FOLATE BASED INHIBITORS OF THYMIDYLATE SYNTHETASE IN EXPERIMENTAL AND CLINICAL CANCER

BY

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A DISSERTATION SUBMITTED TO THE UNIVERSITY OF GLASGOW FOR THE DEGREE OF DOCTOR OF MEDICINE

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ALK P	Alkaline phosphatase
ALT	Alanine transaminase
ATP	Adenosine triphosphate
AUC	Area under concentration versus time curve
BH_2	Dihydrobiopterin
BH_4	Tetrahydrobiopterin
CH_2FH_4	5,10-methylene tetrahydrofolate
CH_3FH_4	5'-methyl tetrahydrofolate
Ci	Curie
CLR	Clearance
CNS	Central nervous system
CR	Complete response
CSF	Cerebrospinal fluid
DAO	Diamine oxidase
DDMP	2,4-diamino-5-(3',4'-dichlorophenyl)-6-methyl pyrimidine
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DTIC	Dimethyl triazine imidazole carboxamide, dacarbazine
dTMP	Deoxythymidine monophosphate
dTTP	Deoxyuridine triphosphate
dUMP	Deoxyuridine monophosphate
dUTP	Deoxyuridine triphosphate
EDTA	Ethylene diamine tetra-acetic acid
FdUMP	5-Fluorodeoxyuridine monophosphate
FH_2	Dihydrofolate
FH_4	Tetrahydrofolate
FPGS	Folyl polyglutamate synthetase
FU	5-Fluorouracil
FUdR	5-Fluoro-2'-deoxyuridine
GFR	Glomerular filtration rate

GSH	Glutathione (reduced)
GTP	Guanosine triphosphate
Hb	Haemoglobin
HMT	Histamine methyltransferase
HPLC	High performance liquid chromatography
ID_{50}	Concentration of a drug required to slow the rate of reaction by 50%
i.p.	Intraperitoneal
i.v.	Intravenous
K_{el}	Elimination rate constant
K_i	Inhibition constant
K_{12}, K_{21}	Rate constants for transfer of drug between compartments 1 and 2 in a two compartment system
LAP	Leucine aminopeptidase
LD10	Dose of drug required to kill 10% animals
LD20	Dose of drug required to kill 20% animals
LD50	Dose of drug required to kill 50% animals
LFT	Liver function tests
MTX	Methotrexate
NAG	N-acetyl glucosaminidase
NADPH	Nicotinamide adenine dinucleotide (reduced)
NaOH	Sodium hydroxide
$NaHCO_3$	Sodium bicarbonate
PR	Partial response
PTFE	Polytetrafluoroethylene
RNA	Ribonucleic acid
SAM	S-adenosyl methionine
TdR	Deoxythymidine
TK	Thymidine kinase
TMQ	Trimethoxybenzylaminoquinazoline
TRIS	2-amino-2-hydroxy-methyl propane-1,3-diol
TS	Thymidylate synthetase
UV	Ultraviolet

V_1	Volume of compartment number one (central compartment)
V_D	Volume of distribution
WBC	White blood cell count
WISP	Waters Intelligent Sample Processor
W/V	Weight per volume

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SUMMARY

CB3717 (N-(4-(N-((2-amino-4-hydroxy-6-quinazolinyl) methyl)prop-2-ynylamino)benzoyl)-L-glutamic acid) is a novel quinazoline folate analogue developed at the Institute of Cancer Research and selected on the basis of its potent inhibition of thymidylate synthetase. The introduction of a compound capable of producing a pure "thymineless" death was sought in the belief that this might confer it with advantageous properties and hence the departure from the traditional enzymic locus of classical antifolates, dihydrofolate reductase. Fluorinated pyrimidine compounds, though capable of metabolism to the thymidylate synthetase inhibitor, 5-fluorodeoxyuridine monophosphate, probably owe their cytotoxicity to a combination of factors including incorporation into RNA and this cannot be used to test the effects of an uncomplicated thymineless state.

Pre-clinical studies confirmed that the cytotoxic effect of CB3717 in tissue culture was associated with the inhibition of thymidylate synthetase and significant antitumour activity was demonstrated in mice. At lethal doses in mice, renal and hepatic toxicity occurred but no evidence of gastrointestinal or bone marrow toxicity was found. An extensive phase I clinical trial incorporating 99 patients is described, with a dose escalation from 100 to 600mg/m^2 and treatments being administered by a one hour or 12 hour infusion at 3 weekly intervals. The dose limiting

toxicity was found to be renal at doses in the region of 500-600mg/m^2 when substantial reductions in glomerular filtration rates were noted. A dose of 400mg/m^2 was recommended for phase II evaluation using this schedule. Transient abnormalities in serum alanine transaminase levels typified the hepatic toxicity which occurred in ~80% of patients at all dose levels and the association of this with variable malaise led to attempts at amelioration by prolongation of the infusion to 12 hours or the administration of prednisolone for one week after treatment. Neither manoeuvre was successful in preventing the enzyme elevation, but steroid therapy improved malaise in some patients. Several patients who received multiple courses of CB3717 (>4) appeared to develop tolerance with a return of transaminase levels towards normal values. Other toxicities observed were rashes (~10%) and sporadic myelotoxicity affecting leucocytes and platelets with a 10-12 day nadir. Neither of these effects were dose related. Gastrointestinal toxicity was not observed. Responses were seen in the following tumours: ovary (1 CR, 1 PR), breast (3 PR), large cell bronchus (1 PR) and mesothelioma (1 PR).

A complementary pharmacokinetic evaluation was performed during the phase I study which established that 25-30% of CB3717 was excreted in the urine in the first 24 hours after treatment, implying that the majority was eliminated in the bile. The finding of an estimated 5% of total drug administered within the renal tissue of a patient who died 8 days after a treatment at 330mg/m^2 gave support to the hypothesis that in man, as well as in mouse, renal toxicity was seconary to precipitation of CB3717. Peak plasma levels of drug were within the concentration range that was cytotoxic to L1210 cells *in vitro* and *in vivo*. In 11 patients who were studied after their first treatment

there was a weak correlation between peak plasma drug levels and the increment in plasma alanine transaminase levels ($r=0.839$, $p=0.01$). Additionally, CB3717 was found to be highly protein bound ($97.6\pm0.1\%$) and no metabolites were detected in plasma or urine.

Investigation of the hepatic toxicity observed in the phase I study was pursued using an animal model after it was established that plasma alanine transaminase levels increased in mice also, after an intraperitoneal injection of a therapeutic dose, reaching a peak by 6 hours and returning to normal by 24 hours. A slight and rather variable depletion of liver glutathione was observed but this was probably not causally related since a trial of N-acetylcysteine as an antidote was unsuccessful. Thymidine, folinic acid or 5-methyltetrahydrofolate similarly did not prevent CB3717 hepatitis. Treatment with the quinazoline analogues, CB3705 (N^{10}-unsubstituted) and CB3714 (N^{10}-ethyl) did not cause liver enzyme elevation suggesting that the N^{10}-propargyl group of CB3717 may be culpable.

The effects of CB3717 on histamine and phenylalanine metabolism in man were investigated as potential mechanisms for its toxicity. Some antifolates inhibit the enzymes of histamine catabolism (histamine methyltransferase and diamine oxidase) and therefore cause skin rashes and central nervous system effects. CB3717 did not inhibit either enzyme and plasma histamine levels were unperturbed from the normal range in 5 patients after treatment. Rises in plasma phenylalanine levels up to 3 times normal were detected in some patients. This may be related to depletion of tetrahydrobiopterin pools (which is a cofactor in the hydroxylation of the aromatic amino acids), mediated by inhibition of dihydrobiopterin reductase. The clinical

significance of this is uncertain but there is a possibility of an effect on neurotransmitter levels.

In parallel with the clinical investigations, cell lines cloned from an L1210 tumour line with acquired resistance to CB3717 were characterised and found to have increased thymidylate synthetase activity. The pattern of cross-resistance to other antimetabolites, which may have therapeutic implications, was also studied. No significant cross-resistance to methotrexate, 5-fluorouracil or 5-fluorodeoxyuridine was found. It was concluded that further clinical and biochemical studies with this promising new antitumour agent are merited.

CHAPTER I

INTRODUCTION

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I.1 Cancer - Some General Considerations

Cancer, as a disseminated disease, is a major cause of morbidity and mortality. Twenty two percent of the total deaths registered in England and Wales in 1982 were attributable to neoplasms ("Mortality Statistics Cause" 1982, Office of Population Censuses and Surveys, Series DH2 No. 9, H.M.S.O.), the greater proportion of which comprised solid tumours arising from the lung, breast, gastrointestinal tract and ovary. Continued research into the causes and predisposing factors is undoubtedly required with the optimistic aim of prevention supervening treatment.

The situation is reminiscent of an earlier fight against infectious diseases where realisation of the contributory role of unsanitary, crowded living conditions and contaminated water supplies led to improvements which were of major importance in the control of microbial illnesses. In this respect, antibiotic chemotherapy was, and still is, of secondary importance, and relevant to the control of bacterial disease in individuals not in populations. Unfortunately, an awareness of preventative measures is not always sufficient and lack of financial resources, amongst other factors, has restricted the introduction of even simple public health measures in some developing countries where infectious diseases are still rife.

The parallel with cancer is exemplified by the depressing facts concerning lung cancer where age-standardized death rates in men increased from 27/100,000 in 1941-45 to 92/100,000 in 1976-78 (Cancer Statistics Incidence, survival and mortality in England and Wales, H.M.S.O., No 43 - Office of Population Censuses and Surveys 1981). Smoking is of course the most important aetiological factor in the development of lung cancer and it is strongly implicated in the cause of tumours of the head and neck (Shedd D.P. 1982). Widespread publicity of the harmful effects of smoking has so far failed to make any real impression on the population of smokers in Great Britain, where sadly the number of females who smoke continues to rise as do the numbers who die from lung cancer (Doll R. 1982). Thus the frailty of human nature coupled with ignorance of the factors which initiate many human tumours supplies an urgent need for more effective remedies.

Cytotoxic chemotherapy presently provides the mainstay of systemic cancer treatment, the numbers of antineoplastic agents having grown exponentially since 1946 when the first clinical reports of the use of nitrogen mustard alkylating agents appeared (Goodman L.S. et al 1946, Jacobson L.O. et al 1946, Rhoads C.P. 1946). Antifolates joined the pharmacopoeia shortly afterwards with aminopterin (4-amino-4-deoxypteroylglutamic acid) being the first folate analogue to show clinical activity by producing temporary remissions in children with acute leukaemia (Farber S. et al, 1948). Methotrexate (amethopterin, 4-amino-4-deoxy-10-methylpteroylglutamic acid) was synthesised a year later by the same group (Seeger D.R. et al, 1949) and was thereafter established (and remains) in the treatment regimens for a variety of tumour types including choriocarcinoma (Hertz R., Lewis J.R. & Lipsett M.B., 1961) acute lymphoblastic

leukaemia of childhood (Acute Leukaemia Group B 1965, Djerassi I. et al 1967, Holland J.F. & Glidewell O. 1972, Wang J.J., Freeman A.I., & Sinks L.F. 1976), carcinoma of the breast (Eastern Cooperative Group In Solid Tumour Chemotherapy 1967 and reviewed by Carter S.K., 1976), epidermoid cancer of the head and neck (Capizzi R.L. et al 1970, Levitt M. et al 1973) and osteogenic sarcoma (Jaffe N. et al 1974).

In 1974, in an article entitled "The Rational Design of Folic Acid Antagonists" Mead wrote; "After this auspicious beginning to the antifolate era it was to be expected that more and better antifolates would soon be available. This was not to be and, twenty five years after the synthesis of aminopterin, the folic acid antagonist of choice in the treatment of human malignant disease remains the closely related compound methotrexate, amethopterin." (Mead J.A.R. 1974). The purpose of this chapter is to review the properties of the classical antifolate methotrexate and then describe one rational approach to the design of a novel antifolate with emphasis on the different locus of action. The preclinical antitumour and toxicology data of this new compound,CB3717 (N-(4-(N-[(2-amino-4-hydroxy-6-quinazolinyl) methyl]prop-2-ynylamino)benzoyl)-L-glutamic acid) which is a quinazoline folate analogue, will be summarised as a preface to the early clinical trial described in chapter II.

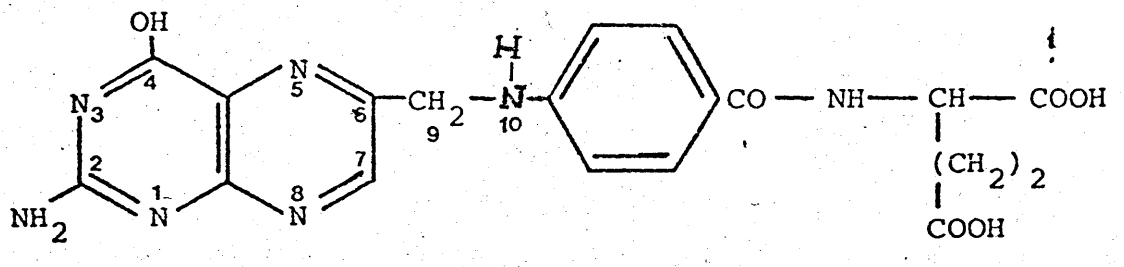
I.2 Methotrexate - Locus of Action

An antimetabolite has been defined as a compound structurally related to a metabolite, which prevents its further utilisation by competing with it for an enzyme (Connors T.A. 1976) Antifolates form part of this group, their relevance to cancer treatment being discovered by

Farber, who noted that the injection of folic acid conjugates (pteroyltriglutamic acid and pteroyldiglutamic acid) gave rise to an "acceleration phenomenon" in the leukaemic process seen in the marrow and viscera of children with acute leukaemia and coupled this with some observations on folic acid deficiency to form the hypothesis that folic acid antagonists might be of use in acute leukaemia (Farber S. 1947, Farber S. et al 1949). This empirical approach led to the introduction of aminopterin and methotrexate (amethopterin), the structures of which are shown in Figure I.1 along with the parent compound folic acid. Nichol and Welch showed that methotrexate blocked the activation of folic acid to "citrovorum factor"in 1950 (Nichol C.A. & Welch A.D. 1950), but it was several years later that the complexities of folate metabolism were elucidated and the potent inhibition of dihydrofolate reductase (DHFR, E.C.1.5.1.3) (the enzyme catalysing the reduction of dihydrofolate to tetrahydrofolate) by methotrexate was described (Futterman S. & Silverman M. 1957).

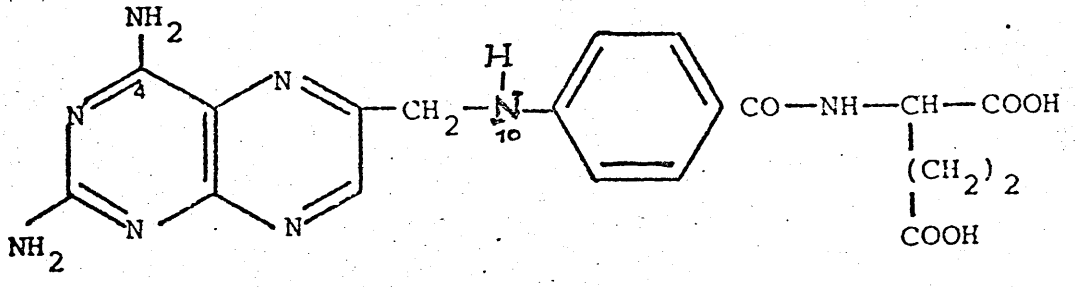
It is useful at this point to consider the interconversions which take place in the folate metabolic pathways which allow the transfer of 1-carbon fragments during the biosynthesis of purines, thymidine and amino acids. Details are shown in Figure I.2.

The kinetics of the inhibition of mammalian dihydrofolate reductase by methotrexate although originally described as "stoichiometric" by Werkheiser (1961) are now recognised to be competitive with respect to dihydrofolate although extremely tight binding. A pH dependence was elaborated by Bertino who found that inhibition was weaker at pH 7.6 than at pH 5.9 (Bertino J.R. 1967, Hillcoat B.L. & Johns D.G. 1967). The net effect of methotrexate is thus to

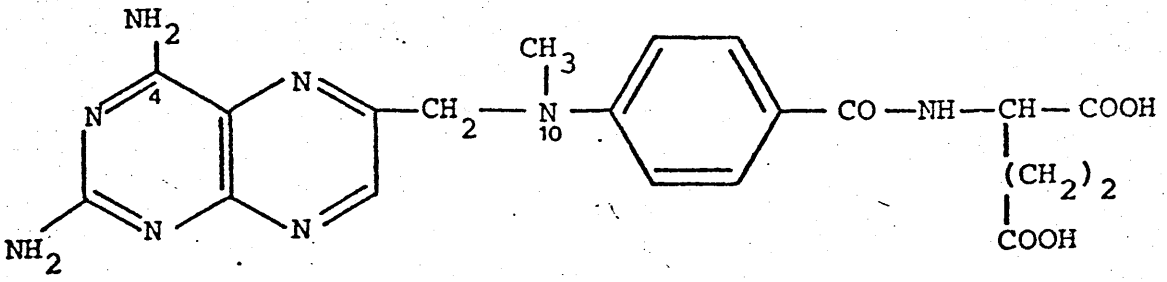


2 amino 4 hydroxy pteridine Para amino benzoic acid Glutamic acic

FOLIC ACID



AMINOPTERIN



METHOTREXATE

FIGURE I.1: The Structures of Folic Acid, Aminopterin and Methotrexate (Amethopterin)

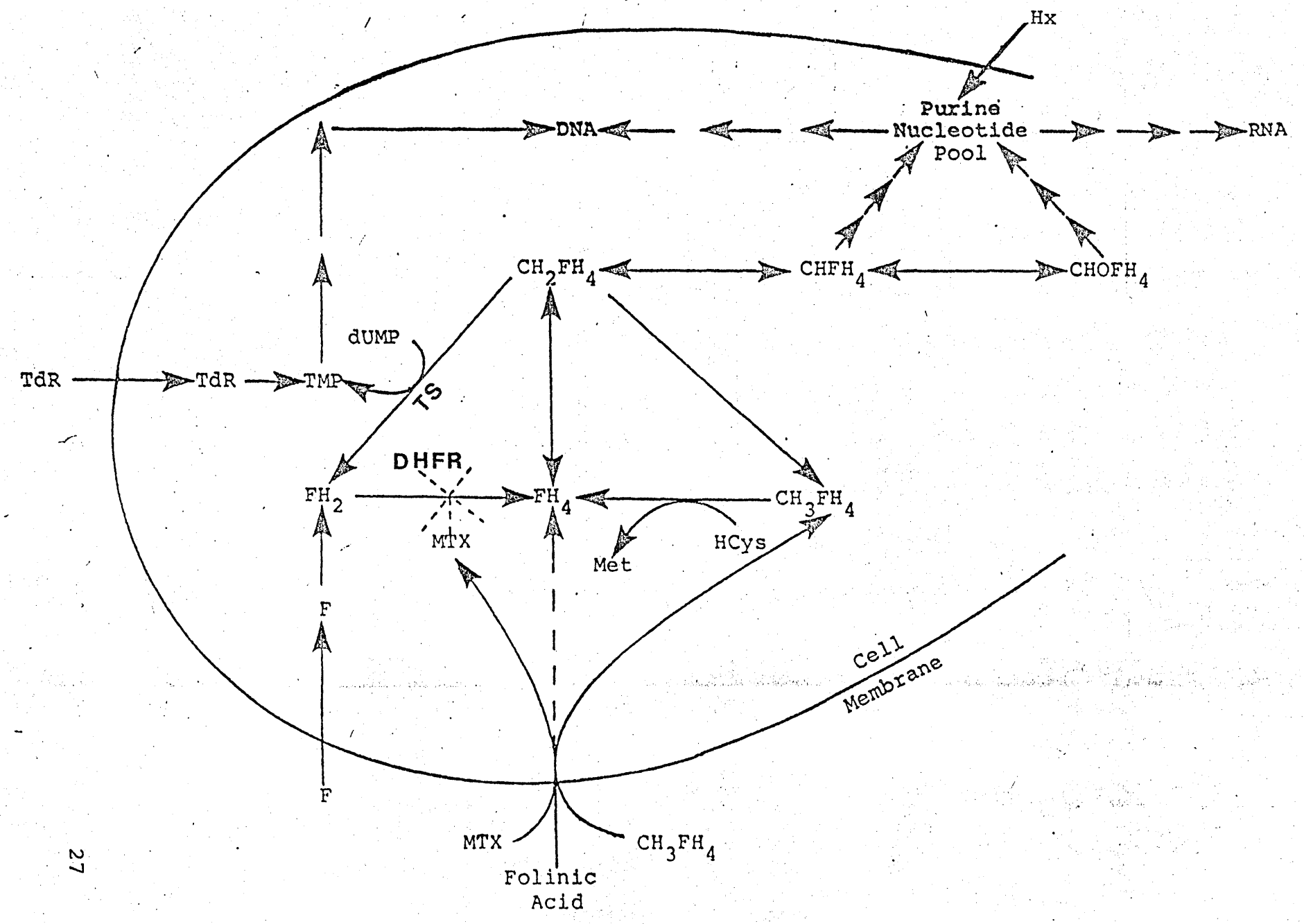


FIGURE I.2: Pathways of Folate Metabolism Relevant to Nucleotide Synthesis

LIST OF ABBREVIATIONS FOR FIGURE I.2

F = Folic acid

FH_2 = 7,8-dihydrofolic acid

FH_4 = 5,6,7,8-tetrahydrofolic acid

CH_3FH_4 = 5-methyltetrahydrofolic acid

CH_2FH_4 = 5,10-methylenetetrahydrofolic acid

$CHOFH_4$ = 10-formyltetrahydrofolic acid

$CHFH_4$ =5,10-methenyltetrahydrofolic acid

MTX = Methotrexate

Hx = Hypoxanthine

TdR = Thymidine

dUMP = 2'-deoxyuridylic acid

TMP = Thymidylic acid

HCys = Homocysteine

Met = Methionine

DHFR = Dihydrofolate reductase

TS = Thymidylate synthetase

deplete the cell of reduced folates and thereby secondarily impair the synthesis of thymidylate and purines (see Figure I.2). However, other biochemical loci have been implicated, the most relevant being direct inhibition of thymidylate synthetase in some cell lines (Borsa J. & Whitmore G.F. 1969a), and it has been suggested that both loci may be of importance in cell killing (Borsa J. & Whitmore G.F. 1969b, McBurney M.W. & Whitmore G.F. 1975).

An examination of the data on the reversibility of methotrexate toxicity provides no simple answer to defining the cytotoxic locus during methotrexate treatment. Goldin's pioneering studies, with delayed administration of citrovorum factor (folinic acid) after aminopterin treatment of tumour bearing mice (Goldin A. et al 1955), led to the introduction of clinical regimens utilising this principle with methotrexate (Capizzi R.L. et al 1970, Djerassi I. et al 1972, Levitt M. et al 1973 and reviewed by Bertino J.R. 1981). Similarly thymidine has been used as a rescue agent in methotrexate-treated tumour bearing mice (Tattersall M.H.N., Brown B., & Frei E. 1975) although other workers found that non-tumour bearing mice required a purine source (hypoxanthine) in addition to thymidine to ensure protection from acute toxicity (Straw J.A. et al 1977). Interestingly, in leukaemic patients successful rescue with high doses of thymidine has been achieved (Howell S.B. et al 1978). None of these data are inconsistent with DHFR being the primary locus of methotrexate action. For example, the differential ability of cells to salvage preformed purines and the differential supply of purines as a result of cell death may contribute to the varied response when thymidine is used as the sole rescue agent (reviewed by Jolivet et al 1982). Indeed, there is some evidence to suggest that impairment of purine synthesis is primarily responsible for the

gastrointestinal toxicity while that of thymidylate relates more to its myelosuppressive effects (Straw J.A. et al 1977.)

The subject is complex but in a recent review Jackson and Grindey concluded that the interaction of methotrexate with DHFR alone can probably account for its observed effects (Jackson R.C. and Grindey G. 1984) "although the exact sequence of events that leads to irreversible damage is still far from clear." If the unbalanced growth caused by cessation of DNA synthesis in the presence of continued RNA and protein synthesis is lethal to cells this might be sufficient explanation (Cohen S.S. 1971). Whatever the mechanism, it is apparent that methotrexate exerts its damaging effects on cells during the S-phase when, necessarily, DNA replication is in process. The rapidly dividing cells of the gastrointestinal tract and bone marrow are thus particularly susceptible to this agent and this is evident in its clinical toxicity profile where mucositis and myelosuppression are dominant (reviewed by Chabner B.A. 1982).

I.3 The Design of a Novel Antifolate

Considerable effort has been expended on attempts to produce an antifolate with superior activity, increased tumour selectivity and an improved therapeutic index compared to methotrexate. A detailed review of the compounds developed is outside the scope of this thesis but it is generally true that agents with altered physicochemical and pharmacokinetic properties have been sought rather than agents with differing cyctotoxic loci. One example is metoprine (DDMP) which is one of several lipophilic 2,4-diamino pyrimidines shown to inhibit DHFR

(Greco W.R. & Hakala M.T. 1980, Browman G.P. et al 1980). Several cell lines resistant to methotrexate are sensitive to metoprine but this is probably accounted for by differences in membrane transport of the two compounds (Sirotnak F.M. et al 1982). Clinical trials showed that the lipophilic properties of metoprine did enable it to cross the blood-brain barrier more effectively, but also led to a very prolonged half life and an extended toxicity risk (Miller D.S. et al 1976). Antitumour activity was rather limited. Additionally it was noted that triazinate (a triazine folate analogue) and metoprine gave rise to central nervous system toxicity and dermatological toxicity (Skeel R.T. et al 1976, Currie V.E., Kempin S.T. & Young C.W. 1980) which are thought to be mediated by inhibition of histamine metabolising enzymes (Duch D.S., Bowers S.W. & Nichol C.A. 1978). A more recently developed lipophilic DHFR inhibitor, trimethoxybenzyl aminoquinazoline (TMQ, or trimetrexate) has a different transport mechanism from methotrexate (reviewed by Jackson R.C. et al 1984) but in preclinical studies was found to be a potent inhibitor of histamine metabolism (Duch D.S., Edelstein M.P. & Nichol C.A. 1980). This topic is discussed more fully in Chapter IV but clearly the current clinical trials of trimetrexate will be of great interest. Some other antifolate compounds include 10 propargyl aminopterin (and alkyl homologues of methotrexate) and 10 deaza-aminopterin, but once again in vitro and in vivo studies suggest that there is no substantial advantage of these over methotrexate (Piper J.R. et al 1982, Sirotnak F.M. et al 1982).

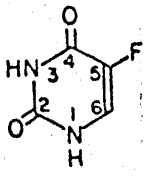
I.4 The Rationale For A Folate Based Inhibitor of Thymidylate Synthetase

In 1981 T.R. Jones and co-workers presented the *in vitro* and *in vivo* results of studies with a quinazoline folate analogue, CB3717, which had been specifically sought and selected from a series of similar compounds for its potent inhibition of thymidylate synthetase (E.C.2.1.1.45) (Jones T.R. *et al* 1981). The reasons for such a novel approach, which departed from the dogma of DHFR inhibition, were made evident in this paper with some of the ground having been prepared by an earlier publication of Calvert *et al* in which antifolates with dual biochemical loci of action were described (Calvert A.H. *et al* 1980). The case for direct inhibition of thymidylate synthetase by a folate analogue will be re-presented here.

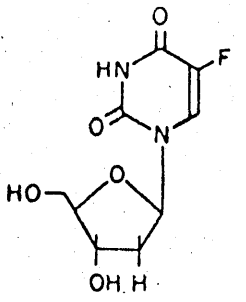
Firstly, there is circumstantial evidence to suggest that a differential response of tumour and host gastrointestinal tissue to the antipurine effect of methotrexate may exist such that avoiding it, may result in diminished gastrointestinal toxicity (Harrap K.R., Taylor G.A. & Browman G.P. 1977) whilst preserving an antitumour effect (Tattersal M.H.N. *et al* 1974). Secondly, Borsa and Whitmore proposed that impairment of purine synthesis by methotrexate, in some systems, may be deleterious to its cytotoxicity (Borsa J. & Whitmore G.F. 1969c). Thirdly, cells with acquired resistance to methotrexate, due either to elevation of DHFR levels or altered DHFR kinetics (reviewed by Bertino J.R. *et al*, 1981), should not be cross-resistant to an inhibitor of TS.

Reflection on the characteristics of established fluorinated pyrimidines which were designed to be metabolically activated to TS inhibitors, highlights the

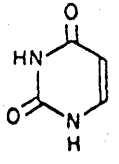
desirability of a folate analogue for this purpose. Figure I.3 shows the structure of the two best studied (and to date most widely used) fluorinated pyrimidines, 5-fluorouracil and 5-fluoro-2'-deoxyuridine, alongside the naturally occurring pyrimidines and nucleosides which they mimic. On Figure I.4 their metabolic pathways are detailed. There is extensive literature on these compounds which were first synthesised in 1957 (Duschinsky R., Plevan E. and Heidelberger C. 1957) and for some years it was believed that their antitumour effect was a result of thymine deficiency, caused by inhibition of TS by a metabolic product, 5-fluorodeoxyuridine monophosphate (Cohen S.S. et al 1958, Danneberg P.B., Montag B.J. & Heidelberger C. 1958) (see figure I.4). It is now appreciated that other loci are probably of importance including incorporation of 5'-FUTP (another metabolic product) into RNA (Martin D.S. 1978 and reviewed by Chabner B.A. 1981). Incorporation into DNA has also been reported (Danenberg P.V. et al 1981, Major P.P. et al 1982). Additionally, FdUMP inhibition of TS is compromised in whole cells by the development of raised levels of dUMP, the competing substrate (Lockshin A. & Danenberg P.V. 1981). In the case of inhibition mediated by a folate analogue, the competing substrate for TS is a vitamin and therefore only able to accumulate to a limited extent. Further, the many anabolic and catabolic routes available to the fluorinated pyrimidines provides several ways of resistance development by appropriate enzyme deletion or overproduction (reviewed by Ardalan B., Cooney D. and MacDonald J.S. 1980). Folate analogues are active per se and generally little catabolised (Jacobs S.A. et al 1976). In summary then, it can be strongly argued that the introduction of a folate analogue inhibitor of thymidylate synthetase might provide an important addition to the cytotoxic armoury as well as a unique opportunity to test



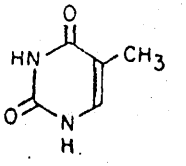
5-Fluorouracil
(FU)



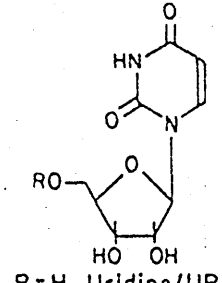
5-Fluoro-2'-deoxyuridine
(FUDR)



Uracil(U)

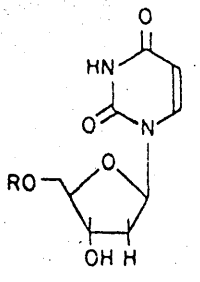


Thymine(T)



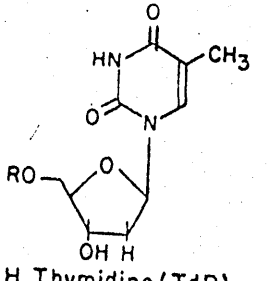
R=H, Uridine(UR)

R=$P(OH)_2$, Uridylate(UMP)



R=H, Deoxyuridine(UdR)

R=$P(OH)_2$, Deoxyuridylate(dUMP)

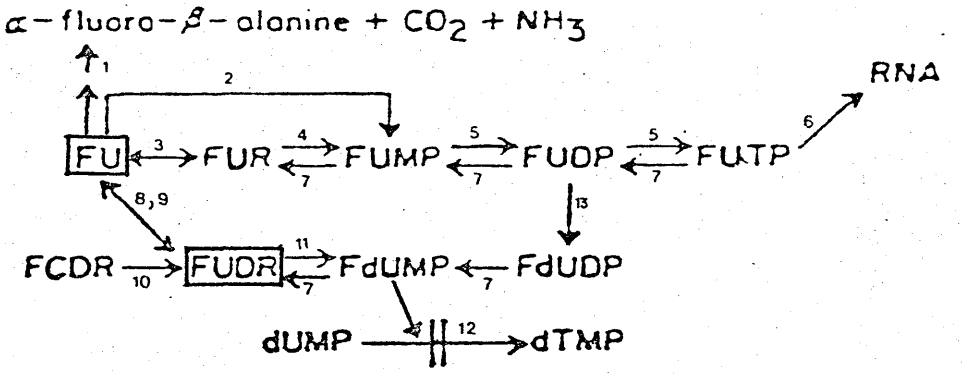


R=H, Thymidine(TdR)

R=$P(OH)_2$, Thymidylate(dTMP)

FIGURE I.3: Structures of Naturally Occurring Pyrimidines and Nucleosides With Two Synthetic Fluorinated Derivatives

FLUOROPYRIMIDINE METABOLISM



1 = Uracil and thymine degrading enzymes
2 = Phosphoribosyl transferase
3 = Uridine phosphorylase
4 = Uridine and cytidine kinase
5 = Uridylate kinase
6 = RNA polymerase
7 = Phosphatases
8 = Deoxyuridine phosphorylase
9 = Thymidine Phosphorylase
10 = Deoxycytidine deaminase
11 = Thymidine Kinase
12 = Thymidylate synthetase
13 = Ribonucleotide reductase

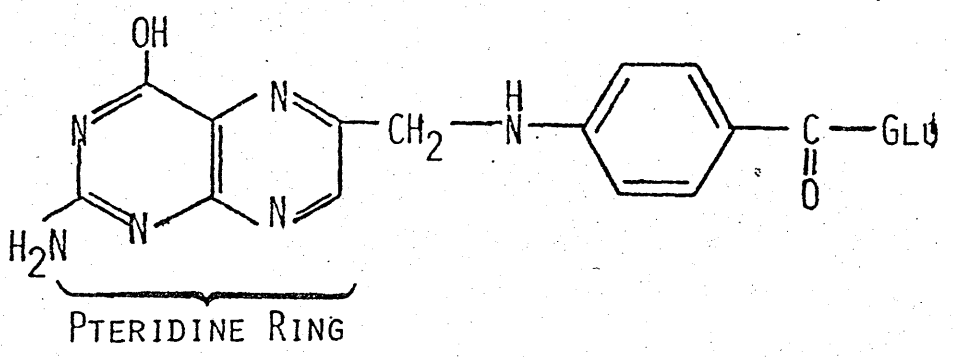
FU = 5-Fluorouracil
FUDR = 5-Fluoro-2'-deoxyuridine

FIGURE I.4: Pathways of Fluorinated Pyrimidine Metabolism

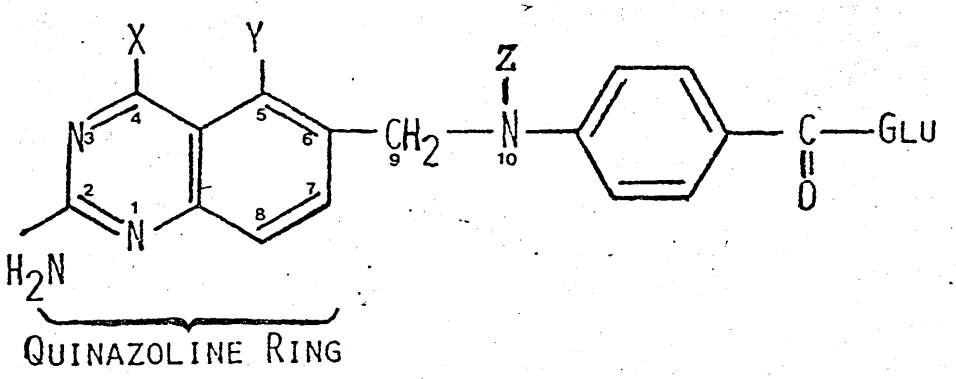
the efficacy of pure thymineless death.

