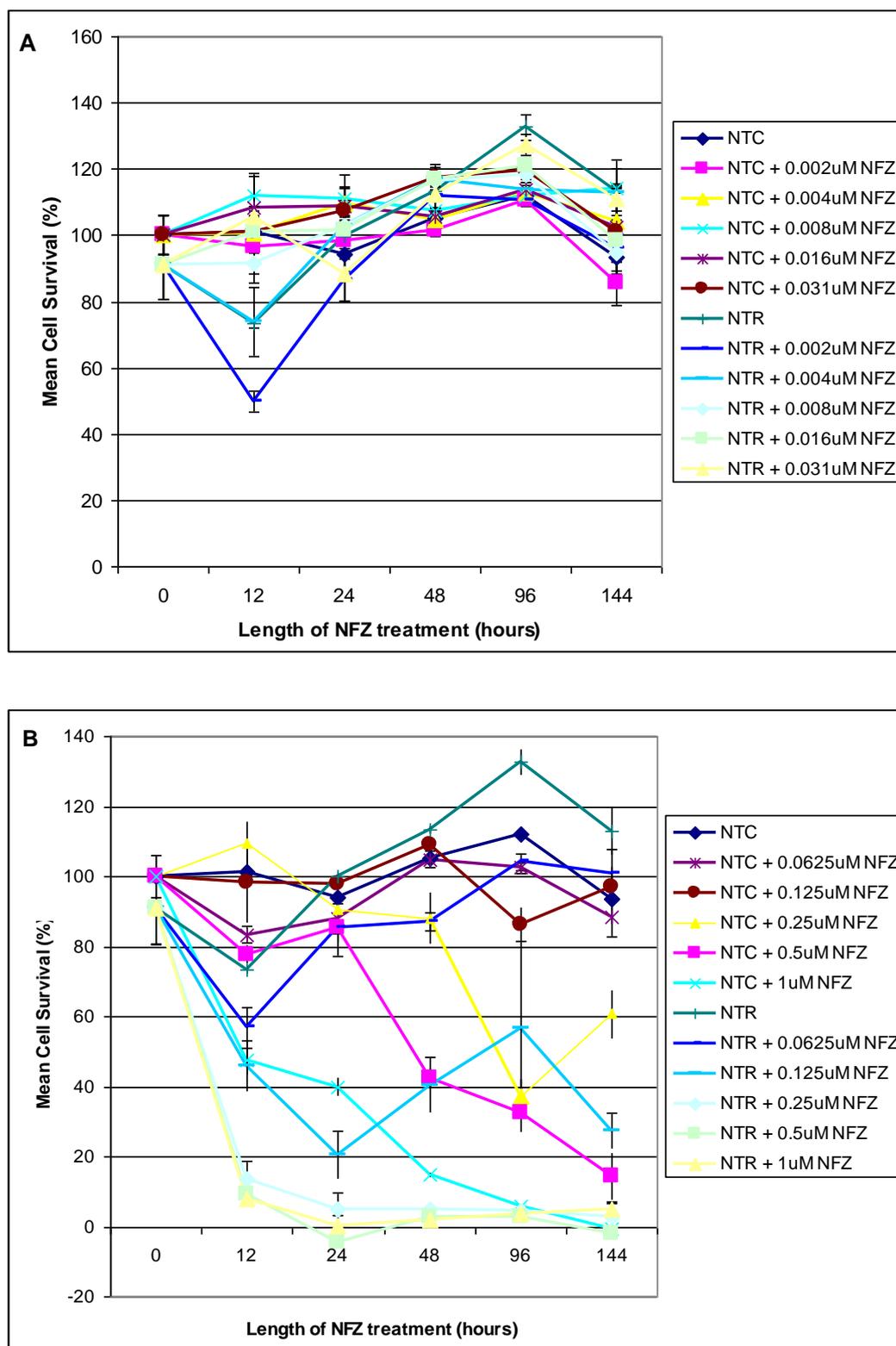


Figure 5.6: The effect of 0.002 - 1 μ M nitrofurazone (NFZ) on survival of D17 cells, following the transfection of pCMVNTR (NTR), over 144 hours: (A) 0.002, 0.004, 0.008, 0.016 and 0.031 μ M NFZ; (B) 0.063, 0.125, 0.25, 0.5 and 1 μ M NFZ. ('NTC', non-transfected cells; each time point was repeated in quadruplicate; bars represent +/- SE).

5.3.3.1.3 D17 experiment

The data from this experiment are summarized in Figure 5.7 and the post hoc pairwise comparison results are displayed in Table 5.8. There were no statistically significant differences in mean cell survival percentages after 96 hours between the NTC, pCMVNTR- and pCMVCAT-transfected cells without NFZ treatment. Hence, cell growth was not affected by the transfection of pCMVNTR and pCMVCAT. However there were statistically significant decreases between the untreated NTC, pCMVNTR- and pCMVCAT-transfected cells with the NTC, pCMVNTR- and pCMVCAT-transfected cells treated with 0.125 μ M and 0.5 μ M NFZ. There were no statistical differences between the NTC and pCMVCAT-transfected cells, following treatment with 0.125 μ M and 0.25 μ M NFZ, showing that the observed decrease in cell survival was due to NFZ treatment. However there were statistical differences between the NTC and pCMVNTR-transfected cells treated with 0.125 μ M and 0.25 μ M NFZ. There were also statistical differences between the NTC and pCMVNTR-transfected cells treated with 0.125 μ M to those treated with 0.25 μ M but not between these treatments in pCMVCAT-transfected cells.

The largest reduction in cell survival was observed in the pCMVNTR-transfected cells treated with 0.25 μ M NFZ. After 12 hours, <20% cells had survived, and by 96 hours, only a small percentage remained. ~40% of the pCMVNTR-transfected cells treated with 0.125 μ M NFZ and the NTC and pCMVCAT-transfected cells treated with 0.25 μ M NFZ, survived 24hours, but after 96 hours, cell survival was reduced to <10%. The smallest decrease in cell survival following NFZ treatment, was observed in the NTC treated with 0.125 μ M NFZ, in which ~70% were alive after 24 hours and ~35% after 96 hours.

Hence, NFZ was seen to reduce survival in pCMVNTR-transfected D17 cells and to a slightly lesser extent in NTC and pCMVCAT-transfected D17 cells. This suggests that NFZ is activated by endogenous enzymes in these canine cells.

Group	Treatment	D17
1	NTC	2, 3, 5, 6, 8, 9
2	NTC + 0.125 μ M NFZ	All except 5
3	NTC + 0.25 μ M NFZ	1, 2, 4, 7, 9
4	CAT	2, 3, 5, 6, 8, 9
5	CAT + 0.125 μ M NFZ	1, 4, 7, 9
6	CAT + 0.25 μ M NFZ	1, 2, 4, 7, 9
7	NTR	2, 3, 5, 6, 8, 9
8	NTR + 0.125 μ M NFZ	1, 2, 4, 7, 9
9	NTR + 0.25 μ M NFZ	All other treatments

Table 5.8: Post hoc pairwise comparisons of treatment groups in Figure 5.7, listing the treatment groups which were significantly different from each group over the 96hour treatment period (significance was set at $p = 0.05$).

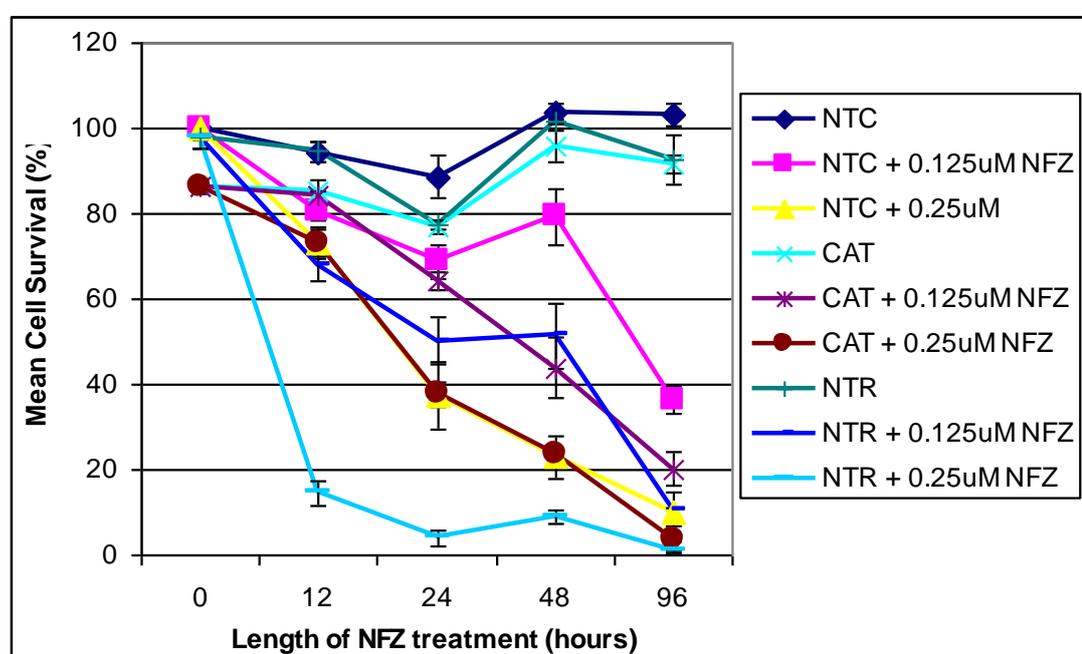


Figure 5.7: The effect of 0.125 μ M and 0.25 μ M nitrofurazone (NFZ) on survival of D17 cells, following the transfection of pCMVNTR (NTR), and pCMVCAT6, over 96 hours. ('NTC', non-transfected cells; this assay and each time point was repeated in quadruplicate; bars represent \pm SE).

5.3.3.2 CMT3, CMT7 and CMT8 assays

5.3.3.2.1 Preliminary experiments

5.3.3.2.1.1 CMT3 experiment 1

The data from this experiment are summarized in Figure 5.8. There were no significant interactions found between post hoc pairwise comparisons of treatment groups shown in Figure 5.8. Cell survival was observed to increase

after 96 hours in untreated NTC and pCMVNTR-transfected cells and NTC and pCMVNTR-transfected cells treated with 0.062, 0.125 and 0.25 μ M NFZ.

Treatment with 0.5 μ M NFZ achieved ~40% reduction in cell survival in NTC and pCMVNTR-transfected cells after 96 hours.

Hence, it appears that CMT3 cells are not as sensitive to NFZ as D17 cells and so a second preliminary assay was performed using higher concentrations of NFZ.

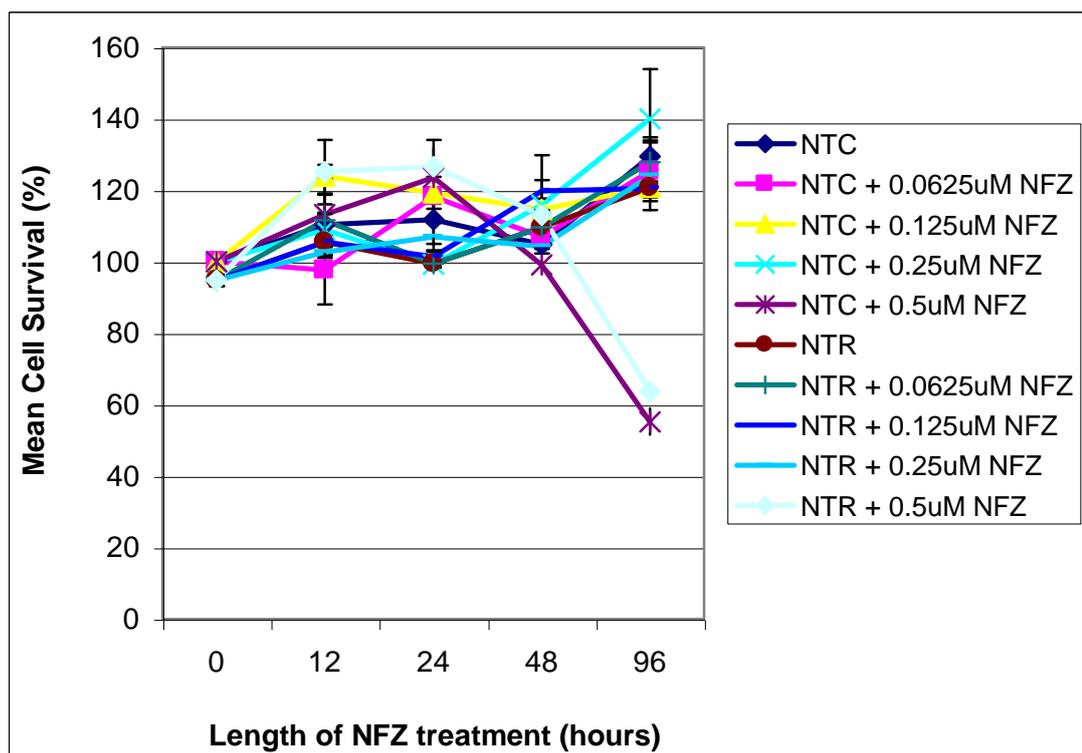


Figure 5.8: The effect of 0.0625 - 0.5 μ M nitrofurazone (NFZ) on survival of CMT3 cells, following the transfection of pCMVNTR (NTR), over 96 hours. ('NTC', non-transfected cells; each time point was repeated in quadruplicate; bars represent +/- SE).

5.3.3.2.1.2 CMT3 experiment 2

The data from this experiment are summarized in Figure 5.9 and the post hoc pairwise comparison results are displayed in Table 5.9. There were no statistically significant differences in mean cell survival percentages after 96 hours between the NTC and pCMVNTR-transfected cells without NFZ treatment. Hence, cell growth was not affected by the transfection procedure. There were also no significant differences between untreated NTC and pCMVNTR-transfected cells and those treated with 0.5 μ M and 1 μ M NFZ.

Statistically significant decreases were found between the untreated NTC following treatment with 2 μ M and 4 μ M NFZ, in both the NTC and pCMVNTR-transfected cells. The cells treated with 4 μ M NFZ were significantly different to those treated with 0.5, 1 μ M and 2 μ M NFZ. However, there were no statistical differences between the 2 μ M NFZ treated cells and those treated with 0.5 μ M and 1 μ M NFZ. In addition, there were no significant differences between any of the NFZ treatments in the NTC with the equivalent treatment in the pCMVNTR-transfected cells as the NTC cells had similar growth patterns to the transfected cells.

Following treatment with 1, 2 and 4 μ M NFZ, NTC and NTR-transfected cells, ~100% reduction in cell survival was observed after 96 hours. 0.5 μ M NFZ produced similar effects on cell survival to that observed in the previous preliminary assay with pCMVNTR-transfected cells and NTC displaying ~60% cell survival after 96 hours. However due to the similar cell survival curves observed between the NTC and NTR-transfected cells for the 4 different NFZ treatments, this suggests that either the transfection of pCMVNTR in CMT3 cells does not increase predisposition to larger decreases in cell survival following NFZ treatment, than in their non-transfected counterparts, or an experimental error occurred. This assay should have been repeated as it was only performed once.

Group	Treatment	CMT3
1	NTC	4, 5, 9, 10
2	NTC + 0.5 μ M NFZ	5, 10
3	NTC + 1 μ M NFZ	5, 10
4	NTC + 2 μ M NFZ	1, 5, 10
5	NTC + 4 μ M NFZ	All except 10
6	NTR	5, 10
7	NTR + 0.5 μ M NFZ	5, 10
8	NTR + 1 μ M NFZ	5, 10
9	NTR + 2 μ M NFZ	1, 5, 10
10	NTR + 4 μ M NFZ	All except 5

Table 5.9: Post hoc pairwise comparisons of treatment groups in Figure 5.9, listing the treatment groups which were significantly different from each group over the 96 hour treatment period (significance was set at $p = 0.05$).

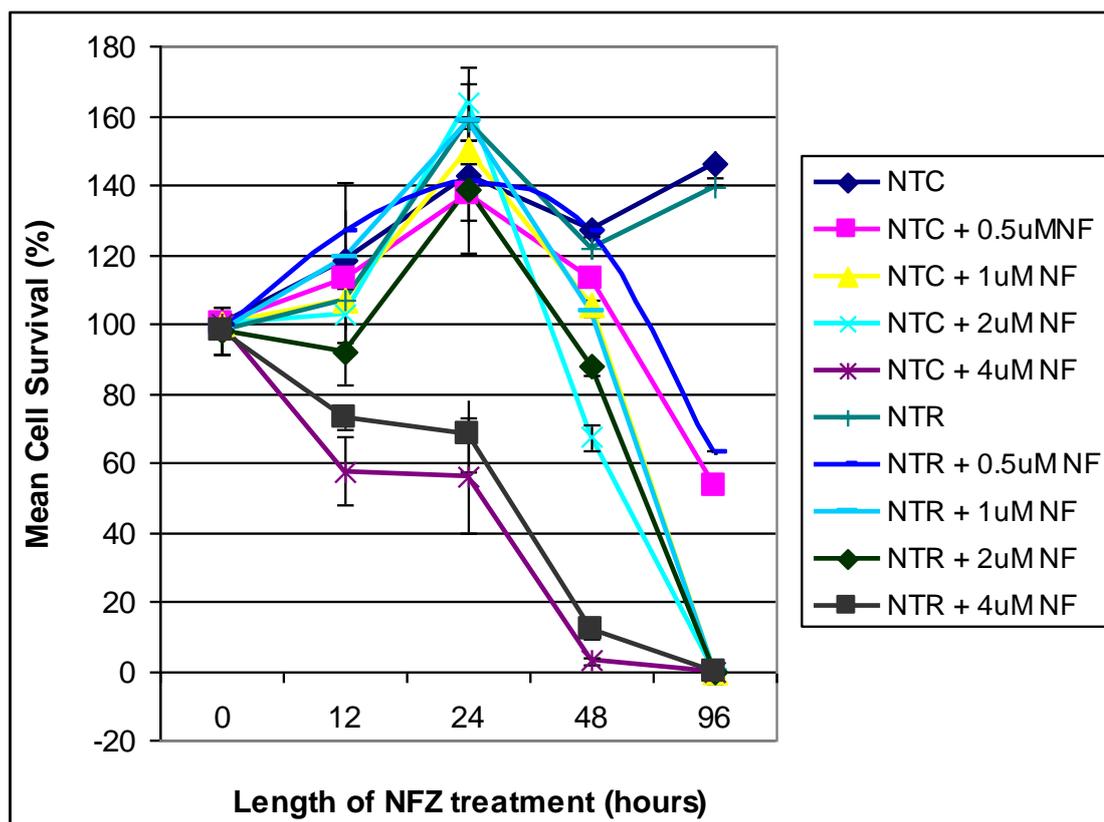


Figure 5.9: The effect of 0.5 - 4 μ M nitrofurazone (NFZ) on survival of CMT3 cells, following the transfection of pCMVNTR (NTR), over 96 hours. ('NTC', non-transfected cells; each time point was repeated in quadruplicate; bars represent +/- SE).

5.3.3.2.1.3 CMT7 experiment

The data from this experiment are summarized in Figure 5.10 and the post hoc pairwise comparison results are displayed in Table 5.10. There were no statistically significant differences in mean cell survival percentages after 144 hours between the NTC and pCMVNTR-transfected cells without NFZ treatment. Hence, cell growth was not affected by the transfection procedure. There were also no significant differences between untreated NTC and pCMVNTR-transfected cells and NTC treated with 0.0625, 0.125 and 0.25 μ M NFZ and pCMVNTR-transfected cells treated with 0.0625 μ M and 0.125 μ M NFZ.

Statistically significant decreases were found between the untreated NTC and pCMVNTR-transfected cells with NTC treated with 0.5 μ M NFZ and pCMVNTR-transfected cells treated with 0.25 μ M and 0.5 μ M NFZ. There were also significant differences between the NTC treated with 0.25 μ M and 0.5 μ M NFZ

with the equivalent treatment in the pCMVNTR-transfected cells. The NTC treated with 0.5 μ M were not statistically different to pCMVNTR-transfected cells treated with 0.25 μ M NFZ, which indicates that the expression of NTR in CMT7 cells decreases the amount of NFZ required to reduce cell survival.

After 96 hours of 0.25 μ M NFZ treatment, the survival of pCMVNTR-transfected cells was ~60%, whereas no decrease in survival was observed in NTC. Treatment with 0.5 μ M NFZ resulted in ~50% reduction in cell survival occurring after 12 hours in the pCMVNTR-transfected cells and nearly 100% at 96 hours. In the NTC, 0.5 μ M NFZ treatment decreased cell survival by ~20% and ~75% after 48 and 96 hours, respectively.

Thus as 0.25 μ M and 0.5 μ M NFZ induced ~ 60% and 100% reductions in cell survival, respectively, in the pCMVNTR-transfected cells, after 96 hours, these 2 concentrations were used in the subsequent CMT 7 experiment (5.3.3.1.2.2).

Group	Treatment	CMT7
1	NTC	5, 9, 10
2	NTC + 0.0625 μ M NFZ	5, 8, 9, 10
3	NTC + 0.125 μ M NFZ	5, 9, 10
4	NTC + 0.25 μ M NFZ	9, 10
5	NTC + 0.5 μ M NFZ	1, 2, 3, 6, 7, 10
6	NTR	5, 9, 10
7	NTR + 0.0625 μ M NFZ	5, 9, 10
8	NTR + 0.125 μ M NFZ	2, 9, 10
9	NTR + 0.25 μ M NFZ	All except 5
10	NTR + 0.5 μ M NFZ	All other treatments

Table 5.10: Post hoc pairwise comparisons of treatment groups in Figure 5.10, listing the treatment groups which were significantly different from each group over the 144 hour treatment period (significance was set at $p = 0.05$).