I.5 Development of CB3717

In 1968 Hutchinson reported the antifolate properties of a new series of 2,4-diamino quinazoline folate analogues, inhibition of DHFR being a primary locus (Hutchinson D.J. 1968) (The quinazoline ring structure and numbering system is shown in Figure I.5). Later, compounds with this configuration were demonstrated to be capable of thymidylate synthetase inhibition (McCuen R.W. & Sirotnak F.M. 1975, Calvert A.H. et al 1980) although tight binding to DHFR had also been noted (Folsch et al 1971, Calvert A.H. et al 1980a). Workers at the Institute of Cancer Research (England) then pursued a study of 2-amino-4-hydroxy quinazolines and discovered that the incorporation of a methyl group into the N^5 position produced weaker TS inhibition (Jones T.R. 1980, Calvert A.H. et al 1980a) whilst in the N^{10} position, improved TS inhibition occurred (Calvert A.H. et al 1980b). The latter observation confirmed some earlier studies of Bird (Bird O.D., Vaitkus J.W. & Clarke J. 1970). By substituting aliphatic chains of increasing length it was then discovered that an ethyl group gave optimal inhibition of TS (Calvert A.H. et al 1980b). Jones and co-workers subsequently introduced the unsaturated allyl and propargyl groups at the N^{10} position and found that this improved the inhibition of TS by 3 and 30 fold respectively (Jones T.R. et al 1981). Figure I.6 shows the structures of these compounds with details of their TS inhibitory properties. Sixty four analogues of CB3717 (the N^{10}-propargyl compound) were evaluated in total, but none displayed superior properties and hence CB3717 was chosen for further investigation and clinical study. A review of the in vitro and in vivo findings will follow.



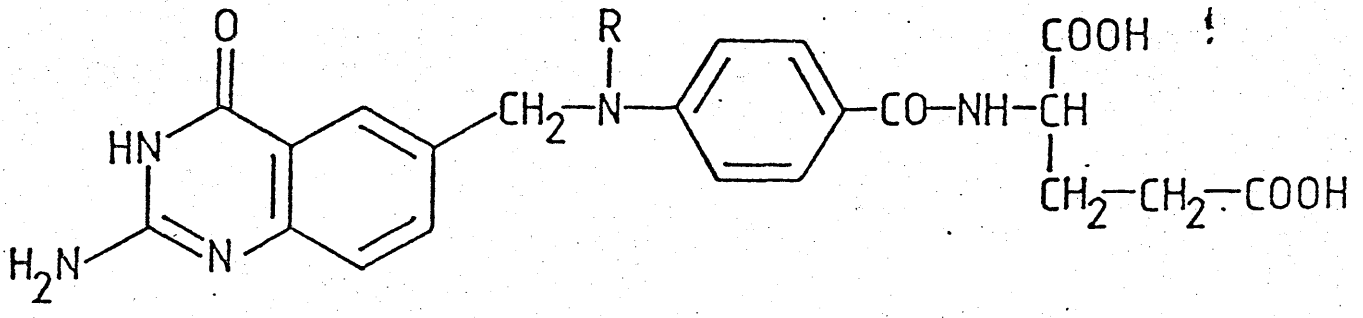
FOLIC ACID



A QUINAZOLINE COMPOUND

(X, Y & Z mark positions of substituent groups)

FIGURE I.5: Structures of a Pteridine Ring Compound (Folic Acid) and a Quinazoline Ring Compound



House Number	R	Thymidylate Synthetase I_{50} (nM)
CB 3715	$-CH_2-CH_2-CH_3$	170
CB 3716	$-CH_2-CH=CH_2$	69
CB 3717	$-CH_2-C\equiv CH$	5

FIGURE I.6: Inhibition of Thymidylate Synthetase by Quinazolines

From Jones T.R. et al, 1981

I.6 Biochemical Properties of CB3717

The enzyme locus of prime importance in this discussion is thymidylate synthetase (E.C.2.1.1.45) which catalyses the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP) as discovered in 1957 (Friedkin M. and Kornberg A. 1957). The methyl group is derived from the cosubstrate 5,10-methylene tetrahydrofolate which is itself oxidised to dihydrofolate during the reaction (Fig. I.7) This reaction provides the only source of thymidine nucleotides synthesised de novo in a cell. In the initial studies with CB3717 Michaelis-Menten kinetics were assumed and it was found that inhibition of TS from L1210 cells appeared to be competitive with respect to 5,10 methylenetetrahydrofolate with a Ki in the region of 1nM (Jones T.R. et al 1981). Subsequently, Zone B analysis for tight-binding inhibition has been applied to CB3717 inhibition of TS from highly purified enzyme derived from L1210 cells (Jackman et al 1984) and WIL2 lymphoblastoid cells (Jackson R.C., Jackman A.L. & Calvert A.H. 1983) leading to the conclusion that CB3717 does inhibit TS competitively with respect to 5,10-CH_2FH_4 with a Ki of 4.2nM and 4.9nM respectively.

Dihydrofolate reductase is also inhibited by CB3717, the Ki for purified rat liver enzyme being 14nM (Jones T.R. et al 1981). Despite this several pieces of data derived from tissue culture experiments suggest that TS and not DHFR is the cytotoxic locus. Firstly for L1210 leukaemia cells grown in tissue culture the ID_{50} of CB3717 was 5uM and reversal of growth inhibition was achieved with 10uM thymidine whilst folinic acid, up to 100uM, was only capable of partial reversal (Jones T.R. et al 1981). More importantly, delayed rescue from CB3717 toxicity (up to 8

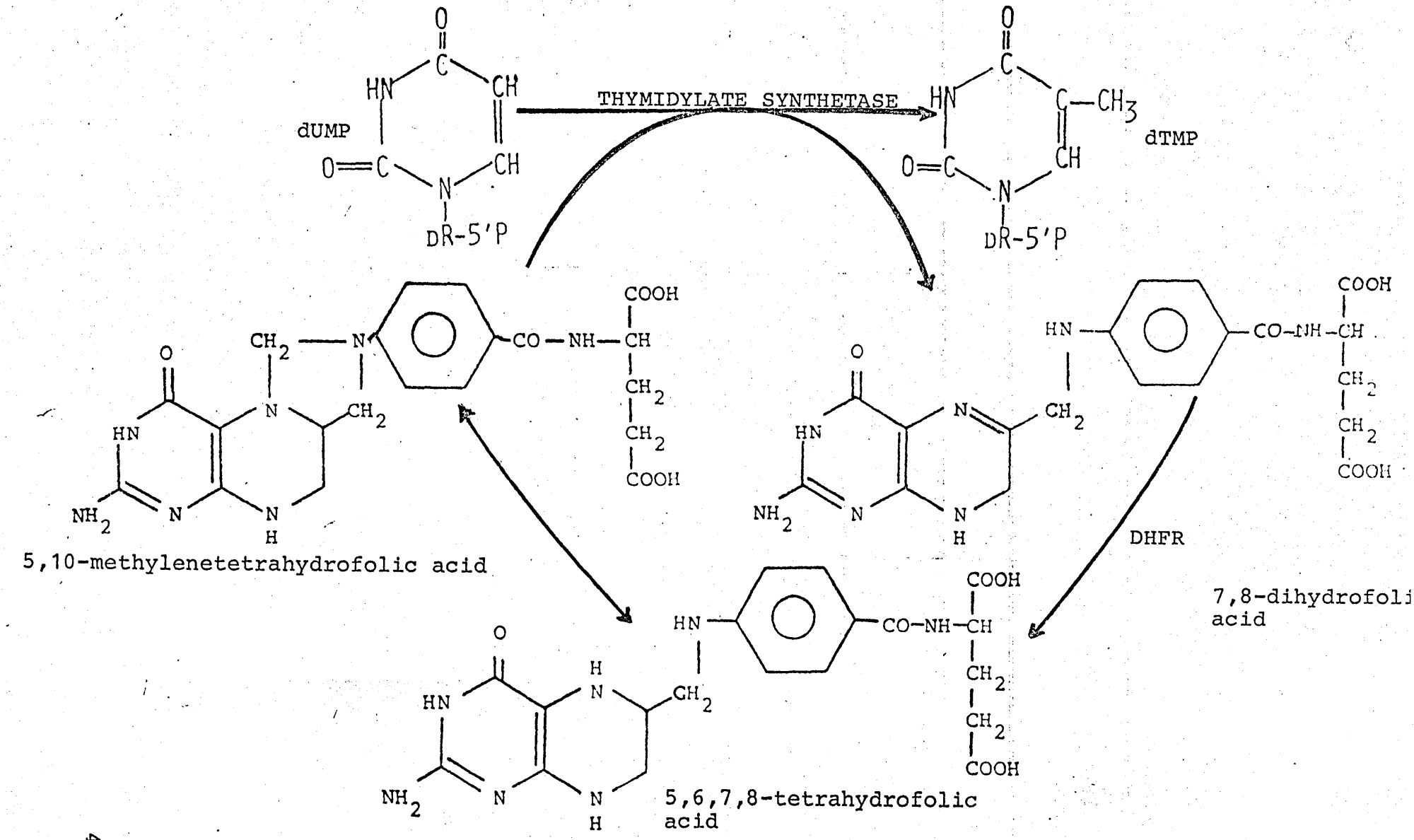


FIGURE I.7: Catalytic Reactions in The Formation of Thymidylate

hours) was possible with thymidine in WIL2 cells (Jackson R.C. et al 1983), while delayed rescue from MTX toxicity was not possible. Parenthetically, a cell line 600 fold resistant to MTX by vitrue of raised DHFR levels (L1210/R71) was also found to be sensitive to CB3717 with an ID_{50} of 14uM (Jones T.R. et al 1981). Secondly, measurement of dihydrofolate pools (FH_2) in WIL2 lymphoblastoid cells treated with equitoxic doses of CB3717 and MTX, gave very different results with no accumulation of FH_2 after CB3717 and marked elevation of FH_2 after MTX (Jackson R.C. et al 1983). Thirdly, in the same paper Jackson and co-workers provided data on purine nucleotide pools, using the same system, and established that at equivalent doses MTX depleted ATP and GTP pools whilst CB3717 did not. Further evidence for CB3717 inhibition of TS was also provided by estimation of pyrimidine deoxynucleotide pools in cells exposed for 16 hours to an IC_{50} concentration of drug. An 88% decrease of cellular dTTP occurred in the presence of a 2,300% increase in dUMP clearly indicating an effect on TS, either directly or indirectly (Jackson R.C. et al 1983). Finally, the elegant mathematical model, developed by Jackson to predict the outcome of compounds which inhibit both DHFR and TS, supported the hypothesis that TS inhibition was rate-limiting for CB3717 (Jackson R.C. et al 1983). As an addendum, some work covered later in this thesis, (Chapter VI) relating to experimental resistance to CB3717, has provided additional support for this belief with the characterisation of an L1210 cell line approximately 100 fold resistant to CB3717 and with up to 40 fold increased TS activity.

I.7 Animal Studies With CB3717

I.7.a. Antitumour Testing

The L1210 tumour test in mice provided the most striking example of *in vivo* antitumour activity. BDFI mice were injected with $5x10^4$ L1210 cells intraperitoneally (i.p.) on day 0 and were treated either by a single dose of CB3717 i.p. or 5 consecutive daily doses beginning on day 3. The results (taken from T.R. Jones *et al* 1981) are shown in Table I.1. A five day schedule was required for the therapeutic effect which showed dose dependence. No significant whole animal toxicity was observed and superiority over methotrexate in the same system was demonstrated (see Figure I.8). Similar results were obtained when L1210 cells ($1x10^4$) were injected intravenously and followed by 5 daily treatments with optimal doses of methotrexate or CB3717 i.p. (see Figure I.9).

Against the ADJ/PC6 tumour model which is normally used as a screen for alkylating agents, CB3717 showed significant activity, causing 84% inhibition of tumour growth at the maximal tolerated dose compared with 42% inhibition by methotrexate (data of A.L. Jackman & P.M Goddard). Other screening tests were disappointing with no significant activity being seen against a TLX5 lymphoma inoculated subcutaneously, or against a human tumour choriocarcinoma xenograft (data of A.L. Jackman & P.M. Goddard).

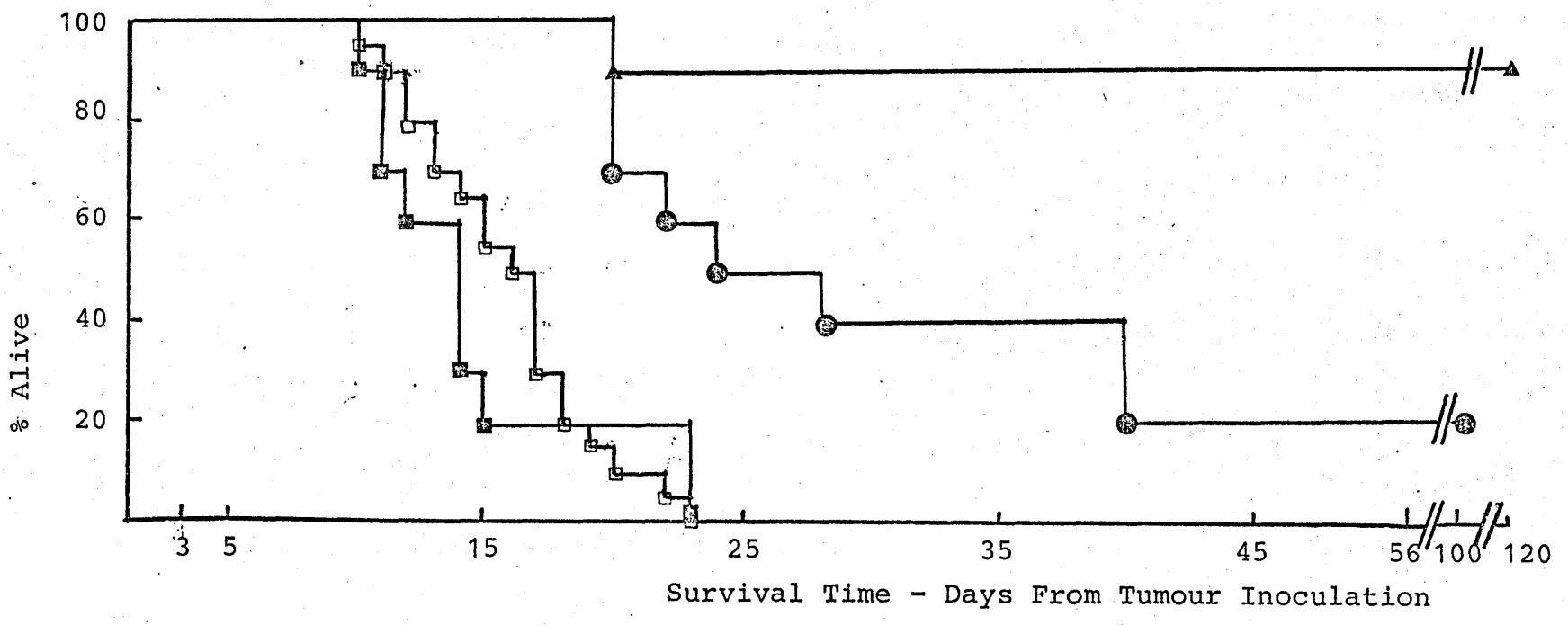
In summary, the unique biochemical locus of CB3717 and its *in vitro* activity against cell lines resistant to methotrexate coupled with evidence of some activity in animal screening models was thought to warrant an evaluation of its capabilities in man. This necessitated preliminary animal toxicology studies, which are described below.

CB 3717 Dose (mg/kg)	Animals per Group	Protocol	% Increase in Mean Survival Time	Long Term Survivors (>120 days)
80	5	Single Dose	14	0
150	5	Single Dose	32	0
8	5	Daily x 5	8	0
16	5	Daily x 5	13	0
16	5	Daily x 5	29	0
32	5	Daily x 5	62	0
64	5	Daily x 5	72	0
128	5	Daily x 5	>1200	4
125	10	Daily x 5	>1200	9
200	10	Daily x 5	>1200	9

The bracketed groups indicate individual experiments

TABLE I.1: Therapeutic Results of CB3717 Treatment in Mice Bearing The L1210 Ascitic Tumour

(Data of Jackman A.L. & Calvert A.H.)



■ No Treatment (10 animals) MTX Control
□ No Treatment (20 animals) CB3717 Control
● MTX 3mg/kg daily x 5 (10 animals)
▲ CB3717 125mg/kg daily x 5 (10 animals)
△ CB3717 200mg/kg daily x 5 (10 animals)

FIGURE I.8: Survival of L1210-Bearing Mice Injected with CB3717 or Methotrexate i.p. (Data of Jones T.R. et al, 1981)

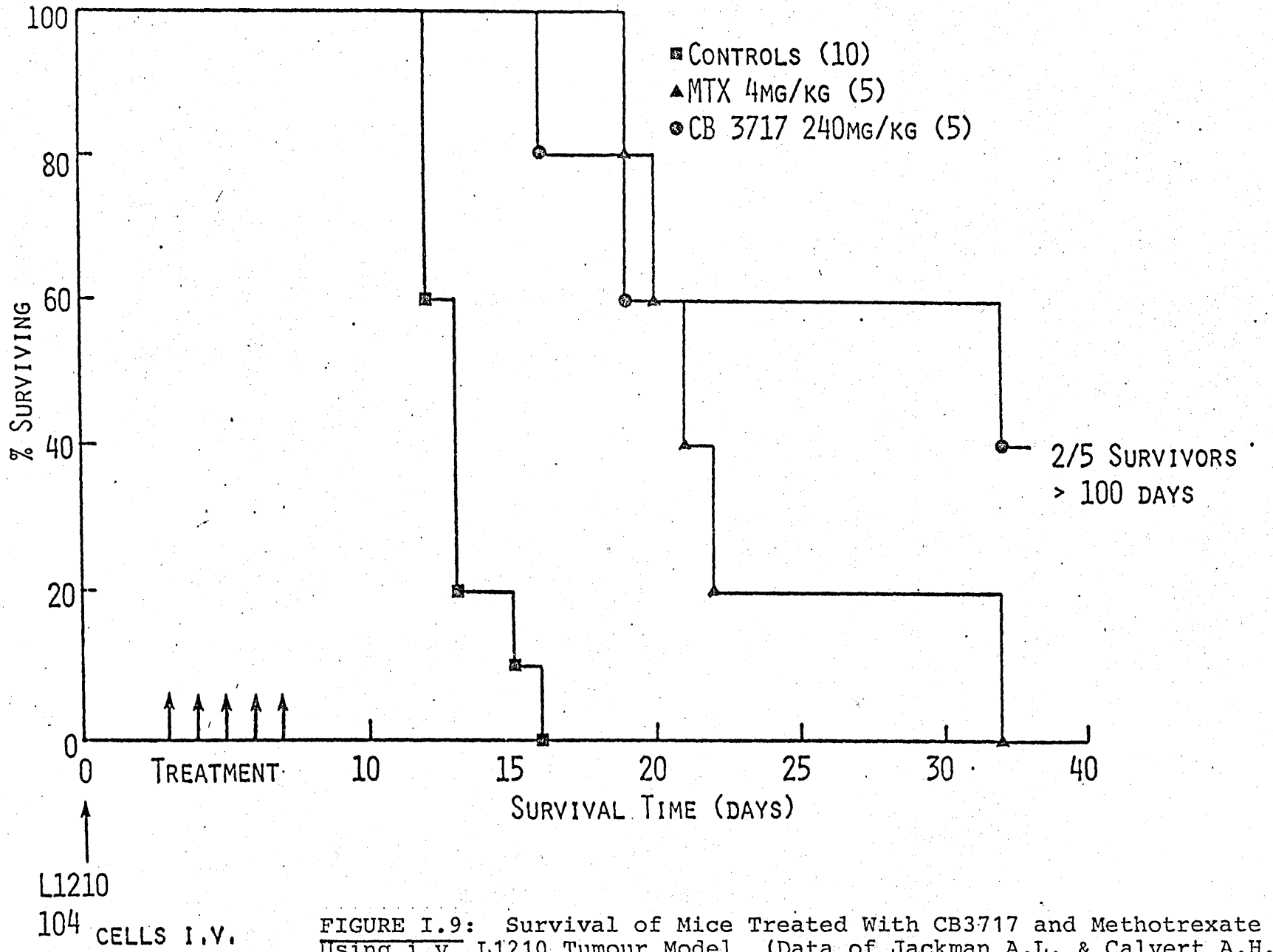


FIGURE I.9: Survival of Mice Treated With CB3717 and Methotrexate Using i.v. L1210 Tumour Model (Data of Jackman A.L. & Calvert A.H.)

I.7.b. Toxicology Studies

These studies were performed by A.L. Jackman and A.H. Calvert using nine week old male and female Alderley Park mice. Two schedules were employed, the first comprising a single 5 day course of treatment with CB3717 at doses of 62.5, 125, 250 and 500mg/kg in direct comparison with methotrexate at doses of 1,2,4 and 8mg/kg. The second schedule involved 4 repeated 5 day courses of treatment with 10 day intervals between each course. The doses on this occasion were 25, 50, 100 and 200mg/kg and 0.75, 1.5, 3 and 6mg/kg for CB3717 and methotrexate respectively. The results are summarised below.

Single 5 day schedule

It was found that animals receiving toxic doses of CB3717 (~300mg/kg) died after the first or second dose of drug and at very high doses (~500mg/kg), the animals became cold and hypokinetic, dying from convulsions within 10-12 hours. In contrast, animals dying from methotrexate toxicity did so 6-9 days after starting treatment with characteristic weight loss and diarrhoea. CB3717 treated animals did not lose weight or develop diarrhoea. The LD50s, calculated from this study, are shown below (measured in mg/kg daily for 5 days)

	Methotrexate	CB3717
males	5.2	330
females	4.6	380

LD50 (mg/kg) in Mice For MTX and CB3717

No haematological abnormalities were noted in CB3717 treated

animals whilst leucopenia was found in female mice treated with 4mg/kg methotrexate. No significant changes were seen in femoral bone marrow preparations.

Macroscopically, irregular pitting of the kidney surfaces of animals receiving an LD20 dose of CB3717 (250mg/kg) was noted. The three animals which survived doses of 500mg/kg and all 10 which survived 125mg/kg showed no obvious ill effects when observed for a further 262 days.

Repeated 5 day course

Deaths occurred only in animals treated at 200mg/kg CB3717 (one male animal on day 4, 2 females on days 3 and 5 all within the first 5 day course). In the methotrexate group, deaths occurred following the first, second and third courses, the calculated LD50 for this protocol being 3.7mg/kg for males and 4.6mg/kg for females. Again weight loss and diarrhoea were features of methotrexate treatments only.

Macroscopically, abnormalities of the kidneys, as before, and testicular atrophy, were observed in animals sacrificed from the 200mg/kg CB3717 group. In the dose group of 100mg/kg CB3717 animals sacrificed after 262 days showed no macroscopic lesions. Again, no significant haematological or bone marrow changes were found.

Histological Examination - Performed by British Industrial Biological Research Association (BIBRA)

CB3717 caused kidney damage in male and female mice in both the single course and repeated course study. This was typified by proximal tubular necrosis with subsequent

regeneration of the epithelium with radial tracts of interstitial fibrosis and a mixed inflammatory cell infiltrate. The severity of the kidney damage was clearly dose related and varied from mild to moderate in animals treated with 62.5mg/kg in the acute study and with 50mg/kg in the repeated dosing study. Severe kidney damage was restricted to animals treated with 250mg/kg or 200mg/kg in the acute and repeated dosing studies respectively. The sequelae to kidney damage involved the development of radial scarring of the cortex.

Testicular atrophy was also observed, being most severe in animals in the repeated dosing study, and was dose related, approaching 100% atrophy in several animals of the high dose group.

I.7c Study to determine the cause of death in animals treated with a lethal dose of CB3717

To complete the toxicological evaluation of CB3717 in mice an additional study was undertaken to determine the cause of death in animals treated with a lethal dose of CB3717. Forty mice were injected intraperitoneally with 300mg/kg CB3717 and ten were sacrificed 24 hours later, two having died within this time period. The remaining twenty eight animals were injected with a second dose of 300mg/kg CB3717 and ten of these were sacrificed 48 hours after the first injection. Pooled blood from the orbital exsanguination of these mice was used to obtain a biochemical profile using the Royal Marsden Hospital autoanalyser. Uninjected animals were used as controls. The results of this study are shown in Table I.2. Most noteworthy was the acute elevation in blood urea levels suggesting acute renal damage which was verified by

macroscopic and microscopic scrutiny of the tissues removed at necropsy from a moribund animal. The kidneys appeared swollen and haemorrhagic. Histopathological examination (performed by Dr. S. Sparrow, MRC Toxicology Unit, Carshalton) revealed damage throughout the nephron and deposits within the lumen. A homogenate of renal tissue was assayed for CB3717 using a high performance liquid chromatography technique (HPLC) (as described in more detail in Chapter III) and revealed 15.5mg CB3717/kg wet weight tissue. This represented the deposition of approximately half the total dose of CB3717 administered to the mouse. This evidence suggested that the most likely cause of death following a lethal dose of CB3717 was due to renal failure caused by drug precipitation in the renal tubule. This is concordant with the poor solubility of CB3717 in acid conditions.

The other interesting feature highlighted in this study was the elevation in plasma alanine transaminase levels accompanied by the histological appearance of periacinar necrosis in hepatic tissue. The desirability of further exploration of CB3717 hepatotoxicity became more apparent when the clinical study was under way. Chapters II and V deal with this subject in more detail.

	Ca^{++}	PO_4^{---}	Urate	Na^+	K^+	HCO_3^-	Urea	Protein	Alan-trans.	Alk-phos.	Bili-rubin	Creat-inine	γGT
Controls (1)	2.49	1.85	0.13	148	4.8	23	5.2	59	32	55	<12	40	5
Controls (2)	2.09	2.35	0.27	145	5.0	24	7.0	60	25	65	<12	35	2
24hr (1)	2.41	2.15	0.06	152	5.2	24	40.0	59	276	50	<12	70	9
24hr (2)	2.73	2.30	0.03	146	5.1	21	44.2	65	158	80	<12	100	2
48hr (1)	2.57	1.60	0.04	151	4.8	21	24.0	62	155	60	<12	55	7
48hr (2)	2.39	2.75	0.02	149	5.0	26	38.5	60	273	80	<12	85	1

Units: Enzymes I.U./litre, Bilirubin μM, Creatinine μM, All Others mM

TABLE I.2: Blood Chemistry of Alderley Park Mice Treated with CB3717 (300mg/kg/day) (Data of Jackman A.L. & Calvert A.H.)

I.8 Summary

Antifolates have a place both in the past and present of cytotoxic chemotherapy. Efforts to produce new drugs with superior properties to the classical dihydrofolate reductase inhibitor, methotrexate, have so far failed to produce any with significant advantages or comparable antitumour activity in man. The concept of exploiting inhibition of the enzyme thymidylate synthetase by a folate analogue represented a new approach and resulted in the development of CB3717 at the Institute of Cancer Research. The evidence to support the belief that TS is the cytotoxic locus of this compound has been reviewed herein along with the animal antitumour and toxicology studies. The further evaluation of this intriguing new drug, both clinically and biochemically is the subject of the rest of this thesis.

CHAPTER II

PHASE I CLINICAL TRIAL OF CB3717

CHAPTER II

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II.1 Introduction

The clinical evaluation of new chemotherapeutic agents has become standardised into four different stages beginning with the Phase I or early trial. During this introductory study one or several dose schedules are investigated to determine the toxicities of the drug in man, with particular reference to their predictability and reversibility. In addition, by virtue of gradual escalation from a starting dose, caclulated from animal toxicology studies, a maximal tolerated dose and the dose limiting toxicity are defined, leading to the recommendation of a dose regimen which is associated with acceptable side effects (Monfardini S. et al 1981, Gehane A. & Schneiderman M.A. 1982). The establishment of antitumour effect is not the purpose of the appraisal at this stage (Carter S.K. Selawry O. & Slavik M. 1977) but is often sought none the less, if only to encourage the investigators. A recent study of a large number of patients receiving "Phase I" drugs under the auspices of the National Cancer Institutes (USA) showed an overall low response rate (~2%). It is perhaps noteworthy that not one of the drugs which was inactive in "Phase I" had become established clinically (Von Hoff D., presented at the Fourth NCI-EORTC Symposium on New Drugs in Cancer Chemotherapy, December 1983).

This generalised plan for transition from animal to patient treatment with cytotoxic agents is useful, but not ideal, and open to criticism. Until recently, in the USA,

preclinical toxicology screens were far more extensive than in the UK, involving several animal species, of which one had to be non-rodent. The starting dose for man was estimated at one third the minimal toxic dose, in milligrams per square metre of body surface area, of the most sensitive large animal species (Carter S.K. et al 1977). Some workers felt that such screening was wasteful and rarely helpful in predicting toxicity in the clinic (Rozencweig M. et al 1981) with sufficient data being obtainable from rodent studies. (Guarino A.M. et al 1979)

The number of different dose schedules to include and the option of dose escalation within patients are other areas open to discussion when a balance between medical resources and scientific exactitude is sought (Carter S.K. et al 1977). The other crucial question is whether or not it is always justifiable to seek to distinguish a maximal tolerated dose or the limiting toxicity if a more precise biochemical end point is relevant. For example, when considering the evaluation of a thymidylate synthetase inhibitor, finding a dose which substantially reduces plasma thymidine levels might be a more satisfactory point at which to terminate the dose escalation than at the development of an unacceptable toxicity, which could be attributable to the physical properties of the compound.

The Phase I trial of CB3717 began in September 1981 at the Royal Marsden Hospital and Charing Cross Hospital. Curative antitumour activity in mice necessitated a five day treatment regimen (see Chapter I) and it is generally believed that cycle specific drugs such as antimetabolites have more therapeutic effect if given in repeated doses or by long infusion when there is the possibility of affecting more cells at the susceptible stage of cell division - S

phase (Connors T.A. 1976). Hence both five day and one day treatment schedules repeated at three week intervals, were investigated in order to balance scientific dictum with practical considerations. The starting dose, calculated as less than one third the LD10 in mice expressed as units/surface area, was 100mg/m^2.

II.2 Materials and Methods

II.2.a Drug Formulation

CB3717 (N-(4-(N-((2-amino-4-hydroxy-6-quinazolinyl)methyl) prop-2-ynylamino)benzoyl)-L-glutamic acid)was synthesised by Imperial Chemical Industries PLC (Macclesfield Park, Wilmslow, Cheshire) as the anhydrous diacid powder which was 96.72% pure as determined by protonmagnetic resonance, ultra-violet spectroscopy and high pressure liquid chromatography. The impurities comprised H_2O (2.06%) Cl^- (0.26%) CB3705, the N^{10} unsubstituted species (0.44%), a first unknown impurity (0.33%) and a second unknown impurity (0.19%). None of the known impurities were considered to have biological effects at the doses to be used. The small quantity of unknown impurities were probably not quinazolines as the spectroscopic properties were different. CB3717 was formulated at a concentration of 10mg/ml in 0.15M sodium bicarbonate adjusted to pH 9.0 with sodium hydroxide and was sterilised by millipore filtration by the pharmacy staff of the Royal Marsden Hospital (Fulham Road, London). It was then stored at 4°C for a maximum of 21 days over which period stability studies had shown no detectable degradation of the material. The dose to be administered to individual patients was diluted to a volume of 250ml in 0.15M sodium bicarbonate (pH 9.0) for infusion into a peripheral vein over 1 hour. Volumes of 1 litre 0.15M

sodium bicarbonate were used for 12 hour infusions.

II.2.b Patient Characteristics

Patients with malignant disease who had failed on conventional chemotherapy and patients for whom no satisfactory chemotherapy existed were recruited to the study. Verbal consent was obtained. Wherever possible the conventional criteria for inclusion were upheld (Hb > 100g/l, WBC >3 x 10^9/l, platelets >100 x 10^9/l, normal blood urea, electrolytes, creatinine and liver function tests, glomerular filtration rate >60ml/min as determined by ^{51}Cr EDTA clearance, performance status WHO grade <2 and absence of serious intercurrent disease). Patients with hepatic dysfunction secondary to metastatic disease or renal impairment were also included at low dose levels, as were a few of poor performance status, subject to the discretion of the physician. The characteristics of the patients included in the study are summarised in Tables II.1 and II.2. The trial was approved by the Ethical Committee of The Royal Marsden Hospital.

II.2.c Dose Escalation

In the early stages of the study two schedules were adopted using a starting dose of 100mg/m^2.

Schedule A - 1 hour i.v. infusion 100mg/m^2 x5 days Q 3 weeks

Schedule B - 1 hour i.v. infusion 100mg/m^2 x1 day Q 3 weeks

Three patients were entered at each dose level and if no

TUMOUR TYPE

Lymphoma	4
Choriocarcinoma	1
Adult Lymphocytic Leukaemia	1
Acute Myeloid Leukaemia	1
Squamous Cell Carcinoma Bronchus	1
Ovarian Carcinoma	1
Teratoma	1
Primary Unknown	1
TOTAL	11

Age Range 13-73 Median Age 42

M:F 7:4

WHO PERFORMANCE STATUS

Grade	
0	1%
1	20%
2	50%
3	0%
4	30%

TABLE II.1: Characteristics of Patients Treated by 5 Day Schedule

TUMOUR TYPE

Ovarian Carcinoma	41
Melanoma	13
Breast Carcinoma	11
Colonic Carcinoma	5
Adenocarcinoma Bronchus	5
Mesothelioma	5
Lymphoma	4
Large Cell Bronchus	3
Hepatoma	1
Myeloma	1
Fibrosarcoma	1
Liposarcoma	1
Osteosarcoma	1
Apudoma	1
Squamous Cell Carcinoma	1
Teratoma/Seminoma	1
Choriocarcinoma	1
Hypernephroma	1
Primary Unknown	2
TOTAL	99

Age Range 22-74 Median Age 52

M:F 31:68

WHO PERFORMANCE STATUS

Grade	
0	7%
1	30%
2	36%
3	22%
4	4%

TABLE II.2: Characteristics of Patients Treated by 1 day Schedule

significant toxicity occurred the dose was escalated in the sequence shown in Tables II.3 and II.4. Doses were escalated in individual patients where possible. Modifications were made to the schedules when the development of toxicity necessitated. The 5 day regimen was used in 11 patients reaching a top dose of 170mg/m^2 x5 but was accompanied by a high incidence of myelosuppression, unpredictable hepatic dysfunction and malaise, so was discontinued (see Results section). In addition, the observation of transient hepatic dysfunction in the majority of patients treated by a single infusion constrained the dose escalation (see Results section) and attempts were made to ameliorate this toxicity, the mechanism of which was unknown. It was postulated that a biliary precipitate might be induced by CB3717 treatment when high peak plasma levels were achieved, as such a process had been observed in rats (Newell DR et al 1982). This could be contributory to disturbance of liver function. Reduction of peak drug levels in the plasma by prolongation of the infusion to 12 hours was thus deemed logical and 18 patients were treated in this manner. Further, two patients concurrently receiving steroid therapy were noted to have been spared the hepatic toxicty and associated malaise and so on an empirical basis another schedule was introduced, comprising the administration of prednisolone 30mg daily for one week after CB3717 treatment, beginning the day after infusion. The outcome of these schedule manipulations is presented in the results section.