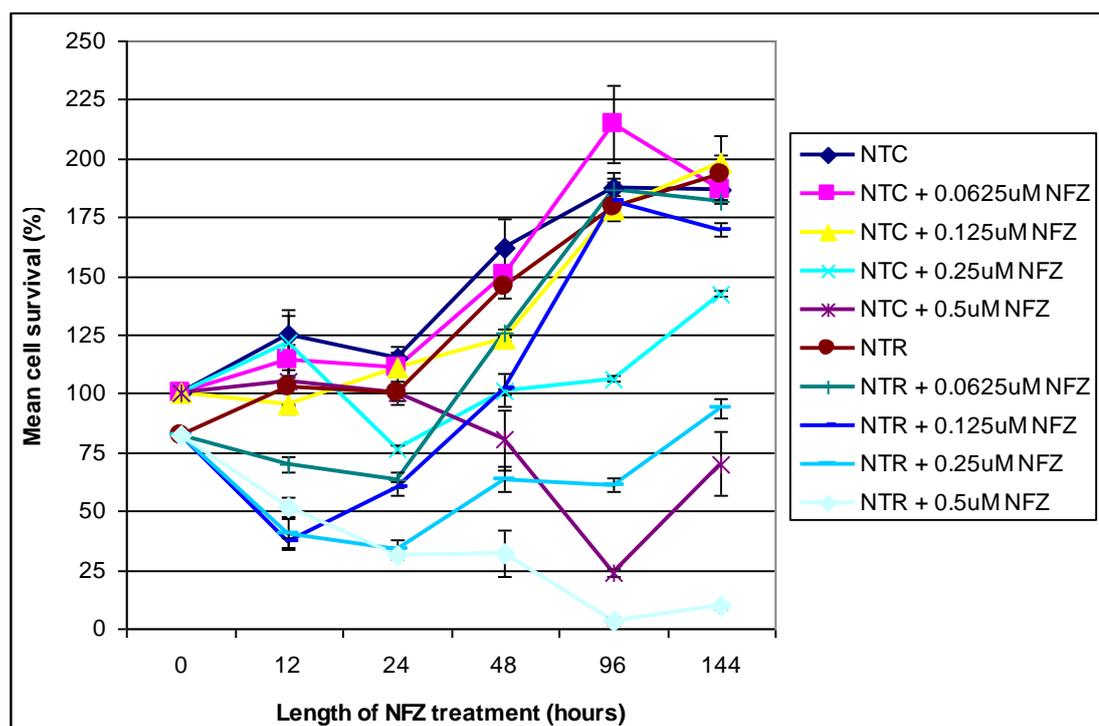


Figure 5.10: The effect of 0.0625 - 0.5 μ M nitrofurazone (NFZ) on survival of CMT7 cells, following the transfection of pCMVNTR (NTR), over 96 hours. ('NTC', non-transfected cells; each time point was repeated in quadruplicate; bars represent +/- SE).

5.3.3.2.1.4 CMT8 experiment

The data from this experiment are summarized in Figure 5.11 and the post hoc pairwise comparison results are displayed in Table 5.11. There were no statistically significant differences in mean cell survival percentages after 96 hours between the NTC and pCMVNTR-transfected cells without NFZ treatment. Hence, cell growth was not affected by the transfection procedure. There were also no significant differences between untreated NTC and pCMVNTR-transfected cells and NTC and pCMVNTR-transfected cells treated with 0.0625 μ M and 0.125 μ M NFZ.

Statistically significant decreases were found between the untreated NTC and pCMVNTR-transfected cells with NTC and pCMVNTR-transfected cells treated with 0.25 μ M and 0.5 μ M NFZ. There were however no significant differences between the NTC treated with 0.25 μ M and 0.5 μ M NFZ with the equivalent treatment in the pCMVNTR-

transfected cells treated with 0.5 μ M NFZ was statistically lower than NTC treated with 0.25 μ M NFZ.

Following 96 hours of 0.25 μ M NFZ treatment, ~75% NTC and pCMVNTR-transfected cells survived. Treatment with 0.5 μ M NFZ, resulted in ~25% survival in the pCMVNTR-transfected cells after 48 hours, compared to ~50% in the NTC. After 96 hours 0.5 μ M NFZ treatment, no NTC and pCMVNTR-transfected cells survived.

Thus, as demonstrated previously in the other cell lines, a decrease in survival of CMT8 cells does not appear to exclusively occur in the pCMVNTR-transfected cells treated with NFZ, as similar reductions also occur in the NFZ treated NTC. 0.25 μ M and 0.5 μ M NFZ were used in the subsequent CMT8 experiment as a means of validating the results from this experiment.

Group	Treatment	CMT8
1	NTC	4, 5, 9, 10
2	NTC + 0.0625 μ M NFZ	4, 5, 9, 10
3	NTC + 0.125 μ M NFZ	5, 10
4	NTC + 0.25 μ M NFZ	1, 2, 6, 10
5	NTC + 0.5 μ M NFZ	1, 2, 3, 6, 7, 8
6	NTR	4, 5, 9, 10
7	NTR + 0.0625 μ M NFZ	5, 9, 10
8	NTR + 0.125 μ M NFZ	5, 10
9	NTR + 0.25 μ M NFZ	1, 2, 6, 7
10	NTR + 0.5 μ M NFZ	1, 2, 3, 4, 6, 7, 8

Table 5.11: Post hoc pairwise comparisons of treatment groups in Figure 5.11, listing the treatment groups which were significantly different from each group over the 96 hour treatment period (significance was set at $p = 0.05$).

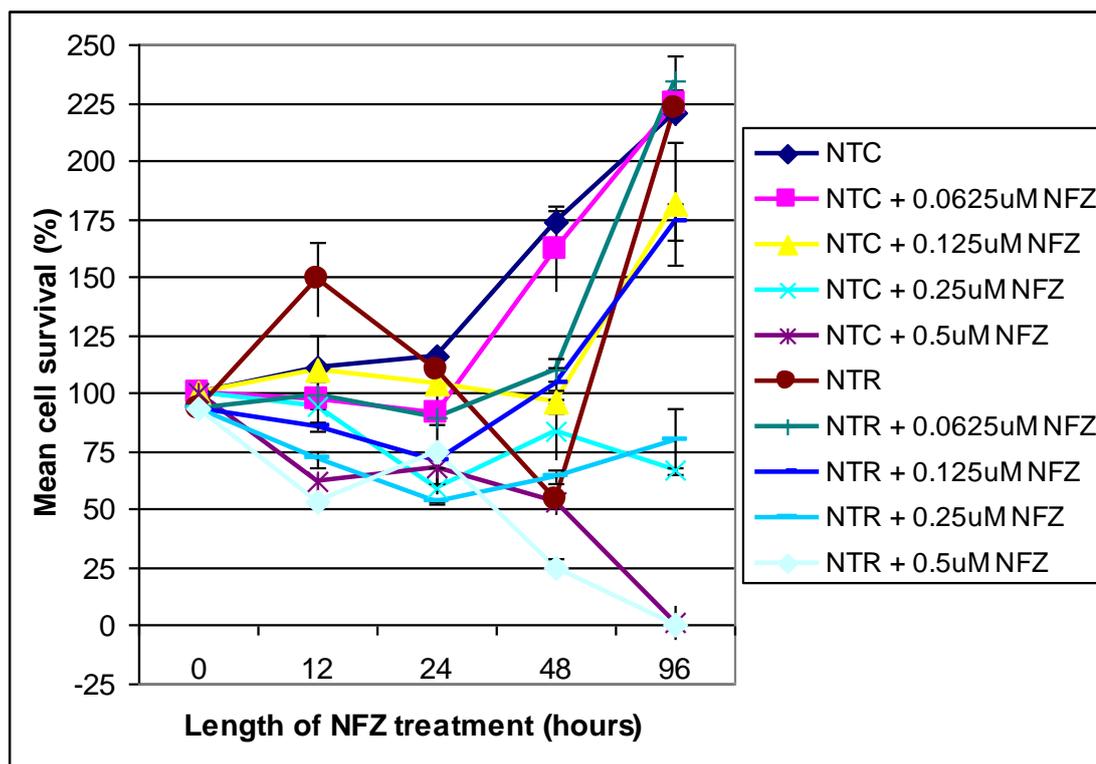


Figure 5.11: The effect of 0.0625 - 0.5 μ M nitrofurazone (NFZ) on survival of CMT8 cells, following the transfection of pCMVNTR (NTR), over 96 hours. ('NTC', non-transfected cells; each time point was repeated in quadruplicate; bars represent \pm SE).

5.3.3.2.2 CMT3, CMT7 and CMT8 experiments

The data from this experiment are summarized in Figure 5.12 and the post hoc pairwise comparison results are displayed in Table 5.12. There were no statistically significant differences in mean cell survival percentages after 144 hours between the NTC and pCMVNTR-transfected cells without NFZ treatment. Hence, cell growth in all 3 cell lines was not affected by the transfection procedure.

Statistically significant decreases were found between the untreated NTC and pCMVNTR-transfected cells with NTC and pCMVNTR-transfected cells treated with 0.5 μ M and 1 μ M NFZ in the CMT3 cells and 0.25 μ M and 0.5 μ M NFZ in the CMT7 and CMT8 cells. There were however no significant differences between the NTC treated with 0.5 μ M and 1 μ M NFZ in the CMT3 cells and 0.25 μ M and 0.5 μ M NFZ in the CMT7 and CMT8 cells with the equivalent treatment in the pCMVNTR-transfected cells. There were statistical differences between the NTC or pCMVNTR-transfected cells treated with 0.25 μ M NFZ and those treated with

0.5 μ M in the CMT3 cells, and 0.25 μ M and 0.5 μ M NFZ in the CMT7 and CMT8 cells, except between the pCMVNTR-transfected CMT8 cells treated with 0.25 μ M and 0.5 μ M NFZ. The NTC treated with 0.5 μ M in CMT3 cells and 0.25 μ M in CMT7 and CMT8 cells were statistically different to pCMVNTR-transfected cells treated with 1 μ M and 0.5 μ M NFZ, respectively. This indicates that in these 3 cell lines, to induce statistically increased reductions in cell survival, a larger amount of NFZ has to be applied to the pCMVNTR-transfected cells than the NTC, which is practically impossible.

Following 96 hours of 0.5 μ M NFZ treatment in the CMT3 cells, ~75% NTC and pCMVNTR-transfected cells survived. Treatment with 1 μ M NFZ, resulted in ~35% cells surviving in the pCMVNTR-transfected cells, compared to ~45% in the NTC. In the CMT7 cells, after the NTC and pCMVNTR-transfected cells had been treated with 0.25 μ M and 0.5 μ M NFZ for 96 hours, ~20% and no cells survived, respectively. Treatment with 0.25 μ M and 0.5 μ M NFZ treatment in the CMT8 cells resulted in 100% decrease in survival after 96 hours.

The increases in cell survival observed at 96 hours were likely due to the removal of NFZ and replacement with fresh medium at 72 hours. Thus showing that these cells are able to recover and increase in number once NFZ is removed.

These results suggest that the expression of NTR in CMT3, CMT7 and CMT8 cells does not effectively predispose them to selective cell kill by NFZ, also indicating that endogenous enzymes are present in these canine cells, as in D17 cells, capable of activating NFZ.

Group	Treatment	CMT3	CMT7	CMT8
1	NTC	2, 3, 5, 6	2, 5, 7, 8	2, 5, 7, 8
2	NTC + 0.5 μ M NFZ	1, 3, 4, 6	1, 4, 7	1, 4, 7
3	NTC + 1 μ M NFZ	1, 2, 4	NA	NA
4	NTR	2, 3, 5, 6	2, 5, 7, 8	2, 5, 7, 8
5	NTR + 0.5 μ M NFZ	1, 4, 6	1, 4, 7, 8	1, 4, 7
6	NTR + 1 μ M NFZ	1, 2, 4, 5	NA	NA
7	NTC + 0.25 μ M NFZ	NA	1, 2, 4, 5	1, 2, 4, 5
8	NTR + 0.25 μ M NFZ	NA	1, 4, 5	1, 4

Table 5.12: Post hoc pairwise comparisons of treatment groups in Figure 5.12, listing the treatment groups which were significantly different from each group over the 144 hour treatment period (significance was set at $p = 0.05$).

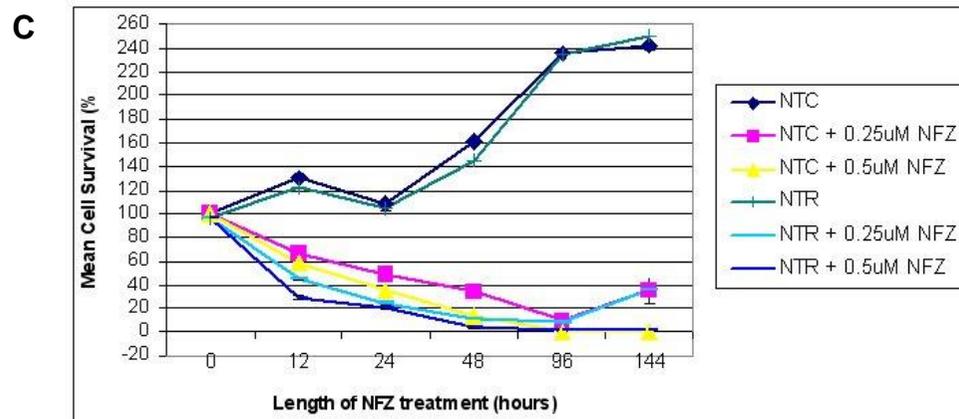
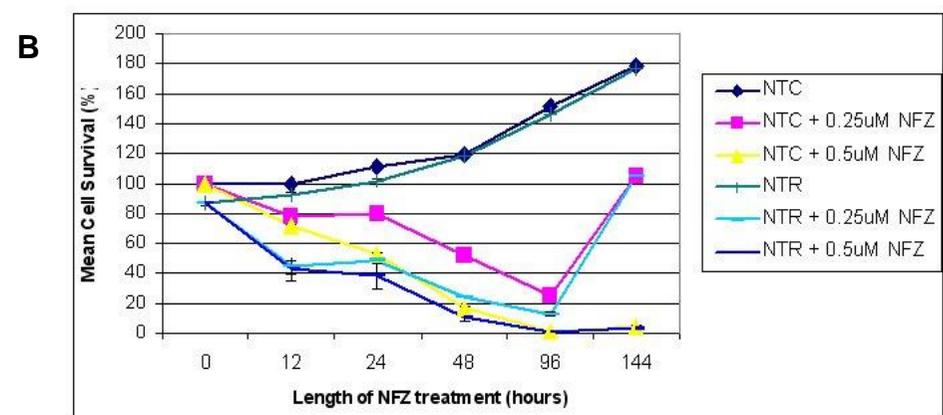
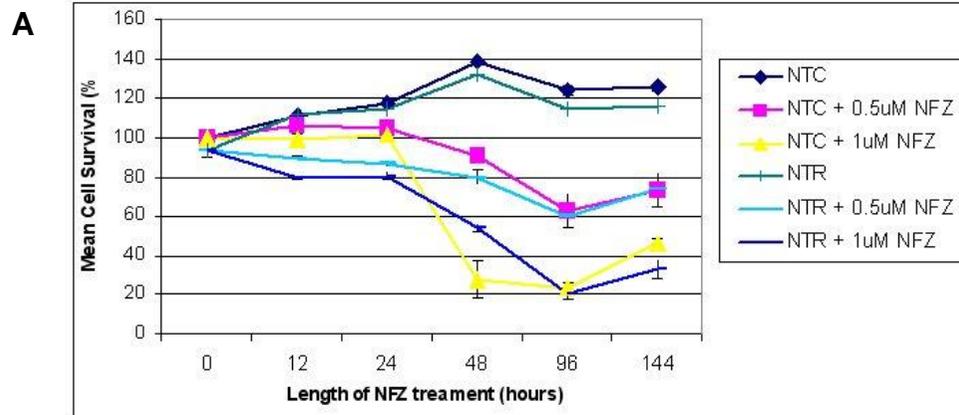


Figure 5.12: The effect of nitrofurazone (NFZ) over 144 hours, following the transfection of pCMVNTR (NTR), on survival of CMT3 cells (A) treated with 0.5µM and 1µM NFZ, and CMT7 (B) and CMT8 (C) cells treated with 0.25µM and 0.5µM NFZ. ('NTC', non-transfected cells; each assay was repeated in triplicate and each time point in quadruplicate; bars represent +/- SE). Please note different scales on Y-axes.

5.3.3.3 Non-osteosarcoma cell line assays

The findings from the NFZ assays suggest that NFZ reduces the survival of canine OS cells regardless of NTR expression. This raised the question as to whether this effect is cell type specific and NFZ was subsequently analysed in 2 non-OS cell lines, MDCK and CML-10.

5.3.3.3.1 MDCK experiment

The data from this experiment are summarized in Figure 5.13 and the post hoc pairwise comparison results are displayed in Table 5.13. There were no statistically significant differences in mean cell survival percentages after 144 hours between the NTC and pCMVNTR-transfected cells without NFZ treatment. Hence, cell growth was not affected by the transfection procedure.

Statistically significant decreases were found between the untreated NTC and pCMVNTR-transfected cells with NTC and pCMVNTR-transfected cells treated with 0.25 μ M and 0.5 μ M NFZ. There was also a significant difference between the NTC treated with 0.5 μ M NFZ with the equivalent treatment in the pCMVNTR-transfected cells, but no difference between the 0.25 μ M NFZ treated cells. There was a statistical difference between the NTC or pCMVNTR-transfected cells treated with 0.25 μ M NFZ and those treated with 0.5 μ M. These results indicate that the expression of NTR in MDCK cells reduces cell survival following 0.5 μ M NFZ treatment.

Following 24 hours treatment with 0.5 μ M NFZ, <20% pCMVNTR-transfected cells survived, compared to ~35% in the NTC. However by 96 hours, ~100% decrease in survival was observed in both sets of cells. In the cells treated with 0.25 μ M NFZ, ~95% NTC and ~65% pCMVNTR-transfected cells survived after 24 hours, which was reduced to ~15%, for both sets of cells, after 144 hours.

Hence, this shows that MDCK cells behave similarly, following treatment with NFZ, to the responses previously seen in the canine OS cell lines.

Group	Treatment	MDCK
1	NTC	All except 4
2	NTC + 0.25 μ M NFZ	All except 5
3	NTC + 0.5 μ M NFZ	All other treatments
4	NTR	All except 1
5	NTR + 0.25 μ M NFZ	All except 2
6	NTR + 0.5 μ M NFZ	All other treatments

Table 5.13: Post hoc pairwise comparisons of treatment groups in Figure 5.13, listing the treatment groups which were significantly different from each group over the 144 hour treatment period (significance was set at $p = 0.05$).

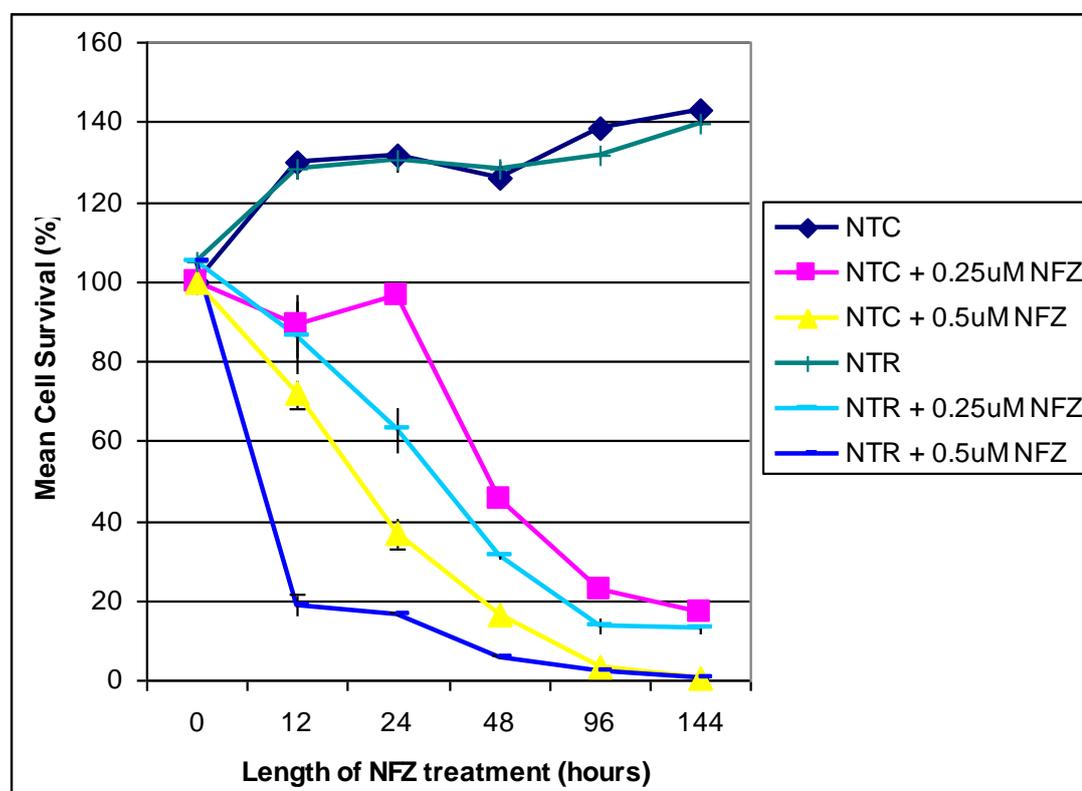


Figure 5.13: The effect of 0.25 μ M and 0.5 μ M nitrofurazone (NFZ), on survival of MDCK cells, following the transfection of pCMVNTR (NTR), over 144 hours. ('NTC', non-transfected cells; each time point was repeated in quadruplicate; bars represent \pm SE).

5.3.3.3.2 CML-10 experiment

Although the CML-10 cells were not transfected, it was still considered worthwhile to determine the effect of NFZ on NTC. The data from this experiment are summarized in Figure 5.14 and the post hoc pairwise comparison results are displayed in Table 5.14. There were statistically significant

differences in mean cell survival percentages after 144 hours between the NTC without NFZ treatment and those treated with 0.5 μ M and 0.25 μ M NFZ. There was not a significant difference between the cells treated with 0.5 μ M and 0.25 μ M NFZ.

After only 12 hours, both 0.5 μ M and 0.25 μ M NFZ treatments induced ~100% reductions in cell survival. Thus in the absence of NTR transfection, NFZ is highly cytotoxic to the CML-10 cell line. It is unlikely that a difference in amounts of cell survival would be observed between NTC and pCMVNTR-transfected cells if lower concentrations of NFZ were used.

Group	Treatment	CML-10
1	NTC	All other treatments
2	NTC + 0.25 μ M NFZ	All except 3
3	NTC + 0.5 μ M NFZ	All except 3

Table 5.14: Post hoc pairwise comparisons of treatment groups in Figure 5.14, listing the treatment groups which were significantly different from each group over the 96 hour treatment period (significance was set at $p = 0.05$).