Patients with impaired renal function (glomerular filtration rate (GFR) <60ml/min) and grossly abnormal liver function tests (alkaline phosphatase >200 IU/l) were treated at reduced levels. These precautions were introduced in the face of the anticipated development of renal toxicity (as

Dose (mg/m^2)	Total No. Doses Given
75[a]	3
100	5
140	9
170	1

[a]Dose reduced to $75mg/m^2$ in 3 patients because of unacceptable toxicity at higher doses.

TABLE II.3: Dose Escalation in 5 Day Schedule

Dose (mg/m^2)	Total No. Doses Given
140	3
200	41
280	13
300	63
330	12
360	16
400	70
450	36
500	24
550	6
600	11
[a]700	1

[a]$700 mg/m^2$ Administered in error

TABLE II.4: Dose Escalation in 1 Day Schedule

predicted by the animal toxicology study described in Chapter I) and the early evidence of hepatic toxicity during the trial.

The 99 patients who entered the single dose schedule trial received a total of 296 doses. Fifty four patients were treated by 1 hour infusion (172 courses), eighteen by 12 hour infusions (41 courses) and twenty seven by 1 hour infusion with steroid cover (83 courses). Twenty nine patients received only 1 course, thirty three patients received two courses, and the rest received between 3-20 courses.

II.2.d Patient Monitoring

Full blood counts, serum urea, electrolytes, creatinine, liver function tests, protein, albumin, calcium, phosphate and prothrombin times were measured prior to treatment and twice weekly thereafter (extended to weekly at later stages of the trial). Renal function was monitored by estimation of the glomerular filtration rate by ^{51}Cr EDTA clearance (Chantler C. et al 1969) prior to starting treatment and 2-3 weeks after each treatment. In 14 patients the enzymes N-acetyl β-glucosaminidase (NAG) and leucine aminopeptidase (LAP) in addition to β_2 microglobulin levels were measured in urine collected pre-treatment and on days 1,3,5,7,10,14 and 20 post treatment (Jones B.R. et al 1980 and using radioimmunoassay test Pharmacia, GB Ltd). Disease response was evaluated weekly, when feasible, by clinical examination and measurement of tumour masses. Radiography, ultrasonic, isotopic and computer assisted tomographic scanning were repeated at appropriate intervals.

II.3 Results of 5 Day Schedule

II.3a Myelotoxicity

This treatment regimen, using a starting dose of 100mg/m^2 administered on 5 consecutive days, was associated with considerable toxicity of an unpredictable pattern and necesitated dose reduction in 3 patients. Myelotoxicity, affecting mainly leucocytes and platelets, occurred after 7 of the total 18 courses and was contributory to the death of 4 patients. Details of the leucopenia and thrombocytopenia observed are shown in Table II.5 and II.6. One patient with squamous cell bronchial carcinoma tolerated his first course of 100mg/m^2 x5 but developed myelosuppression on the second course of 140mg/m^2 x5. His third course of 100mg/m^2 x5 was also associated with myelotoxicity as was his fourth dose of 75mg/m^2 x5. Examination of his marrow revealed no carcinomatous infiltration, and he had received no prior chemotherapy or radiotherapy. Another patient received only 3 days of treatment at at a dose of 100mg/m^2 and developed leucopenia ($0.2x10^9$/l) and thrombocytopenia ($93x10^9$/l) on day 10. She subsequently received a second course of 75mg/m^2 on 3 days and died 9 days later with profound leucopenia ($<0.1x10^9$/l) and thrombocytopenia ($9x10^9$/l). She had previously been treated with adriamycin, cyclophosphamide, cisplatinum, and chlorambucil and had undergone whole abdomen irradiation so that her marrow was undoubtedly compromised at the outset. The one patient treated at 170mg/m^2 had been subjected to 6 cycles of

Dose mg/m^2	No. of Courses Associated with Leucocyte Toxicity Grade:			
	0-1*	2	3	4
75	0	0	1[a]	2[bc]
100	3	0	0	2
140	8	0	0	1
170	0	0	0	1[d]

Toxicity Grade: 0-1 = $>3x10^9$ cells/l*
2 = $2-2.9x10^9$ cells/l
3 = $1-1.9x10^9$ cells/l
4 = $<1x10^9$ cells/l

Nadir Day 9-13 $\bar{x}$ 11
Recovery Day 14-18 $\bar{x}$ 17

[a]Toxic death day 9
[b]Toxic death day 10
[c]Toxic death day 12
[d]Toxic death day 13

*Criteria for treatment WCC $>3.0x10^9/l$

TABLE II.5: Toxicity To Leucocytes - 5 Day Schedule (2 Leukaemia Patients Excluded From The Table)

Dose mg/m^2	Number of Courses Associated With Platelet Toxicity Grade:				
	0	1	2	3	4
75	0	0	1	0	2[ab]
100	3	1	0	1	0
140	8	0	0	0	0
170	0	0	0	0	1[c]

Toxicity Grade 0 = $>100x10^9$ platelets/l
1 = $75-99x10^9$ platelets/l
2 = $50-74x10^9$ platelets/l
3 = $25-49x10^9$ platelets/l
4 = $<25x10^9$ platelets/l

Nadir Day 8-12 $\bar{x}$ = 10
Recovery Day 12

[a]Toxic death day 9
[b]Toxic death day 10
[c]Toxic death day 13

TABLE II.6: Toxicity To Platelets - 5 Day Schedule (2 Leukaemia Patients Excluded From The Table)

cisplatinum, vinblastine and bleomycin chemotherapy followed by abdominal radiotherapy for a testicular teratoma. He became severely myelosuppressed (leucocytes $0.1 \times 10^9/l$, platelets $9 \times 10^9/l$) and died on day 12.

Haemoglobin values fell appreciably after 6 courses, (details Table II.7) which in one case was definitely associated with gastrointestinal bleeding due to thrombocytopenia. It may be significant that the 4 non leukaemic patients who experienced myelotoxicity had elevated plasma alkaline phosphatase levels before treatment started (245, 375, 500, 900 IU/l).

II.3.b Hepatotoxicity

The first patient treated with CB3717 developed elevation of plasma alanine transaminase (ALT) levels heralding the appearance of mild hepatic dysfunction as the most frequently observed toxicity in the whole study. The pattern of ALT elevation in this choriocarcinoma patient is shown on Fig II.1 along with the β human chorionic gonadotrophin (βHCG) levels which were used as a marker for her tumour. After the more pronounced rise in plasma ALT (peak value 769 IU/l) following the second treatment at $140 mg/m^2$ x5, a third course was administered at the same total dose by a continuous 120 hour infusion. On this occasion, ALT levels did not rise further and gradually returned towards normal.

Fourteen of the eighteen courses of treatment were associated with abnormalities of transaminase levels (as detailed in Table II.8) with no apparent dose relationship. Alkaline phosphatase values were unaffected in patients with normal pre-treatment levels but in 3 patients with raised

Dose mg/m^2	Number of Courses Associated With Haemoglobin Toxicity Grade:				
	0	1	2	3	4
75	0	0	3[a]	0	0
100	0	4	1	0	0
140	6	1	1	0	0
170	0	0	0	1	0

[a]Fall in haemoglobin associated with gastrointestinal blood loss secondary to thrombocytopenia

Toxicity Grade 0 = >110g/l
1 = 95-109g/l
2 = 80-94g/l
3 = 65-79g/l
4 = <65g/l

TABLE II.7: Haemoglobin Toxicity - 5 Day Schedule

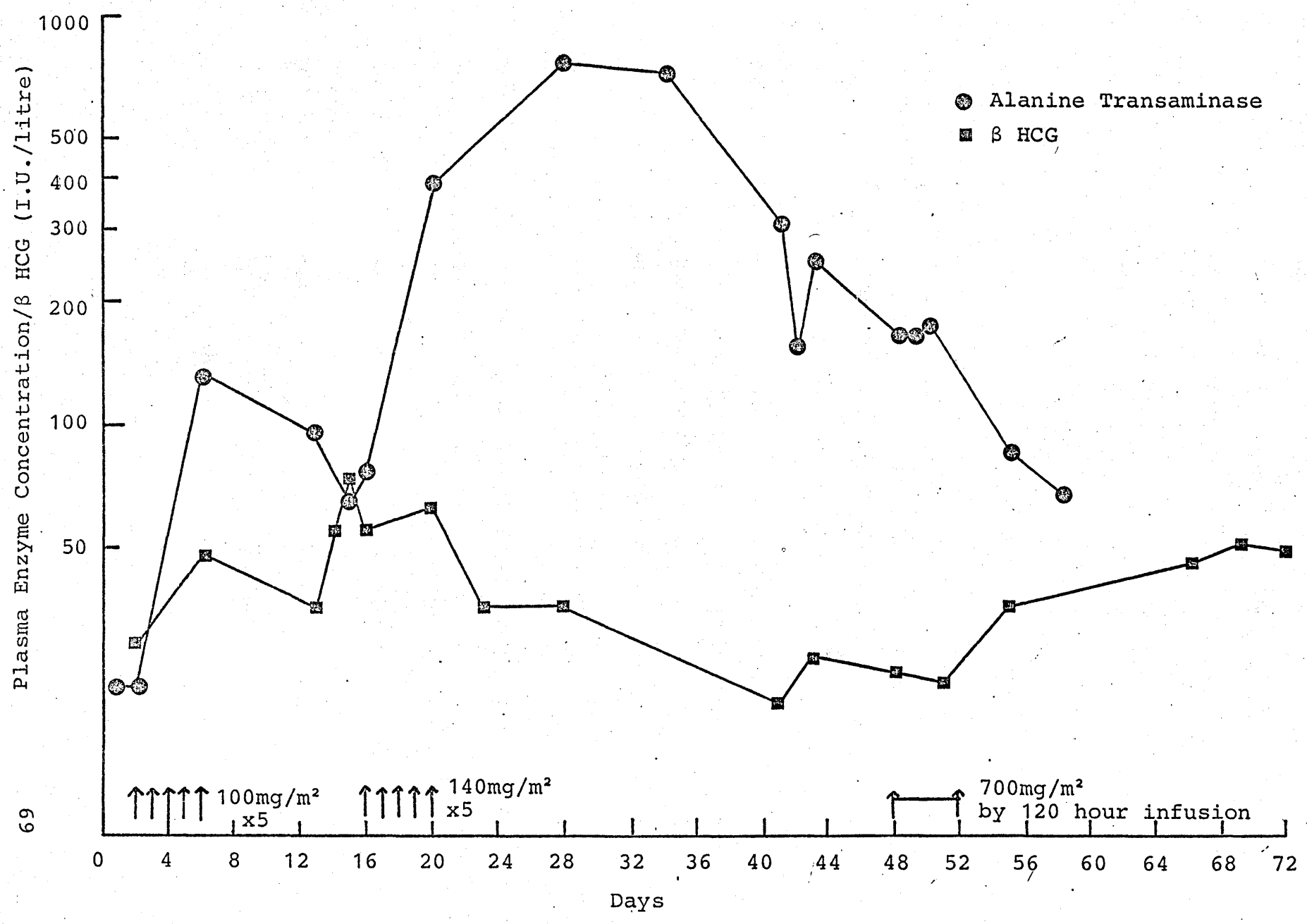


FIGURE II.1: Plasma Alanine Transaminase and β Human Chorionic Gonadotrophin Levels In A Choriocarcinoma Patient Receiving CB3717

Dose mg/m^2	No. of Courses Associated With Alanine Transaminase Elevations:				
	0	1	2	3	4
75	2	1	0	0	0
100	1	2[e]	1[c]	2[b]	0
140	1	3	0	1	3[ad]
170	0	0	0	1	0

Toxicity Grade
0 = <1.25x normal (<27.5IU/l)
1 = 1.25-2.5x normal (27.5-55IU/l)
2 = 2.5-5x normal (55-110IU/l)
3 = 5-10x normal (110-220IU/l)
4 = >10x normal (>220IU/l)

[a]Elevation to 769IU/l
[b]Patient had ALT of 46IU/l before treatment
[c]Patient had ALT of 55IU/l before treatment
[d]Patient had ALT of 43IU/l before treatment
[e]Patient had ALT of 28IU/l before treatment

TABLE II.8: Peak Plasma Alanine Transaminase Levels After CB3717 Treatment - 5 Day Schedule

levels (245, 340 and >500 IU/L) two fold elevations occurred after CB3717. Hyperbilirubinaemia was seen after one course at 75mg/m^2 x5, (60 IU/l), and after another course at 170mg/m^2 x5, (72 IU/l) in two patients with massive metastatic involvement of the liver and abnormal pre-treatment alkaline phosphatase levels.

II.3.c Malaise

The development of abnormal liver function tests coincided with feelings of malaise, lethargy and anorexia necessitating bed rest in 8 patients, although in some this was also associated with periods of myelosuppression.

II.3.d Dermatological Toxicity

Five patients developed rashes which, in three cases, were itchy, erythematous and maculopapular, localised to the trunk and proximal part of the arms and legs and appeared 3-4 days post treatment. Figure II.2 is a photograph of one such patient. Symptomatic relief was obtained with oral antihistamines and local hydrocortisone cream. One patient later tolerated a further course of CB3717 without the development of a rash after he had been started on prednisolone 10mg b.d as a means of controlling hypercalcaemia and improving appetite.

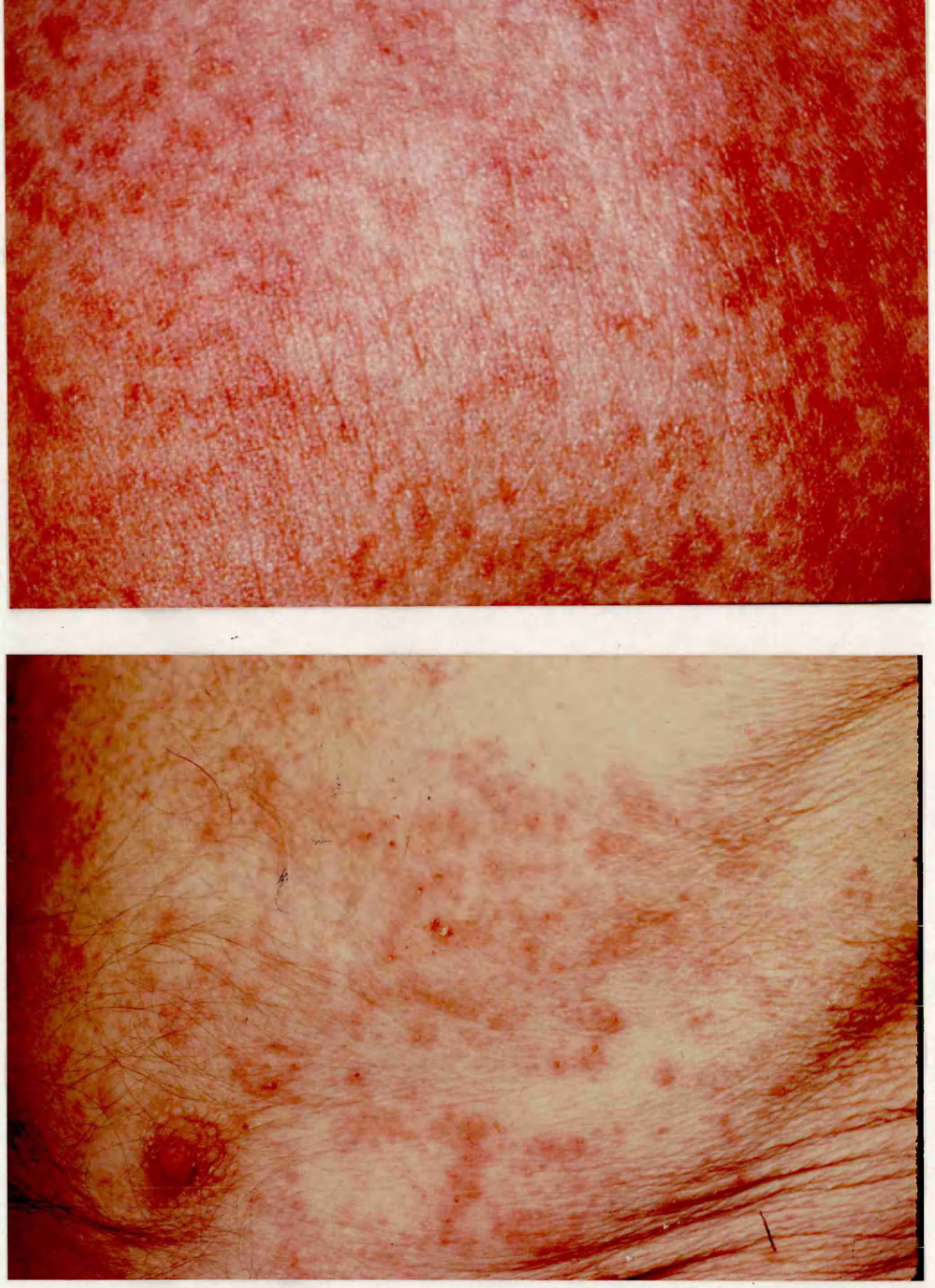


FIGURE II.2: Erythematous Maculopapular Rash In a Patient Treated With 140mg/m² CB3717 for 5 Days

A patient with adult lymphocytic leukaemia noticed a slight acneiform rash on his face, similar to one he had previously experienced when on methotrexate therapy. One other patient, with acute myeloid leukaemia, developed a purpuric rash on his abdomen and the flexures of his limbs two days after finishing the treatment course. Thrombocytopenia was a feature of his disease (pre-treatment platelets $80x10^9/l$, platelet nadir $19x10^9/l$ day 4, platelets $27x10^9/l$ on the day of rash appearance).

II.3.e Gastrointestinal Toxicity and Mucositis

Mucositis with oral ulceration occurred in two patients coincidentally with periods of myelosuppression and was associated with oral fungal infection.

Two patients with lymphomatous bowel involvement developed diarrhoea and in one this continued from day 1 of treatment until his death (attributed to progressive disease) 8 days later.

II.3.f Miscellaneous Toxicities

Pancreatitis, with the formation of a pancreatic pseudocyst, complicated the second course of treatment of a patient with extensive intra-abdominal lymphoma. This was most probably related to enlarging peri-pancreatic lymph nodes and pancreatic metastatic involvement or possibly to concurrent dexamethasone treatment (Cluff L.E. and Caldwell J.R. 1977). Another patient developed an unexplained pyrexia on day 10 with an exacerbation of the erythematous maculopapular rash which had appeared on day 7.

II.3.g Renal Toxicity

Elevation of blood urea or creatinine levels was not seen except in two patients succumbing to progressive disease and myelotoxicity. They had pre-terminal rises in blood urea levels (16mmol/l) but this was not thought to be drug related. ^{51}Cr EDTA clearances were not repeated, unfortunately, in 8 patients because of disease progression, or patient death.

II.3.h Antitumour Effects

Two patients with lymphoma had short-lived, minor responses with some reduction (<50%) in the size of involved lymph node masses. One patient with squamous cell bronchial carcinoma experienced disease stabilisation for 10 weeks. The patient with adult acute lymphocytic leukaemia developed a hypocellular marrow with regrowth of blasts subsequently.

II.4 Results of 1 Day Schedule

II.4.a Renal Toxicity

Renal toxicity was assessed by measurement of glomerular filtration rates, (GFR), as this is a much more sensitive indicator of renal function than blood urea or creatinine. Seventy patients with adequate renal function (GFR >60ml/min) were treated and repeat ^{51}Cr EDTA clearances were obtained on forty four at cessation of treatment, which involved between 1 and 20 courses. Fourteen patients with pre-treatment GFR 41-60ml/min and fifteen patients with pre-treatment GFR <40ml/min were also treated at doses up to 400mg/m^2 and 300mg/m^2 respectively. Follow-up GFR data was

collected on 7 and 12 patients from these groups. Table II.9 shows the reductions in GFR which occurred at different dose levels. Excluding patients who developed worsening renal function associated with pelvic disease, the majority had slight reductions in GFR amounting to <20%. Five of the good risk patients (GFR >60ml/min) treated at doses up to 450mg/m^2 had reductions of 20-50% pre-treatment GFR values. At doses of 500-600mg/m^2, reductions in GFR of 20-50% and >50% occurred more frequently indicating that renal toxicity was dose limiting for CB3717 (using this schedule of administration) with a maximal tolerated dose in the region of 600mg/m^2. Some variability of response to the higher doses was observed, possibly related to the state of hydration of individual patients over the 24 hours post treatment. One patient (pre-treatment GFR 119ml/min) treated at 600mg/m^2 on 4 occasions showed no elevation of serum urea or creatinine until the day of her final treatment (urea 14.5 mmol/l, creatinine 204 mmol/l) and died of acute renal failure 3 weeks later (see Toxic Deaths section). In another patient the GFR fell from 95ml/min to 60ml/min after one dose at 600mg/m^2 but increased to 83ml/min after two further doses at 400mg/m^2. The numbers of patients in the poor risk groups were small but doses of 200-300mg/m^2 were associated with GFR reductions of <20% in 11/16 patients and reductions of 20-50% in 5/16 patients.

Urinary Enzymes

The levels of urinary enzymes (NAG and LAP) and β_2 microglobulin which were measured in fourteen patients before and after doses of 300-500mg/m^2 CB3717 are shown in Figures II.3 II.4 and II.5 with a legend giving some details of the patients. Reported normal means (+2 SD) have been taken from BR Jones et al 1980. Some of the patients had

Dose	Pre Treatment GFR >60ml/min				Pre Treatment GFR 41-60ml/min				Pre Treatment GFR <40ml/min)			
(mg/m^2)	Total No. Patients	Reduction in GFR 0-20%	21-50%	>50%	Total No. Patients	Reduction in GFR 0-20%	21-50%	>50%	Total No. Patients	Reduction in GFR 0-20%	21-50%	>50%
140	1	1	0	0	-	-	-	-	1	1	0	0
200	3	2	1	0	-	-	-	-	4	3	1	0
280	0	0	0	0	1	1	0	0	-	-	-	-
300	14	10	2	2[ab]	3	2	1	0	7	4	3[ef]	0
330	3	3	0	0	1	0	1	0				
360	1	1	0	0	-	-	-	-				
400	11	7	2	2[cd]	2	1	0	1				
450	2	2	0	0	-	-	-	-				
500	4	2	1	1	-	-	-	-				
550	3	0	2	1	-	-	-	-				
600	3	1	1	1[g]	-	-	-	-				

[a] Patient developed severe dysphagia and probable dehydration
[b,c,d,e,f] Patients developed obstructive uropathy secondary to progressive pelvic disease
[g] Patient died of renal failure with myelosuppression after 4 doses at $600mg/m^2$

TABLE II.9: Renal Toxicity After CB3717 Treatment (1 day schedule)

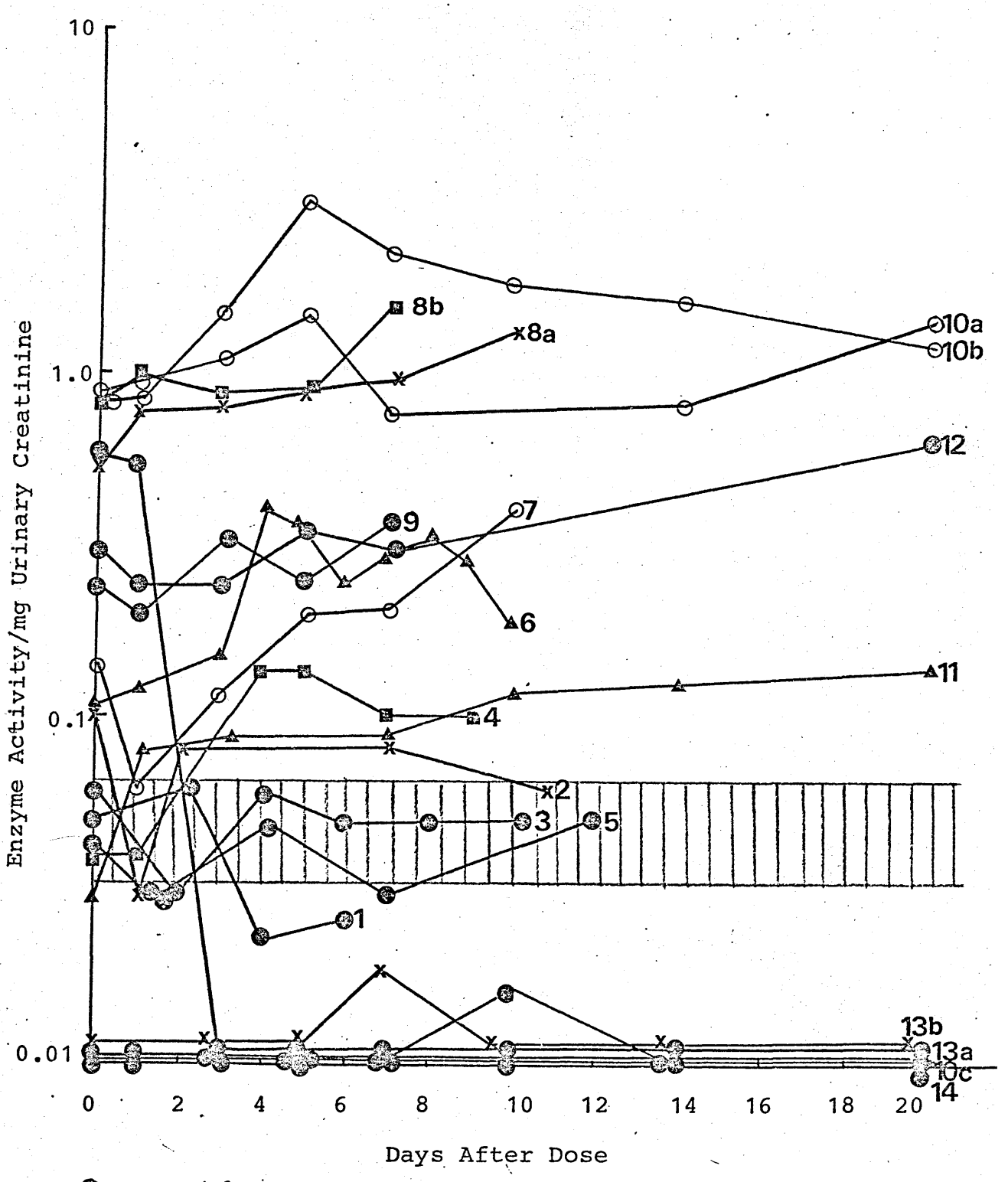
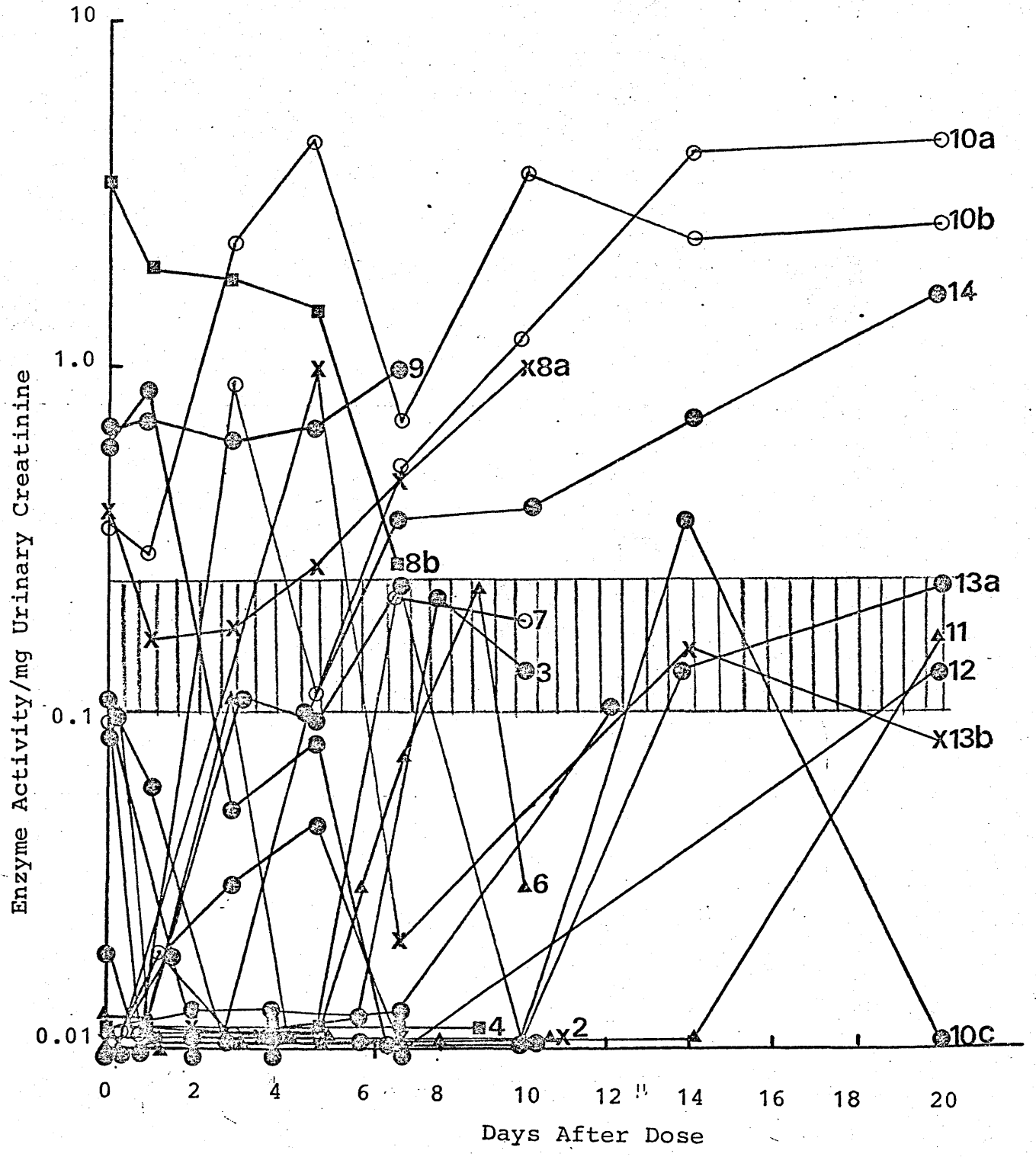


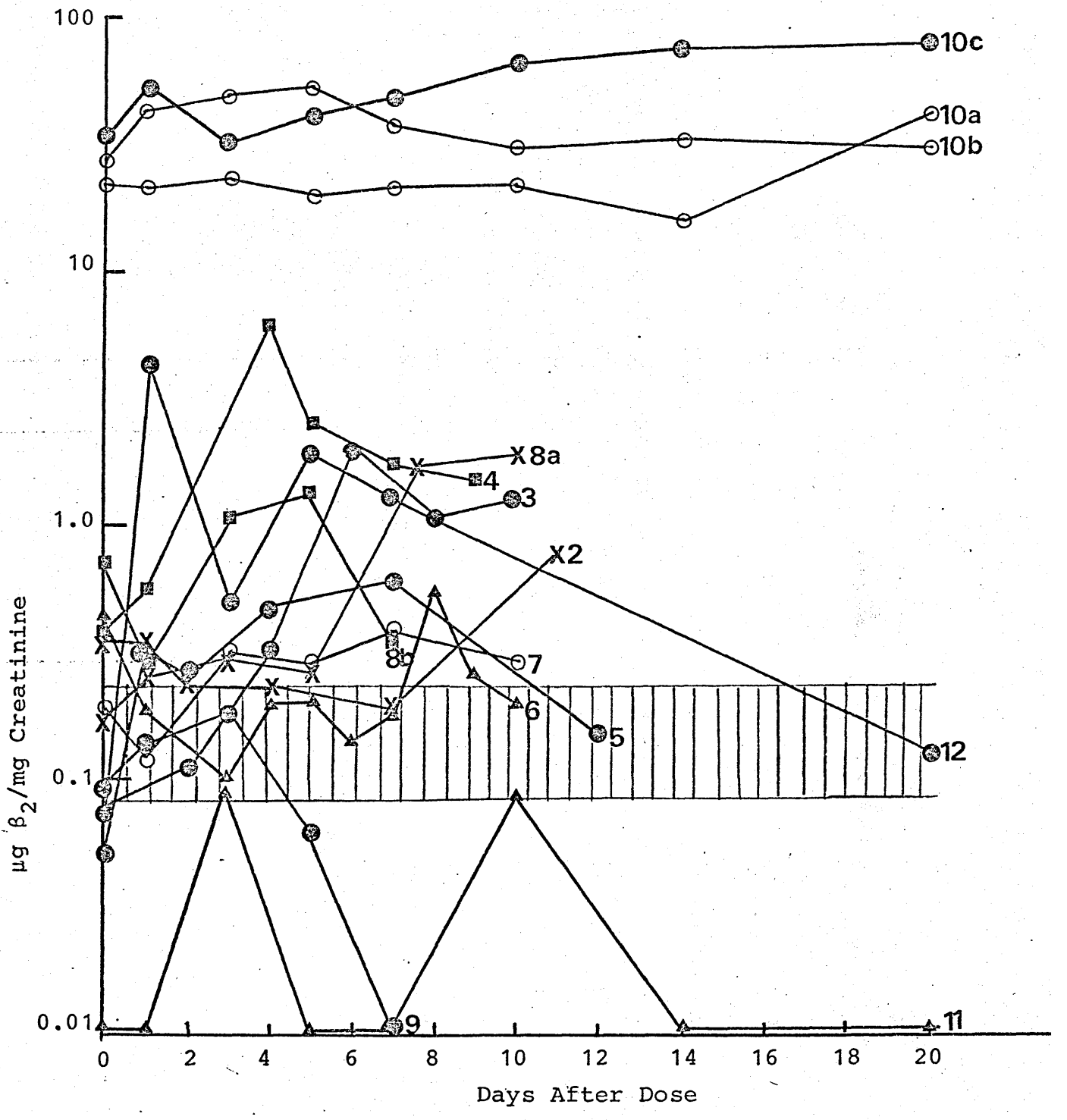
FIGURE II.3: Urinary N-Acetyl Glucosaminidase Excretion In Patients Treated With CB3717



● 300mg/m²
■ 350mg/m²
X 400mg/m²
▲ 450mg/m²
O 500mg/m²

Hatched area represents reported mean + 2 S.D. of values from normal subjects

FIGURE II.4: Urinary Leucine Aminopeptidase Excretion in Patients Treated With CB3717



- ● 300mg/m²
- ■ 350mg/m²
- X 400mg/m²
- ▲ 450mg/m²
- O 500mg/m²

Hatched area represents reported mean + 2 S.D. of values from normal subjects

FIGURE II.5: Urinary β_2-Microglobulin Excretion In Patients Treated With CB3717

Patient Number	Pre-Treatment GFR ml/min	Course Number
1	35	8 (previous Cisplatin, carboplatin)
2	116	5
3	172	1
4	76	3
5	95	2
6	104	1
7	93	2
8	61	3 & 4
9	66	1
10	100 68 47	1, 3 & 5
11	61	1
12	128	1
13	50	3 (previous cisplatin, carboplatin)
14	37	1 (previous cisplatin, carboplatin)

Key For Urinary Enzyme Data (Figures II.3, II.4, II.5)

raised levels of one or more of these parameters at the start of the treatment courses shown and in the case of patients 2,4,7 and 8 this may have been due to previous treatment with CB3717. A consistent pattern of disturbance was not apparent but approximately 50% of the treatments were associated with some elevation of urinary enzymes indicative of renal damage. The numbers were too small to establish a dose relationship.

II.4.b Hepatic Toxicity

Transient elevations in plasma alanine transaminase levels (similar to those described following 5 day treatment schedules) occurred frequently (see Table II.10) after single infusions of CB3717, usually beginning on day 2-3 and peaking on days 10-21. In addition, ALT levels generally rose higher with successive courses of CB3717, but in thirteen patients who received more than 4 treatments, enzyme levels began to return towards normal by the fifth to sixth course (example shown in Fig.II.6). No dose relationship was evident as demonstrated in Figure II.7 and Table II.11. Comparison of the group of patients who had near normal liver function tests prior to treatment (ALT and alkaline phosphatase <1.25 x normal) with the group who had abnormal liver function tests (alkaline phosphatase >1.25 x normal but ALT normal) did not reveal any significant difference in ALT elevation after treatment (x^2=4.86 p=0.4) (see Table II.12).

Increases in plasma alkaline phosphatase levels were also induced by CB3717 treatment but to a lesser extent than for ALT. Table II.13 gives details of this in those patients whose pre-treatment alkaline phosphatase levels were <1.25 x normal. Neither prolongation of the infusion

	Number of courses associated with ALT toxicity grades:					
	0	1	2	3	4	Total
Number of courses in all patients	65	92	70	43	16	288
Number of courses excluding patients with increased ALT at start	65	91	67	36	18	277

Toxicity Grade 0 = <1.25 x normal (<27.5IU/l)
1 = 1.25-2.5 x normal (21.5-55IU/l)
2 = 2.5-5.0 x normal (55-110IU/l)
3 = 5.0-10 x normal (110-220IU/l)
4 = >10 x normal (>220IU/l)

TABLE II.10: Hepatic Toxicity - Elevation of Plasma Alanine Transaminase (1 Day Schedule)

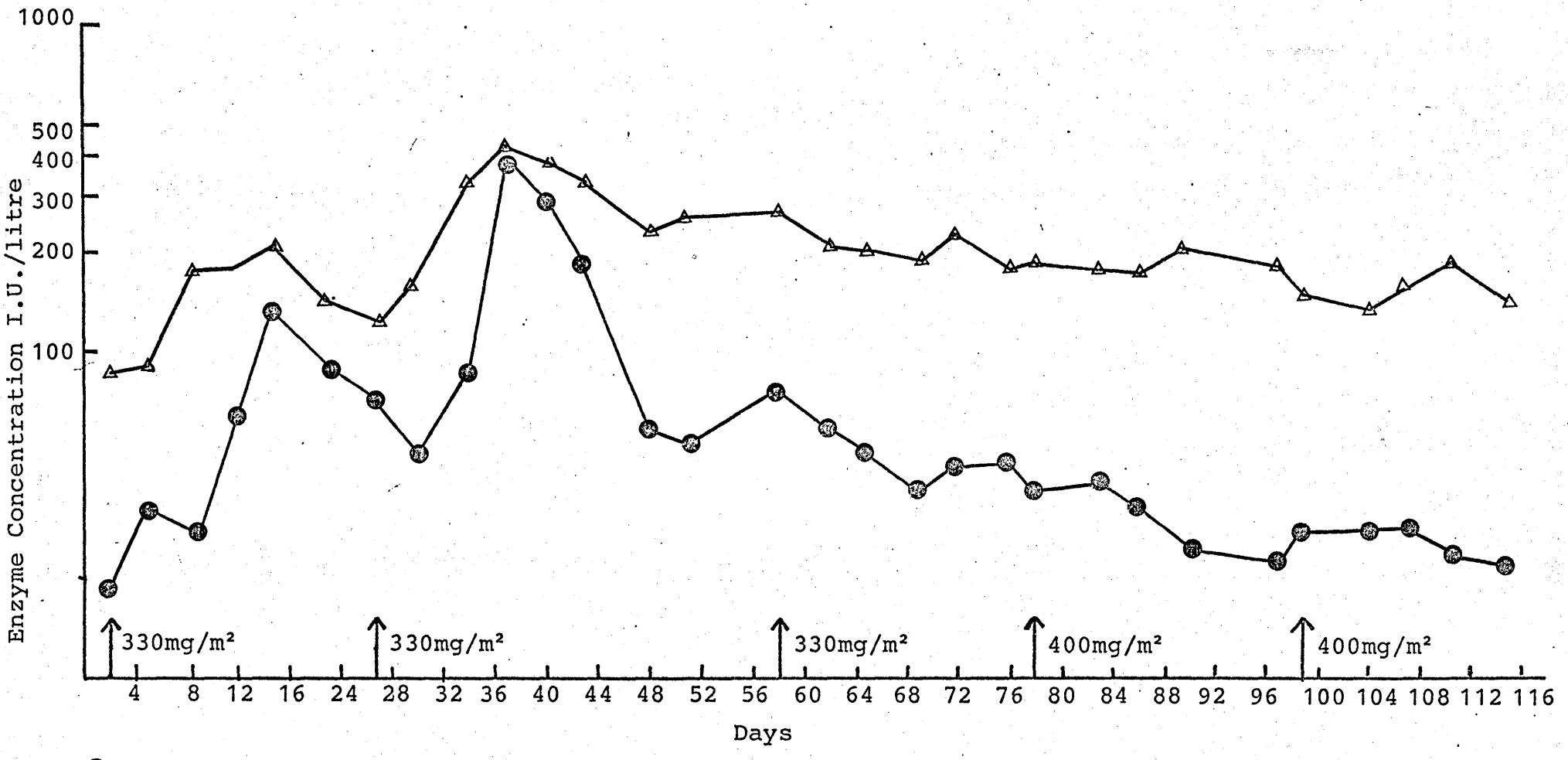


FIGURE II.6: Plasma Liver Enzyme Levels In A Patient Treated With 5 Doses of CB3717

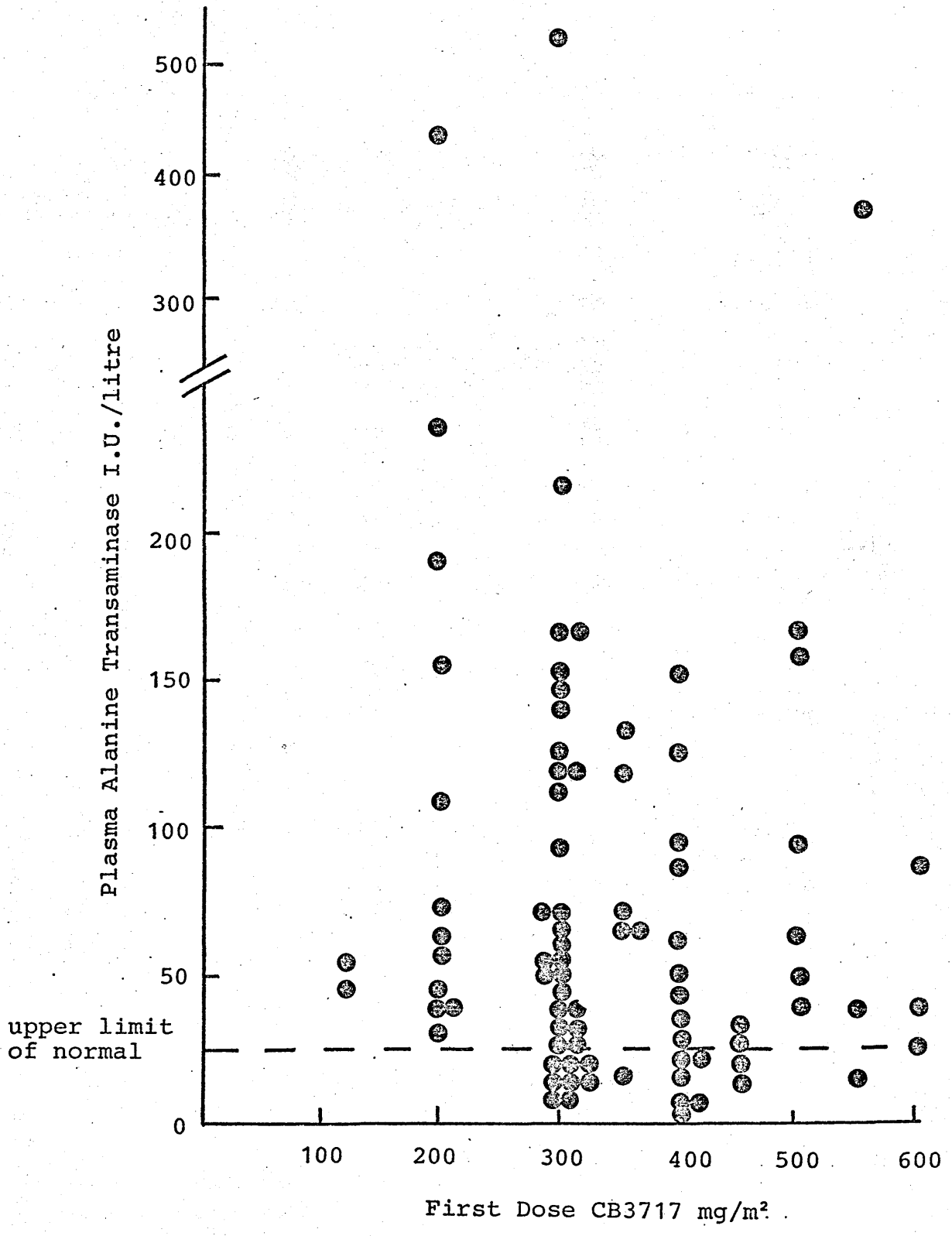


FIGURE II.7: Peak Values of Plasma Alanine Transaminase After First Courses of CB3717 at Doses of 140-600mg/m²

First Course Dose (mg/m^2)	Number of Courses With ALT Toxicity Grade:				
	0	1	2	3	4
140	-	2	-	-	-
200	-	5	3	2	1
280	2	-	1	1	-
300	9	9	9	9	3
330	1	1	1	1	-
360	-	-	2	1	-
400	4	6	4	3	-
450	1	2	-	-	-
500	-	2	2	3	1
600	2	1	1	-	-

Toxicity Grade: 0 = <1.25 x normal (<27.5 IU/l)
1 = 1.25-2.5 x normal (27.5-55 IU/l)
2 = 2.5-5 x normal (55-110 IU/l)
3 = 5-10 x normal (110-220 IU/l)
4 = >10 x normal (>220 IU/l)

TABLE II.11: Peak Plasma Alanine Transaminase Levels After First Courses of CB3717 - 1 Day Schedule

	ALT Toxicity Grade				
	0	1	2	3	4
Pre-treatment ALT & Alk. P. Normal (<1.25 x N)	54	65	46	25	11
Pre-treatment ALT Normal, but Alk P Abnormal (>1.25 x N)	9	23	17	11	4

x^2 test = 4.86 P = 0.4

Toxicity grades
0 = <1.25x normal (<27.5 IU/l)
1 = 1.25-2.5x normal (27.5-55 IU/l)
2 = 2.5-5x normal (55-110 IU/l)
3 = 5-10x normal (110-220 IU/l)
4 >10x normal (>220 IU/l)

TABLE II.12: Comparison of Plasma Alanine Transaminase Elevation After CB3717 Treatment in Groups of Patients With Normal and Abnormal Alkaline Phosphatase Levels Prior to Therapy

to 12 hours nor the administration of prednisolone for one week after treatment made a significant difference to the patterns of enzyme abnormality observed (see Table II.14) (x^2 = 2.96, 0.9>p>0.5, x^2=5.23, 0.5>p>0.1)

Hyperbilirubinaemia (mainly conjugated) occurred after 18 courses (details on Table II.15) in seventeen patients. Three patients were retreated with no further episodes of hyperbilirubinaemia but one other patient experienced elevation in bilirubin after 2 courses. Ten of these patients had pre-treatment alkaline phosphatase levels >300 IU/l and metastatic hepatomegaly.

Prothrombin times were not prolonged except in jaundiced patients. Serum albumin levels were low prior to treatment in the majority of patients and this made it difficult to interpret the effect of CB3717, although no marked fluctuations in levels occurred.

II.4.c Myelotoxicity

Myelosuppression developed sporadically throughout the dose escalation and, as in the 5 day schedule, affected the leucocytes and platelets. Leucopenia (WBC $<3.0\times10^9$/l) occurred in seventeen patients and thrombocytopenia in six, with mean nadir on days 10 and 9 and recovery by days 15 and 13 respectively (see Table II.16 for details). Impaired renal function was a possible risk factor for myelosuppression as of the eight patients with nadir WBC $<1\times10^9$/l, three had pre-treatment GFR of 41-60ml/min and three had pre-treatment GFR 21-40ml/min. Eight of the seventeen patients had alkaline phosphatase levels of >200IU/l (4 of which were >400IU/l) and one patient had a raised serum bilirubin.

	Alk. P. Toxicity Grade:				
	0	1	2	3	4
No. of Courses	78	97	28	1	0

Toxicity Grades
0 = <1.25 x normal (<118 IU/l)
1 = 1.25-2.5 x normal (118-315 IU/l)
2 = 2.5-5 x normal (315-475 IU/l)
3 = 5-10 x normal (475-950 IU/l)
4 = >10 x normal (>950 IU/l)

TABLE II.13: CB3717-Induced Elevation of Plasma Alkaline Phosphatase Levels in Patients With Normal Pre-Treatment Alkaline Phosphatase Levels (<1.25 x normal)

Treatment Schedule	ALT Toxicity Grade				
	0	1	2	3	4
A 1 hour	11	15	10	10	3
B 1h + 1 week prednisolone	10	6	5	1	1
C 12 h	2	5	5	3	3

A vs. B $x^2 = 5.23$ $p > 0.05$ $(0.5 > p > 0.1)$
A vs. C $x^2 = 2.96$ $p > 0.05$ $(0.9 > p > 0.5)$

Toxicity Grades 0 = <1.25 x normal
1 = 1.25-2.5 x normal
2 = 2.5-5 x normal
3 = 5-10 x normal
4 = >10 x normal

TABLE II.14: CB3717 Hepatotoxicity - Comparison of The Effect of 1 Hour Infusions, 12 Hour Infusions And 1 Hour Infusions With Steroid treatment On Peak Plasma Alanine Transaminase Levels After First Courses

	Bilirubin Toxicity Grade				
	0	1	2	3	4
No. of Courses	270	11	5	1	1

0 = <1.25 x normal (<21mmol/l)
1 = 1.25-2.5 x normal (17-42.5mmol/l)
2 = 2.5-5 x normal (42.5-85 mmol/l)
3 = 5-10 x normal (85-170 mmol/l)
4 = >10 x normal (>170 mmol/l)

TABLE II.15: Hyperbilirubinaemia After CB3717 Treatment (One Day Schedule)

Haemoglobin values fell below 100g/l with 47 courses of treatment (16%) but generally recovered in time for further treatment and rarely necessitated transfusion (details on Table II.17).

II.4.d Malaise

Approximately 75% patients experienced malaise with symptoms of anorexia, lethargy, depression and occasionally mild nausea, rather similar to the prodromal phase of hepatitis. In most cases the severity of malaise parallelled the disturbance in liver enzyme levels and lasted between days 3-14 but a few patients who had only trivial or no elevation in ALT levels still experienced considerable constitutional upset. Conversely a few patients with marked enzyme elevations were apparently unaffected in this respect. A one week course of prednisolone, 30mg daily, was of some help in preventing malaise (although not universally) and was administered to patients who were particularly distressed by the symptoms.

II.4.e Dermatological Toxicity

Twelve patients developed an irritating erythematous maculopapular rash of varying severity on the trunk and limbs (see Fig. II.2) which progressed to exfoliation on one occasion. One patient experienced an immediate urticarial reaction lasting for 2 hours after an infusion of CB3717 and later developed a purpuric rash on the extensor surface of her limbs. Interestingly, no patient receiving prednisolone for one week after their CB3717 treatment developed a rash.

A radiation recall rash was seen in 2 patients, one of whom had been irradiated with inverted Y and axillary fields

Dose[b] mg/m^2	Leucocyte Toxicity Grade 0 - 1	2	3	4	Platelet Toxicity Grade 0	1	2	3	4
200	39	-	1	1	40	1	-	-	-
300	57	1	2	3	61	-	2	-	-
330	10	1	-	1	11	-	-	1	-
360	15	-	-	1	16	-	-	-	-
400	67	2	1	-	69	-	-	-	-
550	5	1	-	-	6	-	-	-	-
600	9	-	-	2	9	-	1	-	1

Nadir day 7-16, $\bar{x}$ = 10
Recovery Day 11-19 $\bar{x}$ = 15

Nadir day 7-11, $\bar{x}$ = 9
Recovery Day 11-15 $\bar{x}$=13

Toxicity Grades:	Leucocytes	Platelets
0	>3.0x10^9/l[a]	>100x10^9/l
1		75-99x10^9/l
2	2-2.9x10^9/l	50-74x10^9/l
3	1-1.9x10^9/l	25-49x10^9/l
4	<1.0x10^9/l	<25x10^9/l

[a]Criteria for inclusion to study WCC >3.0x10^9/l
[b]Dose levels not associated with leucopenia or thrombocytopenia are omitted

TABLE II.16: Leucocyte and Platelet Toxicity After CB3717 - One Day Schedule

	Haemoglobin Toxicity Grade				
	0[a]	1[a]	2	3	4
No. of Courses	164	80	41	5[b]	0

[a]Criteria for inclusion to study Hb >100g/l
[b]One patient with sickle cell trait

Toxicity Grades: 0 = >110g/l
1 = 95-110g/l
2 = 80-94g/l
3 = 65-79g/l
4 = <65g/l

TABLE II.17: Haemoglobin Toxicity After CB3717 Treatment (One Day Schedule)

six years previously. Her first treatment of CB3717 at 200mg/m^2 gave rise to a slight burning sensation only but a second dose resulted in vivid erythema over the radiation sites (see Fig. II.8). The dermatological toxicities were not dose related.

II.4.f Gastrointestinal Toxicity and Mucositis

Nausea and vomiting were not problematic acutely but three patients experienced nausea with intermittent vomiting and diarrhoea for 2-3 days in the first week after treatment, which may have been associated with infective episodes. Two patients complained of a sore mouth with some mucosal erythema and three patients complained of gritty eyes although their conjunctivae appeared normal.

II.4.g Miscellaneous Toxicities

Two patients experienced 'flu-like symptoms with myalgia and pyrexia for 2-3 days after treatment and one case was associated with a rash. Severe abdominal pain occurred in two patients, one of whom had massive hepatomegaly and one other patient, treated with 600mg/m^2 of CB3717, experienced loin pain between days 2-7. Another patient, with a pleural mesothelioma extending into the peritoneum, developed hepatic and pleuritic pain with rubs heard over these areas, in association with profound malaise, on two occasions, 1-2 days post treatment. He tolerated 3 further courses, with prednisolone cover, uneventfully.

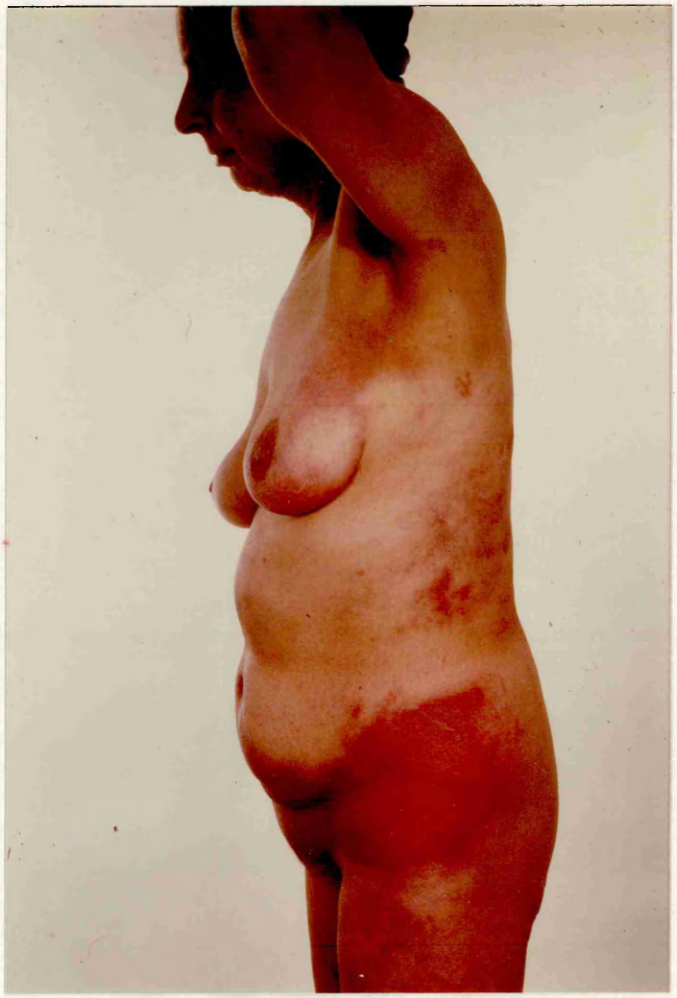


FIGURE II.8: Radiation Recall Reaction In A Patient Treated With Two Doses of CB3717 at 200mg/m²

II.4.h. Toxic and Early Deaths

Two deaths were attributable to drug related toxicities.

Patient 1. A 72 year old man with recurrent colonic carcinoma causing sub-acute intestinal obstruction, who had hepatic metastases, received two doses of CB3717 at 330mg/m^2 by 12 hour infusions. The first treatment was well tolerated but gave rise to modest elevation of plasma ALT to 67 IU/l. After the second dose, a period of myelosuppression ensued (WBC 0.6 x10^9/l, platelets 34 x10^9/l day 7), associated with his general decline and obvious disease progression. Supportive measures were not instituted. Post mortem examination confirmed the presence of large tumour masses in the pelvis, abdomen and liver. Microscopic examination of liver tissue distant from metastases revealed pathology characterised by fatty change, cholestasis and the presence of Mallory bodies (mainly centrilobular in distribution). General liver architecture was well maintained. Professor P.J. Scheuer (Royal Free Hospital, London) reviewed these sections and confirmed the findings to be similar to those seen with alcoholic hepatitis, diabetes and obesity or caused by the drugs perhexilene maleate and stilboestrol. Macroscopically the kidneys were normal, but microscopic examination was unsatisfactory due to severe autolysis.

Patient 2. A female with stage III ovarian carcinoma (resistant to cisplatinum, chlorambucil, treosulphan and carboplatin (JM8)), giving rise to a large abdomino-pelvic mass, was treated with the top dose of CB3717 used (600mg/m^2), infused over 1 hour. Her pre-treatment ^{51}Cr EDTA clearance was 119ml/min. Unfortunately, an exceptional

breach of protocol resulted in her being retreated with 3 more doses of 600mg/m^2 before her glomerular filtration rate was reassessed and was found to be 25ml/min. She had shown signs of disease response and her serum urea and creatinine values had remained normal until the day of her fourth treatment when urea was 14.5mmol/l and creatinine 204mmol/l. Ten days later the patient was admitted to hospital with worsening renal function (urea 33.4 mmol/l, creatinine 447mmol/l), hyperbilirubinaemia (46mmol/l), and myelosuppression (WBC nadir 0.2 x10^9/l, platelet nadir 69 x10^9/l). The period of leucopenia lasted 10 days and the development of septicaemia necessitated the administration of gentamicin and piperacillin. Her renal function did not recover and her serum bilirubin increased to 77 mmol/l although ALT and alkaline phosphatase levels returned to normal values. She died 21 days after her final treatment (urea 45.7mmol/l, creatinine 537mmol/l, bilirubin 70mmol/l). Post mortem examination revealed well differentiated mucinous cystadenocarcinoma of the ovary with widespread intraperitoneal tumour and hepatic metastases. A 3cm mass was present in the pelvis posterior to the bladder and in the right subdiaphragmatic area. A 2cm mass was found adjacent to the common bile duct, but there was no evidence of obstruction. Disseminated candidiasis gave rise to granulomata in the lungs, heart and liver but no other hepatic pathology was described. The kidneys showed changes of ischaemic-type tubular necrosis with additional granulomatous abscesses. Marked hypoplasia of all elements in the bone marrow was observed.

Eleven patients died within 3 weeks of treatment but this could not be attributed to any drug associated toxicity and was thought to be due to progressive disease in all cases.

II.4.i Antitumour Effects

Patients were deemed evaluable for response assessment if they had measurable tumour masses (clinically or radiographically) and received two or more courses of treatment. In addition, patients who received only one treatment but who had clearly progressive disease were included. Details of the responses are shown in Table II.18. Antitumour effects were observed at doses as low as 200mg/m^2. The case histories of the patients attaining complete and partial responses are described below.

II.4.j Case Histories of Complete and Partial Respnse Patients

I.G. was a 54 year old woman with stage III poorly differentiated adenocarcinoma of the ovary who had received previous treatment with cis-platinum, melphalan, carboplatin (JM8) and medroxyprogesterone. On beginning treatment with CB3717 she had a large abdomino-pelvic mass causing intestinal obstruction. She received 13 courses of CB3717 (1 at 400mg/m^2, 11 at 450mg/m^2 and 1 at 700mg/m^2 in error) by 1 hour infusions and with prednisolone cover days 2-8, which were well tolerated. Her tumour mass and ascites gradually resolved and after 9 treatments she attained a complete response as documented by abdomino-pelvic ultrasound and clinical examination. Two months after cessation of treatment she relapsed with the development of ascites and multiple abdominal masses and failed to respond to further treatment at a dose of 600mg/m^2, dying of progressive disease 12 days later. Her complete response lasted approximately 16 weeks.

Tumour Type	CR	PR	MR	NC	PD	Total Evaluable
Ovary	1	1	7	11	10	30
Breast	-	3	1	2	2	8
Colon	-	-	2	3	-	5
Adenocarcinoma Bronchus	-	-	1	2	2	5
Large Cell Bronchus	-	1	1	1	-	3
Lymphoma	-	-	-	-	1	1
Mesothelioma	-	1	1	1	2	5
Melanoma	-	-	-	3	9	12
Hepatoma	-	-	-	1	-	1
Primary Unknown	-	-	-	-	1	1
Choriocarcinoma	-	-	-	-	1	1
Fibrosarcoma	-	-	-	-	1	1
Osteosarcoma	-	-	-	-	1	1
Apudoma	-	-	1	-	-	1
Hypernephroma	-	-	-	1	-	1

CR = Complete Response - The disappearance of all known disease for at least four weeks.

PR = Partial Response - 50% or more decrease in total tumour size of the lesions which have been measured to determine the effect of therapy by two observations at least four weeks apart.

MR = Minor Response - 25-50% decrease in total tumour size of the lesions which have been measured to determine the effect of therapy.

NC = No Change - Less than 25% decrease in total tumour size and <25% increase in the size of one or more measurable lesions.

PD = Progressive Disease - A 25% or more increase in size of one or more measurable lesions, or the appearance of new lesions.

TABLE II.18: Responses To CB3717 (One Day Schedule)

S.B. was a 66 year old female with recurrent breast carcinoma and a large left supraclavicular node mass. Treatment with tamoxifen failed and the combination of cyclophosphamide, methotrexate and 5-fluorouracil was poorly tolerated. Two courses of CB3717 at a dose of 400mg/m^2 were given (the second with prednisolone days 2-8) and she gained a partial response three weeks after the first treatment but relapsed six weeks later.

M.D. was a 53 year old female with recurrent breast carcinoma and multiple cutaneous erythematous lumps on her trunk and neck. Prior therapy comprised tamoxifen, aminoglutethimide, mitoxantrone, chlorambucil, prednisolone and vindesine. Apart from a minor response to aminoglutethimide her disease had relentlessly progressed. She received seventeen courses of CB3717 (1 at 140mg/m^2, 2 at 200mg/m^2, 1 at 280mg/m^2, and a further 13 at 200mg/m^2 with prednisolone days 2-8) whilst continuing her part-time job as a nurse. The skin nodules gradually flattened and became paler. After seven courses she achieved a good partial response although cytology of the lesions remained positive (see Figures II.9 and II.10). The response lasted 46 weeks but relapse in the central nervous system became evident 4 months after cessation of CB3717 therapy. Re-treatment was not instituted and she died 4 months later.

B.B. was a 57 year old female with recurrent breast carcinoma who had soft tissue involvement in both breasts and metastatic hepatomegaly. Her disease was resistant to tamoxifen, adriamycin and vincristine. She had completely responded to cyclophosphamide, methotrexate, and 5-fluorouracil combinations initially but on relapse her disease proved refractory to it. She received 3 doses of CB3717 (200mg/m^2 plus prednisolone days 2-8) and after six

weeks she had a greater than 50% reduction in both soft tissue disease and hepatomegaly. Shortly after her third course she became ill at home and died 4 weeks later. The cause of death was thought to be progressive carcinomatosis.

E.M. was a 71 year old female with stage IV ovarian carcinoma. Initial treatment with high dose cisplatinum (100mg/m^2 x5) resulted in a partial response but severely impaired renal function (GFR 35ml/min). The residual disease was resistant to chlorambucil, prednisolone and the platinum analogue, carboplatin (JM8). At the start of CB3717 therapy she had a central abdominal mass and clinical ascites. Ultrasonic examination revealed pelvic and hepatic masses. Treatment with six courses of CB3717 (1 at 200mg/m^2, 1 at 280mg/m^2, 3 at 330mg/m^2 and 1 at 200mg/m^2) caused the disappearance of the abdominal and pelvic masses although a metastasis could still be visualised by ultrasound in the right lobe of the liver. The patient also received continuous prednisolone (20mg daily) which was instituted some months before CB3717 therapy was started. The ascites did not reaccumulate and after three treatments she was classed as a partial responder. Nine weeks after the last dose, she returned with ascites, an abdominal mass and multiple pulmonary metastases which proved resistant to two further doses of CB3717 at 300mg/m^2. She died of progressive disease having sustained a partial response for 18 weeks.

B.E. was a 42 year old female with large cell bronchial carcinoma unresponsive to vindesine. A large subcutaneous tumour mass in the region of the left shoulder shrank by greater than 50% after the first treatment with CB3717 at 300mg/m^2, given by 12 hour infusion. She received one more dose at 300mg/m^2 and one at 360mg/m^2. Her response

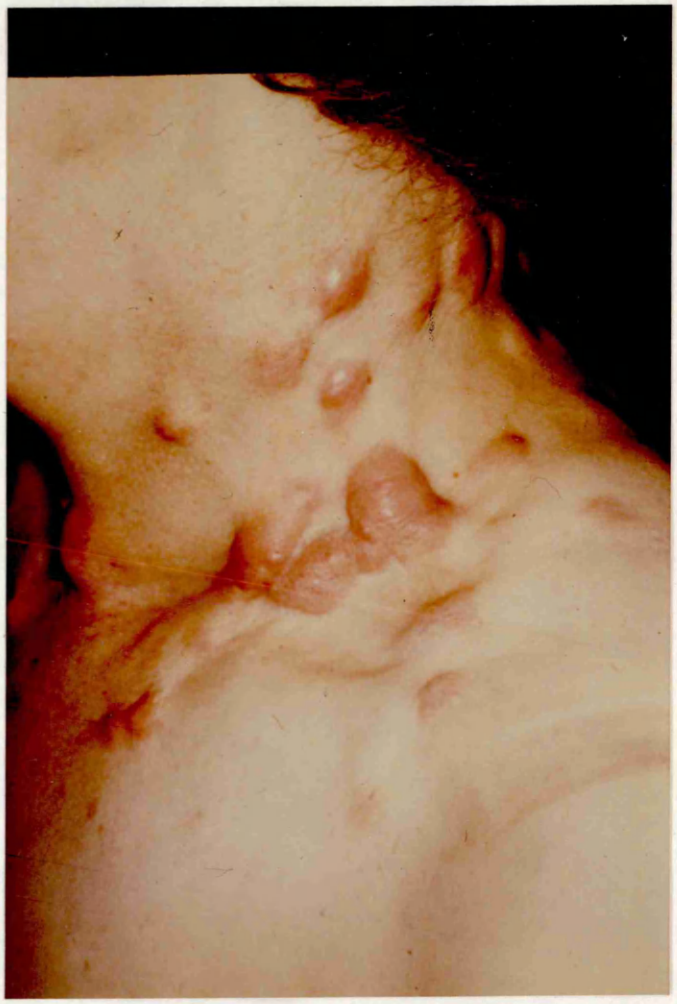


FIGURE II.9: Pre-Treatment Photograph of Patient With Metastatic Breast Carcinoma

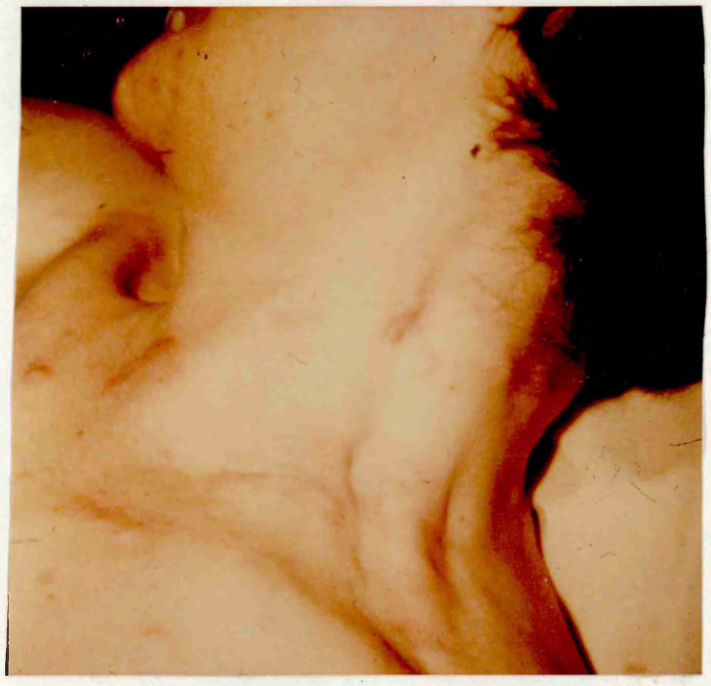


FIGURE II.10: Photograph of Patient With Metastatic Breast Carcinoma After 14 Treatments With CB3717 (140-280mg/m²)

was short-lived with regrowth occurring after 7-8 weeks.

D.A. was a 46 year old man with a pleural mesothelioma diagnosed in November 1981. After a period of disease stasis whilst receiving 14 monthly courses of carboplatin (JM8) enlargement of the mass visualised by chest x-ray led to the start of treatment with CB3717. Two treatments (1 at 400mg/m^2, 1 at 450mg/m^2) resulted in a partial remission of the lung mass as determined radiologically and considerable symptomatic relief. A further 18 treatments at 450mg/m^2 were well tolerated whilst the patient carried out his full time job as a lorry driver. The partial response was maintained for 1 year, following which his disease progressed rapidly leading to his death.

Minor antitumour responses were seen in a further 14 patients (details on Table II.18). The majority of ovarian carcinoma patients had previously received large cumulative doses of cisplatinum, or carboplatin (JM8) followed by an alkylating agent.

II.5 Discussion

The expectation that CB3717 would have toxicities in man quite distinct from those of the classic antifolate, methotrexate, and the reputed thymidylate synthetase inhibitor, 5-fluourouacil, was realised in part, but some similarities were observed.

In the early part of the study, the 5 day treatment schedule proved to be considerably more toxic than the 1 day schedule particularly with respect to the bone marrow. Myelosuppression occurred on repeated dosing even when dose

reduction was instituted. The higher cumulative dose administered of 500mg/m^2 - 700mg/m^2 is one probable explanation. Hepatic toxicity and associated malaise were troublesome in both regimens but more severe constitutional disturbance was apparent after 5 daily treatments. For all these reasons the 5 day schedule was abandoned as it seemed likely that the starting dose of 100mg/m^2 x5 was near to the maximal tolerated dose of this regimen. The appearance of antitumour effects early in the 1 day schedule (200mg/m^2) influenced this decision.