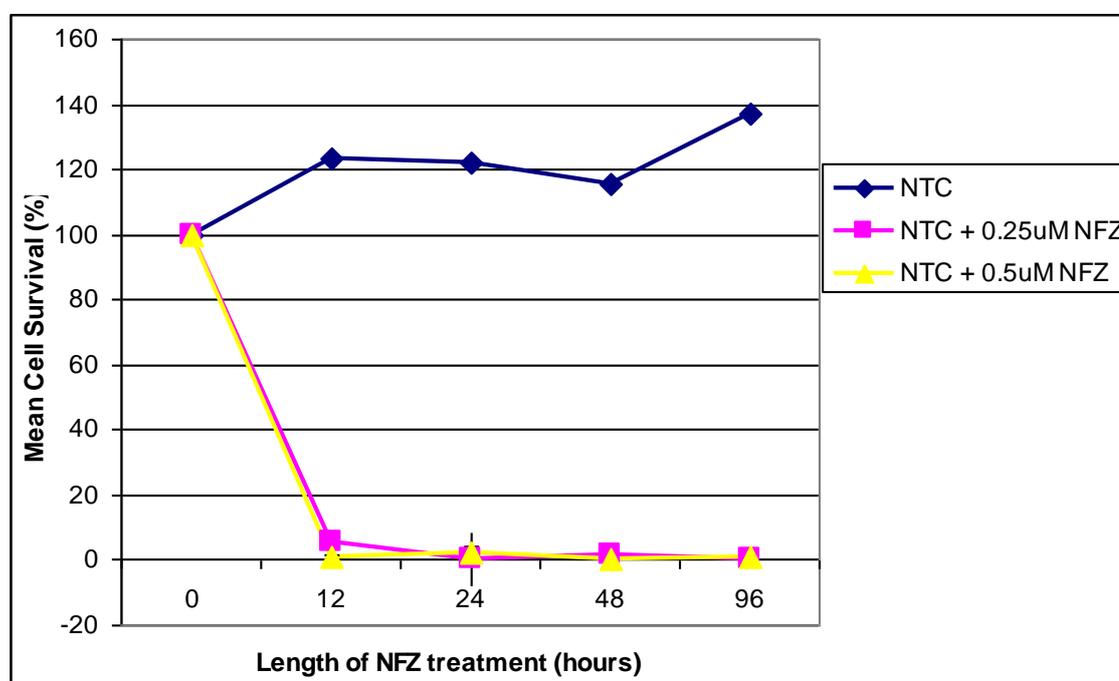


Figure 5.14: The effect of 0.25 μ M and 0.5 μ M nitrofurazone (NFZ), on survival of non-transfected (NTC) CML-10 cells, over 96 hours. (Each time point was repeated in quadruplicate; bars represent \pm SE).

5.3.4 Analysis of pCMVNTR and TP53-targeted suicide gene therapy strategy in combination with CB1954

Due to the limited amount of CB1954 available, the D17 and CMT7 cells were chosen to be used in the CB1954 assays as a result of the findings from the DLR experiments.

5.3.4.1 Preliminary experiment

The data from this experiment are summarized in Figure 5.15 and the post hoc pairwise comparison results are displayed in Table 5.15. There were no statistically significant differences in mean cell survival percentages after 48 hours between the NTC and pCMVNTR-transfected cells without CB1954 treatment, in D17 cell line but there was a difference in the CMT7 cell line. Hence, cell growth was not affected by the transfection procedure in D17 cells but may have been in CMT7 cells.

No statistically significant decreases were found between the untreated NTC with any of the NTC treated with CB1954 in both cell lines. There were statistical differences in the D17 and CMT7 cells between the untreated NTC and all the CB1954 treated NTC, with the pCMVNTR-transfected D17 cells treated with 2.5 - 160 μ M CB1954 and all the CB1954 treated pCMVNTR-transfected CMT7 cells. Significant differences were present between the untreated pCMVNTR-transfected D17 and CMT7 cells, pCMVNTR-transfected D17 cells treated with 0.15 - 1.25 μ M CB1954 and pCMVNTR-transfected CMT7 cells treated with 0.15 - 0.625 μ M CB1954 with pCMVNTR-transfected D17 and CMT7 cells treated with 40, 80 and 160 μ M CB1954. However there were no significant differences between the pCMVNTR-transfected D17 cells treated with 2.5 - 160 μ M CB1954 and the pCMVNTR-transfected CMT7 cells treated with 1.25 - 160 μ M CB1954, demonstrating no clear dose effect at these concentrations. There were significant differences between the D17 NTC treated with 2.5 - 160 μ M CB1954 and all the CMT7 NTC treated with CB1954 with the equivalent treatment in the pCMVNTR-transfected D17 and CMT7 cells. A statistical difference was found between D17 NTC treated with 2.5 μ M CB1954 with pCMVNTR-transfected D17

cells treated with 1.25 μ M CB1954. This shows that the expression of NTR in D17 cells decreases the amount of CB1954 required to significantly reduce cell numbers. Although, this was not able to be demonstrated in the CMT7 cells due to the untreated pCMVNTR-transfected cells being significantly different to untreated NTC and all the treated NTC.

After 48 hours CB1954 treatment, ~60% of the pCMVNTR-transfected D17 cells treated with 2.5 - 20 μ M CB1954 survived and with increasing CB1954 concentration, survival was reduced to 20% with 160 μ M CB1954. In the CMT7 cells after 48 hours treatment with CB1954, ~60% of the pCMVNTR-transfected cells treated with 2.5 μ M CB1954 survived, which also, with increasing CB1954 concentrations, was reduced to ~10% with 160 μ M CB1954.

The subsequent assays were to be performed over 144 hours. CB1954 concentrations of 5 μ M and 20 μ M were chosen to be used, because after 48 hours, both D17 and CMT7 NTC treated with 5 μ M and 20 μ M CB1954 possessed similar survival curves to the untreated NTC, ~60% pCMVNTR-transfected D17 cells survived 5 μ M and 20 μ M CB1954 treatment and 50% and 10% of pCMVNTR-transfected CMT7 cells survived 5 μ M and 20 μ M CB1954 treatment, respectively. Hence by 144 hours, it was assumed that very few of the pCMVNTR-transfected cells, in particular CMT7 cells, treated with 5 μ M and 20 μ M CB1954 would survive.

Thus, the results from this preliminary experiment show that pCMVNTR encodes a functional NTR, which can be expressed in canine cells and is capable of activating CB1954 to its cytotoxic metabolite. In addition, it appears that there is no activation of CB1954 by endogenous enzymes, unlike that observed in the NFZ assays.

Group	Treatment	D17	CMT7
1	NTC	12, 19, 20, 21, 22, 23, 24	7, 8, 9, 10, 11, 12, 19, 20, 21, 22, 23, 24
2	NTC + 0.15 μ M CB	12, 19, 20, 21, 22, 23, 24	7, 8, 9, 10, 11, 12, 19, 20, 21, 22, 23, 24
3	NTC + 0.3 μ M CB	12, 19, 20, 21, 22, 23, 24	7, 8, 9, 10, 11, 12, 19, 20, 21, 22, 23, 24
4	NTC + 0.625 μ M CB	12, 19, 20, 21, 22, 23, 24	7, 8, 9, 10, 11, 12, 19, 20, 21, 22, 23, 24
5	NTC + 1.25 μ M CB	12, 19, 20, 21, 22, 23, 24	7, 8, 9, 10, 11, 12, 19, 20, 21, 22, 23, 24
6	NTC + 2.5 μ M CB	7, 11, 12, 19, 20, 21, 22, 23, 24	7, 8, 9, 10, 11, 12, 19, 20, 21, 22, 23, 24
7	NTR	6, 22, 23, 24	1, 2, 3, 4, 5, 6, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23, 24
8	NTR + 0.15 μ M CB	19, 21, 22, 23, 24	1, 2, 3, 4, 5, 6, 13, 14, 15, 16, 17, 18, 21, 22, 23, 24
9	NTR + 0.3 μ M CB	19, 21, 22, 23, 24	1, 2, 3, 4, 5, 6, 13, 14, 15, 16, 17, 18, 21, 22, 23, 24
10	NTR + 0.625 μ M CB	22, 23, 24	1, 2, 3, 4, 5, 6, 13, 14, 15, 16, 17, 18, 22, 23, 24
11	NTR + 1.25 μ M CB	6, 22, 23, 24	1, 2, 3, 4, 5, 6, 13, 14, 15, 16, 17, 18
12	NTR + 2.5 μ M CB	1, 2, 3, 4, 5, 6, 13, 14, 15, 16, 17, 18	1, 2, 3, 4, 5, 6, 7, 13, 14, 15, 16, 17, 18
13	NTC + 5 μ M CB	12, 19, 20, 21, 22, 23, 24	7, 8, 9, 10, 11, 12, 19, 20, 21, 22, 23, 24
14	NTC + 10 μ M CB	12, 19, 20, 21, 22, 23, 24	7, 8, 9, 10, 11, 12, 19, 20, 21, 22, 23, 24
15	NTC + 20 μ M CB	12, 19, 20, 21, 22, 23, 24	7, 8, 9, 10, 11, 12, 19, 20, 21, 22, 23, 24
16	NTC + 40 μ M CB	12, 19, 20, 21, 22, 23, 24	7, 8, 9, 10, 11, 12, 19, 20, 21, 22, 23, 24
17	NTC + 80 μ M CB	12, 19, 20, 21, 22, 23, 24	7, 8, 9, 10, 11, 12, 19, 20, 21, 22, 23, 24
18	NTC + 160 μ M CB	12, 19, 20, 21, 22, 23, 24	7, 8, 9, 10, 11, 12, 19, 20, 21, 22, 23, 24
19	NTR + 5 μ M CB	1, 2, 3, 4, 5, 6, 8, 9, 13, 14, 15, 16, 17, 18	1, 2, 3, 4, 5, 6, 13, 14, 15, 16, 17, 18
20	NTR + 10 μ M CB	1, 2, 3, 4, 5, 6, 13, 14, 15, 16, 17, 18	1, 2, 3, 4, 5, 6, 7, 13, 14, 15, 16, 17, 18
21	NTR + 20 μ M CB	1, 2, 3, 4, 5, 6, 8, 9, 13, 14, 15, 16, 17, 18	1, 2, 3, 4, 5, 6, 7, 8, 9, 13, 14, 15, 16, 17, 18
22	NTR + 40 μ M CB	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15, 16, 17, 18	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 14, 15, 16, 17, 18
23	NTR + 80 μ M CB	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15, 16, 17, 18	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 14, 15, 16, 17, 18
24	NTR + 160 μ M CB	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15, 16, 17, 18	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 14, 15, 16, 17, 18

Table 5.15: Post hoc pairwise comparisons of treatment groups in Figure 5.15, listing the treatment groups which were significantly different from each group over the 48 hour treatment period (significance was set at $p = 0.05$; CB1954 - 'CB').

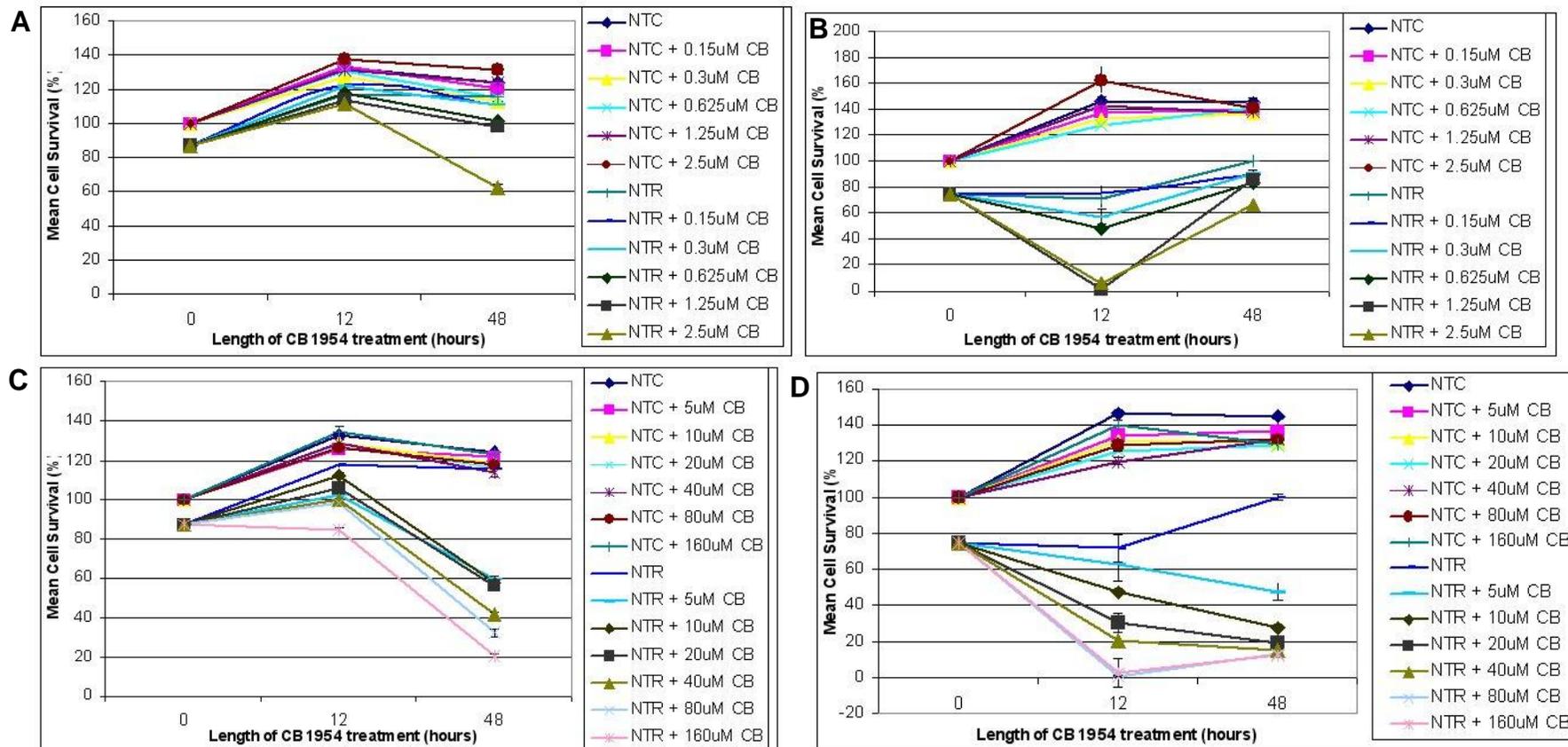


Figure 5.15: The effect of 0.15 - 160 μ M CB1954 (CB) over 48 hours, following the transfection of pCMVNTR (NTR) on survival of D17 (A) and CMT7 (B) cells treated with 0.15 - 2.5 μ M CB1954, and D17 (C) and CMT7 (D) cells treated with 5 - 160 μ M CB1954. ('NTC', non-transfected cells; each time point was repeated in triplicate; bars represent +/- SE). Please note different scales on Y-axes.

5.3.4.2 In the absence and presence of NADH

For ease of displaying the effects of CB1954, on the survival of D17 and CMT7 cells using pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR in combination with pCMVCAT6 or pTargetLex-p53, the data from the experiment in the:

- (i), absence of NADH are summarized in Figures 5.16, 5.17 and 5.18 (respectively) and the post hoc pairwise comparison results are displayed in Tables 5.16, 5.17 and 5.18 (respectively);
- (ii), presence of NADH are summarized in Figures 5.19, 5.20 and 5.21 (respectively) and the post hoc pairwise comparison results are displayed in Tables 5.19, 5.20 and 5.21 (respectively).

In both experiments, there were no statistically significant differences in mean cell survival percentages after 144 hours, in both D17 and CMT7 cell lines, between the NTC and cells transfected with pCMVNTR-, pCMVCAT6-, and pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR in combination with pCMVCAT6 or pTargetLex-p53, without CB1954 treatment. Hence, cell growth was not affected by the transfection procedure in both cell lines.

In the absence and presence of NADH, there was a statistically significant difference in survival between the D17 cells transfected with pCMVNTR and treated with 20 μ M CB1954, to the D17 cells transfected with all the other constructs and treated with 5 μ M and 20 μ M CB1954. In addition, in the absence of NADH, there was also a statistically significant difference in survival between the D17 cells transfected with pCMVNTR and treated with 5 μ M CB1954, to the D17 cells transfected with all the other constructs and treated with 5 μ M and 20 μ M CB1954. There was however no difference between the pCMVNTR-transfected D17 cells treated with 5 μ M and 20 μ M CB1954 in the absence of NADH. Hence, survival in the absence of NADH, was reduced in D17 cells transfected with pCMVNTR and treated with 5 μ M and 20 μ M CB1954 and in the presence of NADH, was only significantly reduced in D17 cells transfected with pCMVNTR and treated with 20 μ M CB1954.

In the absence and presence of NADH, there was a statistically significant decrease between the pCMVNTR-transfected CMT7 cells treated with 20 μ M CB1954 with all the other treatment groups. Except for, in the 'absence of

NADH' experiment, CMT7 cells treated with 20 μ M CB1954 and transfected with pGL3-P8LA-NTR alone, and pGL3B8LA-NTR and pGL3-P8LA-NTR, in combination with pTarget-Lexp53. However, a 90% difference in cell survival existed between the pGL3B8LA-NTR- and pCMVNTR- transfected cells treated with 20 μ M CB1954 after 144 hours. This result was probably due to survival of pGL3B8LA-NTR+pTarget-Lexp53 transfected cells being lower at 12 and 24 hour time points than for pCMVNTR-transfected cells as the statistical tests employed compared overall cell numbers. Additionally, except for, in the 'presence of NADH' experiment, pCMVNTR-transfected CMT7 cells treated with 5 μ M CB1954 and pGL3P8LA-NTR transfected cells, in combination with pTargetLex-p53, treated with 20 μ M CB1954. There was also a significant difference between pCMVNTR-transfected CMT7 cells treated with 5 μ M CB1954 with untreated NTC and pCMVNTR-transfected cells, NTC treated with 5 μ M and 20 μ M CB1954, and pGL3B8LA-NTR treated with 5 μ M CB1954.

These results show that in the absence or presence of NADH, transfection of pGL3B8LA-NTR or pGL3E8LA-NTR in combination with pTargetLex-p53, does not appear to reduce survival of D17 and CMT7 cells following treatment with CB1954. In addition, pGL3P8LA-NTR in combination with pTargetLex-p53, does not appear to reduce survival of D17 cells, with CB1954 treatment.

In the absence of NADH, the survival of pCMVNTR-transfected D17 and CMT7 cells after 144 hours 5 μ M CB1954 treatment was ~25% and ~50%, respectively. 20 μ M CB1954 treatment in the pCMVNTR-transfected D17 cells after 96 hours, resulted in <10% survival and in the pCMVNTR-transfected CMT7 cells after 144 hours, ~15% survival. In the presence of NADH, after 5 μ M CB1954 treatment for 144 hours, ~80% and 35% pCMVNTR-transfected D17 and CMT7 cells survived, respectively. 20 μ M CB1954 treatment, almost reduced cell survival to 0% in both the pCMVNTR-transfected D17 and CMT7 cells. These results suggest that NADH may reduce the survival of pCMVNTR-transfected CMT7 cells treated with CB1954. However, in the D17 cells, an experimental error may have occurred, which would have been repeated if more CB1954 was available, hence requires further investigation.

In the absence of NADH, there was also a significant difference between CMT7 cells treated with 20 μ M CB1954 and transfected with pGL3P8LA-NTR in combination with pTargetLex-p53 to the untreated CMT7 cells transfected with pGL3P8LA-NTR alone or in combination with pTargetLex-p53. This shows that overall, similar levels of reduction in cell survival were achieved in the pGL3P8LA-NTR transfected cells treated with 20 μ M CB1954 alone or in combination with pTargetLex-p53 to those of pCMVNTR-transfected cells. Although no significant difference existed between the pGL3P8LA-NTR transfected cells alone or in combination with pTargetLex-p53, survival was ~20% less in those transfected with pTargetLex-p53 (~30% survival vs. ~50% survival) after 144 hours. Similarly, in the presence of NADH, there was also a significant difference between CMT7 cells treated with 20 μ M CB1954 and transfected with pGL3P8LA-NTR in combination with pTargetLex-p53 to the untreated NTC and NTC treated with 5 μ M and 20 μ M CB1954. This also shows that overall, similar levels of reduction in cell survival were achieved in the pGL3P8LA-NTR transfected cells in combination with pTargetLex-p53 and treated with 20 μ M CB1954 to those of pCMVNTR-transfected cells. Although no significant difference existed between the pGL3P8LA-NTR transfected cells alone or in combination with pTargetLex-p53, survival was ~30% less in those transfected with pTargetLex-p53 (~50% survival vs. ~80% survival).

This suggests that in both the absence and presence of NADH, a decrease in survival of CMT7 cells may occur by pGL3P8LA-NTR in combination with pTargetLex-p53 following treatment with 20 μ M CB1954 after 144 hours. Cell survival also appears to be higher in the presence of NADH (50%) than in the absence of NADH (30%).

Group	Treatment	D17	CMT7
1	NTC	6, 13	13
2	NTC + 5 μ M	6, 13	13
3	CAT	6, 13	13
4	CAT + 5 μ M	6, 13	13
5	NTR	6, 13	13
6	NTR + 5 μ M	All except 13	13
7	B8LA	6, 13	13
8	B8LA + 5 μ M	6, 13	13
9	B8LA + Lexp53	6, 13	13
10	B8LA + Lexp53 + 5 μ M	6, 13	13
11	NTC + 20 μ M	6, 13	13
12	CAT + 20 μ M	6, 13	13
13	NTR + 20 μ M	All except 6	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14
14	B8LA + 20 μ M	6, 13	13
15	B8LA + Lexp53 + 20 μ M	6, 13	

Table 5.16: Post hoc pairwise comparisons of treatment groups in Figure 5.16, listing the treatment groups which were significantly different from each group over the 144 hour treatment period (significance was set at $p = 0.05$).

Group	Treatment	D17	CMT7
1	NTC	6, 13	13
2	NTC + 5 μ M	6, 13	13
3	CAT	6, 13	13
4	CAT + 5 μ M	6, 13	13
5	NTR	6, 13	13
6	NTR + 5 μ M	All except 13	13
7	E8LA	6, 13	13
8	E8LA + 5 μ M	6, 13	13
9	E8LA + Lexp53	6, 13	13
10	E8LA + Lexp53 + 5 μ M	6, 13	13
11	NTC + 20 μ M	6, 13	13
12	CAT + 20 μ M	6, 13	13
13	NTR + 20 μ M	All except 6	All other treatments
14	E8LA + 20 μ M	6, 13	13
15	E8LA + Lexp53 + 20 μ M	6, 13	13

Table 5.17: Post hoc pairwise comparisons of treatment groups in Figure 5.17, listing the treatment groups which were significantly different from each group over the 144 hour treatment period (significance was set at $p = 0.05$).