It is impossible to be emphatic in any statement concerning the renal toxicity caused by CB3717 as patients varied considerably in their susceptibility. Doses of 200-400mg/m^2 were generally associated with modest reductions in GFR (mean 17%). At levels of 500-600mg/m^2, precipitous reductions (>50%) occurred on 3 out of 10 occasions, suggesting that nephrotoxicity was dose limiting using this schedule of drug administration. The perturbations in urinary enzyme levels observed are further evidence for this. Tubular precipitation of CB3717 due to poor solubility at acid pH is considered the most likely mechanism of renal damage with high dose methotrexate providing a precedent (Stoller R.G. et al 1975, Jaffe N. & Traggis D. 1975, Milano G. et al 1983) and mouse toxicology studies supporting the theory (see Chapter I, and Newell DR et al 1982). This issue will be discussed further in Chapter III. Other mechanisms of toxicity to the kidney may also be implicated.

The occurrence of drug induced hepatotoxicity necessitated caution in dose escalation, bearing in mind the unpredictable syndrome of liver failure which can ensue, as described with several drugs including halothane and isoniazid in combination with rifampicin (reviewed by Pessayre D. et al 1977, Sherlock S. 1981). In this study, the disturbance of liver function was always reversible and repeated courses appeared to induce tolerance. Patients with very high levels of alkaline phosphatase were more prone to develop hyperbilirubinaemia than those with normal levels (10 out of 18 had pre-treatment alkaline phosphatase >300IU/l). In the four patients who were treated for longer than 6 months there was no biochemical evidence of cumulative toxicity.

Methotrexate can also cause hepatic toxicity which presents differently depending on the dose schedule (Hersh E.M. et al 1965, Djerassi I. et al 1967, Gottlieb JA & Serpick A.A. 1970,). In particular, high dose methotrexate causes a pattern of liver enzyme abnormalities reminiscent of those seen with CB3717 (Rosen G. et al 1974) but which appears to be dose related in indicence and severity (Pratt C.B. et al 1975, Perez C. et al 1979,). Reports of associated malaise are rare, but Rosen et al describe anorexia in all patients receiving high dose methotrexate (100-750mg/kg via 4 hour infusion) all of whom had some degree of ALT elevation (Rosen G. et al 1974). However other authors (Hersh E.M. et al 1966) stated that no symptoms or signs could be correlated wtih abnormal liver function tests.

Treatments involving infusions of 5-fluorouracil and FUdR directly into the hepatic artery have been reported as causing chemical hepatitis in 10% and 52% of patients

respectively (Oberfield R.A. et al 1979). CB3717 hepatotoxicity is discussed more fully in Chaper V which describes the investigations into the possible mechanisms involved. The two therapeutic manoeuvres pursued clinically (prolongation of the infusion to 12 hours and administration of prednisolone) were clearly of no benefit in averting elevation of transaminase levels but some improvement in patient "well-being" was apparent with steroid treatment Objective assessment of malaise is difficult and although the symptoms described could be entirely attributable to liver disturbance the contribution of other CB3717 induced biochemical abnormalities has to be considered. In particular, disturbance of histamine metabolism, which is a feature of some antifolates especially DDMP (metoprine, 2,4-diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine) and TMQ (trimethyoxybenzylaminoquinazoline) (Duch D.S. et al 1978, Duch DS, et al 1980), could play a role in causing anorexia, mild nausea and dermatological toxicity. Chapter IV deals with this subject in more depth.

The rather unpredictable occurrence of myelotoxicity after CB3717 is comparable to that induced by methotrexate at low doses and had a similar time course. Hansen et al concluded that the individual toxic dose of a single 1 hour infusion of methotrexate, in a series of 49 patients, varied by a factor of 18 (50-900mg/m^2, median 120mg/m^2) (Hansen H.H. et al 1971). In the present study with CB3717, reduced renal function and severely impaired hepatic function seemed to be risk factors for myelotoxicity.

Methotrexate also causes rashes in 10-15% of patients which may be unassociated with other toxicities (Bertino J.R. 1981b) and can give rise to erythema at previous irradiation sites (Jaffe N. et al 1975). This effect is

comparable to that of CB3717.

Overall, with regard to toxicity, CB3717 behaves in a manner much more similar to methotrexate than to 5-fluorouracil or FUdR. These pyrimidine analogues produce more consistent and predictable myelotoxicity and stomatitis along with gastrointestinal disturbances (anorexia, nausea and vomiting and diarrhoea) (Heidelberger C. 1982). Stomatitis and gastrointestinal toxicity are also features of methotrexate treatment (reviewed by Bertino J.R. 1981b). In this respect CB3717 differs from both these compounds causing mucositis and diarrhoea rarely which correlates with the preclinical rationale for its development.

The antitumour responses observed were encouraging and somewhat surprising considering the population under study. Further evidence of the activity of CB3717 will be sought in Phase II trials. In addition, the early relapse of several patients and their failure to respond on retreatment with CB3717 prompted the study of mechanisms of drug resistance. Here the development of a resistant L1210 mouse leukaemia cell line proved helpful and this work is elaborated in Chapter VI.

II.6 Summary

The administration of CB3717 in 0.15M sodium bicarbonate as a 1 hour intravenous infusion at 3 weekly intervals has a maximal tolerated dose in the range of 550-600mg/m^2 at which renal toxicity becomes dose-limiting. A recommended dose for use in Phase II trials is 400mg/m^2 in patients with GFR >60ml/min, which is relatively well tolerated. Careful evaluation of renal function prior to and during therapy is recommended especially as some decline

in renal function (<20%) was observed in most patients receiving CB3717. Hepatic toxicity occurs frequently and requires monitoring by assessment of liver function tests at least twice between treatments. Tolerance may develop after several courses. Dose reductions to 300mg/m^2 should be instituted when the pre-treatment GFR is less than 60ml/min or alkaline phosphatase levels are greater than twice the upper limit of normal. Phase II trials are already in progress at three centres in the UK (RMH Sutton & London, Glasgow and Newcastle) using these recommended doses.

There are several possibilities for future study with this new antifolate in the field of clinical cancer. The use of higher doses of CB3717 in combination with hydration or alkalinisation regimens in an effort to avoid renal toxicity is one obvious scheme. Exploration of protocols employing lower doses given on five consecutive days or on a weekly basis would also be logical. However the most outstanding and pertinent question still to be answered concerns the ability of CB3717 to establish a 'thymineless' state in man in association with its antitumour effect. It was not possible to do this within the Phase I trial as the methodology for measuring plasma nucleoside levels and intracelullar nucleotide pools was incompletely developed. Fortunately, current studies are now able to address this problem.

CHAPTER III

THE CLINICAL PHARMACOKINETICS OF CB3717

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III.1 Introduction

The importance of pharmacokinetic evaluation during early clinical studies with a new anticancer drug has been discussed by many authors (Nichol C.A. 1977, Erlichman C. Donehower R.C. & Chabner B.A. 1980). It is widely believed that this is the most useful stage at which to establish the absorption, distribution, elimination and metabolism of the drug in man. Knowledge of these factors may suggest ways in which to alter administration schedules to improve the therapeutic index and may help define groups of patients in whom dose modification would be prudent. This is particularly relevant to a population of cancer patients where renal and hepatic function are frequently impaired.

The development of a sensitive high performance liquid chromatography (HPLC) assay for CB3717 in plasma, urine and faecal homogenates led to the study of its pharmacokinetics in mice and rats (Newell D.R. et al 1982). In these species biliary excretion was the major route of drug elimination. The desglutamyl metabolite, CB3751 was detected in faecal extracts and was attributed to colonic bacterial action as only the parent compound was detected in plasma and urine samples (Newell D.R. et al 1982, Newell D.R. et al 1983). With the discovery of hepatic dysfunction as the most prevalent toxicity in man, further experiments in rats established that a dose related reduction in bile flow rate occurred (Newell D.R. et al 1982). A precipitate in the bile was also observed which raised the possibility of this being either the parent drug or a related product.

Extrapolation to man begged a trial of the prolongation of the infusion time from 1 to 12 hours in an attempt to reduce peak plasma drug levels and avert the presumed biliary stasis and thus perhaps avoid hepatic toxicity. The pharmacokinetics of both 1 hour and 12 hour intravenous infusions of CB3717 in man were investigated therefore, and attempts to correlate the pharmacokinetic parameters with the toxicities have been made. Finally a study of the urinary solubility of CB3717 within a wide range of pH values was undertaken to establish support for the proposal of drug precipitation in the kidney tubules as the mechanism of renal toxicity.

III.2 Materials and Methods

III.2.a Chemicals

CB3717 was supplied by ICI Pharmaceuticals (Alderley Park, Macclesfield, Cheshire) and CB3751 (4-(N-((2-amino-4-hydroxy-6-quinazolinyl)methyl)prop-2-ynylamino)benzoic acid) by Dr. T.R. Jones (Institute of Cancer Research, Sutton, Surrey). All other reagents were analytical grade and were obtained from Fisons (Loughborough, Leics.), BDH Chemicals (Poole, Dorset) or East Anglia Chemicals (Hadleigh, Ipswich, Suffolk).

III.2.b Patients, Protocol and Sample Collection

Verbal consent was obtained from patients selected for study whose characteristics are presented in Table III.1. Anti-emetics were not routinely used and analgesic preparations were avoided where possible for the 24 hour period of study. Plasma levels of CB3717 were measured in twenty different patients (sixteen following 1 hour

Patient	Dose (mg/m^2)	i.v. Infusion Schedule hrs x days	Treatment Course	Pre Treatment			Post Treatment
				GFR ml/min	Liver Function Tests Alanine Transaminase (normal <27 IU/l)	Alkaline Phosphatase (normal <95IU/l)	Peak Rise in Alanine Transaminase (IU/l)
1	100	1 x 5	1	138	↑	↑	-
2	100	1 x 5	1	99	↑	N	-
3	140	1 x 5	1	66	N	N	-
4	140	1 x 5	1	-	N	N	-
5	300	1	1	128	↑	↑	133
6	300	1	1	72	N	N	21
7	300	1	1	89	N	↑	55
8	300	1	3	48	↑*	↑	-
9	300	1	1	49	N	N	157
10	330	1	1	124	N	N	116
11	450	1	1	61	N	N	4
12	500	1	8	76	N	N	-
13	500	1	1	100	N	↑	152
14	500	1	1	142	N	↑	57
15	500	1	1	76	N	N	35
16	550	1	1	65	N	↑	221
17	550	1	1	117	N	↑	355
18	300	12	1	172	N	↑	435
10	330	12	2	107	↑*	↑	-
19	330	12	7	31	↑*	N	-
20	330	12	4	64	↑*	N	-

N = Normal; ↑ = Raised; * = CB3717 induced elevation ALT

TABLE III.1: Characteristics of Patients Undergoing Pharmacokinetic Evaluation During Treatment With CB3717

infusion, three following 12 hour infusion, one following both 1 and 12 hour infusion). Blood samples (5-10ml) were collected into heparinised tubes from an intravenous cannula sited in the contralateral arm from that receiving the drug infusion. Patency was maintained by the injection of 1ml heparin (10 units/ml) after each sampling. The diacid of CB3717 was dissolved in 250ml or 1 litre of 0.15M Sodium bicarbonate ($NaHCO_3$), adjusted to pH9.0 for infusion over 1 or 12 hours respectively. Sample collection times were as follows: 1 hour infusion - pre, mid, end infusion, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, and 23 hours post infusion. 12 hour infusion - pre, mid, end infusion, 0.5, 1.0, 6, 12 and 24 hours post infusion. Plasma was separated immediately by centrifugation and stored frozen (<-20°C).

Urine samples were taken from random pre-treatmemt specimens and 24 hour collections of urine were made, starting at the time of treatment, on 57 occasions (31 following 1 hour infusion in twenty five patients and 26 following 12 hour infusions in twenty one patients). 20ml aliquots were taken from the 24 hour urine samples and stored frozen (<-20°C). An incomplete collection of faeces was made from one patient (No. 4) during a 5 day treatment schedule. Cerebrospinal fluid (CSF) was also acquired from this patient by lumbar puncture one hour after the completion of his drug infusion on day 1. Post mortem tissue and bile samples were retained for analysis from a patient who died from progressive disease 8 days after his second dose of CB3717 at 330mg/m^2. In addition, 15ml volumes of ascitic fluid were aspirated from a patient with ovarian carinoma at 1,2,4,6 and 23 hours after a 1 hour i.v. infusion, and were stored frozen (<-20°C).

III.2.c Sample Preparation

All samples were thawed at room temperature.

URINE - The samples were adjusted to pH 9.0 by the dropwise addition of 1M Sodium hydroxide (NaOH) to ensure the complete dissolution of any CB3717 present.

FAECES - The total faecal collection was weighed, diluted to a 20% (w/v) mixture with 0.1M trishydroxymethyl ammonium-HCL buffer pH 9.0, left for 24 hours at 4^{o} and then stirred for 30 minutes.

TISSUES - The tissues available from post mortem (primary tumour, metastatic tumour, small bowel, spleen, pancreas, kidney, bone marrow, liver, thyroid, cerebellum, cerebrum, myocardium, skin, adipose, large bowel) were homogenised in a teflon glass homogeniser in 0.1M trishydroxymethylammonium-HCL buffer pH 9.0 as 5% (w/v) homogenates.

Two volumes of methanol were added to one volume (routinely 1ml) of plasma, CSF, urine, faecal and tissue homogenates, bile and ascitic fluid. These were mixed thoroughly and centrifuged at 1,000g for 5 minutes at 4^{o}. The supernatants were decanted into glass vials for analysis by HPLC within 12 hours.

III.2.d HPLC Analysis

All HPLC analyses were performed on a Waters Associates Chromatograph (Waters Associates, Northwich, Cheshire) which consisted of two model 6000A HPLC pumps, a model 660 solvent programmer, a model 440 fixed wavelength detector (254nm and

280nm and a model 710 (A or B) Waters Intelligent Sample Processor. The outputs from the detectors were recorded on a standard chart recorder and, for some analyses, on an automatic integrator (Trivector Ltd., Sandy, Beds,). Peaks were measured either by peak height, determined manually, or by peak area, determined by automatic integration. HPLC solvents were filtered and degassed prior to use by passage through a 0.45 uM filter; polypropylene backed PTFE for organic solutions and cellulose nitrate for aqueous solutions (Whatman Ltd., Maidstone, Kent).

Separations were performed on a uBondapak C18 column (25 x 0.46cm, Waters Associates) fitted with a CO:Pell ODS precolumn (6.5 x 0.21cm, Whatman Ltd.). Samples were eluted isocratically at a flow rate of 2ml/min with a solvent mixture containing, by volume, 1 part glacial acetic acid, 64 parts water and 35 parts methanol. Sample volumes were within the range 10-50ul.

Recovery of CB3717 from human plasma was essentially complete (94.4 $\pm$ 3.4% $\bar{x}$ $\pm$ SD) and linear (r=>0.999) over the concentration range 2-400uM (coefficients of variation; 20uM = 3.5%, 100uM = 5.3%, 200uM = 2.2%). Similarly, CB3717 estimation in urine was linear over the range 2-100uM (r=>0.999) (coefficients of variation; 20uM = 4.1%, 100uM = 1.0%, 200uM = 1.0%). Quantification of CB3717 and CB3751 in CSF, ascites, kidney and faecal homogenates was achieved by comparison with standard solutions dissolved in 0.15M $NaHCO_3$, pH 9.0 and recovery taken as 100%. Chromatograms illustrating the analysis of CB3717 in plasma are shown in Figure III.1. Peaks present in the HPLC effluent were identified by co-chromatography with CB3717 and CB3751 standards and by their 254nm/280nm absorbance ratios.

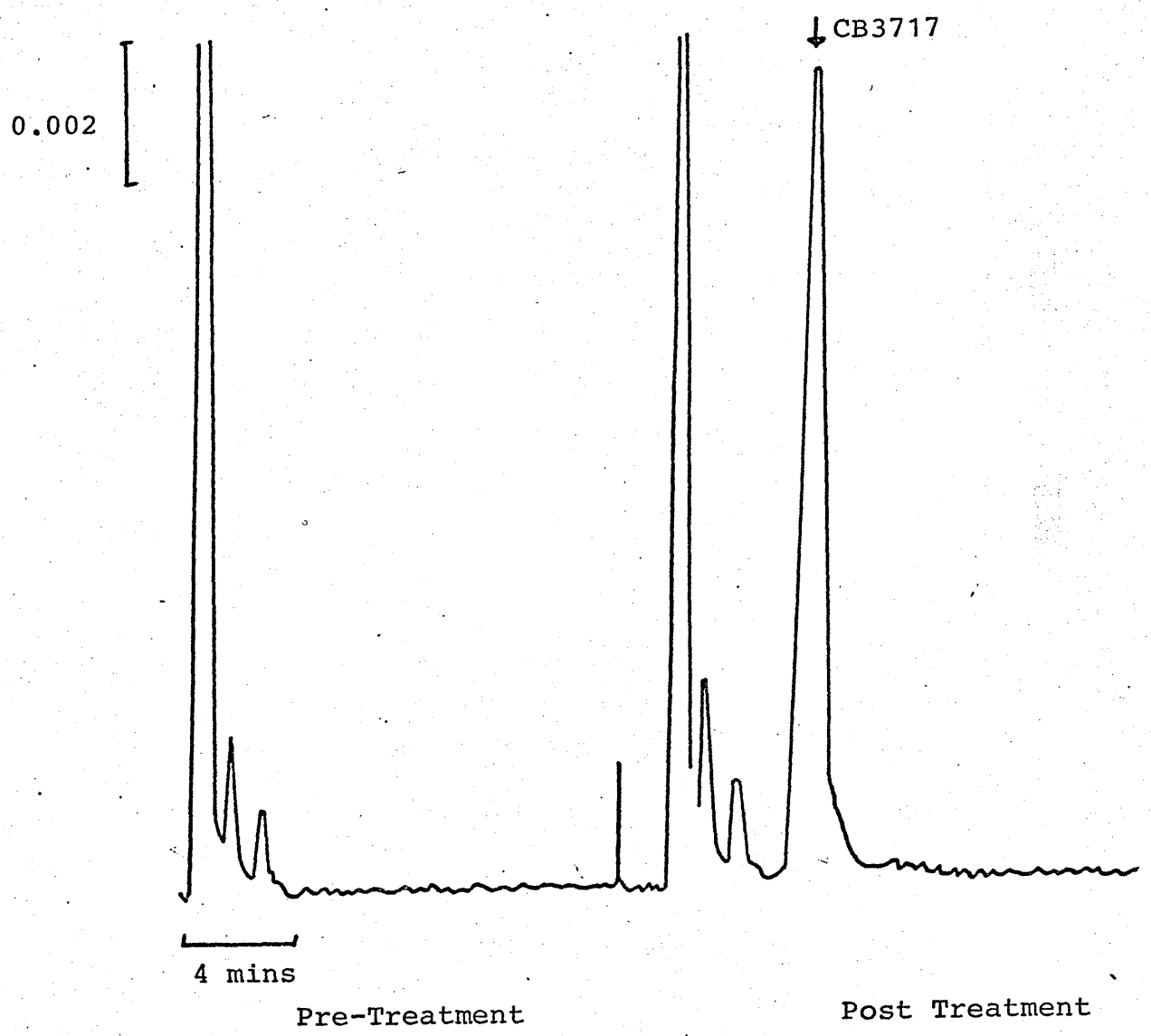


FIGURE III.1: HPLC Trace of Plasma Samples Pre and Post CB3717 Treatment by 1 Hour i.v. Infusion of 400mg/m² (Wavelength 280 nm)

III.2.e Protein Binding Estimation

20ml of plasma was separated from blood venesected from a normal volunteer. Standard solutions of CB3717 in 0.15M sodium bicarbonate, adjusted to pH 9.0, were further diluted in 1ml aliquots of 0.15M sodium bicarbonate or 1ml aliquots of plasma to give final concentrations of 1uM-200uM. These bicarbonate and plasma dilutions were ultrafiltered using an AMICON micropartition system with YMT membranes (Amicon House, Woking, Surrey). Centrifugation was at 1500g for 10 min at 15°. The ultrafiltrates were then analysed by HPLC.

III.2.F Urine Solubility Studies

Urine was collected from a healthy volunteer (pH 5.54). The pH was adjusted to 3.0 by the addition of concentrated hydrochloric acid (approximately 5 drops/100ml urine). The solid diacid of CB3717 was added to 100ml of this urine and mixed to produce a super-saturated solution from which a 7 ml aliquot of the suspension was decanted into a test tube. The remaining urine suspension was adjusted to pH 4.0 with 5M, 1M and 0.1M sodium hydroxide and a further aliquot was removed. This process was repeated adjusting the pH by increments of 1.0 up to pH 9.0, with addition of further CB3717 solid to ensure a super-saturated solution. Duplicate samples were prepared by repetition of the whole procedure using a fresh urine collection. The aliquots of CB3717 suspension were then centrifuged at 2,000 r.p.m. for 10 minutes at 4° and the resulting supernatants were filtered through 0.22uM MILLEX-GS filters (Millipore) into WISP vials. A standard solution of 1mg/ml CB3717 in 0.15M sodium bicarbonate was made and HPLC analysis was performed on 5ul sample volumes using the method previously described.

The solutions remained clear throughout the analysis.

III.2.g Pharmacokinetic Analyses

Following the end of the infusion plasma levels of CB3717 were fitted to a two compartment open model using a non-linear least squares analysis (Sampson J. 1969). The data were weighted either with the function $(1/\hat{y} + yi)^2$ or with the function $1/(\hat{y})^2$ (Ottaway J.H. 1973, Storer A.C. Darlison M.G. & Cornish-Bowden A. 1975). The values obtained for the two-compartment model were corrected for the infusion period as described by Loo and Riegelman (Loo J.C.K. & Riegelman S. 1970). From the corrected values the alpha and beta phase half lives, volume of the central compartment, steady state volume of distribution, total plasma clearance, area under the plasma concentration versus time curve (AUC) and the rate constants k_{12}, k_{21} & k_{el} were calculated using the equations described by Wagner (Wagner J.C. 1975). In two patients, following a 12 hour infusion, a one compartment model was fitted to the data.

III.3 Results

III.3.a CB3717 Plasma Pharmacokinetics

Following a 1 hour infusion of CB3717, plasma levels decayed biphasically with first order kinetics as shown in Figures III.2, III.3 and III.4, where the computer fits of the plasma decay curves from patients treated at doses of 100-500mg/m^2 are depicted. The plasma pharmacokinetic parameters determined for 17 patients are given in Table III.2. Peak plasma CB3717 concentration correlated with dose ($r=0.839$ $p=0.01$) and there was a weak correlation with plasma AUC ($r=0.473$ $p=0.05$) (see Figures III.5, III.6).

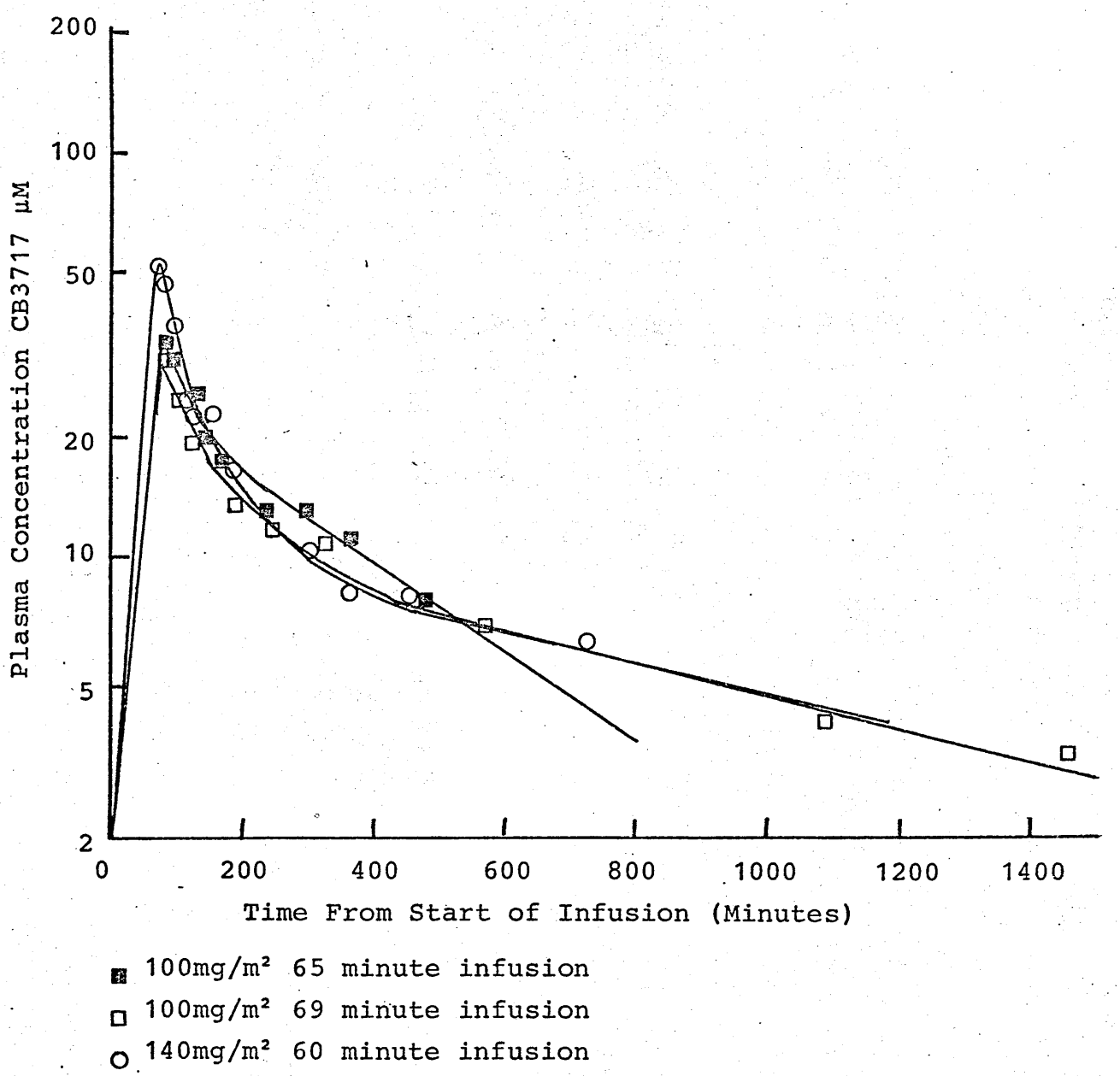


FIGURE III.2: Plasma Levels of CB3717 in Three Patients After An Infusion of 100-140mg/m²

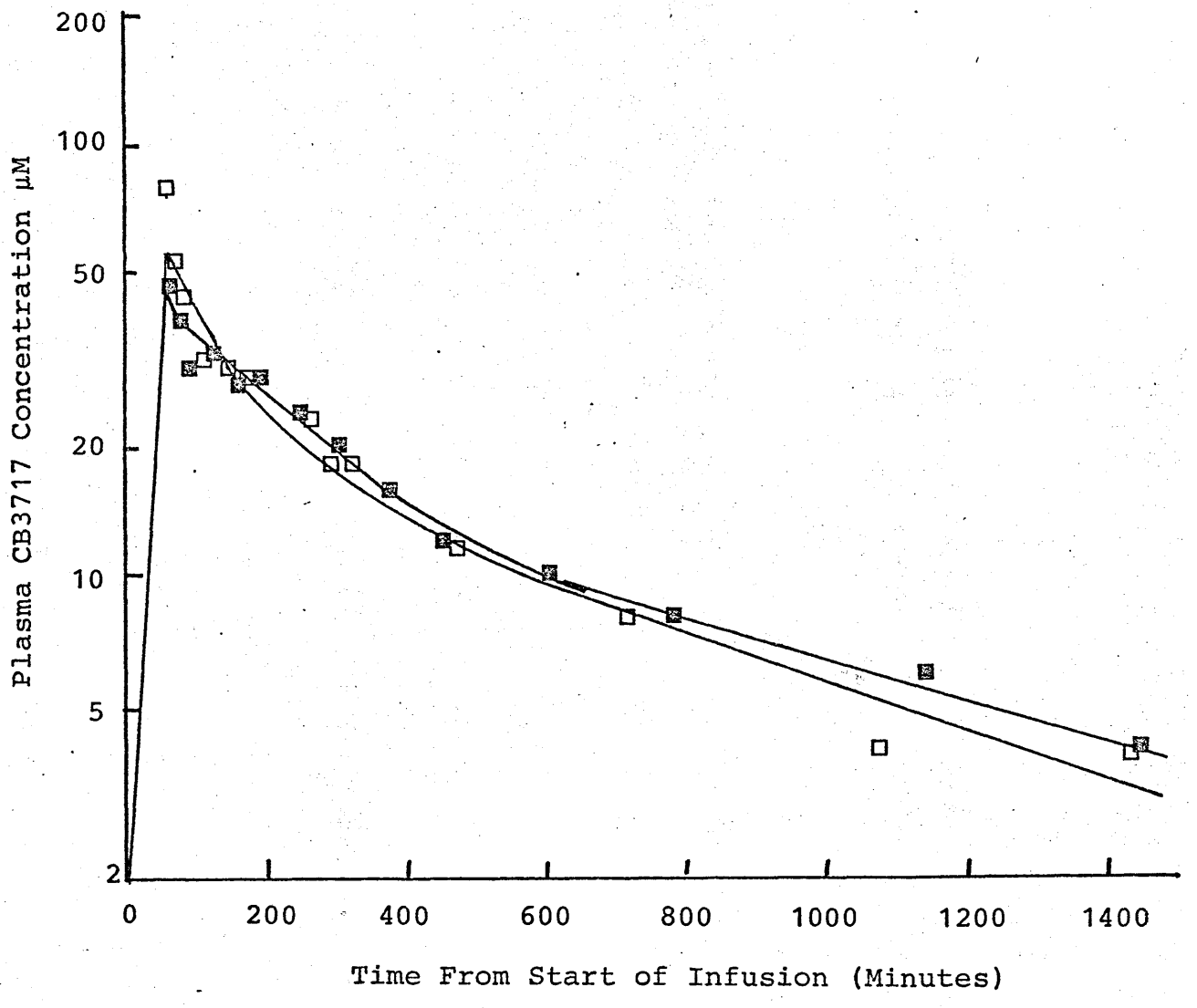
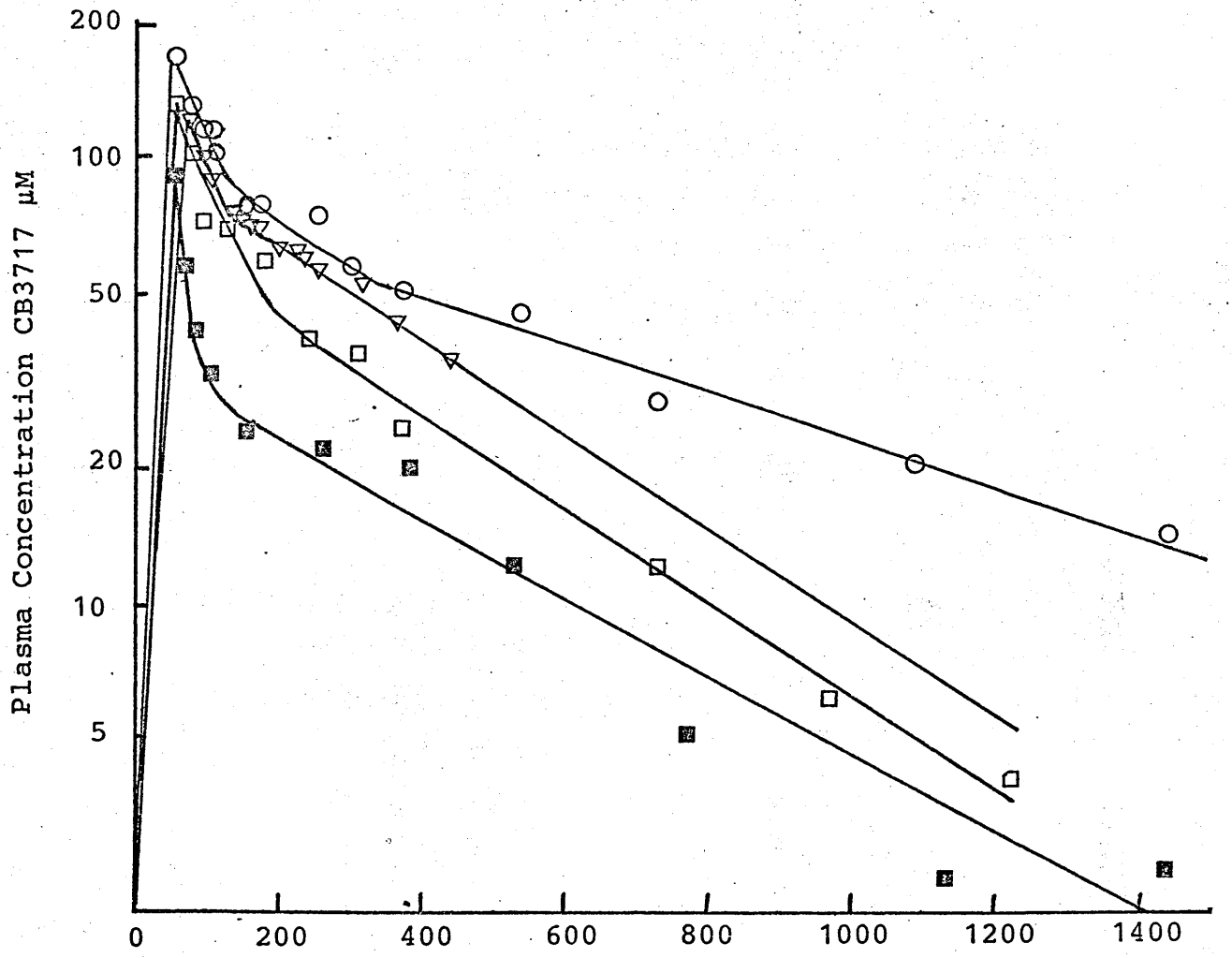


FIGURE III.3: Plasma Levels of CB3717 In Two Patients After An Infusion of 285-330mg/m²



■ 450mg/m² 50 minute infusion

□ 500mg/m² 60 minute infusion

▽ 500mg/m² 80 minute infusion

○ 500mg/m² 60 minute infusion

Lines are computer generated fits

FIGURE III.4: Plasma Levels of CB3717 In Four Patients After An Infusion of 450-500mg/m²

Patient	Dose mg/m^2	$t_{\frac{1}{2}}\alpha$ (min)	$t_{\frac{1}{2}}\beta$ (min)	Vi (l/m^2)	V_D (l/m^2)	CLR $(ml/min/m^2)$	AUC $(uMxhr/m^2)$	Peak Plasma $Conc^n$ (uM)	K_{el} min^{-1}	K_{12} min^{-1}	K_{21} min^{-1}
1	100	32	301	3.4	6.7	17.8	98	42			
2	100	58	770	4.6	12.6	13.7	128	39			
3	140	43	770	3.8	14.7	17.0	192	53			
4	140	96	658	6.7	13.9	19.8	144	45			
5	300	131	767	13.0	23.8	26.6	235	50	0.00205	0.00187	0.00225
6	300	109	1188	7.5	16.4	11.1	533	90	0.00149	0.00296	0.00249
7	300	87.5	3850	7.2	49.3	10.6	541	76	0.00146	0.00566	0.00098
8	300	14.5	747	2.8	9.9	9.7	855	128	0.00342	0.03230	0.0129
9	300	20	385	3.1	7.9	15.9	361	114	0.00505	0.01885	0.01225
10	330	63	533	8.9	19.8	32.7	177	81	0.00365	0.00473	0.00392
11	450	13.3	341	4.6	19.4	46.4	215	92	0.00994	0.0336	0.01065
12	500	14.8	289	4.1	9.3	23.9	201	123	0.00576	0.0258	0.0208
13	500	36	577	4.7	10.2	13.5	924	175	0.00286	0.00937	0.00796
14	500	33	289	5.7	11.2	31.2	304	142	0.00551	0.00874	0.00911
15	500	7.8	383	4.3	12.8	24.3	429	96	0.00563	0.0565	0.0286
16	550	49.5	364	6.1	10.3	22.3	581	155	0.00368	0.00499	0.00722
17	550	23.4	350	3.4	8.2	18.3	543	203	0.00541	0.0154	0.0108
	$\bar{x} \pm$ S.E.	48.9 ±8.9	738.9 ±203.4	5,52 ±0.63	15.1± 2.42	20.9± 6.2					

TABLE III.2: CB3717 Plasma Pharmacokinetic Parameters in Man Following a One Hour Infusion

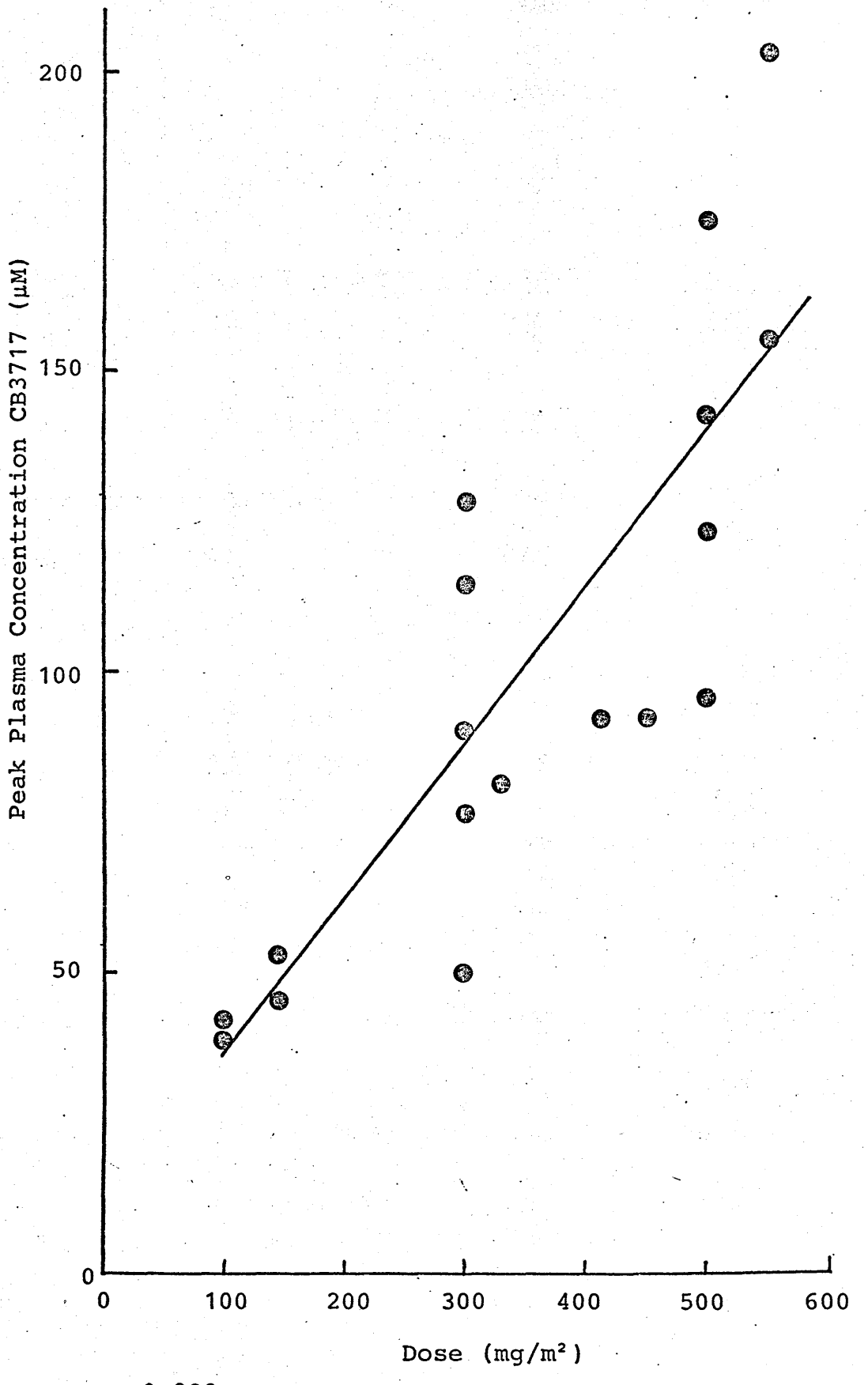


FIGURE III.5: Correlation Between Peak Plasma CB3717 Concentration and Dose

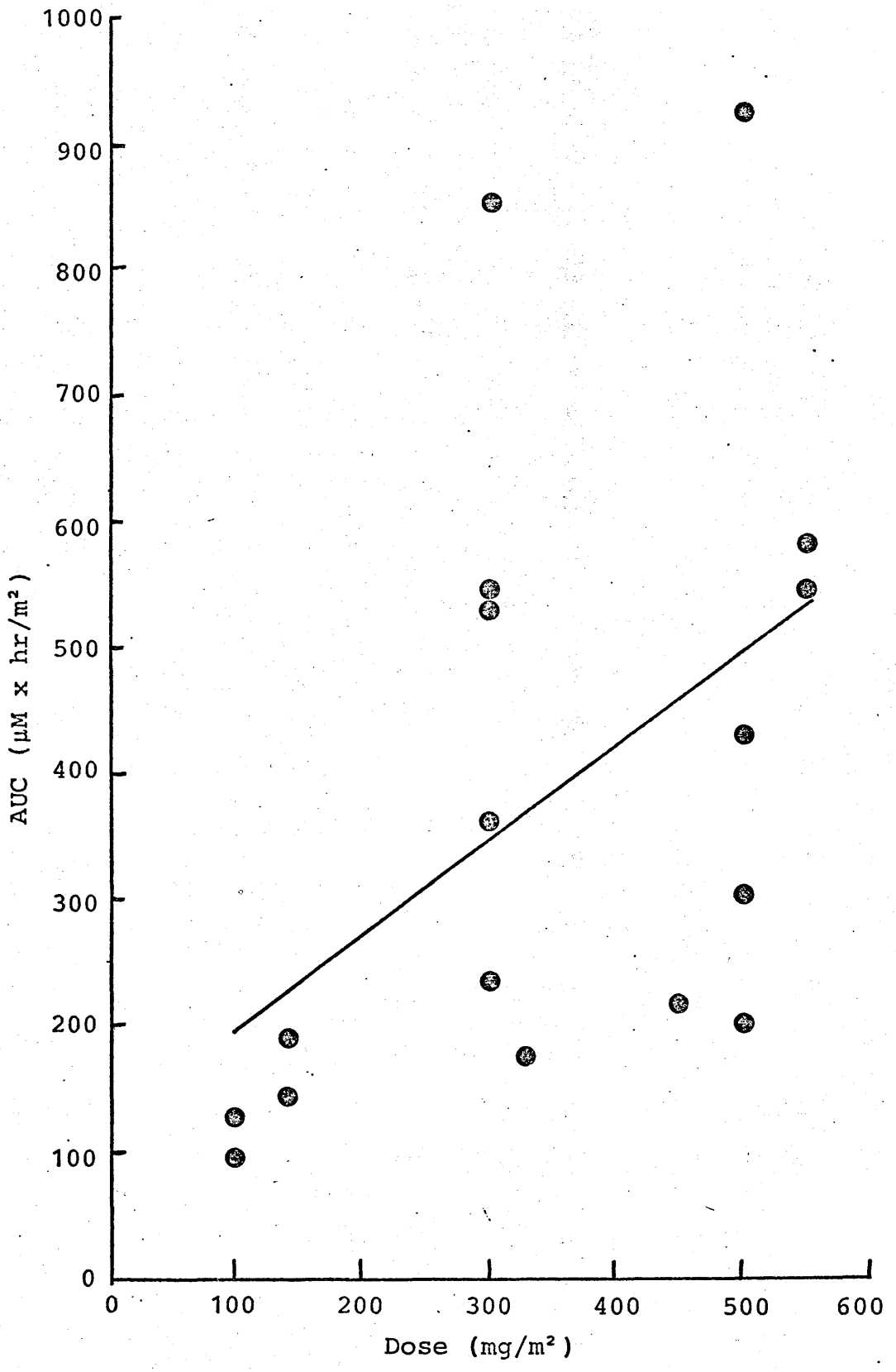


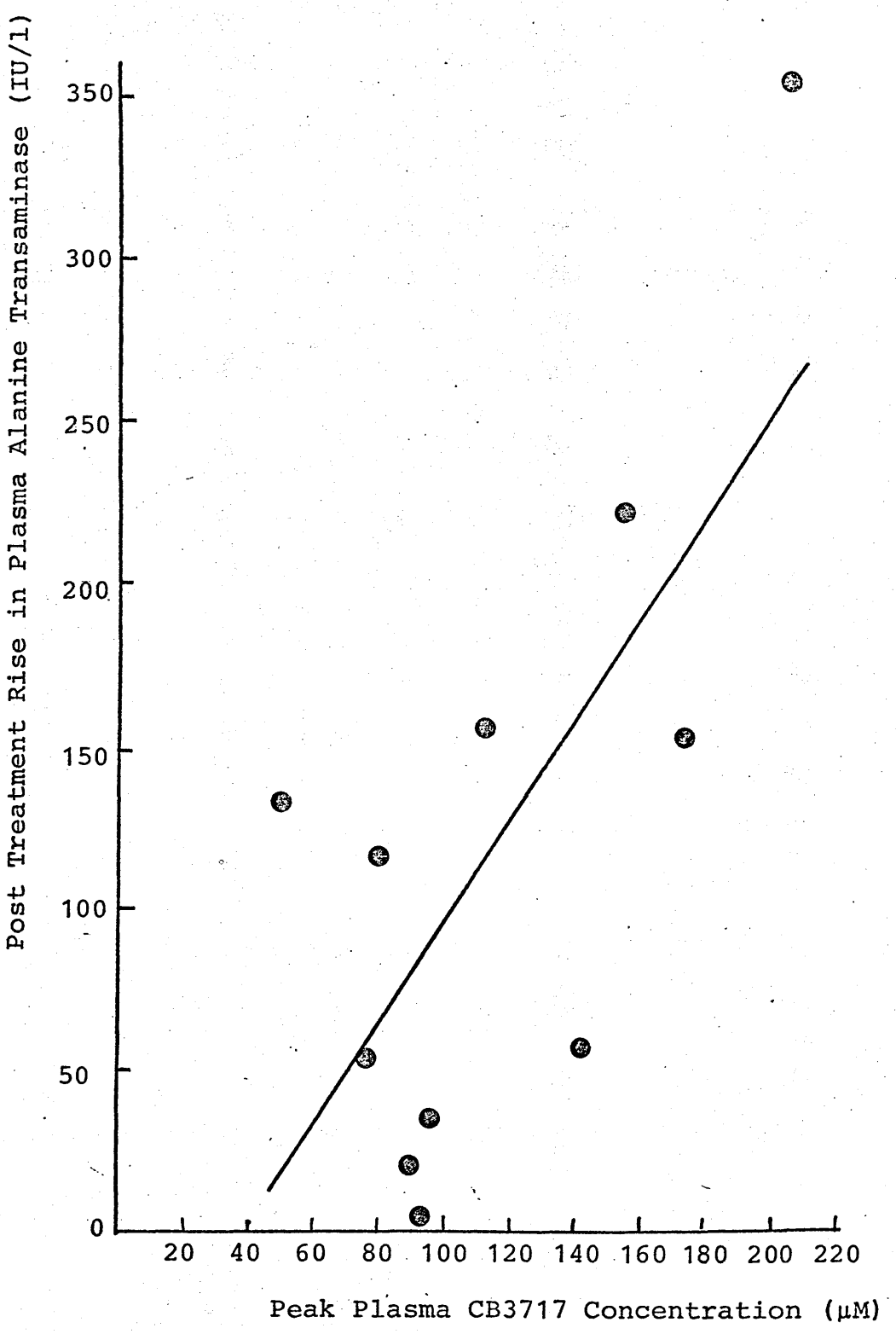
FIGURE III.6: Correlation Between Plasma AUC and Dose in Patients Treated With CB3717

Other parameters were independent of dose. In the eleven patients who were studied during their first course of CB3717 and who received only a single hour infusion, hepatic toxicity, as measured by the peak rise in plasma alanine transaminase level, post treatment (see Table III.1) showed a weak positive correlation with peak plasma CB3717 concentration ($r=0.69$ $p=0.02$, see Figure III.7). There was no correlation between hepatic toxicity and any of the other parameters determined, including k_{21}, k_{12}, k_{el}.

An example of the plasma levels of CB3717 in one of the four patients studied following a 12 hour infusion are shown in Figure III.8. Comparison of Table III.2 and III.3 reveals that the beta phase half lives and total plasma clearances are similar despite the prolonged infusion time. In patient 10, extending the drug infusion time from 1 to 12 hours and using the same total dose decreased the plasma concentration from 81uM to 32uM.

III.3.b CB3717 Excretion and Tissue Distribution

The cumulative urinary excretion data (Table III.4) implied that renal excretion was not the major elimination route for CB3717 with only $27\pm2\%$ of the amount administered excreted in the urine over the first 24 hour period, regardless of the dose. Strict correlation of CB3717 renal clearance with pre-treatment glomerular filtration was not possible as no analysis was made of CB3717 plasma protein binding in individual patients. However, the 24 hour urinary excretion of CB3717 after 57 doses was weakly correlated with pre-treatment GFR and reached significance because of the large numbers studied ($r=0.374$ $p=0.01$, Figure III.9).



r=0.690
p=0.02

FIGURE III.7: Correlation Between Peak Plasma CB3717 Concentration and Rise In Plasma Alanine Transaminase

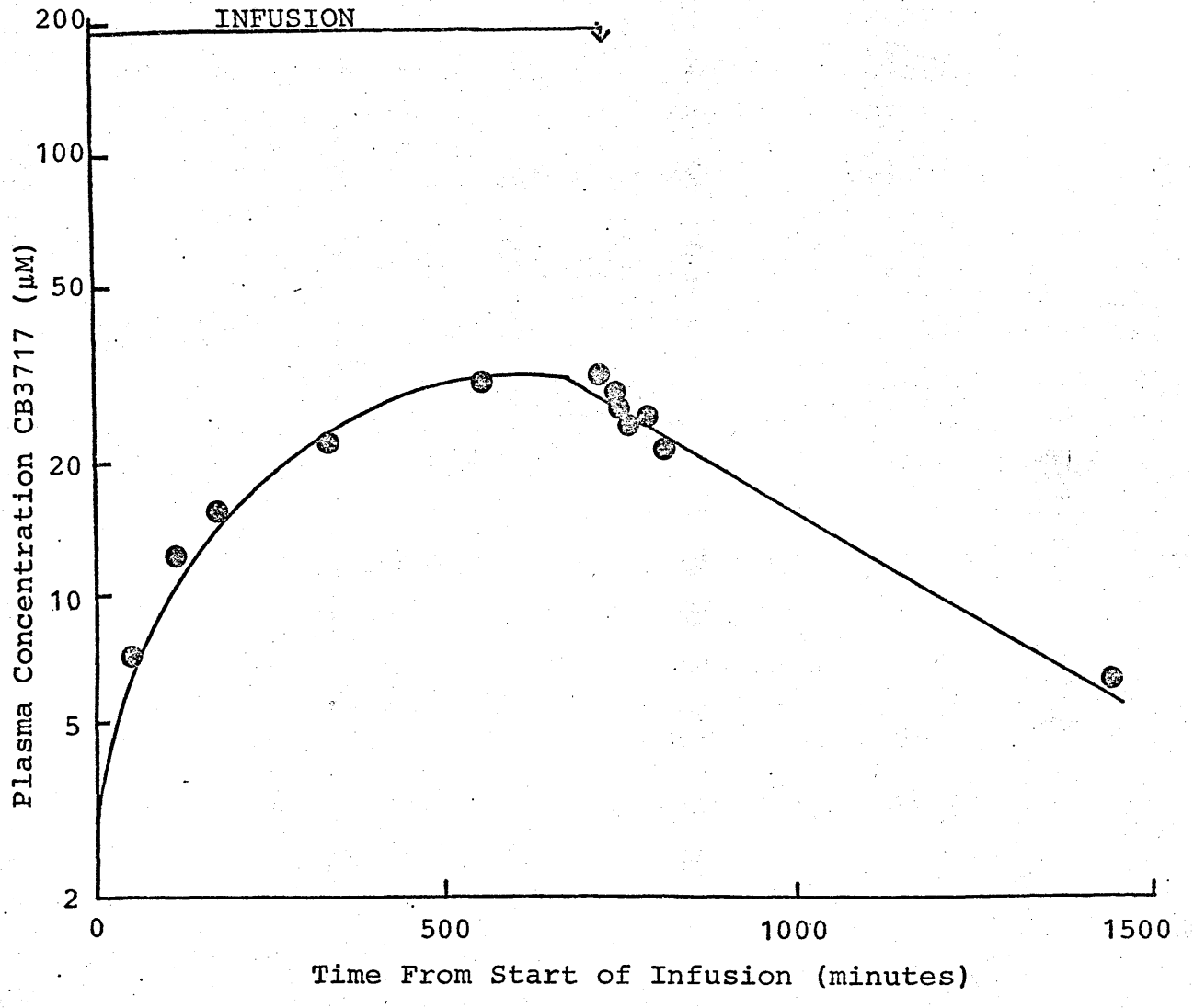


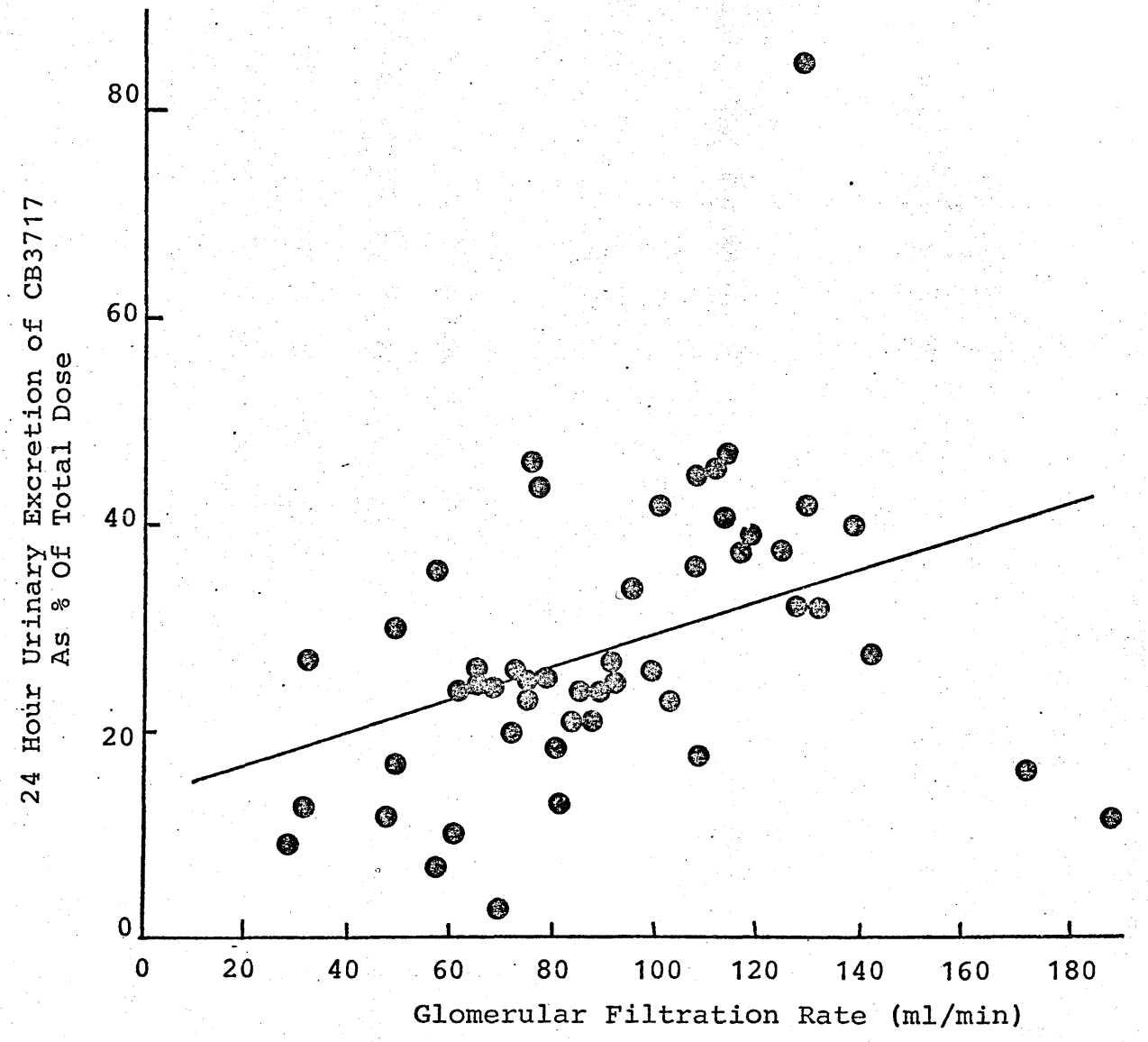
FIGURE III.8: Plasma Levels Of CB3717 During And After A 12 Hour Infusion Of A Dose Of 330mg/m² (The Post Infusion Line is The Computer Generated Fit)

Patient	Dose mg/m^2	$t_{\frac{1}{2}\alpha}$ (min)	$t_{\frac{1}{2}\beta}$ (min)	Vi_2 (l/m^2)	V_{D2} (l/m^2)	CLR $(ml/min/m^2)$	AUC $(uMxhr/m^2)$	Peak Plasma $Conc^n$ (uM)
18	300	121	990	10.2	26.2	33.4	165	17
10	330	-	323	-	12.1	26.1	221	32
19	330	63	866	1.9	6.6	7.3	928	75
20	330	-	770	-	8.5	7.7	691	59
$\bar{x} \pm$ S.E.			737±145		13.3±4.4	18.6±6.6		

TABLE III.3: CB3717 Plasma Pharmacokinetic Parameters In Man Following a 12 Hour Infusion

Dose (mg/m^2)	Urinary Excretion of CB3717 (% dose administered ± S.E.M.)	
	1 Hour Infusion	12 Hour Infusion
100	23 ± 10.9 (n=3)	-
140	26.5 ± 11.4 (n=3)	-
300	33.8 ± 7.6 (n=9)	28.5 ± 4.7 (n=13)
330	32 ± 4.4 (n=4)	27 ± 5.8 (n=6)
360	-	23.5 ± 2.3 (n=6)
400	26 ± 8.2 (n=4)	38 (n=1)
450	28 ± 18 (n=2)	-
500	29.5 ± 4.3 (n=4)	-
550	32.5 ± 6.5 (n=2)	-

TABLE III.4: Cumulative 24 Hour Urinary Excretion of CB3717



r=0.3744
p <0.01

FIGURE III.9: Correlation Between The Pre-Treatment Glomerular Filtration Rate of Patients Treated With CB3717 And The 24 Hour Urinary Excretion of The Drug

Faecal elimination was investigated in one patient where 15% of the total dose of drug administered was detected as unchanged CB3717 in an incomplete faecal collection. In addition, a compound was observed which co-chromatographed with CB3751, the desglutamyl metabolite of CB3717.

The only post mortem tissue found to contain CB3717 was the kidney where the concentration of 140 ug/g wet weight represented 5% of the total dose administered 8 days prior to the patient's death, assuming even drug distribution throughout the kidneys. Figure III.10 displays the data concerning the distribution of CB3717 into the ascitic fluid of a patient with ascites secondary to ovarian carcinoma. Equilibrium between plasma and ascitic fluid was observed 7 hours after the end of the infusion. CB3717 was not detected in the sample of CSF taken one hour post infusion from patient 4 and hence the plasma to CSF ratio at this time point was at least 45:1.

III.3.c Protein Binding

CB3717 was 97.6±0.1% protein bound in human plasma. The free drug concentration was linear over the range 20-200uM CB3717 in plasma ($r=0.99$, $p<0.001$). Below a concentration of 20uM, CB3717 was undetectable in the plasma ultrafiltrates (<1uM).

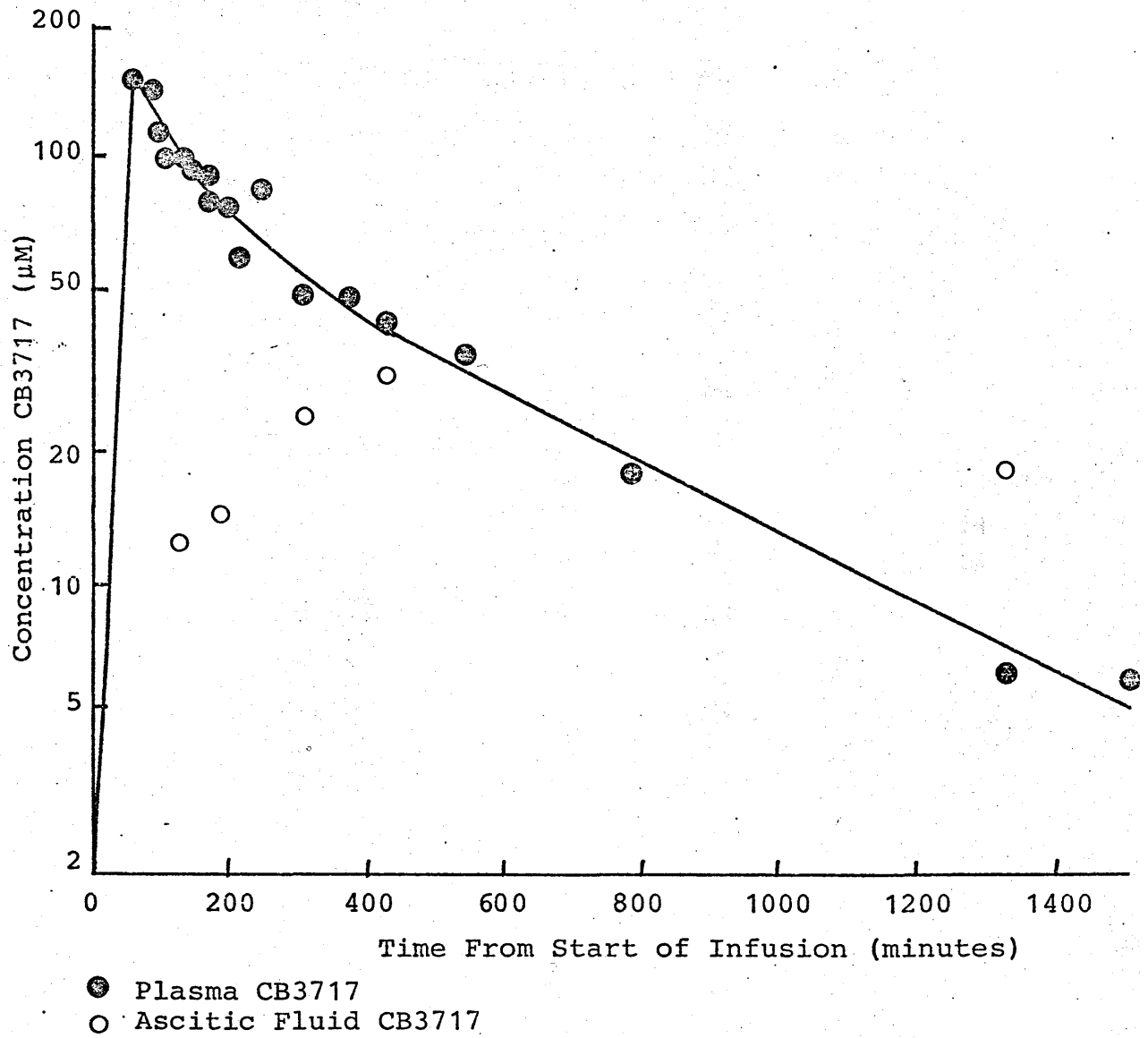


FIGURE III.10: Plasma And Ascitic Fluid Levels Of CB3717 After A 60 Minute Infusion Of 550mg/m²

III.3.d Urinary Solubility of CB3717

Table III.5 shows the maximal concentration of CB3717 which could be attained in urine at pH 3-9. For comparison the results obtained by Stoller *et al*, when studying the solubility of methotrexate in urine, are shown Stoller R.G. *et al* 1975).

III.4 Discussion

The pharmacokinetic studies of CB3717 in man were pertinent to its appraisal in early clinical studies. The peak drug plasma concentrations observed lay within the range that is cytotoxic to cultured cells *in vitro* (I_{50} 1-40uM) (Jones T.R. *et al* 1981, Diddens H. Niethammer D. & Jackson R.C. 1983) and are similar to those which are seen in mice at doses which cure the Chester Beatty L1210 leukaemia (Newell D.R. *et al* 1982). This may be relevant to the antitumour effects observed clinically (described in Chapter II), although the intracellular concentrations achieved are obviously of primary importance.

When CB3717 was administered as a one hour infusion there was a positive correlation between peak plasma CB3717 concentrations and the subsequent elevation of plasma alanine transaminase levels (Figure III.7). This reinforces the hypothesis, which is suggested by the frequency of hepatic disturbance, that the phenomenon is a direct toxic manifestation and not due to an idiosyncratic reaction. Although a dose-hepatotoxicity relationship could not be demonstrated with CB3717 in the larger series of patients included in the Phase I trial (see Chapter II, figure II.4), this may have been a reflection of inter-patient variation in peak CB3717 plasma levels and the relatively small dose

Urine pH	ug/ml	uM
3	<5	<10
4	<5	<10
5	<5	<10
6	11	23
7	45	93
8	415	856
9	4820	9938

TABLE III.5.a: Solubility of CB3717 in Urine

Urine pH	MTX Remaining in Solution (mM)
5.7	2.2
6.3	9.0
6.4	10.0
6.7	11.0
6.9	22.0

TABLE III.5.b: Solubility of Methotrexate in Urine (From Stoller et al 1975)

range used. For example, in this pharmacokinetic study there was no significant difference between the peak CB3717 plasma levels achieved following 300 and 500mg/m^2 (Table III.2, t test p=0.16). Furthermore, prolongation of the infusion to 12 hours was not successful in averting hepatic toxicity. This is perhaps not surprising as the peak plasma CB3717 levels observed at 300-330mg/m^2 (12 hour infusion) were still within the concentration range which was associated with hepatotoxicity after one hour infusions. One patient treated at the same dose level (330mg/m^2) by 1 hour and 12 hour infusions showed only a 60% reduction in the peak plasma CB3717 levels (81uM to 32uM).

Further discussion of CB3717 hepatotoxicity is continued in Chapter V, but it is perhaps relevant to emphasise here that methotrexate especially in high dose regimens (1g-20g/m^2) has also been shown to produce transient rises in alanine transaminase levels (Djerassi I. et al 1967, Rosen G. et al 1974, Jaffe N. & Traggis D. 1975) which are clearly dose related (Pratt C.B. et al, Perez C. et al 1979). However, Pratt reports that at the top dose range, 500mg/kg (~17.5g/m^2), the peak plasma concentration of methotrexate was approximately 1600uM whilst after treatment at 100mg/kg (3.5mg/m^2) it was approximately 160-250uM. By comparison, peak plasma CB3717 concentrations of 20uM have been associated with hepatic dysfunction indicating a considerable difference in hepatotoxic potency.

The 24 hour urinary excretion of CB3717 comprises approximately 30% of the administered dose whereas a larger proportion of a methotrexate dose (up to 90%) can be recovered in the urine within a similar time period (Isacoff W.H. et al 1977 Calvert A.H. Bondy P.K. & Harrap K.R. 1977, Christophidis N. et al 1981, Breithaupt H. & Kuenzlen E.

1982). Significant quantities of 7-hydroxymethotrexate are also found in the urine of patients receiving high dose methotrexate (Jacobs S.A. et al 1976, Breithaupt H. & Kuenzlen E. 1982) and this is considered to be relevant to the renal toxicity seen in such regimens. Methotrexate and 7-hydroxymethotrexate are both relatively insoluble at the acid pH generally present in the distal renal tubule and may precipitate giving rise to crystalluric tubular damage (Stoller R.G. et al 1975, Jacobs S.A. et al 1976). Judicious use of alkalinisation regimens can successfully avoid this problem (Pitman S. et al 1976, Nirenberg A. et al 1977). Although, in the case of CB3717, metabolites have not as yet been found in plasma or urine, the parent compound is a weak acid and insoluble at acid pH (see Table III.5). Studies in mice showed that nephrotoxicity was caused by precipitation of CB3717 in the renal tubules which could be prevented by an alkalinisation regimen (Newell D.R. et al 1982). Significantly, the autopsy kidney specimen from a patient who died 8 days post treatment with 330mg/m^2 CB3717, contained approximately 5% of the total dose although microscopical examination was unsatisfactory due to autolysis and no crystalluria was seen. There had been no evidence of pre-terminal renal failure. The possibility for future dose escalation of CB3717 utilising hydration or alkalinisation regimens has been broached in Chapter II. Indeed this may be a logical step when administering doses similar to those used in this trial in an effort to prevent even minor reductions in glomerular filtration rates (GFR) (<20%) and avoid the occasional more significant reduction in GFR (20-50%) which occurred unpredictably, possibly due to poor hydration of individual patients, in the immediate post treatment period (see Table II.9).

CB3717 is highly protein bound and consideration should therefore be given to the co-administration of other drugs with this property due to the possibility of displacement of CB3717 and consequent increases in the circulating level of free drug. In one patient, good distribution of the drug into ascitic fluid was seen; an observation which may be related to the activity of CB3717 observed against ovarian cancer. In contrast CB3717 did not partition readily into the CSF being similar to methotrexate in this respect. Large systemic doses of methotrexate (500mg/m^2 infused over 24 hours) are required to obtain satisfactory concentrations within the CSF for adequate time periods (Wang J.J. et al 1976) or alternatively, intrathecal or intraventricular routes of administration may be prefered (Shapiro W.R., Young D.F. & Mehta B.M. 1975). The low lipophilicity of both CB3717 and methotrexate accounts for their poor diffusion across the blood brain barrier, whereas more lipid soluble antifolates, for example metoprine (2,4-diamino-5-(3',4'-dichlorophenyl)-6-methyl pyrimidine) are more effective at entering the central nervous system (Stickney R.C. et al 1973).

In conclusion the results of this study have demonstrated that cytotoxic levels of CB3717 can be achieved in human plasma. Biliary excretion is probably the major elimination route for CB3717, a fact which should be borne in mind when prescribing for patients with abnormal liver function. Extensive retention of the drug in the kidney can occur, which may be related to the dose limiting renal toxicity, and prompts pursuit of hydration or alkalinisation regimens. In addition a tentative correlation between the incidence of hepatic toxicity and peak CB3717 concentration has been shown which requires further confirmation.