Group	Treatment	D17	CMT7
1	NTC	6, 13	13
2	NTC + 5 μ M	6, 13	13
3	CAT	6, 13	13
4	CAT + 5 μ M	6, 13	13, 15
5	NTR	6, 13	13, 15
6	NTR + 5 μ M	All except 13	13
7	P8LA	6, 13	13, 15
8	P8LA + 5 μ M	6, 13	13
9	P8LA + Lexp53	6, 13	13, 15
10	P8LA + Lexp53 + 5 μ M	6, 13	13
11	NTC + 20 μ M	6, 13	13
12	CAT + 20 μ M	6, 13	13
13	NTR + 20 μ M	All except 6	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12
14	P8LA + 20 μ M	6, 13	
15	P8LA + Lexp53 + 20 μ M	6, 13	4, 5, 7, 9

Table 5.18: Post hoc pairwise comparisons of treatment groups in Figure 5.18, listing the treatment groups which were significantly different from each group over the 144 hour treatment period (significance was set at $p = 0.05$).

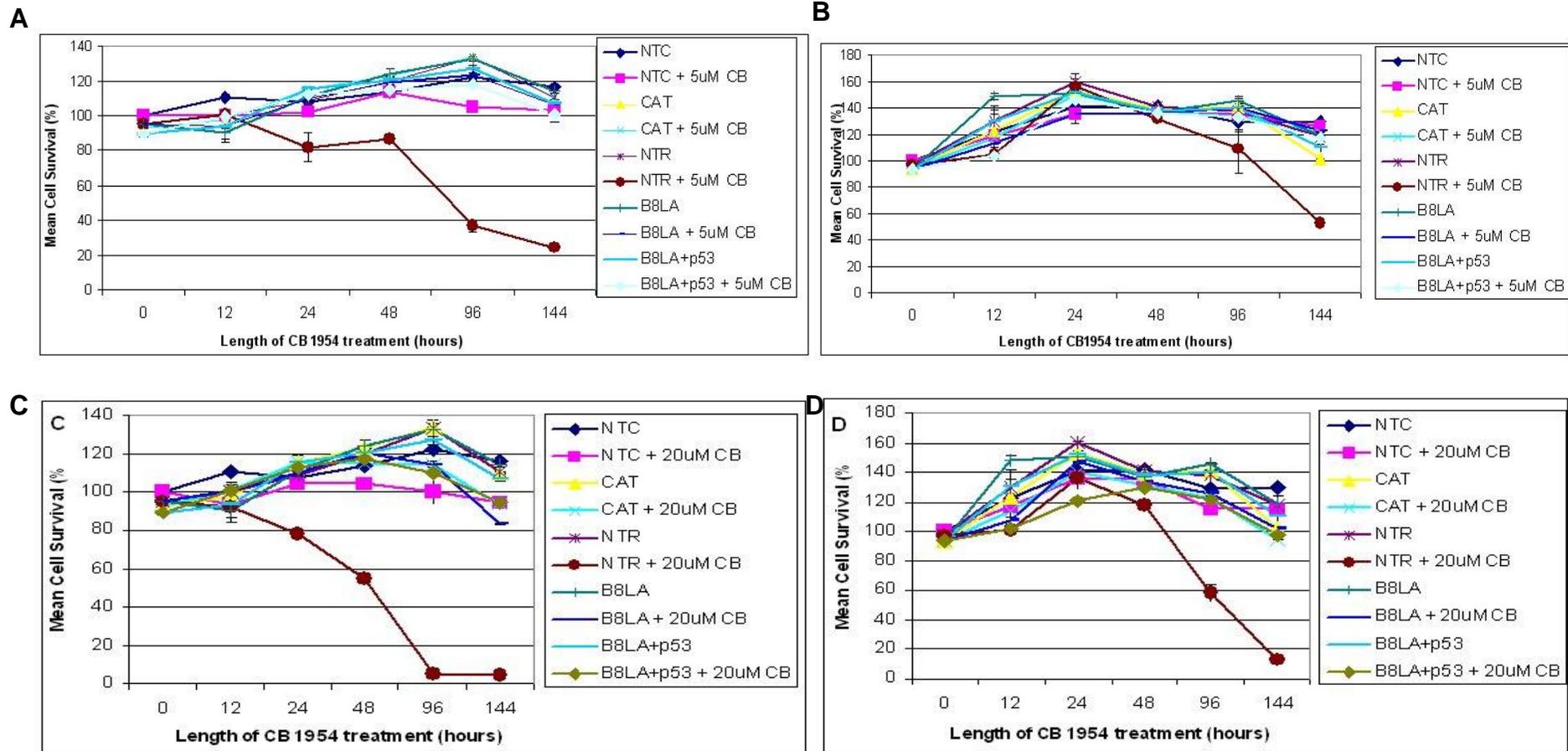


Figure 5.16: The effect of 5µM (A & B) and 20µM (C & D) CB1954 (CB), in the absence of NADH over 144 hours, on survival of D17 (A & C) and CMT7 (B & D) cells transfected with: pCMVCAT6 (CAT); pCMVNTR in combination with pCMVCAT6 (NTR); and pGL3B8LA-NTR, in combination with pCMVCAT6 (B8LA), or pTargetT-Lexp53 (B8LA+p53). (NTC, non-transfected cells; each time point was repeated in triplicate; bars represent +/- SE). Please note different scales on Y-axes.

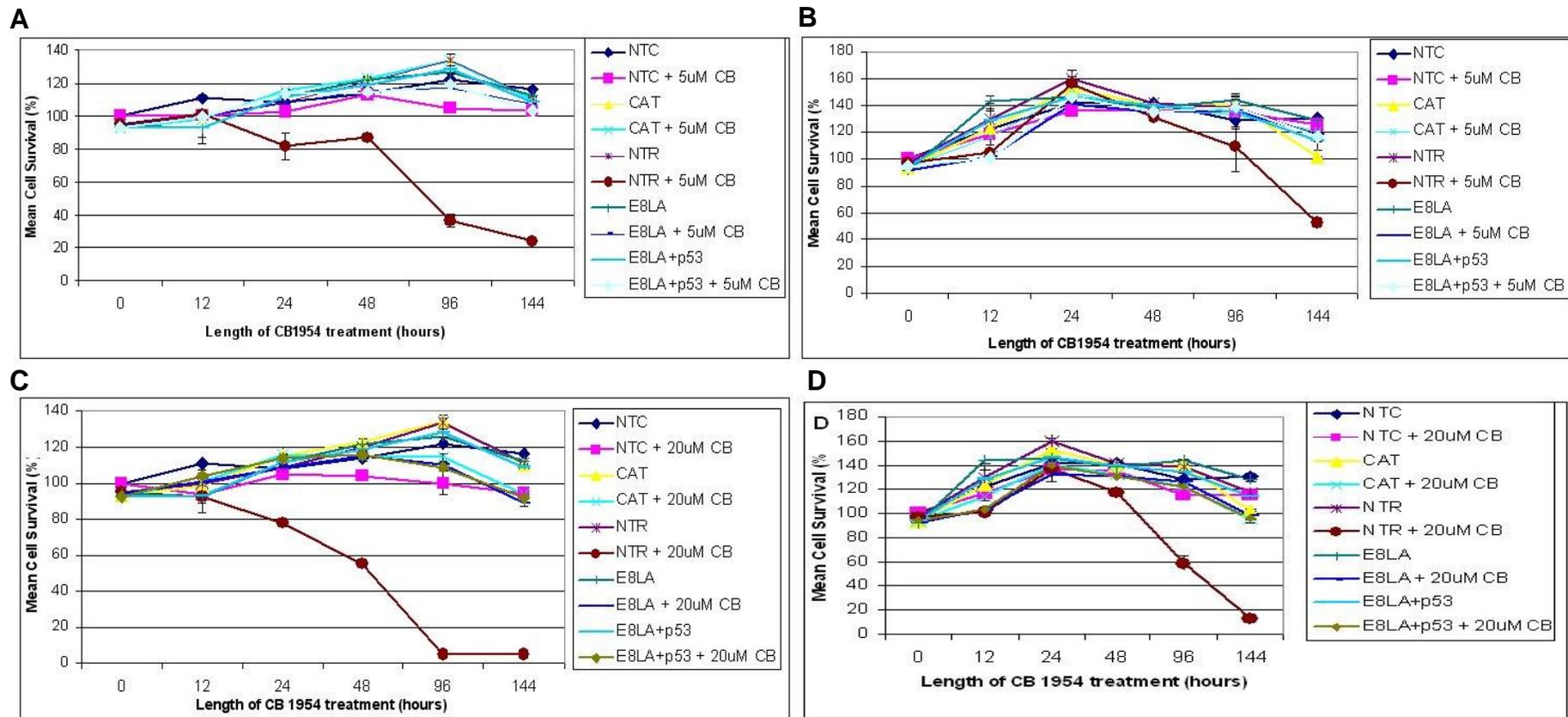


Figure 5.17: The effect of 5µM (A & B) and 20µM (C & D) CB1954 (CB), in the absence of NADH over 144 hours, on survival of D17 (A & C) and CMT7 (B & D) cells transfected with: pCMVCAT6 (CAT); pCMVNTR in combination with pCMVCAT6 (NTR); and pGL3E8LA-NTR, in combination with pCMVCAT6 (E8LA), or pTarget-T-Lexp53 (E8LA+p53). (NTC, non-transfected cells; each time point was repeated in triplicate; bars represent +/- SE). Please note different scales on Y-axes.

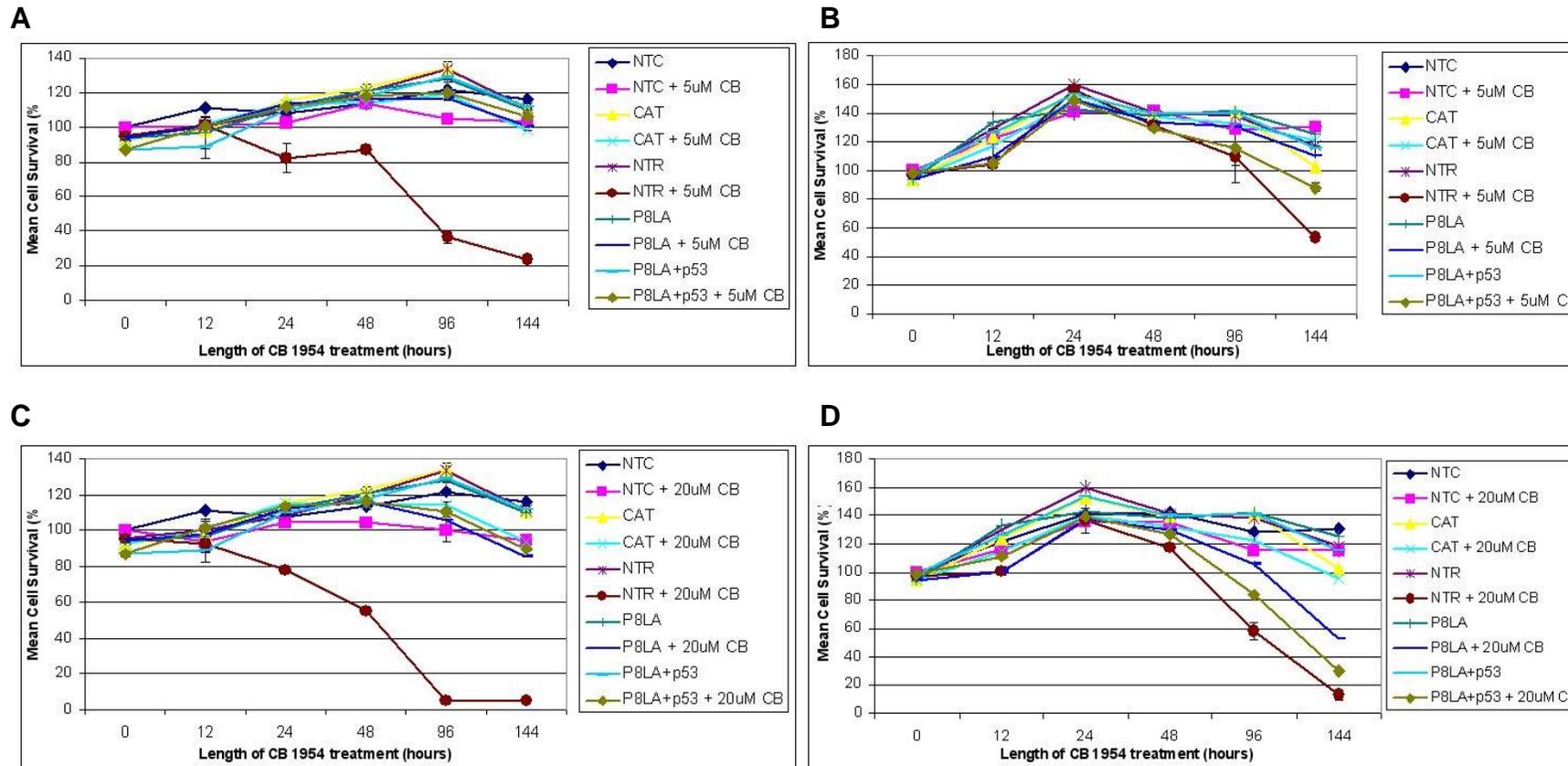


Figure 5.18: The effect of 5µM (A & B) and 20µM (C & D) CB1954 (CB), in the absence of NADH over 144 hours, on survival of D17 (A & C) and CMT7 (B & D) cells transfected with: pCMVCAT6 (CAT); pCMVNTR in combination with pCMVCAT6 (NTR); and pGL3P8LA-NTR, in combination with pCMVCAT6 (P8LA), or pTargetT-Lexp53 (P8LA+p53). (NTC, non-transfected cells; each time point was repeated in triplicate; bars represent +/- SE). Please note different scales on Y-axes.

Group	Treatment	D17	CMT7
1	NTC	13	6, 13
2	NTC + 5 μ M	13	6, 13
3	CAT	13	13
4	CAT + 5 μ M	13	13
5	NTR	13	6, 13
6	NTR + 5 μ M	13	1, 2, 5, 8, 11
7	B8LA	13	13
8	B8LA + 5 μ M	13	6, 13
9	B8LA + Lexp53	13	13
10	B8LA + Lexp53 + 5 μ M	13	13
11	NTC + 20 μ M	13	6, 13
12	CAT + 20 μ M	13	13
13	NTR + 20 μ M	All other treatments	All except 6
14	B8LA + 20 μ M	13	13
15	B8LA + Lexp53 + 20 μ M	13	13

Table 5.19: Post hoc pairwise comparisons of treatment groups in Figure 5.19, listing the treatment groups which were significantly different from each group over the 144 hour treatment period (significance was set at $p = 0.05$).

Group	Treatment	D17	CMT7
1	NTC	13	6, 13
2	NTC + 5 μ M	13	6, 13
3	CAT	13	13
4	CAT + 5 μ M	13	13
5	NTR	13	6, 13
6	NTR + 5 μ M	13	1, 2, 5, 11
7	E8LA	13	13
8	E8LA + 5 μ M	13	13
9	E8LA + Lexp53	13	13
10	E8LA + Lexp53 + 5 μ M	13	13
11	NTC + 20 μ M	13	6, 13
12	CAT + 20 μ M	13	13
13	NTR + 20 μ M	All other treatments	All except 6
14	E8LA + 20 μ M	13	13
15	E8LA + Lexp53 + 20 μ M	13	13

Table 5.20: Post hoc pairwise comparisons of treatment groups in Figure 5.20, listing the treatment groups which were significantly different from each group over the 144 hour treatment period (significance was set at $p = 0.05$).

Group	Treatment	D17	CMT7
1	NTC	13	6, 13, 15
2	NTC + 5 μ M	13	6, 13, 15
3	CAT	13	13
4	CAT + 5 μ M	13	13
5	NTR	13	6, 13
6	NTR + 5 μ M	13	1, 2, 5, 11
7	P8LA	13	13
8	P8LA + 5 μ M	13	13
9	P8LA + Lexp53	13	13
10	P8LA + Lexp53 + 5 μ M	13	13
11	NTC + 20 μ M	13	6, 13, 15
12	CAT + 20 μ M	13	13
13	NTR + 20 μ M	All other treatments	1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 14
14	P8LA + 20 μ M	13	13
15	P8LA + Lexp53 + 20 μ M	13	1, 2, 11

Table 5.21: Post hoc pairwise comparisons of treatment groups in Figure 5.21, listing the treatment groups which were significantly different from each group over the 144 hour treatment period (significance was set at $p = 0.05$).

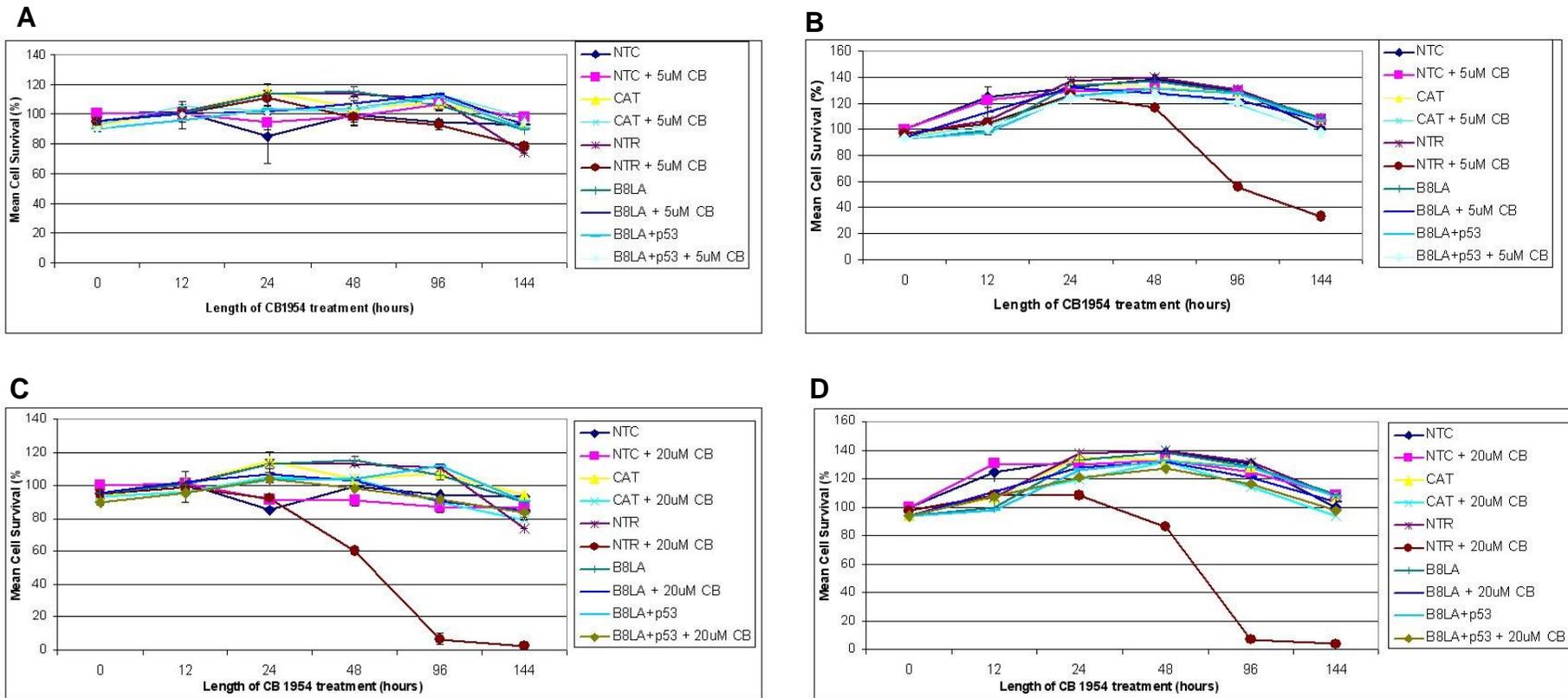


Figure 5.19: The effect of 5 μ M (A & B) and 20 μ M (C & D) CB1954 (CB), in the presence of NADH over 144 hours, on survival of D17 (A & C) and CMT7 (B & D) cells transfected with: pCMVCAT6 (CAT); pCMVNTR in combination with pCMVCAT6 (NTR); and pGL3B8LA-NTR, in combination with pCMVCAT6 (B8LA), or pTargetT-Lexp53 (B8LA+p53). (NTC, non-transfected cells; each time point was repeated in triplicate; bars represent +/- SE). Please note different scales on Y-axes.

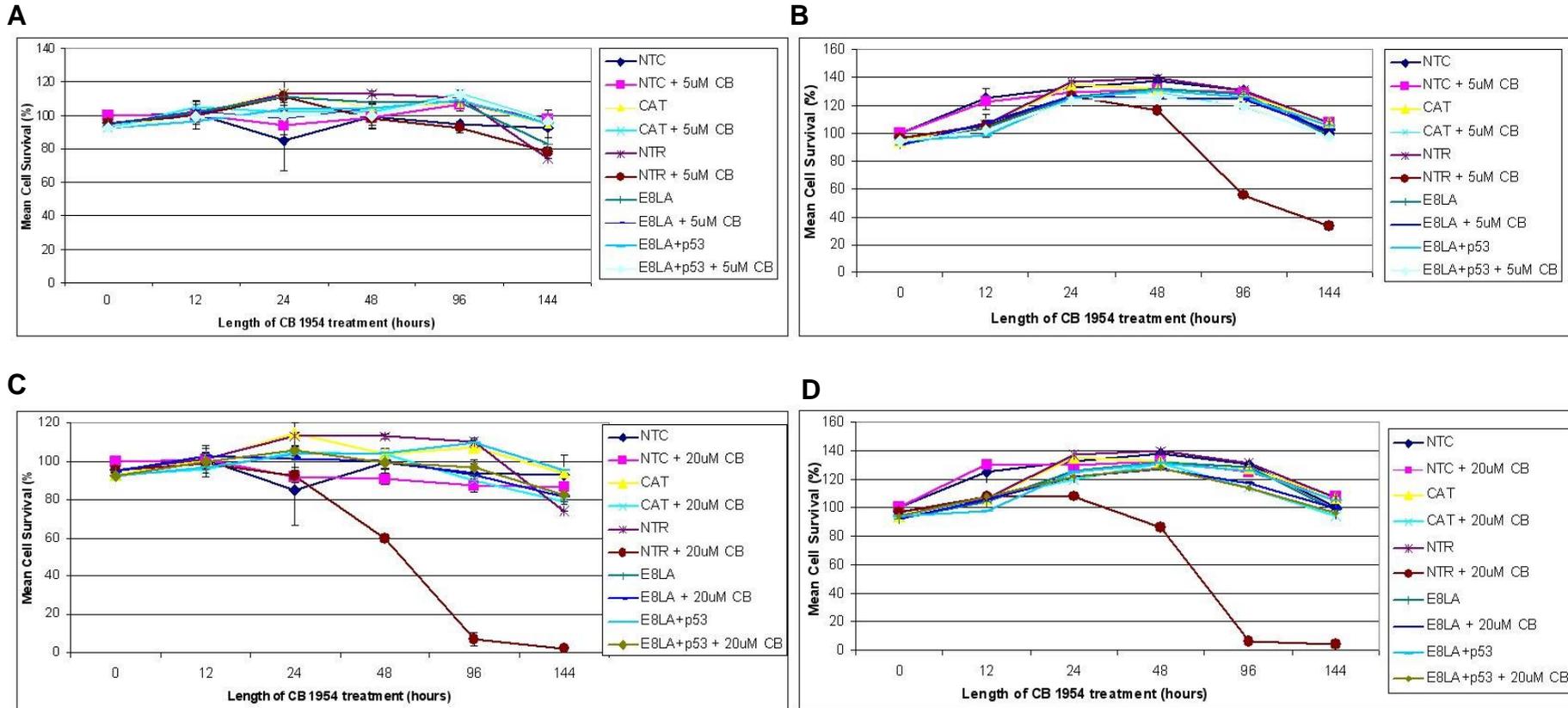


Figure 5.20: The effect of 5 μ M (A & B) and 20 μ M (C & D) CB1954 (CB), in the presence of NADH over 144 hours, on survival of D17 (A & C) and CMT7 (B & D) cells transfected with: pCMVCAT6 (CAT); pCMVNTR in combination with pCMVCAT6 (NTR); and pGL3E8LA-NTR, in combination with pCMVCAT6 (E8LA), or pTargetT-Lexp53 (E8LA+p53). (NTC, non-transfected cells; each time point was repeated in triplicate; bars represent +/- SE). Please note different scales on Y-axes.

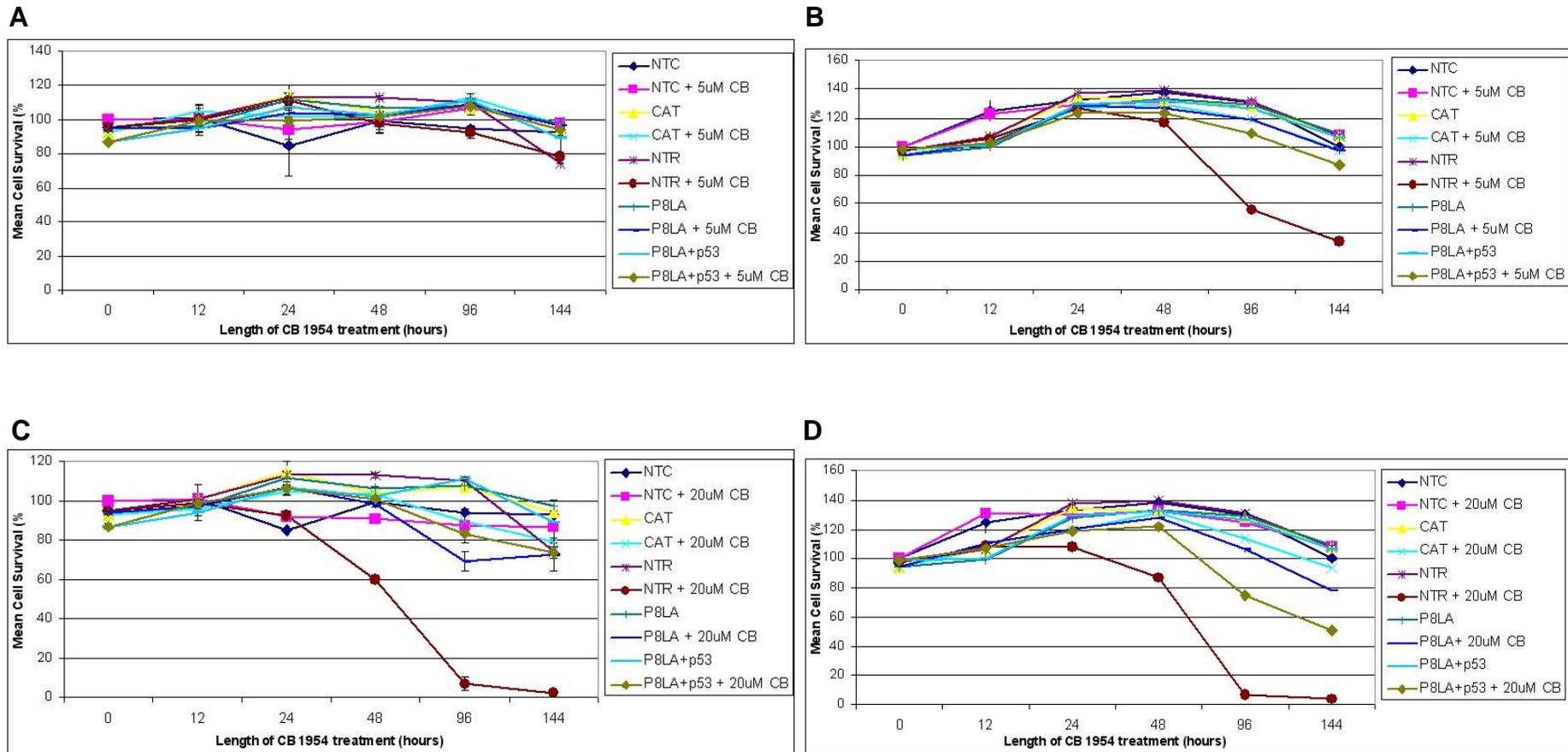


Figure 5.21: The effect of 5 μ M (A & B) and 20 μ M (C & D) CB1954 (CB), in the presence of NADH over 144 hours, on survival of D17 (A & C) and CMT7 (B & D) cells transfected with: pCMVCAT6 (CAT); pCMVNTR in combination with pCMVCAT6 (NTR); and pGL3P8LA-NTR, in combination with pCMVCAT6 (P8LA), or pTargetT-Lexp53 (P8LA+p53). (NTC, non-transfected cells; each time point was repeated in triplicate; bars represent +/- SE). Please note different scales on Y-axes.

5.4 Discussion

5.4.1 Detection of nitroreductase protein expression by pCMVNTR and pGL3B8LA-NTR, pGL3E8LA-NTR and pGL3P8LA-NTR, in combination with pTarget-Lexp53 or pCMVCAT6

The results observed in this study are in agreement with those obtained in the DLR experiments (previously described in 4.3.4), as expression of NTR was detected following transfection of pGL3P8LA-NTR in combination with pTarget-Lexp53 in CMT7 cells possessing accumulated TP53, but not in D17 cells containing wild-type TP53. Thus, further confirming that the TP53-targeted suicide gene therapy system is able to recruit the transactivation domain of the accumulated TP53 present in CMT7 cells to drive the expression of the gene downstream of the upstream activating sequence (i.e. LexA operator site), whether it is a gene for FFluc or NTR.

The lack of detection of NTR expression following transfection of pGL3B8LA-NTR and pGL3E8LA-NTR, in combination with pTarget-Lexp53, in CMT7 cells, could be due to, as previously described in 4.4.1, the sensitivity of the ECLTM western blotting analysis system (Amersham Pharmacia Biotech). The amount of NTR expressed, as a result of pGL3B8LA-NTR and pGL3E8LA-NTR lacking a promoter, may be below the detection limit of this kit. The ECLTM Plus Western Blotting System was previously used by Blackwood *et al.*, (2001), to detect expression of NTR following transfection of pCMVNTR into feline cells (Blackwood *et al.*, 2001). The ECLTM Plus system is 4- to 20-fold more sensitive than the standard kit. Hence, the use of a more sensitive chemiluminescent method (as previously described in 4.4.1), is warranted in subsequent studies.

Although the expression of NTR was only detected after a single time point in this study (48 hours post-transfection), NTR expression by pCMVNTR has been previously shown by Blackwood *et al.*, (2001), to be expressed in feline cells from 24 hours after transfection upto at least 72 hours. Hence, in future studies, the expression of NTR by all the vectors containing NTR, following transfection in the relevant cell lines, should be confirmed over the period of time utilized in the cell survival assays.

5.4.2 Analysis of pCMVNTR in combination with nitrofurazone

Preliminary prodrug activating system cell survival assays were performed in the 4 OS cell lines, D17, CMT3, CMT7 and CMT8, and 2 additional canine cell lines, MDCK and CML-10, using pCMVNTR and the prodrug, NFZ. These assays represent the first demonstration of the activities of the *E. coli* NTR/NFZ system in canine cells. Overall, the reductions in cell survival after treatment with NFZ were similar between the NTC and cells transfected with pCMVNTR, as few significant differences were found. Hence, selective cell kill could not be achieved using NTR in combination with NFZ in these 6 cell lines. Additionally, NTR has demonstrated an apparent K_m value for NFZ at $64\mu\text{M}$ (Roldan *et al.*, 2008), which is at least 10-fold greater than the maximum concentration employed in this study.

These results suggest that there may be endogenous activation of NFZ under the conditions of the experiments carried out. Mammalian cells, including canine, contain for example, NQO1 and xanthine oxidase, which can also reduce NFZ (Roldan *et al.*, 2008). NQO1 is known to be present in a variety of tumours, including OS (Belinsky & Jaiswal 1993; Friedlos *et al.*, 1992a; Hussein *et al.*, 2009; Kumaraguruparan *et al.*, 2006), and normal tissues (Jaiswal 1994; Li *et al.*, 2001), in both dogs and humans. In addition, NQO1 present in breast cancer cells is capable of resulting in high levels of cytotoxicity following exposure to $5\mu\text{M}$ CB1954 for 20 hours (Friedlos *et al.*, 1992a). Furthermore, xanthine oxidase, is thought to play a pivotal role in the bone microenvironment (Kanczler *et al.*, 2003). Thus, it is realistic to expect that D17, CMT3, CMT7, CMT8, MDCK and CML-10 cells also contain endogenous enzymes, such as NQO1, that possess nitroreductase activity. Hence, further studies are warranted to identify the nitroreductases present in these 6 canine cell lines. However, an alternative explanation for the activation of NFZ in canine cells also exists, in that toxicity may occur via another, as yet, unknown pathway.

The concentrations of NFZ employed in this study were based on those previously published by Bailey and colleagues (1996), in which $1\mu\text{M}$ NFZ achieved 100% cell kill in NTR-transfected hamster lung fibroblasts, whereas $100\mu\text{M}$ NFZ was required in NTC (Bailey *et al.*, 1996). Hence, the findings from this study are

in contrast to those obtained by Bailey and co-workers (1996), as no endogenous activation of NFZ was observed in hamster lung fibroblasts (Bailey *et al.*, 1996). As far as I am aware, no other mammalian *in vitro* studies have been reported investigating either the use of *E.coli* NTR/NFZ system or the endogenous activation of NFZ.

Additionally, Bailey *et al.*, (1996) employed the MTT dye reduction assay to determine levels of cell survival (Bailey *et al.*, 1996); the CellTiter 96® AQueous One Solution Cell Proliferation Assay utilized in this study is the improved version of the MTT assay.

Thus, NFZ does not appear to be suitable as a prodrug for NTR in these canine cells. Although NFZ is used in human and veterinary medicine primarily to treat surface infections that have become resistant to antibiotics, the National Toxicology Program has provided clear evidence of carcinogenic activity of NFZ for female rats and mice (National Toxicology Program 1988), its use is prohibited in medicinal products for food-producing species in the UK (EEC 1995), and the International Agency for Research on Cancer (IARC) suspected that NFZ was carcinogenic to humans (Bartsch & Malaveille 1990; Bartsch & Tomatis 1983; Hiraku *et al.*, 2004; Marselos & Vainio 1991); suspected levels are similar to the concentrations used in this study and by Bailey *et al.*, (1996) (Bailey *et al.*, 1996). Hence, the use of a carcinogenic substance should not be used without much consideration in the treatment of tumours.

5.4.3 Analysis of pCMVNTR and TP53-targeted suicide gene therapy strategy in combination with CB1954

Preliminary prodrug activating system cell survival assays were also carried out in the D17 and CMT7 cell lines, using pCMVNTR and TP53-targeted suicide gene therapy strategy, both in combination with the prodrug, CB1954. These assays represent the first demonstration of the activities of the *E.coli* NTR/CB1954 system in canine cells.

The results from the preliminary experiment show that functional NTR can be expressed from the bacterial gene by canine OS cells *in vitro*, which is capable

of activating CB1954 to its cytotoxic metabolite, resulting in significant reductions in cell survival after only 12 hours. This also suggests that the D17 and CMT7 cells are capable of carrying out the final step required to activate CB1954, which requires thioesters endogenously present to react with the reduced CB1954 metabolite, to produce the cytotoxic difunctional alkylating agent. The rapid activation of CB1954 by NTR has been previously reported *in vitro* in a variety of human cancer cells (Bridgewater *et al.*, 1995; Green *et al.*, 1997; McNeish *et al.*, 1998a), and in feline cells using pCMVNTR *in vitro* (Blackwood *et al.*, 2001). In addition, histological changes *in vivo*, have been observed in mammary gland of transgenic mice as soon as 7 hours after treatment with CB1954, and severe pathological changes after 24 hours (Cui *et al.*, 1999). Furthermore, *in vivo* studies have shown the expression of NTR in combination with CB1954 results in significant decreases in survival of human tumour cells (Djeha *et al.*, 2000; McNeish *et al.*, 1998a; Weedon *et al.*, 2000).

CB1954 itself, appeared to be entirely non-toxic to the D17 and CMT7 cells at the concentrations used in the preliminary pCMVNTR experiment, indicating that any endogenous enzymes present are unable to significantly activate CB1954, even after prolonged exposure. This finding is in contrast to the results of the NFZ assays (5.4.2), in which endogenous nitroreductases, such as NQO1, were suggested to be responsible for the lack of a significant difference between pCMVNTR-transfected cells and NTC. The ability of the endogenous nitroreductases to reduce NFZ but not CB1954 is likely to be due to the lower sensitivity of these enzymes to reduce CB1954 to its cytotoxic metabolite (Boland *et al.*, 1991). For example, Boland *et al.*, (1991) reported that in human cells possessing relatively high levels of NQO1, a higher dose of CB1954 (100 to 5000 times, representing concentrations of 100 μ M to 10000 μ M) was required to produce responses comparable to those produced in CB1954-sensitive rat cells (Boland *et al.*, 1991). Additionally, Drabek *et al.*, (1997) reported that concentrations greater than 500 μ M were required to induce toxicity in cells not transfected with NTR, compared to 10 μ M in NTR expressing cells (Drabek *et al.*, 1997). Hence, canine NQO1 may be similar to human NQO1 in that they both poorly reduce CB1954. In addition, the dose required to induce a significant decrease in cell survival by cells not expressing NTR, was not investigated in this

study, as it would have rapidly used up the limited stock of CB1954 available. Therefore, these should be analysed in future studies.

In cells that produce endogenous enzymes capable of CB1954 activation (as previously described in 5.4.2), and exposed to concentrations of CB1954 similar to that used in this study, appreciable levels of cytotoxicity have been reported to occur in less than 24 hours (Friedlos *et al.*, 1992a; Sunters *et al.*, 1991). Thus it would be expected that, if the enzymes present in canine OS cells were able to activate CB1954, as observed with NFZ, some reductions in cell survival would have occurred after maintenance of the cells in the CB1954 treatment medium for up to 72 hours. In all experiments, numbers of NTC treated with CB1954 were not significantly different from untreated NTC.

The concentrations of CB1954 chosen to be used in this study were based on published *in vitro* data, in which 5, 10 and 40 μ M CB1954 were previously employed by Blackwood *et al.*, (Blackwood *et al.*, 2001). The concentrations in the preliminary experiment, except for 160 μ M CB1954, are also below the peak plasma concentrations that can be achieved in dogs and mice *in vivo* (100 μ M and 400 μ M CB1954 following intravenous injection of 25mg/kg in dogs and 50mg/kg in mice, respectively), which do not produce toxicity (Workman *et al.*, 1986b). Concentrations of ~50 - 60% of the plasma levels have been reported in tumours in mice, so the concentrations used *in vitro* are theoretically obtainable *in vivo* (Workman *et al.*, 1986b). Indeed, doses of 20mg/kg (Drabek *et al.*, 1997) and 50mg/kg (Clark *et al.*, 1997) have been successfully used in GDEPT in mice. Furthermore, concentrations of 1 - 100 μ M (Bridgewater *et al.*, 1995) and 1 - 40 μ M (Clark *et al.*, 1997) have been employed *in vitro*, to selectively induce cytotoxicity following expression of NTR.

No significant dose effects were demonstrated in NTR-expressing D17 and CMT7 cells following treatment with 2.5 - 160 μ M and 1.25 - 160 μ M, respectively. This is likely to be due to the final step of converting the reduced CB1954 metabolite into the difunctional alkylating agent, also being the rate-limiting step, rather than an insufficient amount of NTR being available, due to the efficiency of transfection. This is because Blackwood *et al.*, (2001), previously reported a dose response to increasing concentrations of CB1954 at a relatively low

transfection efficiency of 5%, which was absent at a higher transfection efficiency of 28% (Blackwood *et al.*, 2001). The transfection efficiencies for these NTR/CB1954 assays were deemed to be similar to those previously determined in 4.3.1.2, of 18.1% for D17 cells and 10% for CMT7. Hence, a threshold was reached whereby increases in the amount of CB1954 administered did not result in correlating reductions in cell survival. A plateau effect with increasing CB1954 concentrations has been previously reported (Blackwood *et al.*, 2001; Knox *et al.*, 1988). Hence, additional investigations are warranted.

A bystander effect was also observed in this preliminary experiment, as the percentages of surviving cells following transfection of pCMVNTR in combination with 5 μ M and 20 μ M CB1954 after 48 hours were 50% and 10% in CMT7 cells, respectively, and ~60% in D17 cells for both concentrations. The set up of the experiment enabled the cells expressing NTR to be exposed to CB1954 for 72 hours, maximising prodrug activation. Activated CB1954 rapidly diffuses into the cell culture medium, conferring cytotoxicity to cells not expressing NTR. However, the half-life of the diffusible active metabolite is short, hence, build up in the medium will be limited (Knox *et al.*, 1988). The bystander effect and transfection efficiency seen in this study are comparable to that published in an *in vitro* study, in which NTR expressing cells were treated with 10 μ M CB1954 and a 50% cell kill was demonstrated after 72 hours when only 10% of cells expressed NTR (McNeish *et al.*, 1998a).

The effect of NADH, a cofactor for NTR, was also investigated on NTR-expressing D17 and CMT7 cells, in combination with CB1954. It was expected that larger decreases in cell survival would be observed in the presence of NADH, as had previously been reported by Blackwood *et al.*, 2001 (Blackwood *et al.*, 2001). This was observed in the pCMVNTR-transfected CMT7 cells, but not in the D17 cells. This difference may be because of an experimental error occurring in the D17 cells, but may also partly be due to the stability of NADH. NADH is a relatively unstable compound that is metabolized by serum components and at the cell surface (Friedlos & Knox 1992). Friedlos *et al.*, (1992) reported that metabolism of NADH is rapid because they observed that ~50% of NADH (500 μ M) in DMEM medium containing 10% FCS and PBS had disappeared within 6 hours (Friedlos & Knox 1992). Thus, it can be suggested that NADH is more stable in

RPMI 1640 medium containing FCS, used for culturing CMT7 cells, than DMEM, which was employed for D17 cells. Unfortunately, due to the limited availability of CB1954, this experiment could only be performed once, hence, should be repeated in future studies.

No significant toxicity of NADH was observed in this study on the NTC cells, which is in agreement with similar previously published studies (Anlezark *et al.*, 1995; Blackwood *et al.*, 2001). Although nicotinamide riboside (reduced) (NRH) can be generated from the metabolism of NADH, in the presence of FCS in culture medium (Friedlos & Knox 1992), which is more stable than NADH and is a very poor cofactor for NTR, it can act as a cofactor for endogenous NQO1 (Knox *et al.*, 1995). No reductions in cell survival were apparent in NTC treated with 20 μ M CB1954 and NADH. This finding agrees with the preliminary experiment results in which no decreases in cell survival were observed in NTC even treated with 160 μ M CB1954. Hence, the endogenous enzymes present in D17 and CMT7 cells are poor at activating CB1954, but not NFZ, under the conditions of this experiment, even in the presence of a cofactor. This provides a promising basis, on which these experiments may be performed *in vivo*, as NADH is an intracellular factor that does not appear to be a cofactor for endogenous canine nitroreductases. Thus, NTR and CB1954 can be exogenously introduced to selectively kill tumour cells, with hopefully, minimal unwanted toxicity occurring.

The ultimate aim of this study was to use the TP53-targeted suicide gene therapy strategy, using NTR as the suicide gene and CB1954 as the prodrug, to selectively target and reduce cell numbers in canine OS cells possessing accumulated TP53. The results obtained in this experiment, in the presence and absence of NADH, were generally in agreement with the findings of the DLR experiments. Although no significant decreases in survival of CMT7 cells, containing accumulated TP53, occurred following the transfection of pGL3P8LA-NTR in combination with pTargetLex-p53 after CB1954 treatment, to that of pGL3P8LA-NTR transfected alone and treated with CB1954, the percentages of cells surviving were, both in the presence and absence of NADH, 20-30% lower. This non-significant result may be due to the statistical analyses employed, which analysed all the time points together, as it can be seen in Figures 5.18 and

5.21 that the cell survival curves began to separate after 96 hours treatment. Hence, further statistical analysis is warranted. Unfortunately, the significant differences observed in the DLR experiments in the CMT7 cells treated with 'B8LA+Lexp53' and 'E8LA+Lexp53' to those treated with 'Lexp53' did not translate into any significant or apparent decreases in cell survival. Importantly however, no significant reductions in survival of D17 cells, possessing accumulated TP53, occurred following transfection of pGL3P8LA-NTR, pGL3B8LA-NTR or pGL3E8LA-NTR in combination with pTargetLex-p53, after CB1954 treatment. Significant decreases in survival of D17 cells were only observed following transfection with pCMVNTR and CB1954 treatment. Hence, the results demonstrated in this study are encouraging, because the TP53-targeted suicide gene therapy strategy does appear to selectively target and reduce cell numbers in canine OS cells possessing accumulated TP53. Furthermore, similar levels of reductions in survival of CMT7 cells were achieved following transfection with pGL3P8LA-NTR in combination with pTargetLex-p53, to CMT7 cells transfected with pCMVNTR, when treated with 20 μ M CB9154. Additionally, in the absence of CB1954 treatment, the survival of CMT7 cells transfected with pGL3P8LA-NTR in combination with pTargetLexp53 is not statistically different to that of the non-transfected CMT7 cells.