CHAPTER IV

BIOCHEMICAL PROPERTIES OF CB3717 AND OTHER ANTIFOLATES: RELEVANCE TO CLINICAL TOXICITY

CHAPTER IV

BIOCHEMICAL PROPERTIES OF CB3717 AND OTHER ANTIFOLATES: RELEVANCE TO CLINICAL TOXICITY

IV.1a. Introduction

Anticancer chemotherapy is rarely devoid of toxicity to normal host tissue by virtue of its desired effect in preventing cell proliferaton (Connors T.A. 1976). Whilst tumour selectivity and specificity remain the ideal but elusive goals (McElwain T.J. 1976), the possible benefits of treatment have to be balanced against the unpleasant, and sometimes hazardous, side effects of nausea, vomiting, alopecia, mucositis, dermatitis and myelosuppression. In the few cases where cure is the rule, rather than the exception (for example, choriocarcinoma, Burkitt's lymphoma, acute lymphoblastic leukaemia of children and Hodgkins disease, McElwain T.J. 1976), the decision to treat defies argument. Unfortunately many solid tumours remain refractory, or only partially responsive, to present cytotoxic regimens (eg, bronchogenic carcinoma, colorectal carcinoma, melanoma, bladder carcinoma, cervical carcinoma) so that palliation is the realistic aim and hence drug toxicity assumes greater significance. Attention to those features of drug related morbidity, which are not directly associated with its effect at a cytotoxic locus, can, therefore, be helpful. For example, cyclophosphamide and ifosfamide urotoxicity was found to be due to the renally excreted metabolite, acrolein, which is formed during the metabolic activation of cyclophosphamide but does not contribute to its antiproliferative activity (Brock N. Pohl J. & Stekar J. 1981a). Sodium-2-mercapto-ethane sulfonate (MESNA) was discovered to be effective as a uroprotective

agent without impairing the antitumour activity of cyclophosphamide (Brock N. Pohl J. & Stekar J. 1981b) and it is now recommended in Europe with high dose cyclophosphamide or ifosfamide regimens (Falkson G. et al 1982, Souhami R.L. et al 1983). Similarly doxorubicin cardiotoxicity, which becomes apparent at cumulative doses of >550mg/m^2 has been extensively investigated (reviewed by Unveferth D.V. et al 1982) and though debate continues regarding the mechanism, much effort has gone into the development of less toxic analogues (reviewed by Muggia F.M. & Rozencweig M. 1983).

Cisplatinum has proved to be a highly active but toxic compound, causing nephrotoxicity and severe nausea and vomiting. Exploration of hydration and forced diuresis regimens enabled its safer usage (reviewed by Penta J.S. Muggia F.M. & Salem P.A. 1983) and analogue development has been successful in producing at least two compounds with considerably diminished renal toxicity, carboplatin (JM8, CBDCA) (Calvert A.H. et al 1982) and iproplatin (CHIP, JM9) (Creaven P.J. et al 1982. Preliminary reports also suggest that carboplatin has comparable antitumour activity to cisplatin (Wiltshaw E. et al 1983).

The toxicities of CB3717 were defined in the Phase I clinical trial (see Chapter II). The most prevalent toxicity was hepatic and its association with malaise, anorexia and lethargy invited investigation which is described in Chapter V. However the correlation between drug hepatitis and malaise was not absolute, implying that other properties of this quinazoline folate analogue might contribute to the symptoms. One possibility, with the precedent of various other antifolate compounds, was that CB3717 was causing disturbance of histamine metabolism (Duch D.S. et al 1978) and this was supported by the observation

of dermatological toxicity in a proportion of patients. In addition, it was considered prudent to investigate the effect of CB3717 on serum phenylalanine levels as transient hyperphenylalaninaemia has been documented after high dose methotrexate treatment (Hilton M.A. Kmetz D.R. & Patel C.C. 1976). The rationale for pursuing these two areas of research is discussed in more detail below, the primary objective being to elucidate the properties of CB3717 (both biochemical and biophysical) which might contribute to its toxicity profile.

IV.1.b. Histamine Metabolism

Histamine (B-aminoethylimidazole) is a vasoactive amine which is widely distributed in human tissues and involved in diverse physiological processes including gastric secretion and modulation of neurotransmission within the brain. Its release from mast cells during allergic reactions and from skin cells secondary to injurious stimuli gives rise to capillary dilatation and increased permeability, resulting in flushing and oedema. The stimulatory effect of histamine on bronchial smooth muscle and on cutaneous nerve endings causes bronchial constriction and itch or pain (reviewed by Douglas W.W. 1980). Two different receptors, H_1 and H_2, have been identified (Ash A.S. & Schild H.O. 1966, Black J.W. et al 1972) and, subsequently, different classes of antagonists have proved useful in counteracting the unpleasant consequences of inappropriate localised, or systemic, histamine release. The H_1 receptor antagonists are many and include proheptadine, chlorpheniramine and promethazine, being most useful in preventing urticaria and treating allergic rashes, hayfever, vasomotor rhinitis, pruritus and drug allergies. The H_2 receptor antagonists, cimetidine and ranitidine have found a use in the treatment

of peptic ulceration, by their effect on gastric acid output (reviewed by Feely J. & Wormsley K.G. 1983).

The importance of histamine to antifolate therapy arises from its routes of metabolism and a scheme showing the major pathways involved in man is shown on Figure IV.1 (taken from Goodman and Gilman 'The Pharmacological Basis of Therapeutics, Sixth Edition). Histamine-N-methyl transferase (EC.2.1.1.8) was shown to play a major role by Schayer and Cooper who injected volunteers with 1 microgram/kg ^{14}C histamine and found that 46-55% of the urinary metabolites were accounted for by this route (Schayer R.N. & Cooper J.A.D. 1956). Brown et al established S-adenosyl methionine as the methyl donor for histamine methyltransferase (Brown D.D. Axelrod J. & Tomchick R. 1959) and an effective assay was developed using methyl-^{14}C-S-adenosyl methionine with extraction of the ^{14}C methylated histamine product into chloroform (Brown D.D. Tomchick I.R. & Axelrod J. 1959). These workers then developed a purification procedure for the enzyme and investigated its tissue distribution in several mammalian species. They found that histamine methyltransferase was present in many tissues with high concentrations occurring in skin, lung, ileum, stomach and kidney. Guinea pig brain contained the highest concentration (Brown D.D. et al 1959). Inhibition of enzyme activity by the weakly antihistaminic major tranquilliser, chlorpromazine, was noted and later Cohn pursued this and established that amongst the antimalarial compounds several more potent inhibitors of HMT existed including quinacrine, chloroquine, amodiaquine, pyrimethamine and chlorguanide (Cohn V. 1965). As pyrimethamine is thought to exert antimalarial activity by inhibition of dihydrofolate reductase, the established DHFR inhibitor, amethopterin (methotrexate) was also tested and

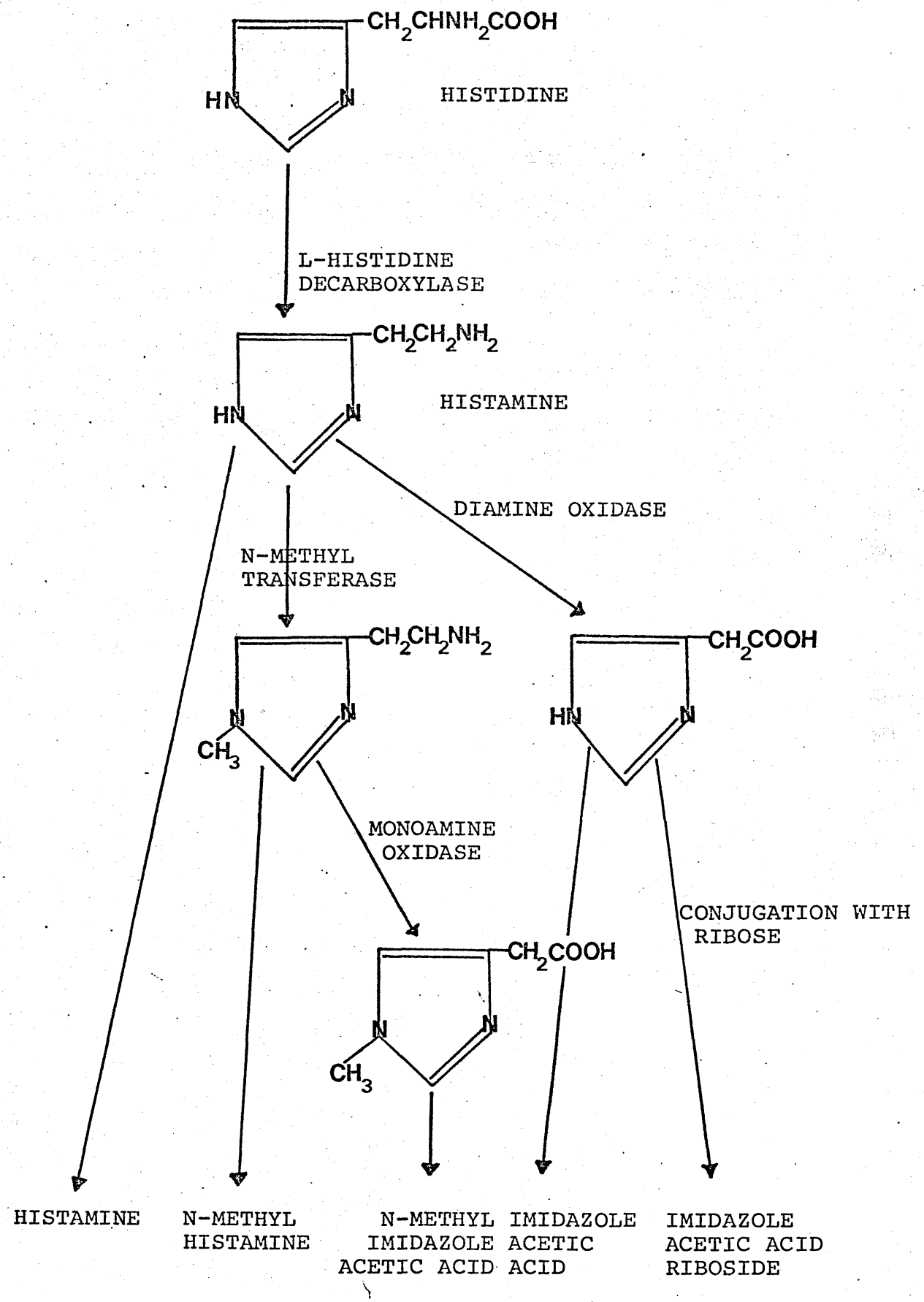


FIGURE IV.1: Synthesis And Catabolism of Histamine (Taken from Douglas W.W., 1980)

was found to be inactive against HMT at concentrations up to 10^{-3}M. Other workers subsequently found that antihistamines could also inhibit and sometimes potentiate HMT (Netter K.J. & Bodenschatz K. 1967, Taylor K.M. & Snyder S.H. 1972, Beaven M.A. & Shaff R.E. 1979) and the list of inhibitors was expanded further by Thithapandha and Cohn to include mercurial diuretics (eg, triamterene) and some local anaesthetics (eg, procaine, dibucaine) (Thithapandha A. & Cohn V.H. 1978).

It was Duch, Bowers and Nichol who were first prompted by the clinical reports of the toxicity of the antifolates metoprine (DDMP), and triazinate to propose a correlation between the inhibition of histamine metabolism by these compounds and the development of skin and central nervous system (CNS) toxicity in some patients (Duch D.S. et al 1978, Duch D.S. et al 1979). Metoprine is a lipophilic diaminopyrimidine antifolate with a long biological half life (Nichol C.A. et al 1977) which was first used clinically in 1954 (Murphy M.L. et al 1954) and reintroduced in 1975 in combination with folinic acid rescue (Price L.A. Goldie J.H. & Hill B.T. 1975). This and later studies (Miller D.S. et al 1976, Price, L.A., Hill B.T. & Goldie J.H. 1977, Alberto P. et al 1978, Currie V.E. et al 1980, Alberto P. et al 1980) reported blotchy erythematous skin rashes, mental haziness, headache and gastritis in a significant proportion of patients. CNS toxicity, typified by frontal headache, lethargy and 'muzziness' was most apparent at higher dose levels (Miller D.S. et al 1976, Hindmarsh J.R. Hall R.R. & Kulatilake A.E. 1979). Triazinate, another DHFR inhibitor, also caused moderate to severe central neurological impairment with light headedness, somnolence, visual disturbances and weakness along with radiation recall dermatitis (Rodriguez V. et al 1975, Skeel R.T. et al 1976).

Duch and coworkers confirmed that triazinate, metoprine and the closely related compound, etoprine, were potent HMT inhibitors and demonstrated that the metoprine concentrations achieved in rat brain ($2x10^{-5}$M) were associated with elevations of brain histamine levels and were comparable with the plasma drug concentrations achieved in man (10^{-5} to 10^{-6}M) (Duch D.S. et al 1978). The brain was felt to be particularly sensitive due to its reliance on inactivation of histamine by methylation (Schayer R.W. & Reilly M.W. 1973) because of its lack of diamine oxidase (E.C.1.4.3.6) which can provide an alternative degradative pathway in other tissues (Schaff R.E. & Beaven M.A. 1976). Thus a strong case was made for an association between disturbed histamine metabolism and some of the toxicities of serveral antifolates.

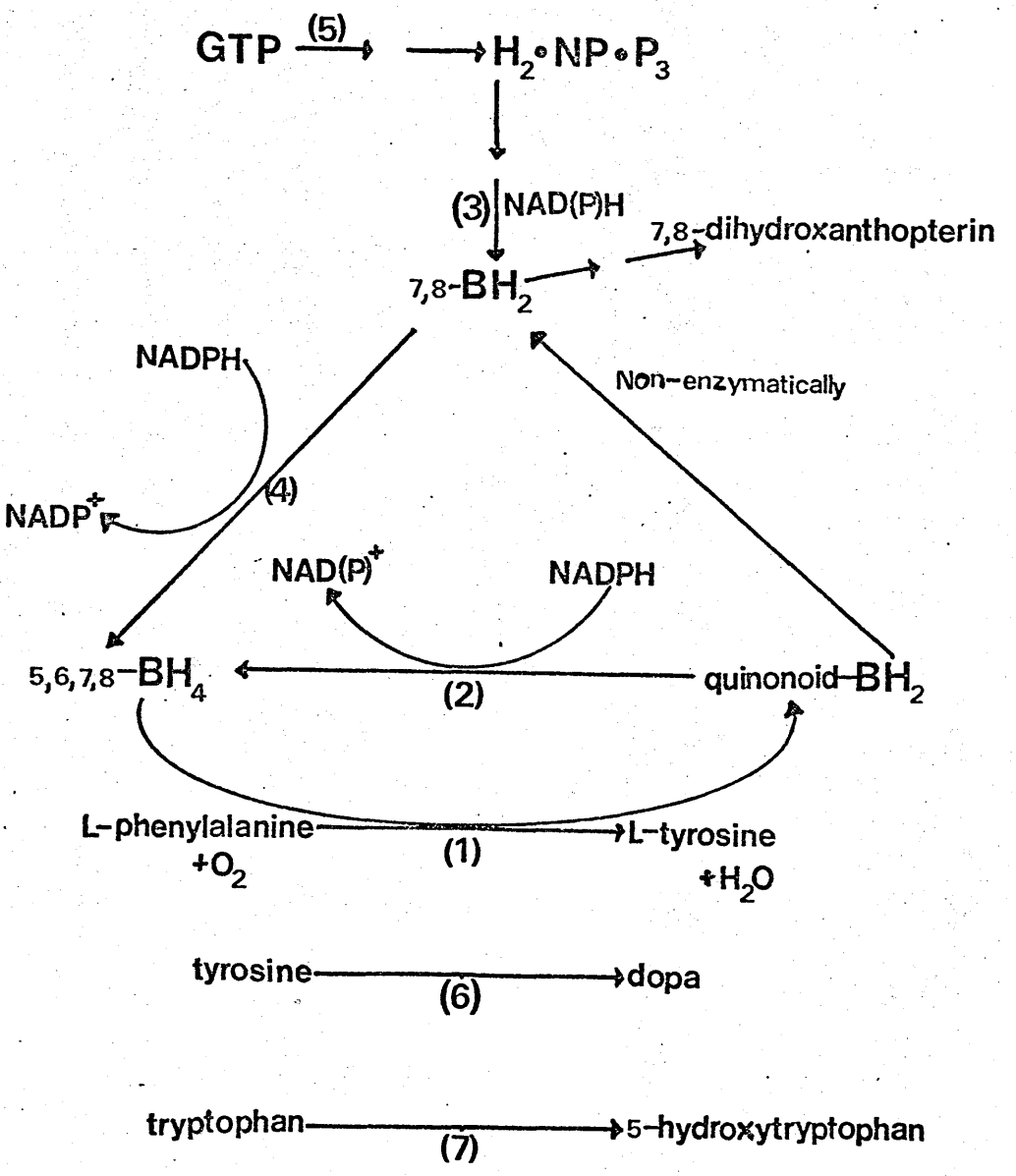
The further possibility of inhibition of diamine oxidase (DAO) by such compounds was also studied (Duch D.S. et al 1980). Metoprine and triazinate weakly inhibited DAO whilst TMQ (trimethoxybenzylaminoquinazoline) proved to be a powerful inhibitor of DAO (88% at 10^{-4}M) and HMT (96% at 10^{-6}M) and caused histamine elevation in rat brain and kidney. The conclusions drawn from this work were that the development of novel antifolates should include assessment of their effect on histamine metabolism (Duch D.S. et al 1980). Indeed these workers have subsequently produced a 2,4-diaminopyrimidine antifolate (BW301U) with favourable properties in this respect (Duch D.S. et al 1982).

In the Phase I trial, CB3717 caused dermatological toxicity in about 10% of patients which was symptomatically relieved by steroids and antihistamines, rather reminiscently of metoprine (Price L.A. et al 1977). Generalised malaise and lethargy occurred frequently,

usually in association with hepatic disturbances (see Chapter II). It was postulated that such features might be related to CB3717-induced disturbances of histamine metabolism. A crude preparation of histamine methyltransferase was therefore used to test the inhibitory effect of CB3717 and, for comparison, known HMT inhibitors were tested in the same system. The activity of CB3717 against commercially prepared diamine oxidase was similarly investigated and an attempt was made to establish some structure activity relationship utilising quinazoline intermediates. Plasma levels of histamine before and after CB3717 treatment were measured by ICI to determine if any *in vivo* effect could be established.

IV.1.c Phenylalanine Metabolism

The effect of methotrexate on folate pools secondary to its inhibition of dihydrofolate reductase is well known. Methotrexate also has the potential to disturb biopterin metabolism via inhibition of dihydropteridine reductase (Craine J.E. Hall E.S. & Kaufman S.J. 1972). The importance of this arises from the essential role played by tetrahydrobiopterin as cofactor in the hydroxylation of the three aromatic amino acids phenylalanine, tyrosine and tryptophan, by their respective hydroxylase enzymes (Kaufman S. 1958). A scheme showing the various pathways involved is shown in Figure IV.2 (adapted from Tanaka T. *et al* 1981). Newly synthesised 7,8-dihydrobiopterin is thought to be reduced to 5,6,7,8-tetrahydrobiopterin by dihydrofolate reductase and is then oxidised to quinonoid dihydrobiopterin as a result of phenylalanine hydroxylation. Quinonoid dihydrobiopterin may be reconverted to the active 5,6,7,8-tetrahydrobiopterin by dihydropteridine reductase or may tautomerise non-enzymatically to 7,8-dihydrobiopterin which



1 Phenylalanine hydroxylase
2 Dihydropteridine reductase
3 Dihydropteridine synthetase
4 Dihydrofolate reductase
5 GTP cyclohydrolase
6 Tyrosine hydroxylase
7 Tryptophan hydroxylase
BH_2 Dihydrobiopterin
BH_4 Tetrahydrobiopterin
H_2•NP•P_3 7,8-dihydroneopterin triphosphate
GTP Guanosine triphosphate

FIGURE IV.2: Enzymatic Hydroxylation of Phenylalanine To Tyrosine And The Biosynthesis of Biopterin And Its Function (Adapted From Tanaka T. et al, 1981)

can be reduced by dihydrofolate reductase as described above. Methotrexate was found to inhibit dihydropteridine reductase purified from sheep liver with a Ki of 4×10^{-5}M and was competitive with the pterin cofactor (Craine J.E. et al 1972). Subsequently, potent inhibition of the phenylalanine hydroxylase activity in rat liver slices, in the presence of methotrexate, was observed (50% at <0.18mM) and intraperitoneal injection of methotrexate to rats gave rise to 90% inhibition of liver phenylalanine hydroxylase within 4 hours of a dose of 10mg/kg (Milstein S. & Kaufman S. 1975). No inhibition occurred when a non enzymatic tetrahydrobiopterin regenerating system was used to supplement the systems.

The condition of phenylketonuria which is characterised by grossly elevated blood phenylalanine levels and the excessive excretion of its metabolites (eg, phenylpyruvate and phenylacetate) in the urine, is associated with a clinical syndrome of mental retardation and frequently central nervous system abnormalities such as convulsions, increased muscle tone, hyperactive tendon reflexes, tremors and inceased irritability. (reviewed by Kaufman S. 1976). The disorder is inherited in an autosomal reccessive manner and in the majority of patients a deficiency of phenylalanine hydroxylase is the causative biochemical lesion, although the reason why elevated phenylalanine levels should produce neurological impariment remains to be elucidated. Most theories are based on the belief that excess phenylalanine interferes with normal metabolism, especially that of tyrosine and tryptophan which are precursors of the neurotransmitters (catecholamines and serotonin) (Kaufman S. 1976). Indeed high levels of phenylalanine do inhibit mammalian tyrosinase, (an enzyme in the pathway convering tyrosine to the pigment melanin,

Miyamoto M. & Fitzpatrick T.B. 1957) which accounts for the fair skin and hair of phenylketonurics. Transport of tryptophan into the brain may also be impaired by competition from elevated blood phenylalanine levels (reviewed by Bender D.A. 1982).

The study of patients with atypical phenylketonuria, who develop neurological impairment despite a restricted phenylalanine diet, elucidated several other contributory biochemical lesions (Kaufman S. 1976). Mild hyperphenylalaninaemia and associated neurological disturbance has been ascribed to both impaired dihydrobiopterin synthesis (Tanaka T. et al 1981) and deficiency of dihydropteridine reductase (Koslow S.H. & Butler J.J. 1977, Brewster G. et al 1979) Koslow also measured serotonin and dopamine levels in brain biopsies from such a patient and found them to be low. Treatment with neurotransmitters or their precursors gave variable responses which was possibly dependent on the age at which it was instituted.

The relevance of this discussion to antifolate therapy is in relation to the development of neurological toxicity. It has been postulated that the somnolence and confusion which sometimes accompany high dose methotrexate therapy may be due to decreased availability of biogenic amines and serotonin (Abelson H.T. 1978), as an indirect result of dihydropteridine reductase inhibition. Certainly, patients on low dose methotrexate developed elevated phenylalanine levels when presented with an oral loading dose of phenylalanine (Goodfriend T.L. & Kaufman S. 1961) and Hilton reports transient hyperphenylalaninaemia (7 x normal) in a 19 year old patient treated with 200mg/kg methotrexate by 4 hour infusion (Hilton M.A. et al 1976). The hypothesis

that CB3717 would also give rise to such an effect was put to the test in patients treated on the Phase I trial.

IV.2 Methods

IV.2.a Materials

The reagents used and the suppliers are listed below: S-adenosyl-L-[methyl-^{14}C]methionine (60mCi/mmol) and [1,4-^{14}C]-putrescine (96.6mCi/mmol) - Amersham International PLC (Amersham, Bucks., England). Histamine dihydrochloride, putrescine dihydrochloride, aminoguanidine dihydrochloride - Sigma London (Poole, Dorset, England). Cold S-adenosyl methionine - Boehringer Ingleheim Ltd. (Bracknell, Berks, England). Sodium phosphate, sodium hydroxide, boric acid - BDH Chemicals Ltd (Poole, Dorset, England). Isoamyl alcohol - Fisons Scientific Apparatus (Loughborough, Leics, England). Toluene - May and Baker Ltd (Dagenham, England).

Compounds screened: Chlorpromazine, quinacrine, chloroquine - Sigma London (Poole, Dorset, England). Pyrimethamine, trimethoprim, amiloride - gift from Merck, Sharp and Dohme (Hoddesdon, Herts, UK). Triamterene - gift from Smith, Kline and French (Welwyn Garden City, Herts, England). CB3717, CB3702, CB3703, CB3704 and quinazoline intermediates prepared by Dr. T.R. Jones, Institute of Cancer Research (Sutton, Surrey). Methotrexate donated by Nils Klauson Kaas (Denmark). TMQ (trimethoxybenzylaminoquinazoline) National Cancer Institute (USA). DDMP (metoprine; 2,4-diamino-5-(3,4-dichorophenyl)-6-methylpyrimidine) donated by Dept. of Pharmacy, University of Aston (Birmingham, England).

Enzymes; Diamine oxidase (0.25units/mg) - Sigma London

(Poole, Dorset, England). Histamine methyltransferase prepared from rat brain as described.

IV.2.b Histamine methyltransferase - Enzyme Preparation

A crude preparation of histamine methyltransferase was prepared from rat brains. Seven Wistar rats were anaesthetised with ether then sacrificed by cervical dislocation. The brains were excised immediately, weighed and homogenised with 2 volumes of 0.1M sodium phosphate buffer adjusted to pH 7.4 (4°C) using 5 strokes of a Potter homogeniser. The homogenate was then centrifuged at 4°C, 25,000g for 90 minutes. The supernatant was divided into 5ml aliquots and stored frozen (-40°C) in sealed tubes. A fresh tube was used for each set of experiments.

IV.2.c Histamine Methyltransferase Assay

Histamine methyltransferase activity was determined by measuring the formation of ^{14}C-N-methylhistamine from an incubation of the crude enzyme preparation with histamine and S-adenosyl-L[methyl-^{14}C]methionine according to a method adapted from Axelrod (Axelrod J. 1971).

Reagents: Crude enzyme preparation in 0.1M sodium phosphate buffer, 0.75mM cold S-adenosyl-methionine, 0.83mM S-adenosyl-L(methyl-^{14}C) methionine (60mCi/mmol), 0.5mM histamine dihydrochloride, 0.5M sodium borate buffer (pH 10), toluene/isoamyl alcohol (1:1 mixture).

In a final volume of the 250ul incubation mixture the following substances were dissolved: enzyme plus 12.5umoles sodium phosphate, 18.75 nmoles cold S-adenosyl-L-methionine, 12.5 nmoles S-adenosyl-L-[methyl-^{14}C]-methionine, 12.5

nmoles histamine. In control tubes histamine was omitted and extra water was added to reach the same final volume. Duplicate mixtures were prepared in glass stoppered centrifuge tubes and the reaction was initiated by the addition of histamine (test) or water (controls). These were then incubated in a pre-heated water bath at 37°C for varying time periods (0,20,40,60 minutes). The reaction was stopped by the addition of 0.25ml of 0.5M borate buffer (pH 10). 3 ml of toluene/isoamyl alcohol (1:1) was then added and the tubes were shaken for 10 minutes in order to extract the products. The tubes were then centrifuged for 5 minutes and 2ml aliquots of the organic extract were added to 8ml scintillant in scintillation vials. Counting was performed in an SL 30 liquid scintillation spectrometer (Intertechnique).

The reaction was found to be linear up to 60 minutes incubation. The effect on the enzyme activity of several compounds (including known inhibitors) was then tested by adding varying amounts of these compounds to the incubation mixture as above (0.25-250nmoles) to give final concentrations from $1x10^{-3}$M to $1x10^{-6}$M. The reaction time was 60 minutes.

IV.2.d Diamine Oxidase Assay

Reagents: 0.2M sodium phosphate buffer (pH 7.2), 0.5M sodium borate buffer (pH 10.5), 0.8mM putrescine hydrochloride, [1,4-^{14}C] putrescine (specific acivity 96.4mCi/mmol) 10mM aminoguanidine hydrochloride, diamine oxidase 2mg/ml (0.25 units/mg) toluene.

Assay: The method of enzyme assay was adapted from Okuyama and Kobayashi (Okuyama T. & Kobayashi Y. 1961) which

measures the radioactivity of toluene extractable ^{14}C products of ^{14}C labelled aliphatic diamines (eg, putrescine, cadaverine). Incubation mixtures consisted of 100 umoles sodium phosphate buffer (pH 7.2), 0.2 umol putrescine, (0.1uCi [1,4-^{14}C] putrescine), 0.125 unit enzyme (500ug) and where appropriate 1nmol-1umol compounds for testing (to give final concentrations of $1x10^{-3}M$ to $1x10^{-6}M$) in a final volume of 1ml. The reactions were initiated by the addition of enzyme and were performed in a pre-heated water bath ($37^{o}C$) for 20 minutes. The addition of 1ml 0.5M sodium borate buffer (pH 10.5) and 20ul of 10mM aminoguanidine terminated the reaction. The radioactive products were extracted by mixing with 6ml toluene. The mixture was centrifuged and 4ml of the toluene extract was counted in 8ml of scintillant mixture using an Intertechnique SL 30 liquid scintillation spectrometer.

IV.2.e Plasma Histamine Levels

Blood samples were collected from 5 selected patients, (treated with CB3717 at doses of 300-400mg/m^2) before treatment and at varying time points after treatment. 5ml of whole blood was collected into tubes containing ethylene diamine tetraacetic acid (EDTA) anticoagulant and was immediately cooled to $4^{o}C$ on ice. The plasma was separated by centrifugation in a pre-cooled centrifuge for 5 minutes. 1ml of plasma was then added to 1.5ml of 0.8M trichloroacetic acid to precipitate the plasma proteins and stabilise the histamine present. After being left overnight at $4^{o}C$ the sample was centrifuged and the supernatant was separated and stored at $4^{o}C$. Histamine assays were performed by Dr. D.S. Thomson (ICI Pharmaceuticals Division) using an automated fluorimetric assay involving condensation of histamine with O-phthalaldehyde (Evans D.P., Lewis J.A. &

Thomson D.S. 1973).

IV.2.f Plasma Phenylalanine Levels

Blood samples were collected into EDTA tubes from 19 patients treated with doses of CB3717 ranging from 140-550mg/m^2 immediately prior to their infusion and at varying time points (12-168 hours) after treatment. The plasma was separated by centrifugation and the samples were stored frozen at -20°C. Phenylalanine assays were performed later by the Biochemistry Dept. of Queen Mary's Hospital for Children, Carshalton, Surrey using a Guthrie technique (Newman R.L. & Starr D.J.T. 1971). No dietary restriction was placed on the patients.