The results obtained in this study are in agreement with those previously published by Costa *et al.*, (1996) (Da Costa *et al.*, 1996). In addition, several *in vitro* and *in vivo* transcriptional targeting studies utilising NTR/CB1954 system have been reported, in which the number of targeted tumour cells were significantly reduced following CB1954 treatment, whilst non-targeted cells remained unaffected (Blackwood *et al.*, 2001).

The effect of NADH had similar effects on D17 and CMT7 cells transfected with pGL3P8LA-NTR, pGL3B8LA-NTR and pGL3E8LA-NTR in combination with and without pTargetLex-p53, to those in pCMVNTR-transfected cells, after CB1954 treatment. No increases in reductions in cell survival were seen in presence of NADH, infact cell survival appeared to be lower in absence of NADH. This is probably due to the stability of NADH, as previously described, or an anomalous result as this experiment was only performed once.

Improvements for modifying the constructs employed in the TP53-targeted suicide gene therapy strategy have been previously described in 4.4.3, which will enable a reduction in the expression of luciferase/NTR gene, in the absence of pTarget-Lexp53, to occur. In particular, the use of pGL4 vectors to reduce the occurrence of any *trans* effects, optimizations into the amounts of different constructs transfected and the number of LexA operator sites employed upstream of the luciferase/NTR gene. Additionally, verification of these results at a future time will be necessary as the assays utilising CB1954 were only performed once.

CB1954 certainly warrants further investigation as the prodrug of choice for the TP53-targeted suicide gene therapy strategy, whereas NFZ does not. Prior to any *in vivo* evaluation of the TP53-targeted suicide gene therapy strategy, a range of canine cell lines possessing wild-type and accumulated TP53, from different types of tissue should be tested, to determine whether activation by endogenous enzymes might occur in other tissues. In particular, hepatotoxicity has been reported as the dose-limiting toxicity of CB1954 administered alone in humans (Chung-Faye *et al.*, 2001), hence, the possibility exists that this may also occur in dogs. However, the dose of CB1954 required for use in combination with NTR was well-tolerated and no dose-limiting toxicities have been reported for NTR given on its own (Chung-Faye *et al.*, 2001; Palmer *et al.*, 2004). Furthermore, minimal levels of toxicity were observed in the recently reported first clinical trial in human prostate cancer patients analysing NTR in combination with CB1954 (Patel *et al.*, 2009). Hence the NTR/CB1954 system may be suitable for use in dogs.

Furthermore, the TP53-targeted suicide gene therapy strategy, in conjunction with the selective bioactivation of CB1954 by NTR, offers the potential of a strategy that can be targeted towards any canine tumour cell, or human cell, possessing accumulated TP53. In recent years, the NTR/CB1954 system has been extensively optimized to increase efficacy. Mutants of NTR have been developed, which include double and triple mutants and unnatural amino acids, that are at least 7-fold more efficient at activating CB1954 than NTR itself (Jackson *et al.*, 2006; Jarrom *et al.*, 2009; Searle *et al.*, 2004). Additionally, different nitroreductases have been evaluated, including *nfsA*, the primary *E.*

coli nitroreductase, which has shown a greater sensitivity to CB1954 as well as a greater bystander effect in human cells, than *nfsB* (Emptage *et al.*, 2009; Vass *et al.*, 2009). NTR has also been analysed with a variety of different substrates to enable new prodrugs to be synthesized, e.g. the 3,5-dinitrobenzamide-2-bromomustard, SN27686, that are more potent at selectively killing cells *in vitro* and/or possess a greater bystander effect than CB1954 (Atwell *et al.*, 2007; Denny 2003; Jiang *et al.*, 2006; Singleton *et al.*, 2007). Furthermore, the actual mechanism of action of CB1954 has recently been elucidated (Christofferson & Wilkie 2009), so it is hoped that together, all this information will enable further progress to be made in developing optimal suicide gene therapy strategies for cancers in both dogs and humans.

Chapter 6

Conclusions & Future Directions

TP53, the most commonly altered and studied gene in canine and human cancers, has also been demonstrated in this study to be important in canine OS. In particular, the canine *TP53* C-terminus domain was shown to be able to recruit the transactivation domain present in canine OS cells possessing accumulated *TP53*, to drive the expression of NTR, acting as a suicide gene, via the use of a Lex-DNA binding domain interacting with its operator site acting as an upstream activating sequence. Thus enabling, following the administration of the prodrug CB1954, the selective targeting and killing of canine OS cells possessing accumulated *TP53*. Importantly, cells possessing wild-type *TP53* did not show any reductions in cell survival.

Hence, a *TP53*-targeting gene therapy suicide strategy has been designed and constructed which has been shown to be functional *in vitro*, which warrants further investigation as a treatment modality for OS, in both dogs and humans. In addition, this system could be used to selectively target and initiate the destruction of any tumour containing cells possessing accumulated *TP53*.

Few suicide gene therapy studies have been published in canine tumour cells *in vitro* or *in vivo* and none as yet in canine OS, or with the *E.coli* NTR/CB1954 system. This study demonstrates, for the first time, that the prodrug CB1954 is non-toxic to canine OS cells '*in vitro*', and that *E.coli* NTR can be expressed in its biologically active form in these cells, resulting in activation of CB1954 to significantly reduce survival of canine OS cells. A marked bystander effect was also observed.

Veterinary treatment for canine cancer, in terms of suicide gene therapy, is well behind the progress that has been made in the treatments for humans, particularly in the number of suicide gene therapy trials that have been performed in cancer patients. Dogs with cancer are gaining acceptance as excellent comparative models for human neoplasia, due to the identification of similarities in the histological types, tumour genetics, molecular targets e.g. *TP53*, response to conventional therapies and biological behaviour of tumours. It is therefore hoped that with the formation of the Canine Comparative Oncology and Genomics Consortium in 2003 (<http://ccr.cancer.gov/resources/cop/>), an increase in the number of clinical trials performed in dogs will occur. Thereby

providing useful information for assessing the efficacy and optimal therapeutic regimens of anti-cancer agents (Figure 6.1).

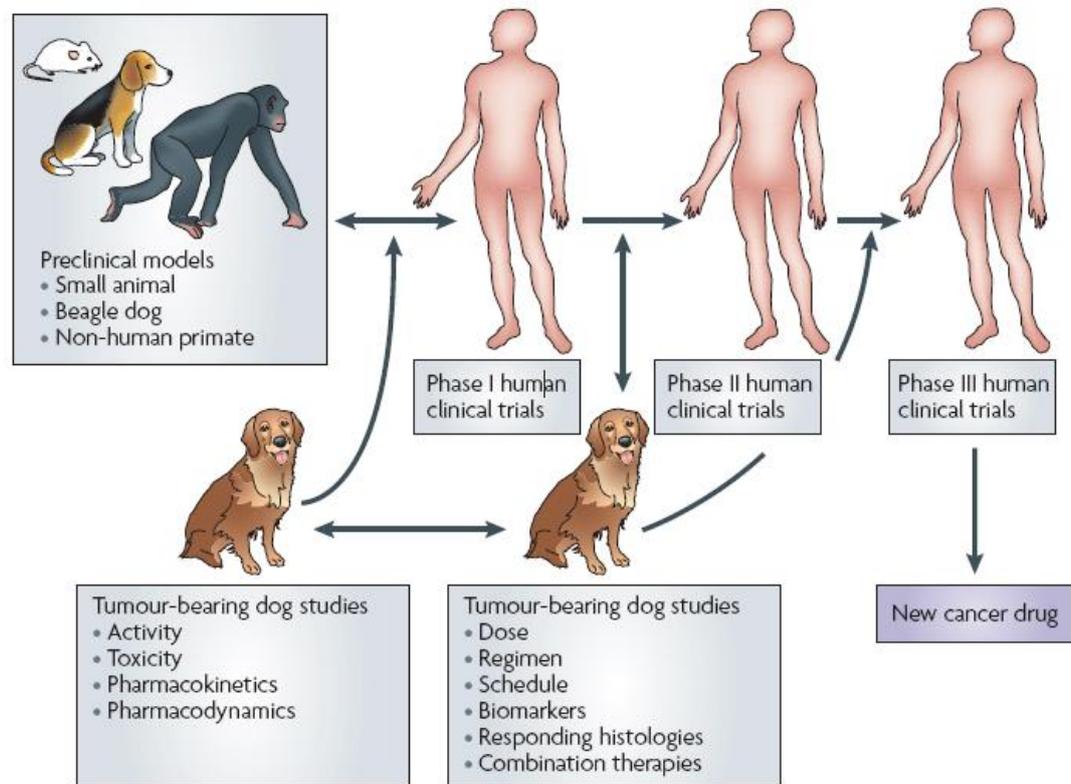


Figure 6.1: Integrated approach. An optimal drug development path would integrate both preclinical and clinical components of drug development so that questions that emerge in the human clinic could be answered in animals. Translational drug development studies in the pet dog with cancer are optimal for such an approach, being an intermediary between conventional preclinical models (mouse, research-bred dog and non-human primate) and the human clinical trial (Paoloni & Khanna 2008).

A major area of gene therapy research is the development of effective gene delivery vectors because for GDEPT to be successful at treating cancers in the clinic, they must exist. Advances in vector technology are progressing at a rapid rate and there is a tremendous effort to increase the ability to express the therapeutic gene of interest at high levels, deliver selectively and efficiently to tumour cells, to reduce levels of toxicity and to increase applicability for systemic administration, enabling the treatment of metastatic disease. Synthetic systems have been designed that combine positive elements from various sources, for example, dioleoyl trimethylammonium propane: Cholesterol

(DOTAP:Chol) liposomes encapsulate adenovectors, thereby possessing the targeting potential of natural receptor ligands, viral ability to enter cells, viral nuclear localization signals and packaging of liposomal envelopes. In preclinical studies, DOTAP:Chol liposomes were demonstrated to efficiently deliver TSG to disseminated lung tumours *in vitro* and *in vivo* (Ramesh *et al.*, 2001; Ramesh *et al.*, 2004). Although, the systemic administration of DNA- DOTAP:Chol liposomes induced multiple signalling molecules associated with inflammation, both *in vitro* and *in vivo*, small molecule inhibitors against these signalling molecules have been successfully used, resulting in their suppression and thus the reduction of inflammation (Gopalan *et al.*, 2004). Thus, if the TP53-targeted suicide gene therapy system continued to produce successful results in future studies, the current method of using cationic liposomes would need to be improved, as well as alternative methods of delivery investigated, in particular for administration systemically. Numerous studies have also demonstrated that gene therapy transfer protocols, optimized for use *in vitro*, including liposome formulations, may not predict the optimal formulation that can be used *in vivo*, hence, optimizations must also be carried *in vivo*, prior to *in vivo* analyses being performed (Danthinne & Imperiale 2000; Egilmez *et al.*, 1996; Nishikawa & Huang 2001; Xu *et al.*, 2001b). However, the use of liposomes has already shown promise as a gene delivery system for dogs with OS, as two separate Phase I studies demonstrated that liposomes are well-tolerated and produce minimal toxicity in dogs with OS lung metastases (Dow *et al.*, 2005; Khanna *et al.*, 1997).

Since factors that influence the efficacy of cancer therapy remain poorly understood, a molecular understanding of neoplasia will certainly improve cancer diagnosis and treatment. However, attempts to translate basic biology to clinically useful information have encountered many pitfalls. While TP53 clearly has an important role in tumourigenesis, it does not function in isolation. To date, many studies attempting to correlate findings to TP53 status have failed to account for other members of the TP53 pathway and hence, interpretations are incomplete. Indeed, tumours containing wild-type TP53, typically possess defects in the TP53-mediated apoptotic pathway. In the past few years, better methods to assess both TP53 status and the TP53 pathway have been developed and are being used to further increase our knowledge of TP53 biology and understand the clinical behaviour of TP53 mutant and wild-type tumours.

However, the preliminary success of approaches, that specifically address TP53 status in tumours, underscores the notion that a molecular understanding of cancer will have a positive impact on treatment.

Cancer, even of a single cell type in a single organ, is a molecularly heterogeneous disease. Although a single therapeutic approach alone, including a fully optimized suicide gene therapy strategy, can induce a tumour response, it is unlikely to be curative and induce cell death in all tumour cells. Instead, it is believed that several different therapeutic methods will need to be combined to enhance efficacy, reduce toxicity, alleviate associated pain, slow the development of metastasis and prevent or delay drug resistance. Multimodality therapy using combinations of conventional therapies are already in use for treating cancer and has, unfortunately, shown that intolerably high levels of toxicity occur in most cancer patients because of the small difference in biochemical signals between normal and malignant cells.

Gene therapies however, in comparison to current cancer therapies, have the great advantage of non-cross-resistance to most conventional treatments, a much lower systemic toxicity and the potential to selectively target micrometastatic deposits that are currently difficult to detect or treat, particularly so in OS. Efforts to combine gene therapies with conventional modalities are proceeding, as are efforts to combine different types of gene therapies themselves. For example, if *TK* gene transfer (followed by GCV) is combined with cytokine gene transfer, the patient may benefit from both a direct cytotoxic and an immune-mediated antitumour effect. NTR has been inserted into oncolytic viruses, which has been demonstrated to further enhance levels of cell kill and increase survival *in vitro* and *in vivo*, following administration of CB1954 and SN27686 (Braidwood *et al.*, 2009; Singleton *et al.*, 2007). The *E. coli* NTR/CB1954 system has also been demonstrated to improve the effect of radiotherapy *in vitro* and *in vivo* (White *et al.*, 2008). Hence, at the current rate of biotechnological development, it is only a matter of time until the development of more efficient vectors and combined modality approaches overcome technical limitations that currently prevent the widespread application of gene therapy to cancer (Roth & Grammer 2004).

In addition to this study, there is a growing body of investigation into molecular targeted therapies for the treatment of canine OS. Several studies have shown that proliferation can be reduced and apoptosis induced in canine OS cell lines *in vitro*, including, multikinase inhibitor, sorafenib (Wolfesberger *et al.*, 2009), small molecule MET inhibitor, PF2362376 (Liao *et al.*, 2007), histone deacetylase (HDAC) inhibitors, OSU-HDAC42 (S(+)-N-hydroxy-4-(3-methyl-2-phenyl-butrylamino)benzamide) and SAHA (suberoylanilide hydroxamic acid) (Kisseberth *et al.*, 2008), HSP90 inhibitor, STA-1474 (McCleese *et al.*, 2009), mammalian target of rapamycin (mTOR) inhibitor, rapamycin (Gordon *et al.*, 2008), and *in vivo*, thalidomide, a drug that is gaining a modest resurgence in oncology as a potential anti-angiogenesis agent (Farese *et al.*, 2004). These have all been suggested to represent potential therapeutic agents that may be used as adjuvant treatments to conventional therapies. However, they all require further investigation to determine if preclinical activity translates into antitumour activity in dogs with OS. Two Phase I clinical trials have been performed in dogs with OS, investigating an IGF-1 suppressant, OncoLAR (octreotide pamoate), in combination with carboplatin following amputation (Khanna *et al.*, 2002), and a protein-kinase C inhibitor, dexniguldipine (Hahn *et al.*, 1997). A third is currently in progress evaluating rapamycin (Paoloni & Khanna 2007). Although the two completed trials reported encouraging results, they did not produce the desired therapeutic benefit. OncoLAR suppressed IGF-1 levels by 43% and minimal levels of toxicity were observed, however survival times were not enhanced when compared to carboplatin treatment alone (Khanna *et al.*, 2002). Dexniguldipine- and cisplatin- treated dogs had longer median remission duration and survival time than untreated dogs; however, dexniguldipine-treated dogs had a shorter survival time than cisplatin-treated dogs (Hahn *et al.*, 1997). Hence, clinical studies investigating alternative molecular targets are still necessary. Furthermore, understanding the mechanisms and interactions of various molecular pathways in OS will hopefully help to identify new targets for therapy that may prove more effective and/or less toxic, especially for the tumours that respond poorly to conventional treatments.

It is hoped therefore, that with future research, a targeted suicide gene therapy approach in combination with other molecular-based treatments will be

developed that is able to successfully treat OS and combat the problem of micro- and macro- metastatic disease, thereby increasing survival rates in both dogs and humans.

Overall, this thesis demonstrates that a TP53-targeted suicide gene therapy strategy may play a role as a treatment modality for OS and other canine and human cancers possessing accumulated TP53.

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Appendices

Appendix A: Solutions & general reagents

CB1954: 10mM solution in DMSO, prepared immediately prior to use. Kindly donated by Dr. N. Keith, University of Glasgow, following manufacture by Cobra Therapeutics, University of Keele. Supplied as a powder and stored at 4°C.

DNA Size Marker: 100-2072 bp DNA ladder consisting of 15 blunt-ended DNA fragments from 100-1500 bp at 100 bp increments and an additional fragment at 2072 bp. 3-5µg loaded per lane.

***E. coli* NTR enzyme:** Donated by Dr. G. Anlezark, Porton Down, Salisbury (Anlezark *et al.*, 1995). Supplied as a 1x stock solution and stored at 4°C.

HiFCS: Heated at 56°C for 30 minutes and then stored in 50ml aliquots at -20°C (GIBCO BRL).

Freezing Media: 40% medium without antibiotics, 50% FCS and 10% dimethyl sulphoxide (DMSO). Prepared immediately before use.

Gel loading buffer: 0.25% bromophenol blue, 30% glycerol and 70% TE buffer (2.1.8 xix). Stored at RT.

Laemmli Running buffer: 0.025M Trizma base, 0.192M glycine, 0.1% sodium dodecyl sulphate (SDS). Stored at RT.

Low DNA Mass Marker: 6 blunt-ended DNA fragments of 2000, 1200, 800, 400, 200 and 100 bp, electrophoresis of 4µl, gives bands containing 200, 120, 80, 40, 20 and 10 ng DNA, respectively. Mixed 4:1 (v:v) with gel loading buffer.

Lysis Buffer for DNA Extractions (300ml): 3ml of 0.5M EDTA, 30ml of 10% SDS, 6ml of 2.5M NaCl, 15ml of 1M Tris pH 8.0, 4.7ml of Proteinase K (20mg/ml; Roche) and 241.3ml of sterile H₂O. Made immediately prior to use.

NADH: 50mM solution prepared immediately before use in PBS, kept on ice and used at 100µM in cell culture medium. Supplied as a powder (Sigma) and stored at 4°C.

NFZ: 1 μ M solution in DMSO made immediately before use (Sigma).

PBS: Each sachet of dry powder (Sigma) produces 1 litre of PBS pH 7.4, containing NaCl (120mM), KCl (2.7mM) and phosphate buffer salts (10mM).

Protein ladder: Pre-stained PAGE standards low range: phosphorylase B 113kDa; BSA 82kDa; ovalbumin 52.4kDa; carbonic anhydrase 36.2kDa; soybean trypsin inhibitor 29.6kDa; lysozyme 20.3kDa.

Protein treatment sample buffer (2x): 0.125M Tris-Cl, 4% SDS, 20% glycerol, 0.2M dithiothreitol, 4% bromophenol blue. Stored at -20°C for up to 6 months.

Protein transfer buffer: 0.016M Trizma base, 0.12M glycine. Stored at RT.

TAE (50x): 2M Tris-HCl pH 8.15, 1.5M NaOAc, 1M NaCl and 0.1M EDTA. Stored at RT.

TBE (10x): 0.9M Tris-HCl, 0.9M Boric acid and 25mM EDTA, pH 8.3. Stored at RT.

TE buffer (10x): 10mM Tris-HCl and 1mM disodium EDTA, pH 8.0. Stored at RT.

Appendix B: Bacterial strains, culture media & antibiotics

***E. coli* DH5 α :** Subcloning MAX Efficiency DH5 α Competent Cells, F⁻ Φ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 *deoR recA1 endA1 hsdR17*(r_K⁻, m_K⁺) *phoA supE44* λ ⁻ *thi-1 gyrA96 relA1* (GIBCO BRL). The Φ 80dlacZ Δ M15 marker provides α -complementation of vectors containing the β -gal gene. Used for blue/white colony screening on plates containing X-gal.

***E. coli* JM109:** Subcloning JM109 High Efficiency Competent Cells, *endA1, recA1, gyrA96, thi, hsdR17* (r_K⁻, m_K⁺), *relA1, supE44, Δ (lac-proAB), [F', traD36, proAB, lacI^qZ Δ M15]* (Promega). Used for blue/white screening with IPTG and X-gal.

***E. coli* TOP10:** Subcloning One ShotTM TOP10 Competent Cells, F⁻ *mcrA Δ (mrr-hsdRMS-mcrBC) Φ 80dlacZ Δ M15 Δ lacX74 recA1 deoR araD139 d(ara-leu)7697 galU galK rpsL* (Str^R) *endA1 nupG* (Invitrogen). Used for blue/white screening with X-gal.

IPTG: 100mM solution in ddH₂O and stored at -20oC in the dark. 40 μ l applied to LB agar plate and spread evenly (in addition to X-gal), then incubated at 37oC for ~30 minutes prior to bacteria being plated out. Supplied as a powder (Sigma) and stored at 4oC.

X-gal: 50mg/ml solution in dimethylformamide and stored at -20°C in the dark. 30 μ l applied to LB agar plate and spread evenly, then incubated at 37°C for ~30 minutes prior to bacteria being plated out. Supplied as a powder (Sigma) and stored at -20°C.

Appendix C: Vector maps

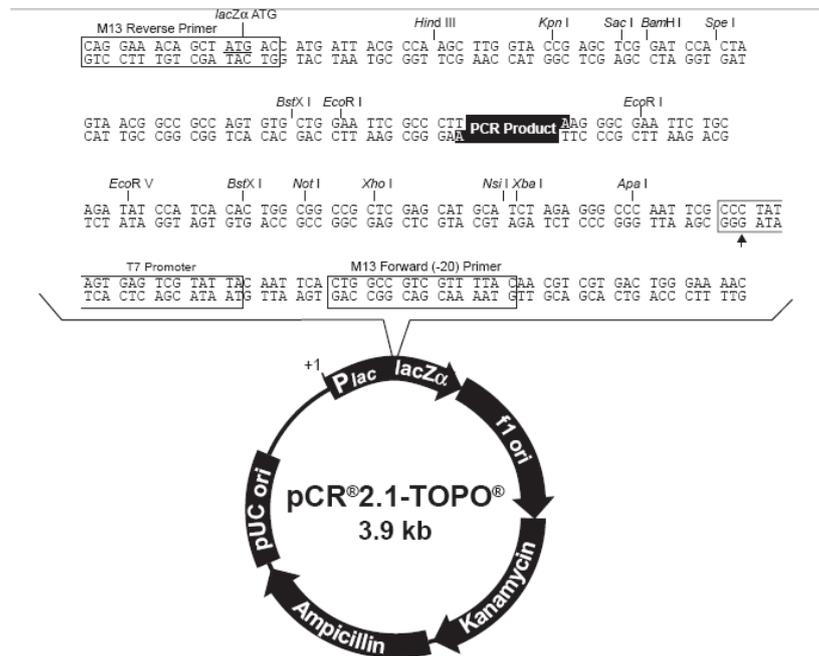


Figure C1: pCR®2.1-TOPO®. Plasmid vector ($lacZ^+$, amp^r , kan^r) with single 3'-thymidine overhangs for TA Cloning® and Topoisomerase I covalently bound to the vector for direct ligation of PCR products (Invitrogen). This is because thermostable enzymes, such as *Taq* polymerase, possess template independent terminal transferase activity, which adds a single deoxyadenosine to the 3'-ends of PCR products. Multiple cloning sites, T7 promoter/priming site, M13 forward and reverse primer annealing sites, pUC origin and f1 origin are present.

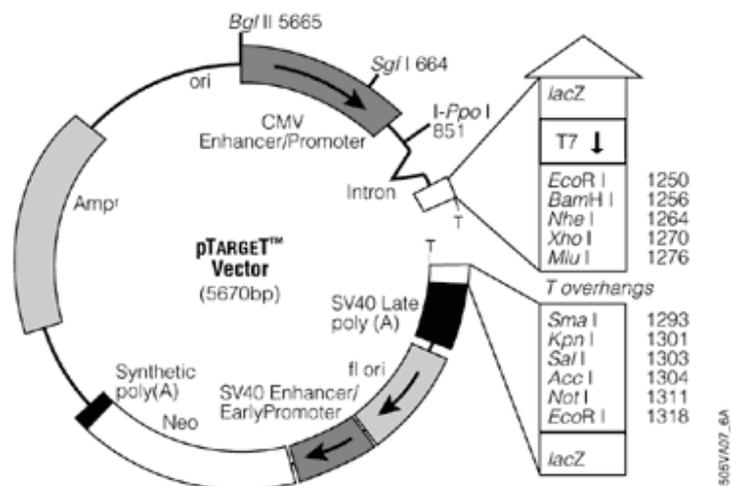


Figure C2: pTarget™ Mammalian Expression Vector. A linearised plasmid vector ($lacZ^+$, amp^r , *Neo*) which facilitates the direct insertion of PCR products by possessing single 3'-thymidine overhangs that can be ligated to the single deoxyadenosine at the 3'-ends of PCR products introduced by thermostable enzymes (Promega). Multiple cloning sites, *CMV* intermediate early enhancer/promoter (IE) region, *SV40* late poly(A) signal and *T7* promoter/priming site are present.

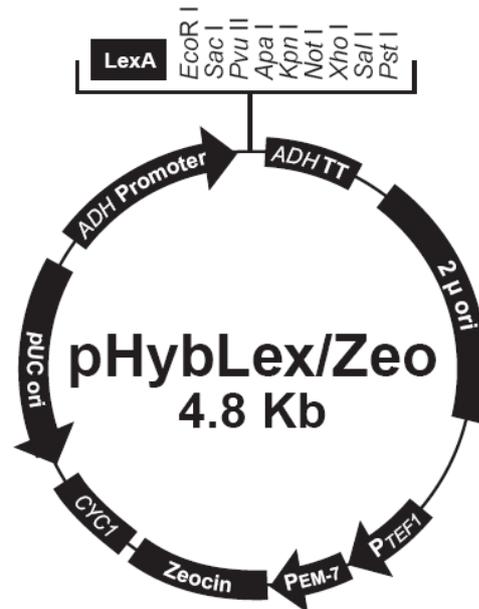


Figure C3: pHybLex/Zeo. A plasmid vector (Zeocin[®]) containing a multiple cloning site upstream of an open reading frame for a LexA DNA binding domain (Invitrogen). pHybLex/Zeo is designed to express LexA fusion bait proteins and can be used with vectors containing LexA operator-based reporters. *ADH* promoter, *ADH* transcription termination region (*ADH* TT), 2 μ origin of replication (2 μ ori), *TEF1* promoter (*PTEF1*), EM-7 promoter (*PEM-7*), *CYC1* transcription termination region (*CYC1*) and pUC origin of replication (pUC ori) are present.

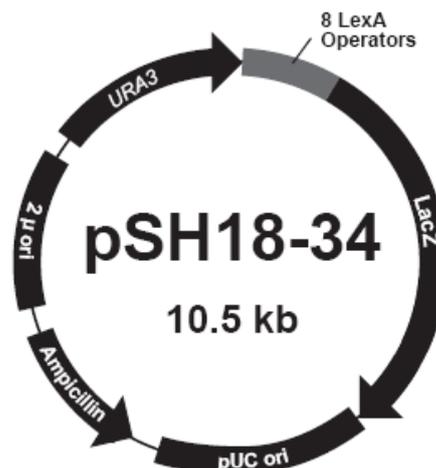


Figure C4: pSH18-34. A pRB322-derived reporter plasmid (*lacZ*⁺, *amp*^r) possessing 8 LexA operator sites which allow the transcriptional activation of the *lacZ* gene by LexA-containing bait plasmids (e.g. pHybLex/Zeo), that facilitate the detection of LexA-mediated protein-protein interactions (Invitrogen). *URA3* gene (*URA3*), 2 μ origin of replication (2 μ ori) and pUC origin of replication (pUC ori) are present.

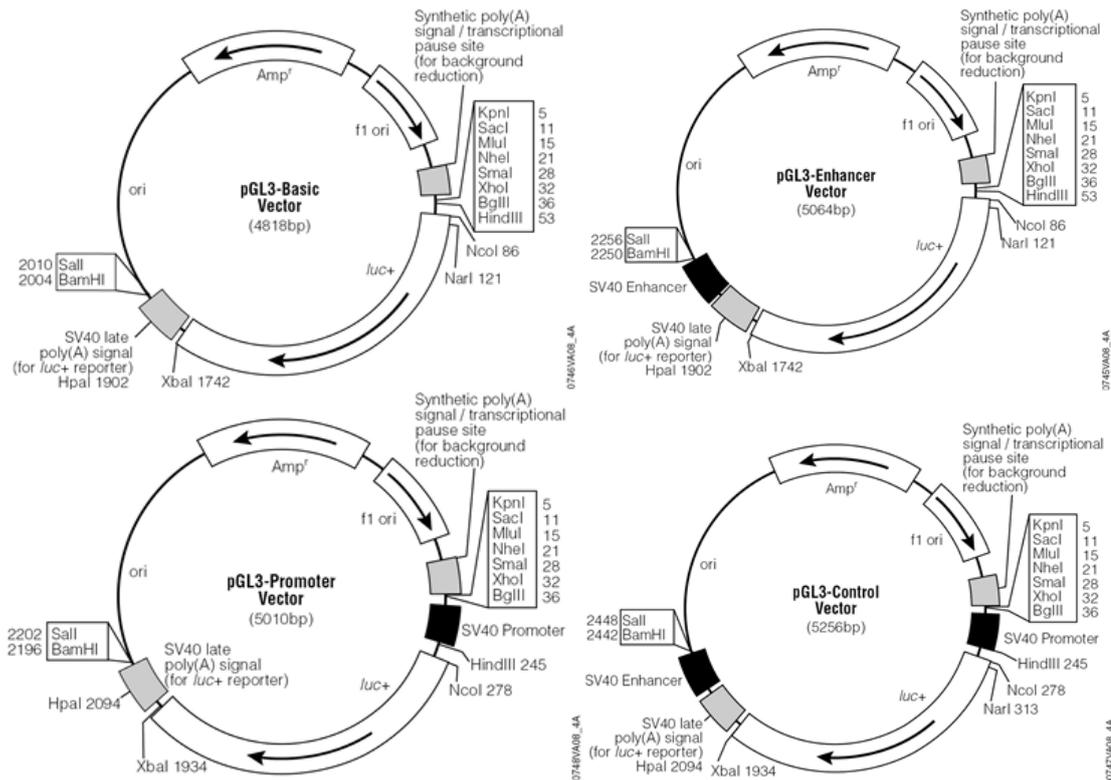


Figure C5: pGL3 Luciferase Reporter Vectors. 4 plasmid vectors, pGL3-Basic, pGL3-Enhancer, pGL3-Promoter and pGL3-Control, which enable the quantitative analysis of factors that potentially regulate mammalian gene expression (Promega). They are structurally identical, each containing cDNA encoding the modified firefly luciferase enzyme (*luc+*), gene conferring ampicillin resistance in *E. coli* (*Amp^r*), origin of replication in *E. coli* (*f1 ori*), origin of plasmid replication in *E. coli* (*ori*), multiple cloning sites and SV40 late poly(A) signal, except for the inclusion/exclusion of SV40 promoter and enhancer sequences: (i), pGL3-Basic - lacks the SV40 promoter and enhancer sequences; (ii), pGL3-Enhancer - contains the SV40 enhancer located downstream of *luc+*; (iii), pGL3-Promoter - possesses the SV40 promoter upstream of *luc+*; (iv), pGL3-Control - contains the SV40 promoter and enhancer sequences upstream and downstream of *luc+*, respectively, which result in the strong expression of *luc+* in many types of mammalian cell types. This plasmid is useful for monitoring transfection efficiency and provides an internal standard for promoter and enhancer activities expressed by pGL3 recombinants. Arrows within the *luc+* and *Amp^r* genes indicate the direction of transcription and the arrow in the *f1 ori* indicates the direction of ssDNA strand synthesis (Promega Corporation 1999).

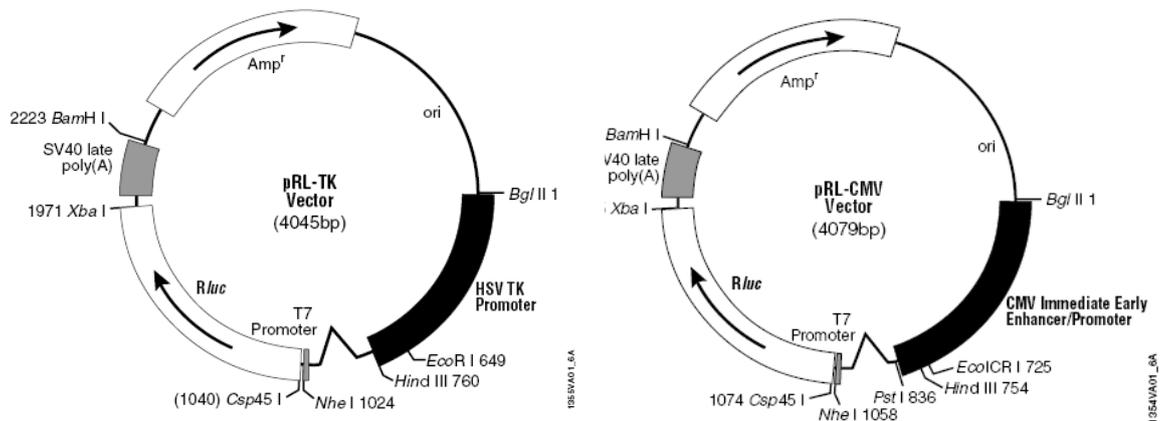


Figure C6: pRL Reporter Vectors. 2 plasmid vectors, pRL-*TK* and pRL-*CMV*, each possessing cDNA encoding *Renilla* luciferase enzyme (*Rluc*), SV40 late poly(A) signal, gene conferring ampicillin resistance in *E. coli* (Amp^r), origin of plasmid replication in *E. coli* (*ori*) and position of intron (_/_) (Promega). They are isolated from a *dam/dcm* *E. coli* K host strain to enable digestion with restriction enzymes that are sensitive to *dam* and *dcm* methylation. The pRL vectors are intended for use as internal control reporters and so may be used in combination with any experimental reporter vector to co-transfect mammalian cells. pRL-*TK* and pRL-*CMV* are structurally identical except for different promoter sequences located upstream of the *Rluc* reporter gene:

- (i), pRL-*TK* - contains the herpes simplex virus thymidine kinase (HSV-*TK*) promoter region which provides a low to moderate level, constitutive expression of the *Rluc* reporter in co-transfected mammalian cells from both embryonic and mature mammalian tissues (GenBank Accession No. AF025846);
- (ii), pRL-*CMV* - possesses the cytomegalovirus (*CMV*) intermediate early enhancer/promoter that provides a strong, constitutive expression of *Rluc* in a variety of cell types, e.g. the transcriptional activity of the *CMV* intermediate early enhancer/promoter has been observed in transgenic mice, where its transcriptional activity was observed in 24 of the 28 murine tissues examined (Schmidt *et al.*, 1990). Arrows within the *Rluc* and Amp^r genes indicate the direction of transcription (Promega Corporation 2001; Sherf *et al.*, 1996).

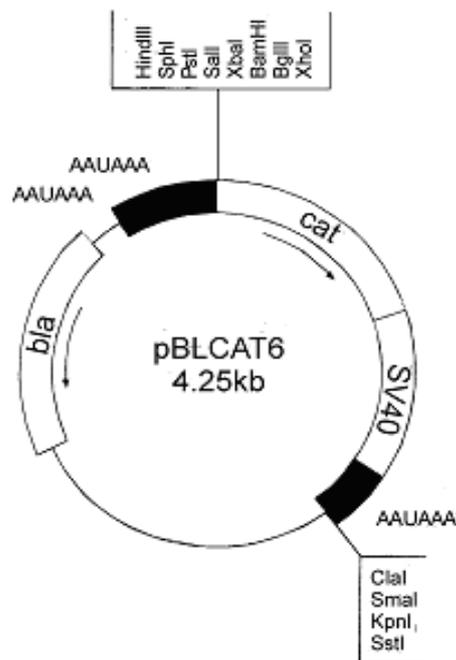


Figure C7: pBLCAT6. A pUC18-derived plasmid (amp^r) which contains the reporter gene chloramphenicol acetyl transferase (*cat*), β -lactamase gene (*bla*), multiple cloning sites, SV40 small t intron sequence (SV40) and SV40 polyadenylation (poly(A)) signals (black boxes, AAUAAA) (GenBank Accession No. M80484). The multiple cloning sites are upstream and downstream of the *cat* gene and the SV40 small t intron is downstream of the *cat* gene (Boshart *et al.*, 1992; Luckow & Schutz 1987). Arrows outside the *cat* and *bla* genes indicate the direction of transcription (Boshart *et al.*, 1992). Kindly donated by Dr. L. Blackwood. This plasmid has been designed for the analysis of promoter and enhancer sequences in mammalian cells (Boshart *et al.*, 1992; Luckow & Schutz 1987).

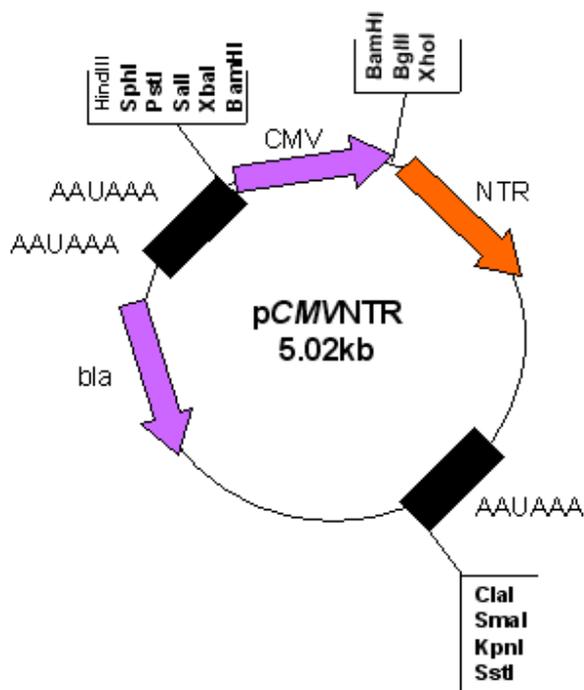


Figure C8: pCMVNTR. A pCMVCAT6-derived plasmid which contains the *E. coli* B nitroreductase (NTR) gene, instead of the *CAT* gene, under the control of CMV IE (Blackwood *et al.*, 2001). Multiple cloning sites and SV40 poly(A) signals are present. Kindly donated by Dr. L. Blackwood, Department of Veterinary Clinical Studies, University of Glasgow.

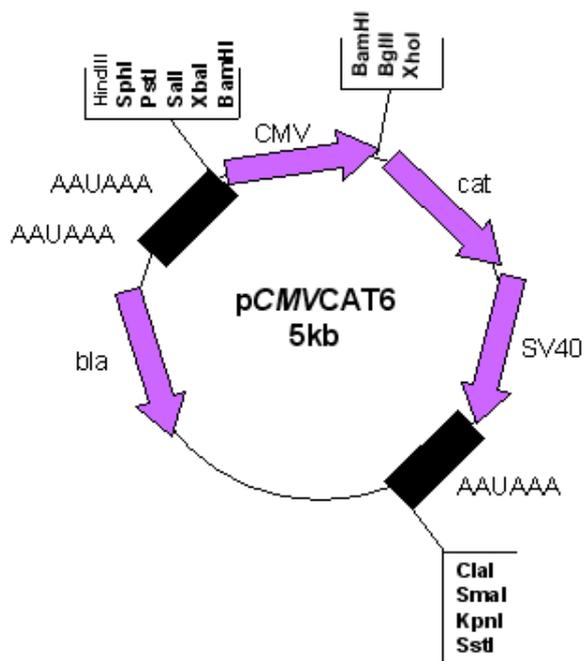


Figure C9: pCMVCAT6. A pBLCAT6-derived plasmid which contains the chloramphenicol acetyl transferase gene (*cat*) gene under the control of CMV IE (Blackwood *et al.*, 2001). Multiple cloning sites and SV40 poly(A) signals are present. Kindly donated by Dr. L. Blackwood, Department of Veterinary Clinical Studies, University of Glasgow.

Appendix D: Alignment of nucleotide sequence for canine TP53 exons 5-8

Control (DNA)	-----CCCTCTCCTCAACAAGTTGTTTTGCCAGCTGGCGAAGACCTGCC	44
U62133 (DNA) (732)	TTTTCTTACAGT---CCCCTCTCCTCAACAAGTTGTTTTGCCAGCTGGCGAAGACCTGCC	788
U51857 (DNA)	-----CAGCTGGCGAAGACCTGCC	19
L27630 (DNA)	-----	
NC_006587 (DNA) (1087)	TTTTCTTACAGT ACT CCCCTCTCCTCAACAAGTTGTTTTGCCAGCTGGCGAAGACCTGCC	1146
S77819 (mRNA) (265)	----- TACT CCCCTCTCCTCAACAAGTTGTTTTGCCAGCTGGCGAAGACCTGCC	313
L37107 (mRNA) (53)	----- TACT CCCCTCTCCTCAACAAGTTGTTTTGCCAGCTGGCGAAGACCTGCC	101
AF159062 (mRNA) (23)	----- TACT CCCCTCTCCTCAACAAGTTGTTTTGCCAGCTGGCGAAGACCTGCC	71
DN444655 (mRNA) (471)	----- TACT CCCCTCTCCTCAACAAGTTGTTTTGCCAGCTGGCGAAGACCTGCC	519
AF060514 (mRNA) (353)	----- TACT CCCCTCTCCTCAACAAGTTGTTTTGCCAGCTGGCGAAGACCTGCC	401
AB020761 (mRNA) (383)	----- TACT CCCCTCTCCTCAACAAGTTGTTTTGCCAGCTGGCGAAGACCTGCC	431
Control (DNA)	CCGTGCAGCTGTGGGTGACGCTCCCCACCCCAACCAATACCTGCGTCCGCGCTATGGCCA	104
U62133 (DNA)	CCGTGCAGCTGTGGGTGACGCTCCCCACCCCAACCAATACCTGCGTCCGCGCTATGGCCA	848
U51857 (DNA)	CCGTGCAGCTGTGGGTGACGCTCCCCACCCCAACCAATACCTGCGTCCGCGCTATGGCCA	79
L27630 (DNA)	-----	
NC_006587 (DNA)	CCGTGCAGCTGTGGGTGACGCTCCCCACCCCAACCAATACCTGCGTCCGCGCTATGGCCA	1206
S77819 (mRNA)	CCGTGCAGCTGTGGGTGACGCTCCCCACCCCAACCAATACCTGCGTCCGCGCTATGGCCA	373
L37107 (mRNA)	CCGTGCAGCTGTGGGTGACGCTCCCCACCCCAACCAATACCTGCGTCCGCGCTATGGCCA	161
AF159062 (mRNA)	CCGTGCAGCTGTGGGTGACGCTCCCCACCCCAACCAATACCTGCGTCCGCGCTATGGCCA	131
DN444655 (mRNA)	CCGTGCAGCTGTGGGTGACGCTCCCCACCCCAACCAATACCTGCGTCCGCGCTATGGCCA	579
AF060514 (mRNA)	CCGTGCAGCTGTGGGTGACGCTCCCCACCCCAACCAATACCTGCGTCCGCGCTATGGCCA	461
AB020761 (mRNA)	CCGTGCAGCTGTGGGTGACGCTCCCCACCCCAACCAATACCTGCGTCCGCGCTATGGCCA	491
Control (DNA)	TCTATAAGAAGTCGGAGTTCGTGACCGAGGTTGTGCGGCGCTGCCCCACCATGAACGCT	164
U62133 (DNA)	TCTATAAGAAGTCGGAGTTCGTGACCGAGGTTGTGCGGCGCTGCCCCACCATGAACGCT	908
U51857 (DNA)	TCTATAAGAAGTCGGAGTTCGTGACCGAGGTTGTGCGGCGCTGCCCCACCATGAACGCT	139
L27630 (DNA)	-----GAAGTCGGAGTTCGTGACCGAGGTTGTGCGGCGCTGCCCCACCATGAACGCT	53
NC_006587 (DNA)	TCTATAAGAAGTCGGAGTTCGTGACCGAGGTTGTGCGGCGCTGCCCCACCATGAACGCT	1266
S77819 (mRNA)	TCTATAAGAAGTCGGAGTTCGTGACCGAGGTTGTGCGGCGCTGCCCCACCATGAACGCT	433
L37107 (mRNA)	TCTATAAGAAGTCGGAGTTCGTGACCGAGGTTGTGCGGCGCTGCCCCACCATGAACGCT	221
AF159062 (mRNA)	TCTATAAGAAGTCGGAGTTCGTGACCGAGGTTGTGCGGCGCTGCCCCACCATGAACGCT	191
DN444655 (mRNA)	TCTATAAGAAGTCGGAGTTCGTGACCGAGGTTGTGCGGCGCTGCCCCACCATGAACGCT	639
AF060514 (mRNA)	TCTATAAGAAGTCGGAGTTCGTGACCGAGGTTGTGCGGCGCTGCCCCACCATGAACGCT	521
AB020761 (mRNA)	TCTATAAGAAGTCGGAGTTCGTGACCGAGGTTGTGCGGCGCTGCCCCACCATGAACGCT	551

Control (DNA)	GCTCTGACAGTAGTGACGGTAAGCCATCGGGGGCTACAGATGGGACAAGGCTGCTCCCCA	224
U62133 (DNA)	GCTCTGACAGTAGTGACGGTAAGCCATCGGGGGCTACAGATGGGACAAGGCTGCTCCCCA	968
U51857 (DNA)	GCTCTGACAGTAGTGACGGTAAGCCATCGGGGGCTACAGATGGGACAAGGCTGCTCCCCA	199
L27630 (DNA)	GCTCTGACAGTAGTGACGGTAAGCCATCGGGGGCTACAGATGGGACAAGGCTGCTCCCCA	113
NC_006587 (DNA)	GCTCTGACAGTAGTGACGGTAAGCCATCGGGGGCTACAGATGGGACAAGGCTGCTCCCCA	1326
S77819 (mRNA)	GCTCTGACAGTAGTGACG-----	451
L37107 (mRNA)	GCTCTGACAGTAGTGACG-----	239
AF159062 (mRNA)	GCTCTGACAGTAGTGACG-----	209
DN444655 (mRNA)	GCTCTGACAGTAGTGACG-----	657
AF060514 (mRNA)	GCTCTGACAGTAGTGACG-----	539
AB020761 (mRNA)	GCTCTGACAGTAGTGACG-----	569
Control (DNA)	GAGG G CCCAAAGCCCTGATTCCTCCCGATGGCTCTTAGGCTTTGCCCTCCTCAGCATC	284
U62133 (DNA)	GAGG G CCCAAAGCCCTGATTCCTCCCGATGGCTCTTAGGCTTTGCCCTCCTCAGCATC	1027
U51857 (DNA)	GAGG G CCCAAAGCCCTGATTCCTCCCGATGGCTCTTAGGCTTTGCCCTCCTCAGCATC	259
L27630 (DNA)	GAGG G CCCAAAGCCCTGATTCCTCCCGATGGCTCTTAGGCTTTGCCCTCCTCAGCATC	172
NC_006587 (DNA)	GAGG G CCCAAAGCCCTGATTCCTCCCGATGGCTCTTAGGCTTTGCCCTCCTCAGCATC	1386
S77819 (mRNA)	-----GTCTTGCCCTCCTCAGCATC	472
L37107 (mRNA)	-----GTCTTGCCCTCCTCAGCATC	260
AF159062 (mRNA)	-----GTCTTGCCCTCCTCAGCATC	230
DN444655 (mRNA)	-----GTCTTGCCCTCCTCAGCATC	678
AF060514 (mRNA)	-----GTCTTGCCCTCCTCAGCATC	560
AB020761 (mRNA)	-----GTCTTGCCCTCCTCAGCATC	590

Appendix D: Alignment of Nucleotide Sequence for Canine TP53 Exons 5-8

Control (DNA)	TCATCCGAGTGAAGGAAATTTGCGGGCCAAGTACCTGGACGACAGAAACACTTTTTCGAC	344
U62133 (DNA)	TCATCCGAGTGAAGGAAATTTGCGGGCCAAGTACCTGGACGACAGAAACACTTTTTCGAC	1087
U51857 (DNA)	TCATCCGAGTGAAGGAAATTTGCGGGCCAAGTACCTGGACGACAGAAACACTTTTTCGAC	319
L27630 (DNA)	TCATCCGAGTGAAGGAAATTTGCGGGCCAAGTACCTGGACGACAGAAACACTTTTTCGAC	232
NC_006587 (DNA)	TCATCCGAGTGAAGGAAATTTGCGGGCCAAGTACCTGGACGACAGAAACACTTTTTCGAC	1446
S77819 (mRNA)	TCATCCGAGTGAAGGAAATTTGCGGGCCAAGTACCTGGACGACAGAAACACTTTTTCGAC	532
L37107 (mRNA)	TCATCCGAGTGAAGGAAATTTGCGGGCCAAGTACCTGGACGACAGAAACACTTTTTCGAC	320
AF159062 (mRNA)	TCATCCGAGTGAAGGAAATTTGCGGGCCAAGTACCTGGACGACAGAAACACTTTTTCGAC	290
DN444655 (mRNA)	TCATCCGA-----	686
AF060514 (mRNA)	TCATCCGAGTGAAGGAAATTTGCGGGCCAAGTACCTGGACGACAGAAACACTTTTTCGAC	620
AB020761 (mRNA)	TCATCCGAGTGAAGGAAATTTGCGGGCCAAGTACCTGGACGACAGAAACACTTTTTCGAC *****	650
Control (DNA)	ACAGTGTGGTGGTGCCTTATGAGCCACCCGAGGTCTGGTTTGGCATCTGAGGGGTCTCTG	404
U62133 (DNA)	ACAGTGTGGTGGTGCCTTATGAGCCACCCGAGGTCTGGTTTGGCATCTGAGGGGTCTCTG	1147
U51857 (DNA)	ACAGTGTGGTGGTGCCTTATGAGCCACCCGAGGTCTGGTTTGGCATCTGAGGGGTCTCTG	379
L27630 (DNA)	ACAGTGTGGTGGTGCCTTATGAGCCACCCGAGGTCTGGTTTGGCATCTGAGGGGTCTCTG	292
NC_006587 (DNA)	ACAGTGTGGTGGTGCCTTATGAGCCACCCGAGGTCTGGTTTGGCATCTGAGGGGTCTCTG	1506
S77819 (mRNA)	ACAGTGTGGTGGTGCCTTATGAGCCACCCGAG-----	564
L37107 (mRNA)	ACAGTGTGGTGGTGCCTTATGAGCCACCCGAG-----	352
AF159062 (mRNA)	ACAGTGTGGTGGTGCCTTATGAGCCACCCGAG-----	322
DN444655 (mRNA)	-----	
AF060514 (mRNA)	ACAGTGTGGTGGTGCCTTATGAGCCACCCGAG-----	652
AB020761 (mRNA)	ACAGTGTGGTGGTGCCTTATGAGCCACCCGAG-----	682
Control (DNA)	GGAGGAGGGGGCTGGAGGGATTTGTGAGCGGGAGTTGAGGGGGCTTTCTCCTCCTTCA	464
U62133 (DNA)	GGAGGAGGGGGCTGGAGGGATTTGTGAGCGGGAGTTGAGGGGGCTTTCTCCTCCTTCA	1207
U51857 (DNA)	GGAGGAGGGGGCTGGAGGGATTTGTGAGCGGGAGTTGAGGGGGCTTTCTCCTCCTTCA	439
L27630 (DNA)	GGAGGAGGGGGCTGGAGGGATTTGTGAGCGGGAGTTGAGGGGGCTTTCTCCTCCTTCA	352
NC_006587 (DNA)	GGAGGAGGGGGCTGGAGGGATTTGTGAGCGGGAGTTGAGGGGGCTTTCTCCTCCTTCA	1566
S77819 (mRNA)	-----	
L37107 (mRNA)	-----	
AF159062 (mRNA)	-----	
DN444655 (mRNA)	-----	
AF060514 (mRNA)	-----	
AB020761 (mRNA)	-----	
Control (DNA)	CCTCCCGCAGCCCTGTGAGGAGTGCAGAGGCAGGTGGGTCTATCCCATTTCTGCGGTTGGG	524
U62133 (DNA)	CCTCCCGCAGCCCTGTGAGGAGTGCAGAGGCAGGTGGGTCTATCCCATTTCTGCGGTTGGG	1267
U51857 (DNA)	CCTCCCGCAGCCCTGTGAGGAGTGCAGAGGCAGGTGGGTCTATCCCATTTCTGCGGTTGGG	499
L27630 (DNA)	CCTCCCGCAGCCCTGTGAGGAGTGCAGAGGCAGGTGGGTCTATCCCATTTCTGCGGTTGGG	412
NC_006587 (DNA)	CCTCCCGCAGCCCTGTGAGGAGTGCAGAGGCAGGTGGGTCTATCCCATTTCTGCGGTTGGG	1626
S77819 (mRNA)	-----	
L37107 (mRNA)	-----	
AF159062 (mRNA)	-----	
DN444655 (mRNA)	-----	
AF060514 (mRNA)	-----	
AB020761 (mRNA)	-----	
Control (DNA)	AAAGACTGAGGCTGATAGACTACAGGCCTGCCAGGGCTGAGTGGCCCGACCTGGGCCT	584
U62133 (DNA)	AAAGACTGAGGCTGATAGACTACAGGCCTGCCAGGGCTGAGTGGCCCGACCTGGGCCT	1327
U51857 (DNA)	AAAGACTGAGGCTGATAGACTACAGGCCTGCCAGGGCTGAGTGGCCCGACCTGGGCCT	559
L27630 (DNA)	AAAGACTGAGGCTGATAGACTACAGGCCTGCCAGGGCTGAGTGGCCCGACCTGGGCCT	472
NC_006587 (DNA)	AAAGACTGAGGCTGATAGACTACAGGCCTGCCAGGGCTGAGTGGCCCGACCTGGGCCT	1686
S77819 (mRNA)	-----	
L37107 (mRNA)	-----	
AF159062 (mRNA)	-----	
DN444655 (mRNA)	-----	
AF060514 (mRNA)	-----	
AB020761 (mRNA)	-----	
Control (DNA)	ACCTTCTACCTCAGGTTGGCTCTGACTATAACCACCATCCACTACAACCTACATGTGTAACA	644
U62133 (DNA)	ACCTTCTACCTCAGGTTGGCTCTGACTATAACCACCATCCACTACAACCTACATGTGTAACA	1387
U51857 (DNA)	ACCTTCTACCTCAGGTTGGCTCTGACTATAACCACCATCCACTACAACCTACATGTGTAACA	619
L27630 (DNA)	ACCTTCTACCTCAGGTTGGCTCTGACTATAACCACCATCCACTACAACCTACATGTGTAACA	532
NC_006587 (DNA)	ACCTTCTACCTCAGGTTGGCTCTGACTATAACCACCATCCACTACAACCTACATGTGTAACA	1746
S77819 (mRNA)	-----GTTGGCTCTGACTATAACCACCATCCACTACAACCTACATGTGTAACA	610
L37107 (mRNA)	-----GTTGGCTCTGACTATAACCACCATCCACTACAACCTACATGTGTAACA	398
AF159062 (mRNA)	-----GTTGGCTCTGACTATAACCACCATCCACTACAACCTACATGTGTAACA	368
DN444655 (mRNA)	-----	
AF060514 (mRNA)	-----GTTGGCTCTGACTATAACCACCATCCACTACAACCTACATGTGTAACA	698
AB020761 (mRNA)	-----GTTGGCTCTGACTATAACCACCATCCACTACAACCTACATGTGTAACA	728

Appendix D: Alignment of Nucleotide Sequence for Canine TP53 Exons 5-8

Control (DNA)	GTTCCCTGCATGGGAGGCATGAACCGGCGGCCCATCCTCACTATCATCACCCCTGGAAGACT	704
U62133 (DNA)	GTTCCCTGCATGGGAGGCATGAACCGGCGGCCCATCCTCACTATCATCACCCCTGGAAGACT	1447
U51857 (DNA)	GTTCCCTGCATGGGAGGCATGAACCGGCGGCCCATCCTCACTATCATCACCCCTGGAAGACT	679
L27630 (DNA)	GT-----	534
NC_006587 (DNA)	GTTCCCTGCATGGGAGGCATGAACCGGCGGCCCATCCTCACTATCATCACCCCTGGAAGACT	1806
S77819 (mRNA)	GTTCCCTGCATGGGAGGCATGAACCGGCGGCCCATCCTCACTATCATCACCCCTGGAAGACT	670
L37107 (mRNA)	GTTCCCTGCATGGGAGGCATGAACCGGCGGCCCATCCTCACTATCATCACCCCTGGAAGACT	458
AF159062 (mRNA)	GTTCCCTGCATGGGAGGCATGAACCGGCGGCCCATCCTCACTATCATCACCCCTGGAAGACT	428
DN444655 (mRNA)	-----	
AF060514 (mRNA)	GTTCCCTGCATGGGAGGCATGAACCGGCGGCCCATCCTCACTATCATCACCCCTGGAAGACT	758
AB020761 (mRNA)	GTTCCCTGCATGGGAGGCATGAACCGGCGGCCCATCCTCACTATCATCACCCCTGGAAGACT	788
Control (DNA)	CCAGGTAGGGACCTGCCTGCCACCCTGCTCCTGGCCACTTCCTT ACTGT ACCCCCCTCCA	764
U62133 (DNA)	CCAGGTAGGGACCTGCCTGCCACCCTGCTCCTGGCCACTTCCTT -CTGT ACCCCCCTCCA	1506
U51857 (DNA)	CCAGGTAGGGACCTGCCTGCCACCCTGCTCCTGGCCACTTCCTT ACTGT ACCCCCCTCCA	739
L27630 (DNA)	-----	
NC_006587 (DNA)	CCAGGTAGGGACCTGCCTGCCACCCTGCTCCTGGCCACTTCCTT ACTGT ACCCCCCTCCA	1866
S77819 (mRNA)	CCAG-----	674
L37107 (mRNA)	CCAG-----	462
AF159062 (mRNA)	CCAG-----	432
DN444655 (mRNA)	-----	
AF060514 (mRNA)	CCAG-----	762
AB020761 (mRNA)	CCAG-----	792
Control (DNA)	TCCTTCCCATCCATTCTGTGCCAATGTGGAATCTCCTCTGCCATCCG -CCTCCCCTGG-	824
U62133 (DNA)	TCCTTCCCATCCATTCTGTGCCAATGTGGAATCTCCTCTGCCATCCG GCCTCCCCTGGG	1566
U51857 (DNA)	TCCTTCCCATCCATTCTGTGCCAATGTGGAATCTCCTCTGCCATCCG -CCTCCCCTGG-	797
L27630 (DNA)	-----	
NC_006587 (DNA)	TCCTTCCCATCCATTCTGTGCCAATGTGGAATCTCCTCTGCCATCCG -CCTCCCCTGG-	1924
S77819 (mRNA)	-----	
L37107 (mRNA)	-----	
AF159062 (mRNA)	-----	
DN444655 (mRNA)	-----	
AF060514 (mRNA)	-----	
AB020761 (mRNA)	-----	
Control (DNA)	TTTTCTTCTCTGGCTTTGG GAC CTCCCTGACCTGTGGCTTCTCAATAGTCTGTAGGCT	884
U62133 (DNA)	TTTTCTTCTCTGGCTTTGG AGC CTCCCTGACCTGTGGCTTCTCAATAGTCTGTAGGCT	1626
U51857 (DNA)	TTTTCTTCTCTGGCTTTGG GAC CTCCCTGACCTGTGGCTTCTCAATAGTCTGTAGGCT	857
L27630 (DNA)	-----	
NC_006587 (DNA)	TTTTCTTCTCTGGCTTTGG GAC CTCCCTGACCTGTGGCTTCTCAATAGTCTGTAGGCT	1984
S77819 (mRNA)	-----	
L37107 (mRNA)	-----	
AF159062 (mRNA)	-----	
DN444655 (mRNA)	-----	
AF060514 (mRNA)	-----	
AB020761 (mRNA)	-----	
Control (DNA)	TTGGCTCTACGTAGGATGAGGGTGGCTAGGAGTCAGTGGGGCCACCACCCTCACGGCCC	944
U62133 (DNA)	TTGGCTCTACGTAGGATGAGGGTGGCTAGGAGTCAGTGGGGCCACCACCCTCACGGCCC	1686
U51857 (DNA)	TTGGCTCTACGTAGGATGAGGGTGGCTAGGAGTCAGTGGGGCCACCACCCTCACGGCCC	917
L27630 (DNA)	-----	
NC_006587 (DNA)	TTGGCTCTACGTAGGATGAGGGTGGCTAGGAGTCAGTGGGGCCACCACCCTCACGGCCC	2044
S77819 (mRNA)	-----	
L37107 (mRNA)	-----	
AF159062 (mRNA)	-----	
DN444655 (mRNA)	-----	
AF060514 (mRNA)	-----	
AB020761 (mRNA)	-----	
Control (DNA)	CCTGCTTCTCTTCTCACCTGGGTAGTGGAACGTGCTGGGACGCAACAGCTTTG AGGT	1004
U62133 (DNA)	CCTGCTTCTCTTCTCACCTGGGTAGTGGAACGTGCTGGGACGCAACAGCTTTG AGGT	1746
U51857 (DNA)	CCTGCTTCTCTTCTCACCTGGGTAGTGGAACGTGCTGGGACGCAACAGCTTTG AGGT	977
L27630 (DNA)	-----	
NC_006587 (DNA)	CCTGCTTCTCTTCTCACCTGGGTAGTGGAACGTGCTGGGACGCAACAGCTTTG AGGT	2104
S77819 (mRNA)	-----TGGAACGTGCTGGGACGCAACAGCTTTG AGGT	707
L37107 (mRNA)	-----TGGAACGTGCTGGGACGCAACAGCTTTG AGGT	495
AF159062 (mRNA)	-----TGGAACGTGCTGGGACGCAACAGCTTTG AGGT	465
DN444655 (mRNA)	-----	
AF060514 (mRNA)	-----TGGAACGTGCTGGGACGCAACAGCTTTG AGGT	795
AB020761 (mRNA)	-----TGGAACGTGCTGGGACGCAACAGCTTTG AGGT	825

Appendix D: Alignment of Nucleotide Sequence for Canine TP53 Exons 5-8

Control (DNA)	ACGCGTTTGTGCCTGTCCCGGAGAGACCGCCGGACTGAGGAGGAGAATTTCCACAAGAA	1064
U62133 (DNA)	ACGCGTTTGTGCCTGTCCCGGAGAGACCGCCGGACTGAGGAGGAGAATTTCCACAAGAA	1806
U51857 (DNA)	ACGCGTTTGTGCCTGTCCCGGAGAG-----	1003
L27630 (DNA)	-----	
NC_006587 (DNA)	ACGCGTTTGTGCCTGTCCCGGAGAGACCGCCGGACTGAGGAGGAGAATTTCCACAAGAA	2164
S77819 (mRNA)	ACGCGTTTGTGCCTGTCCCGGAGAGACCGCCGGACTGAGGAGGAGAATTTCCACAAGAA	767
L37107 (mRNA)	ACGCGTTTGTGCCTGTCCCGGAGAGACCGCCGGACTGAGGAGGAGAATTTCCACAAGAA	555
AF159062 (mRNA)	ACGCGTTTGTGCCTGTCCCGGAGAGACCGCCGGACTGAGGAGGAGAATTTCCACAAGAA	525
DN444655 (mRNA)	-----	
AF060514 (mRNA)	ACGCGTTTGTGCCTGTCCCGGAGAGACCGCCGGACTGAGGAGGAGAATTTCCACAAGAA	855
AB020761 (mRNA)	ACGCGTTTGTGCCTGTCCCGGAGAGACCGCCGGACTGAGGAGGAGAATTTCCACAAGAA	885
Control (DNA)	GGGGGAGCCTTGTCTGAGCCACCCCCGGGAGTACCAAGCG--GTAAGCAAGCAAGACA	1122
U62133 (DNA)	GGGGGAGCCTTGTCTGAGCCACCCCCGGGAGTACCAAGCG--GTAAGCAAGCAAGACA	1864
U51857 (DNA)	-----	
L27630 (DNA)	-----	
NC_006587 (DNA)	GGGGGAGCCTTGTCTGAGCCACCCCCGGGAGTACCAAGCGAGGTAAGCAAGCAAGACA	2224
S77819 (mRNA)	GGGGGAGCCTTGTCTGAGCCACCCCCGGGAGTACCAAGCGAG-----	811
L37107 (mRNA)	GGGGGAGCCTTGTCTGAGCCACCCCCGGGAGTACCAAGCGAG-----	599
AF159062 (mRNA)	GGGGGAGCCTTGTCTGAGCCACCCCCGGGAGTACCAAGCGAG-----	569
DN444655 (mRNA)	-----	
AF060514 (mRNA)	GGGGGAGCCTTGTCTGAGCCACCCCCGGGAGTACCAAGCGAG-----	899
AB020761 (mRNA)	GGGGGAGCCTTGTCTGAGCCACCCCCGGGAGTACCAAGCGAG-----	929

Figure D1: Alignment of the nucleotide sequence for canine *TP53* exons 5-8 from control DNA (normal canine lung tissue) and 10 canine wild-type *TP53* nucleotide sequences accessible in GenBank (accession numbers listed): partial DNA - U62133, U51857, L27630; complete DNA - NC_006587; partial mRNA - S77819, L37107, AF159062, DN444655; complete mRNA - AF060514, AB020761 (differences between nucleotide sequences are highlighted in red; “*” indicate nucleotide similarities between all 11 sequences). Based on NC_006587 sequence: exon 5, 1098-1284 bp; exon 6, 1366-1478 bp; exon 7, 1701-1810 bp; exon 8, 2072-2208 bp.

Appendix E: Oral presentation

E1: AVTRW Conference, April 1999 - Characterisation of p53 (tumour suppressor) mutational status in a canine osteosarcoma cell line (D17).

Sabine VS, Nasir L, Burr PD, McFarlane S, Argyle DJ.

Department of Veterinary Clinical Studies, University of Glasgow Veterinary School

P53 is a tumour suppressor gene which functions to protect the organism from genetic damage by either inducing growth arrest or apoptosis. Alterations of the p53 gene have been shown to occur in many human carcinomas, including osteosarcoma. The loss of the normal function of p53 has also been shown to occur in canine osteosarcoma which is a highly aggressive disease associated with high mortality. The aim of the present study was to characterise the mutational status of p53 in a number of canine cell lines with the view to developing an *in vitro* system to investigate the efficacy of p53 directed gene therapy protocols. The status of p53 in MDCK, D17, CML10 cells was examined by immunocytochemistry using a CM-1 polyclonal anti-p53 antibody.

Immunohistochemical analyses failed to demonstrate aberrant p53 expression in the cell lines and the absence of p53 gene mutations was confirmed by cloning and direct DNA sequence analysis of p53 cDNA isolated from the D17 cell line.

These data suggest that the melanoma cell line (CML-10) and osteosarcoma cell line (D17) harbour wildtype p53.

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