IV.3 Results

IV.3.a Histamine Methyltransferase

The crude preparation of rat liver homogenate exhibited adequate histamine methyltransferase activity which was linear from 0 to 60 minutes (see figure IV.3). The blank/control values increased from 254 - 3,844 cpm (over an instrument background of ~30 cpm) during this time course (see Figure IV.3). This was presumed to be due to methylation of histamine present in the crude tissue extract with a possible contribution from other naturally occurring substrates for histamine methyltransferase such as N^{α} methyl histamine and N^{α}, N^{α}-dimethylhistamine (Barth H. et al 1980). The incubation time chosen for testing the effect of various compounds on histamine methyltransferase activity was 60 minutes. Structures of the antifolates and quinazoline compounds tested are shown in Figures IV.4 and 5. Table IV.1 shows the percent inhibition caused by these

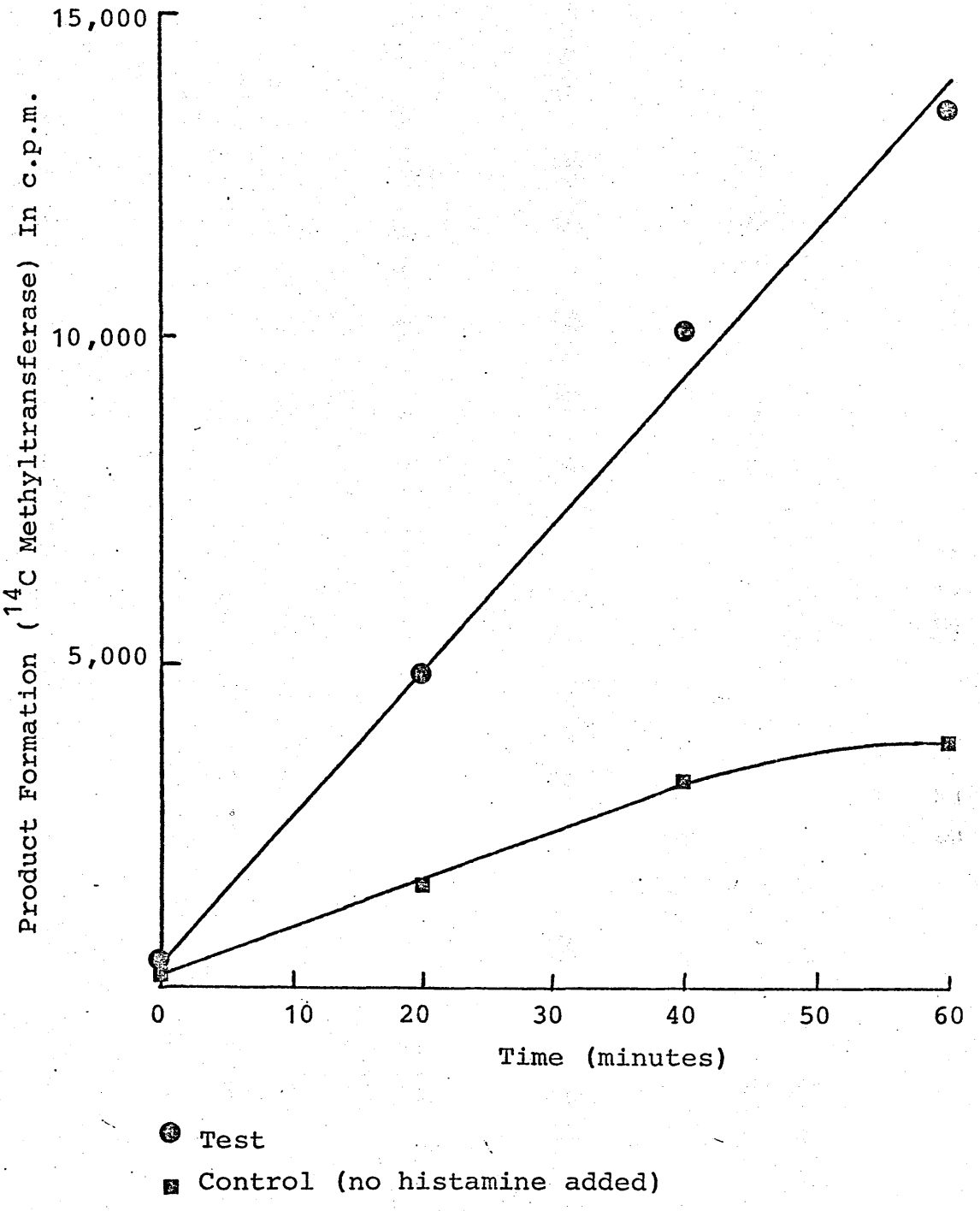
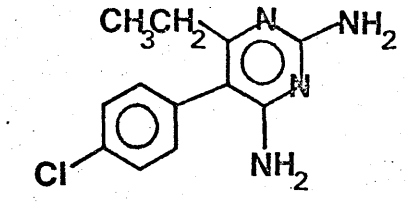
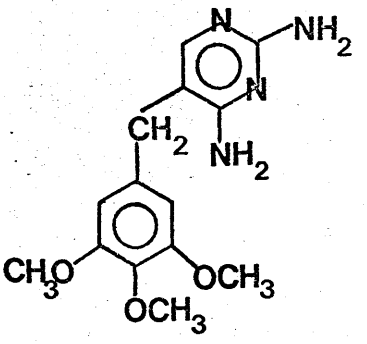


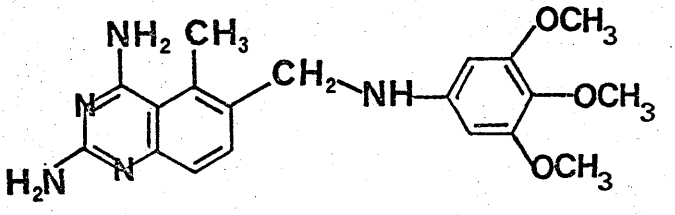
FIGURE IV.3: Time Course For Formation Of ^{14}C Methylhistamine Using Histamine Methyltransferase Prepared From Rat Brain



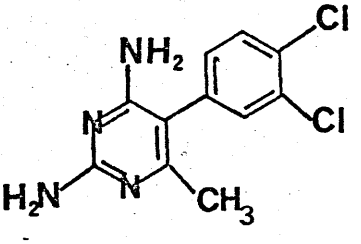
PYRIMETHAMINE



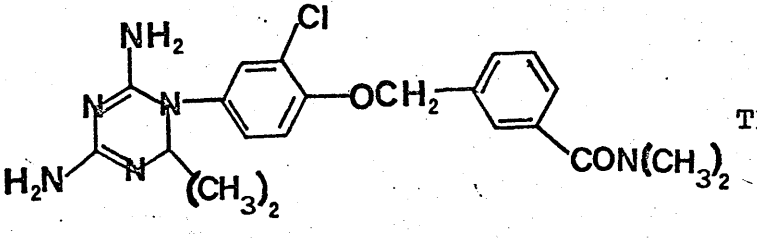
TRIMETHOPRIM



TRIMETREXATE
(TMQ)

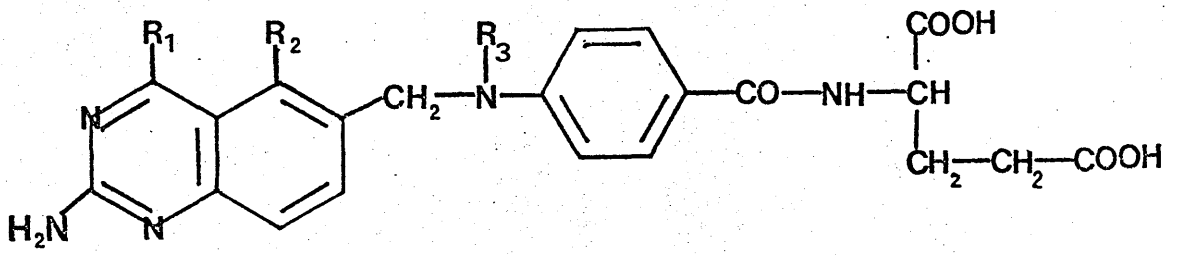


METOPRINE
(DDMP)

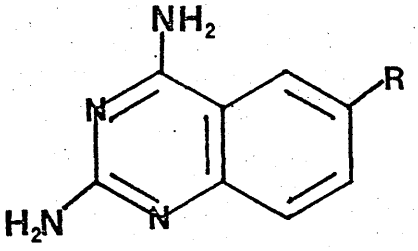


TRIAZINATE

FIGURE IV.4: Structures of Some Antifolate Compounds



	R_1	R_2	R_3
CB 3702	NH_2	H	H
CB 3703	NH_2	CH_3	H
CB 3704	NH_2	Cl	H
CB 3705	OH	H	H
CB 3717	OH	H	CH_2-C≡CH



R	
CH_3	2,4-diamino,6-methyl quinazoline
CN	2,4-diamino,6-cyano quinazoline
Cl	2,4-diamino,6-chloro quinazoline
NH_2	2,4-diamino,6-amino quinazoline

FIGURE IV.5: Structures of Some Quinazoline Compounds And Intermediates

Drug	Concentration			
	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M
CB3717	0 (115)	0 (132)	0 (123)	0 (136)
CB3703	6	0 (138)	0 (156)	-
TMQ	80	-	-	-
DDMP	82	-	-	-
Quinacrine	-	0 (122)	68	-
Chloroquine	-	0 (165)	49	-
Pyrimethamine	-	0 (146)	67	-
Trimethoprim	-	-	0 (137)	-
Amiloride	-	-	0 (155)	-
Triamterene	-	-	0 (148)	-
Chlorpromazine	-	0 (142)	-	-
2,4-diamino-6-amino quinazoline	-	-	0 (132)	-

TABLE IV.1: Percent Inhibition of Histamine Methyltransferase (Rat Brain) (% Control Activity in Brackets)

Drug	Concentration 10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M
TMQ	96[c]	99[c]	-	-
DDMP (Metoprine)	69[b]	93[b]	98[b]	-
Quinacrine	7[a] 25[b] 70[d]	41[a] 79[b] 93[d]	100[a] 97[b] 100[d]	100[a] 100[d]
Chloroquine	0[a] 9[b] 60[d]	39[a] 55[b] 92[d]	79[a] 89[b] 100[d]	100[a]
Pyrimethamine	8[a] 18[b]	29[a] 69[b] 42[d]	91[a] 94[b] 70[d]	100[a] 95[d]
Chlorpromazine	-	-	13[a]	81[a]
Methotrexate	0[c]	0[c]	0[a] 0[c]	0[a] 0[c]
Etoprine	52[b]	84[b]	96[b]	-
Triazinate	46[b]	82[b]	97[b]	-
BW 301U	0[c]	11[c]	91[c]	-

[a] Rat kidney enzyme, Cohn V. (1965) Biochem. Pharmacol. 14 1686-1688
[b] Bovine brain enzyme (partially purified), Duch D.S., Bowers S.W. & Nichol C.A. (1978) Biochem. Pharmacol. 27 1507-1509
[c] Bovine brain enzyme (partially purified), Duch D.S., Edelstein M.P. & Nichol C.A. (1980) Mol. Pharmacol. 18 100-104
[d] Guinea pig brain enzyme (purified) Thithapandha A., Cohn V. (1977) Biochem. Pharmacol. 27 263-271

TABLE IV.2: Percent Inhibition of Histamine Methyltransferase From Published Data (Guinea Pig Brain, Bovine Brain and Rat Kidney)

and it is noticeable that CB3717 did not cause any inhibition of histamine methyltransferase at concentrations up to $1x10^{-3}$M whilst TMQ and DDMP were potent inhibitors at concentrations of $1x10^{-6}$M. In some cases a high concentration of the test drug caused enzyme inhibition whereas a low concentration caused an increase in activity (eg, chloroquine, quinacrine, pyrimethamine). This will be discussed later. Table IV.2 shows cumulative results of compounds causing inhibition of partially purified and purified histamine methyltransferase from bovine brains, guinea pig brain and rat kidney (Thithapandha A. and Cohn V.H. 1978, Duch D.S. et al 1978, Duch D.S. et al 1980, Cohn V.H. 1980).

IV.3.b Diamine Oxidase

Table IV.3 shows the effect of various antifolates, antimalarials and quinazoline intermediates on diamine oxidase activity and for comparison the results obtained by Duch, Edelstein and Nichol using diamine oxidase partially purified from rat ceca are shown in Table IV.4 (Duch D.S. et al 1980). CB3717 was only weakly inhibitory to diamine oxidase at a concentration of $1x10^{-3}$M (26% inhibition) whereas TMQ and DDMP were more potent as inhibitors (29% at $1x10^{-5}$M and 31% at $1x10^{-4}$M) which agreed with the published data.

IV.3.c Plasma Histamine Levels

The levels of histamine in plasma from patients pre- and post CB3717 treatment are shown in Figure IV.6. The mean pre-treament value for the 5 patients was 13.4±4.3ng/ml and the mean post treatment value (6-120 hours) was 14.7±4.4ng/ml ($\bar{x}$ ± S.D., t test, p=0.658) which was not

Drug	Concentration			
	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M
CB3717	-	-	0 (105)	26
CB3702	-	2	50	81
CB3703	-	0 (103)	17	70
CB3704	-	9	20	-
CB3705	-	-	-	0 (107)
2,4-diamino-6-methyl quinazoline	-	-	[$2x10^{-4}$] 87	-
2,4-diamino-6-cyano quinazoline	-	-	54	-
2,4-diamino-6-chloro quinazoline	-	-	50	-
2,4-diamino-6-amino quinazoline	-	-	93	-
TMQ	0 (106)	29	69	-
DDMP (Metoprine)	-	3	31	78
Quinacrine	-	-	85	-
Chloroquine	-	-	95	-
Pyrimethamine	-	-	4	61
Chlorpromazine	-	-	48	-
Methotrexate	-	-	-	4

TABLE IV.3: Percent Inhibition of Diamine Oxidase (Sigma London) (% Control Activity in Brackets)

Drug	Concentration		
	10^{-6}M	10^{-5}M	10^{-4}M
TMQ	19	66	88
DDMP (Metoprine)	0	14	30
Methotrexate	0	0	16
Aminopterin	0	0	0
Triazinate	0	14	43

From Duch D.S., Edelstein M.P., Nichol C.A. (1980) Biochem. Pharmacol. 18 100-104

TABLE IV.4: Percent Inhibition of Diamine Oxidase (Rat Ceca) From Published Data

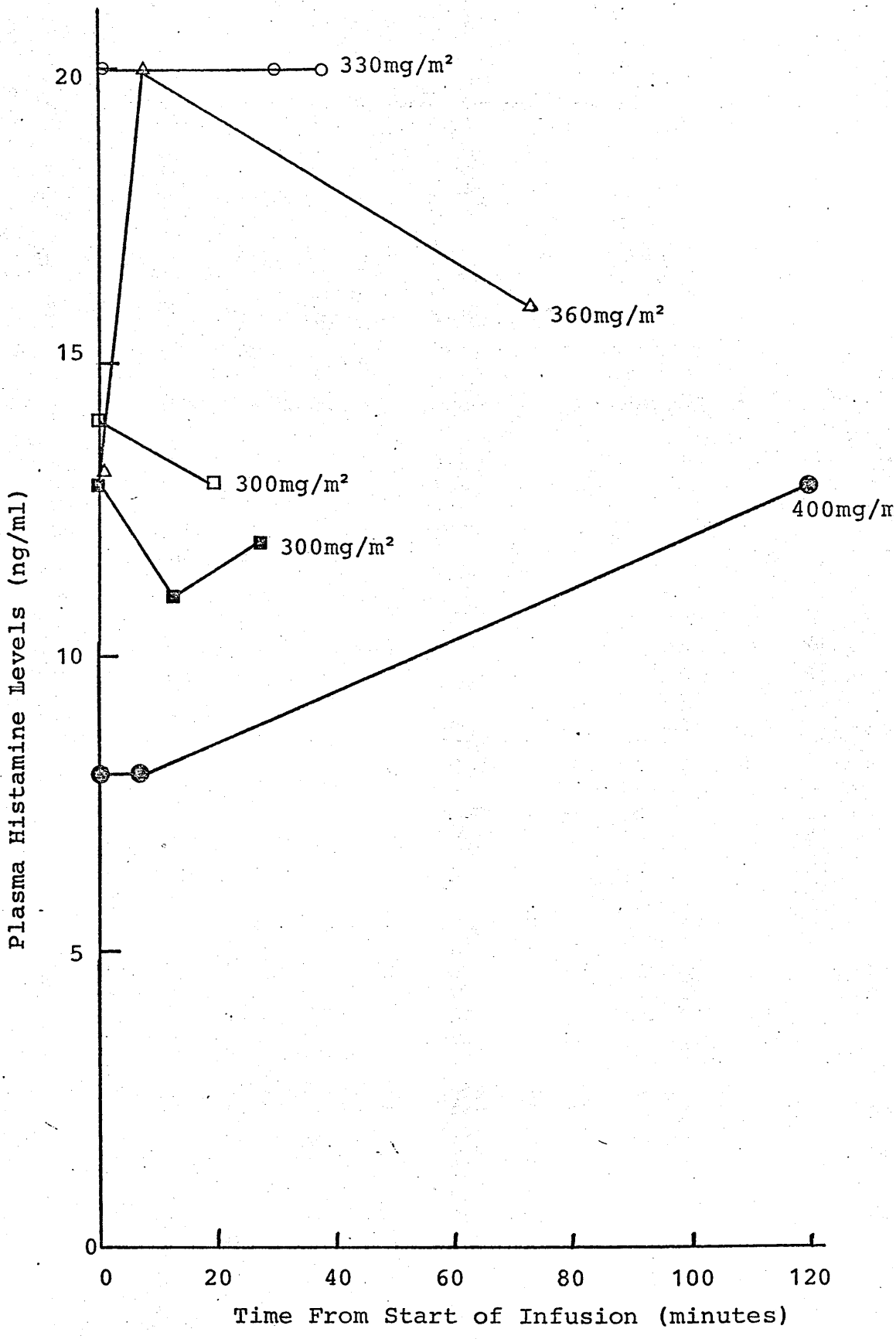


FIGURE IV.6: Plasma Histamine Levels In Five Patients Treated With CB3717 (300-400mg/m²)

significantly different.

IV.3.d Plasma Phenylalanine Levels

The phenylalanine levels pre and post CB3717 treatment are represented graphically in Figure IV.7. The upper limit of normal for non fasting patients is 2.5mg/100ml (Dr. Stern, personal communication) and some diurnal variation in blood amino acid levels is to be expected (Feigin R.D., Klainer A.S. & Beisel W.R. 1967). The majority of these patients were anorexic and eating less than normal amounts. No neurological symptoms or signs were noted except for malaise and lethargy which generally began 24-48 hours after treatment and lasted 7-14 days. Plasma phenylalanine levels rose to above the upper limit of normal in 13 patients at 24-48 hours post treatment and 3 patients were noted to have marginally elevated levels just prior to CB3717 treatment. They were all markedly cachetic, two had ascites and one had massive metastatic hepatomegaly. There was a suggestion of a dose related increase in values with elevations to 3.5-7.3mg/100ml occurring 24 hours after treatment at doses of 400-500mg/m^2.

IV.4 Discussion

IV.4.a Histamine Methyltransferase, Diamine Oxidase and Histamine Levels

The crude preparation of HMT from rat brain displayed similar sensitivity towards the most potent antifolate type inhibitors, TMQ and metoprine, when compared with more purified enzymes (Cohn V. 1965, Thithapandha A. et al 1977, Duch D.S. et al 1978, Duch D.S. et al 1980,). No attempt was made to elicit the type of inhibition involved. The

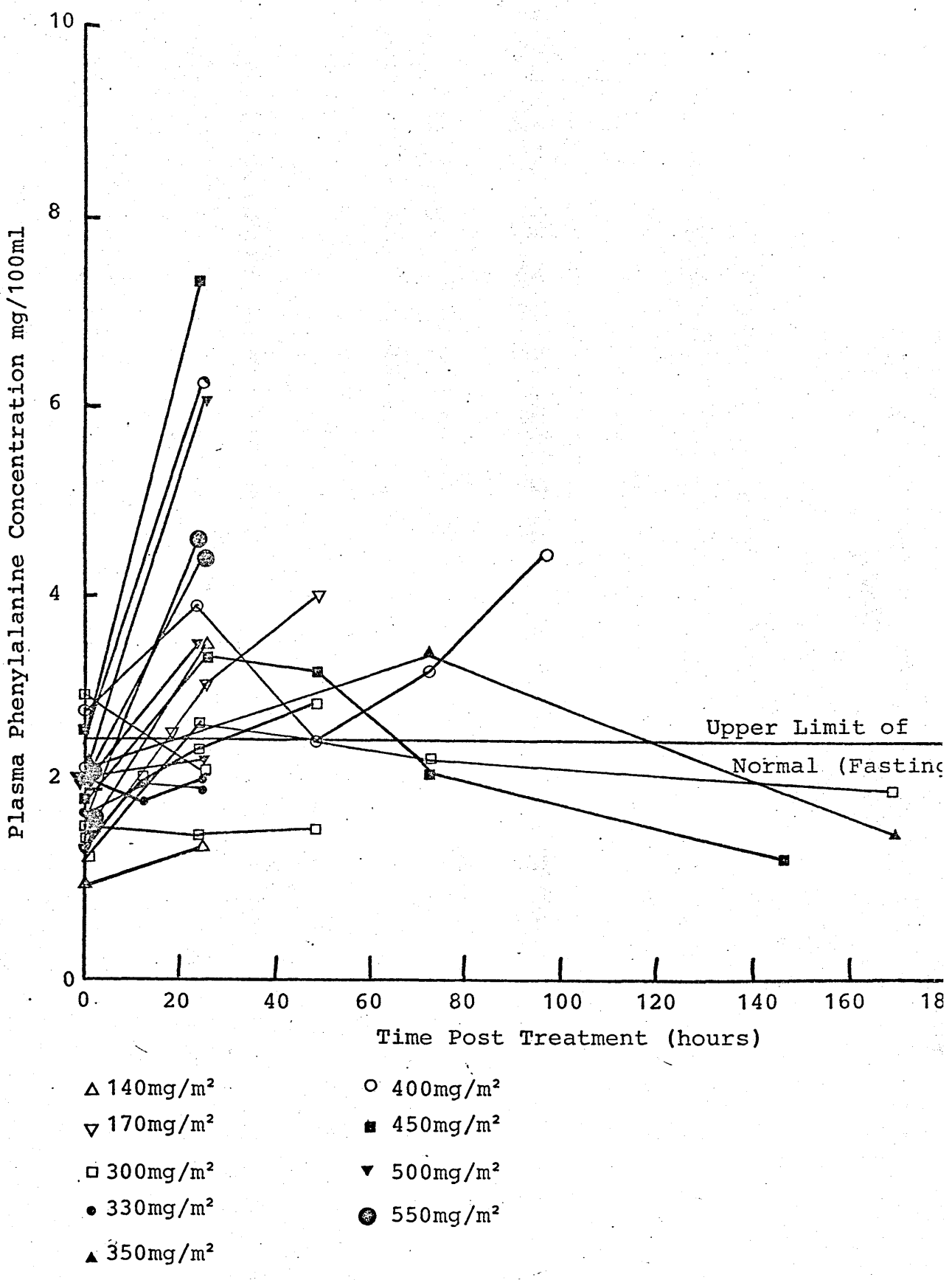


FIGURE IV.7: Plasma Phenylalanine Levels In Nineteen Patients Pre and Post CB3717 Treatment (140-550mg/m²)

antimalarials, quinacrine, chloroquine and pyrimethamine were less effective as inhibitors in this system but even amongst the published data there seems fairly wide variation in the percent inhibition achieved at a particular drug concentration (see Table IV.1). The biphasic effect of some compounds on the crude HMT (i.e. inhibition at high concentrations, stimulation at low concentrations) has been observed by other workers, particularly when using substituted imidazole compounds with antihistamine activity (Taylor K.M. & Snyder S.H. 1972, Barth H. & Lorenz W. 1978). Taylor also found that chlor promazine, thioridazine and desmethylimipramine were capable of this and postulated a reversal of substrate inhibition rather than "activation" of HMT activity as the mechanism of "enhancement". The impure nature of the enzyme preparation used herein may also have been contributory.

The diamine oxidase assay also produced results comparable with those of Duch et al (Duch D.S. et al 1980, see Table IV.3) with TMQ and metoprine being potent inhibitors in both studies. Both assay systems used were therefore suitable for the assessment of the ability of CB3717 to perturb histamine metabolism in vitro. It was conclusively demonstrated that within the drug plasma concentration range found in patients, (20-200uM, see Chapter III) CB3717 did not inhibit histamine methyltransferase or diamine oxidase. The weak inhibition of diamine oxidase by CB3717 at 10^{-3}M (26%) is probably not significant. The limited structure activity relationship showed that the 2,4-diaminoquinazolines (CB3702, CB3703 and CB3704) were somewhat more potent inhibitors of DAO than the 2,amino-4-hydroxy quinazolines CB3705 and CB3717 (see FigureIV.5 for structures). The diamino quinazoline intermediates (which lack a benzoyl glutamate group) were

also capable of inhibiting DAO within a similar concentration range (10^{-3}-10^{-4}M) and thus the benzoylglutamate half of the quinazoline molecules appear to be unnecessary for this effect.

Confirmation of the lack of activity of CB3717 against histamine methyltransferase and diamine oxidase is derived from its failure to alter significantly the plasma histamine levels of the patients studied (although none of these developed rashes). The different methods of measuring plasma histamine levels give widely varying results depending on the assay used (Graham H. et al 1968). Other workers, using a fluorimetric assay similar to that used in this study, reported mean values of 13.6ug/l and 15.7ug/l for normal subjects (Noah J.W. and Brand A. 1961) which are in the range of the mean values for the CB3717 treated patients.

In summary, considering that several structural classes of antifolate drugs (pyrimidine, triazine, quinazoline, and pyridopyrimidine) are capable of inhibiting the histamine metabolising enzymes, an important negative result was achieved with the 2-amino-4-hydroxy quinazoline, CB3717, leading to the conclusion that the development of rashes in some patients treated with CB3717 is not related to reduced histamine catabolism. It now seems more likely that they are caused by localised release of histamine as part of a hypersensitivity reaction, which is not unusual with various types of drug. The poor penetration of CB3717 across the blood brain barrier (see Chapter III) and its inability to inhibit HMT argue strongly against a role for elevated histamine levels in the causation of patient malaise.

IV.4.b Plasma Phenylalanine Levels

The finding that thirteen of the seventeen CB3717-treated patients under study showed transient elevation of plasma phenylalanine levels, up to 3 times the normal range, demonstrated that CB3717 was capable of perturbing phenylalanine metabolism in man. In the case reported by Hilton of a patient treated with methotrexate 200mg/kg, plasma phenylalanine rose up to 7 times the pre-treatment value by 22 hours after the start of the infusion and was still elevated 3 fold by 98 hours. No other amino acid measured showed any marked fluctuations from normal vaues (Hilton M.A. et al 1976). It was presumed that tyrosine levels were maintained by protein catabolism.

As discussed in the introductory section of this chapter, methotrexate is believed to exert its effect on phenylalanine levels by depletion of tetrahydrobiopterin (BH_4) levels, the cofactor required for its hydroxylation. Serum biopterin derivative levels were found to be increased in patients given methotrexate (Leeming R.J. et al 1976, Leeming R.J. & Blair J.A. 1980) which is presumed to be due to dihydropteridine reductase inhibition. More recently the same group reported changes in biopterin pools with BH_4 depletion in rats treated with oral methotrexate 100mg/kg. No effect was seen after doses of 1mg/kg (Blair J.A. Morar C. & Whitburn S. 1984). Work by Duch and coworkers, however, failed to show reductions in BH_4 or total biopterin levels in tissues from rats treated with 10mg/kg methotrexate i.p. and metoprine, or from neuroblastoma cells in culture grown in the presence of methotrexate concentrations sufficient to completely inhibit dihydrofolate reductase (Duch D.S. et al 1983). Treatment of rats with loading doses of phenylalanine along with

methotrexate did reduce the level of tetrahydrobiopterin though. They conclude that the pathway from 7'8 BH_2 to 5,6,7,8 BH_4 catalysed by dihydrofolate reductase is not generally involved in the de novo biosynthesis of BH_4. It may only assume importance if abnormal BH_4 utilisation occurs (for example with phenylalanine loading), producing excessive amounts of quinonoid BH_2 which isomerises to 7,8 BH_2. Clearly, elucidation of the biosynthetic pathways of tetrahydrobiopterin and their regulation in vivo is required.

Some workers feel that disturbance of aromatic amino acid metabolism, as might occur with depletion of tetrahydrobiopterin pools, may be crucial to the development of neurological toxicity which may follow methotrexate therapy. Treatment with neurotransmitters has been recommended (Cotton R.G.H. 1978, Leeming R.J. & Blair J.A. 1978). In the case of CB3717 the mechanism of producing elevated pheylalanine levels is unknown but it is possible that it is due to inhbition of dihydropteridine reductase. Clearly in vitro testing of this hypothesis and measurement of terahydrobiopterin pools in patients before and after CB3717 administration would help establish or refute this. Leeming has reviewed the role of tetrahydrobiopterin in a variety of neurological diseases including maligant hyperphenylalaninaema, senile dementia, Parkinson's disease, Alzheimers disease, Huntington's Chorea and lead poisoning, all of which may be associated with deranged serum biopterin derivative levels. He concludes that no matter how such changes arise, they are likely to be associated with altered neurotransmitter synthesis (Leeming R.J. Pheasant A.E. & Blair J.A. 1982). The rather tentative suggestion that the malaise and lethargy which follow CB3717 treatment is linked to the slight elevation in serum phenylalanine levels

requires further exploration although it is not totally unreasonable to postulate a role for neurotransmitter therapy as an antidote.

CHAPTER V

INVESTIGATIONS INTO THE MECHANISM OF CB3717 HEPATOTOXICITY

CHAPTER V

INVESTIGATIONS INTO THE MECHANISM OF CB3717 HEPATOTOXICITY

V.1 Introduction

Drug related hepatotoxicity is a fairly common occurrence and may complicate cytotoxic chemotherapy with agents such as 6-mercaptopurine, methotrexate, mithramycin, L-asparaginase, mitomycin C, nitrosoureas, DTIC and anthracyclines (reviewed by Woolley III P.V. 1983). It is possible that the biochemical lesions which give rise to the antiproliferative effect of these drugs are responsible for the damage to liver tissue, but the central role of the liver in drug metabolism and excretion is obviously of importance in determining its sensitivity to all classes of drugs. The mechanisms reponsible are generally divided into two broad groups (Sherlock S. 1981). First, a direct interaction of a drug (or more usually a reactive drug metabolite) with cellular macromolecules may result in hepatocyte injury or death. Such an effect is usually dose dependent and will occur frequently in the population treated. For example, paracetamol (acetaminophen) toxicity is believed to be due to the reactivity of one of its minor metabolites (probably N-acetyl-imidoquinone) which is produced by the liver mixed function oxidase system. This metabolite is usually detoxified by glutathione conjugation but, in conditions of excess, the metabolite covalently binds to hepatic tissues causing hepatocellular damage (reviewed by Black M. 1980).

The second mechanism of drug induced hepatic injury involves an immunological attack mounted against the antigenic challenge provided by the drug in combination with

liver cell constituents. This so called "idiosyncratic response" occurs infrequently in a population (~1%) and is not dose related. Re-challenge with the offending drug (for example, halothane) may produce an overwhelming hypersensitivity reaction with liver cell necrosis (Sherlock S. 1981).

The assessment of liver damage relies largely on the measurement of serum bilirubin, liver enzymes (alkaline phosphatase, alanine transaminase, aspartate transaminase, γ glutamyl transpeptidase) and albumin along with estimations of the prothrombin time. Such tests are relatively sensitive, but are limited in specificity (Zilva J.F. & Pannall P.R. 1975). Intrahepatic or extrahepatic cholestasis is generally associated with rises in alkaline phsophatase levels, whilst hepatocellular damage or necrosis results more often in elevated transaminase values. Small rises in all the enzymes may occur with either process.

The clinical and biochemical features of the hepatic disturbance caused by CB3717 treatment in man were described in Chapter II, the common finding being that of perturbation of plasma liver enzyme levels (in particular alanine transaminase) between days 4-21 after an infusion. The frequency of this phenomenon (~80%) strongly suggested that this was due to a direct toxic manifestation of CB3717. Mechanistically, the distinction between cholestasis and hepatocellular damage was not clear. On the occasions when hyperbilirubinamia was found, the majority was conjugated, suggesting cholestasis but this does not exclude an element of hepatocellular necrosis. The other puzzling characteristics of CB3717 hepatotoxicity were the apparent lack of dose dependency within the range 140-600mg/m^2, and the return towards normal of liver enzyme levels after 3-4

treatments (see Chapter II). Liver biopsy was not justified in this population of patients but the post mortem histological examination of liver tissue from one elderly patient revealed only the non-specific appearance of fatty change, cholestasis, and Mallory bodies in a centrilobular distribution with no disturbance of general liver architecture. A significant interpretation of this is impossible with the knowledge that the patient had received prior treatment with 5-fluorouracil and had required intravenous hydration due to intestinal obstruction for 5 weeks prior to death.

During the Phase I trial no evidence was accrued to support the belief that CB3717 caused an irreversible or progressive hepatic lesion, despite six patients receiving treatment for 6-15 months. On the other hand, methotrexate given in low dose chronically for psoriasis (2.5-50mg weekly) may give rise to hepatic fibrosis or cirrhosis and this may not be accompanied by plasma liver enzyme abnormalities (Coe R.O. & Bull F.E. 1968, Filip D.J. et al 1971, Robinson J.K. et al 1980). However, the main incentive in pursuing the aetiology of the CB3717-induced hepatic disturbance was the belief that there existed a direct relationship between this and the malaise syndrome which occurred in a similar proportion of patients. Efforts to prevent the 'hepatitis' were thus expected to influence the acceptability of this new antifolate in a clinical setting.

To investigate the liver toxicity an animal model was required. Pre-clinical toxicology studies in mice had only demonstrated abnormality in liver function tests (i.e. alanine transaminase increased 8 fold) when lethal doses were used (300mg/kg i.p.). Histologically, periacinar

necrosis was seen but the cause of death in these mice was thought to be extensive renal damage. A review of the literature revealed that the time course for elevation of liver enzymes after exposure to a hepatotoxin can differ markedly between rodents and man. Both paracetamol and morphine are examples of this and cause elevations of transaminases within 3-6 hours of treatment (James R.C. Goodman D.R. & Harbison R.D. 1982). Therefore, further experiments in mice were undertaken using therapeutic (or sub-lethal) doses of CB3717. Blood sampling within the first 24 hour period after treatment established that elevation of plasma alanine transaminase did occur but with a return to normal values by 24 hours, (described later in detail) so it seemed that an experimental system for studying the liver toxicity had been found.

The work described in this chapter attempted to test several hypotheses concerning the mechanism of CB3717 hepatotoxicity and its possible prevention, by exploiting the mouse model. First, the extended biological half life of $^{2\text{-}14}C$ CB3717 in mouse liver and kidney was determined as a development of the tissue distribution studies performed by other workers (Newell D.R. and Manteuffel-Cymborowska M., personal communication) which had shown that these organs were exposed to the majority of the administered drug. As mentioned earlier, glutathione depletion had been described in liver toxicity induced by paracetamol and some narcotics (Mitchell J.R. et al 1973, Davis D.C. et al 1974, James R.C. et al 1982) and therefore the effect of CB3717 on liver glutathione levels was studied. CB3705 (the N^{10}-unsubstituted 2-amino-4-hydroxy quinazoline) and CB3714 (the N^{10}-ethyl-2-amino-4-hydroxy quinazoline) were similarly investigated for their effect on plasma liver enzyme levels in an attempt to determine the importance, if any, of the

N^{10}-propargyl group of CB3717 in causing toxicity.

The success of N-acetylcysteine as an antidote to paracetamol poisoning, when administered sufficiently early (Piperno E. & Berssenbruegge D.A. 1976, Peterson R.G. & Rumack B.H. 1977, Prescott L.F. et al 1977, Walker R.M. et al 1982) suggested a trial of its effectiveness against CB3717 hepatotoxicity. Other compounds thought likely to be capable of preventing the toxicity were those which might be expected to reverse the activity of CB3717 against thymidylate synthetase and dihydrofolate reductase and other folate dependent enzymes (that is thymidine, folinic acid, 5-methyltetrahydrofolate). The mouse model was used to gauge the influence of any of these agents on the CB3717-induced elevations in alanine transaminse levels. The experimental methods and results of all the experiments are described below. This work was performed in conjunction with Dr. D.R. Newell.

V.2 Materials

The mice used throughout the experiments were C57 black/DBA_2F_1 male animals. The reagents used and their suppliers are listed below. Analar grade was used unless specified to the contrary.

CB3717 (diacid), ICI Pharmaceuticals Division (Alderley Park, Macclesfield, Cheshire). CB3705 and CB3714, Dr. T.R. Jones, Institute of Cancer Research (Sutton, England). Paracetamol (N-acetyl-p-aminophenol), trishydroxymethyl ammonium hydrochloride (TRIS), 5 sulfosalicylic acid, glutathione (reduced), folinic acid (calcium leucovorin) - Sigma London (Poole, Dorset, England). Urea, sodium hydroxide, sodium hydrogen phosphate, sodium bicarbonate

(hydrogen carbonate), methanol, acetic acid, L-ascorbic acid - BDH Chemicals Ltd., (Poole, Dorset, England). Ellmans reagent [5,5'-dithiobis-(2-nitrobenzoic acid)], Aldrich Chemical Company Inc, (Milwaukee, Wisconsin, USA). Diethyl ether, May & Baker (Dagenham, Essex, England). ^{14}C-CB3717 (5.9uCi/mg) ^{14}C-hexadecane (CFR 65, batch 62 868,000 DPM/ml), Amersham International PLC (Amersham, Bucks, England). Combustaid, permafluor V, carbosorb, Packard Instruments Ltd (Reading, Berks, England). Sodium heparin, Duncan Flockhart & Co Ltd (London, England).

V.3 Methods

V.3.a CB3717 Hepatotoxicity in Mice

CB3717 for injection was dissolved at a concentration of 10mg/ml in 150mM sodium bicarbonate and was adjusted to pH 9.0. The mice were weighed and then injected intra-peritoneally with CB3717 at a dose of 100mg/kg (260mg/m^2) which falls within the dose range causing hepatotoxicity in man (100-600mg/m^2). Control mice were injected with 150mM sodium bicarbonate. Groups of three control mice and three CB3717-treated mice were sacrificed by exsanguination via cardiac puncture, under diethyl ether anaesthesia at the time points 1,3,6,9,24,48 and 72 hours. Blood was withdrawn into a 1ml syringe rinsed with sodium heparin (50 I.U. diluted in 1N saline). Plasma was separated by centrifugation in microfuge tubes, (700g for 2 minutes at room-temperature) pipetted into plastic vials then stored immediately frozen (-20^o). The samples were thawed at room temperature prior to measurement of alanine transaminase and alkaline phosphatase levels (within one week) by the Biochemistry Department of The Royal Marsden Hospital using

a Roche Cobas Bio Analyser.

V.3.b Experiment to Determine The Long Term Exposure of Liver, Kidney and Plasma To ^{14}C-CB3717 Following Intra-peritoneal Injection in Mice

Standardization of Packard Sample Oxidiser

Three mice were sacrificed by exsanguination under diethyl ether anaesthesia. Portions from the excised livers and the whole kidneys were weighed, wrapped in Whatman No. 1 5.5cm filter paper and placed in Combusto Cones. 20ul of ^{14}C-hexadecane was added to each of these either before or after combustion. Blanks of filter paper alone were similarly treated. 0.25ml of Combustaid was added to the samples prior to burning for 1 minute in the Packard Oxidiser 306. Collection was into 9ml scintillant and 9ml carbosorb with all machine settings as recommended by the manufacturer. Counting was performed in an SL30 liquid scintillation spectrometer (Intertechnique, St. Albans).

Standardization of CB3717 Injection Solutions

Total Radioactivity

Duplicate dilutions (1 in 50) of the 2mg/ml, 10mg/ml and 20mg/ml ^{14}C-CB3717 solutions were prepared in 150mm sodium bicarbonate. 100ul aliquots of these were added to 8ml scintillant in scintillation vials. No evidence of immiscibility was seen. Blanks were also prepared using 100ul 150mM sodium bicarbonate and 100ul 150mM sodium bicarbonate plus 50ul ^{14}C-hexadecane with 8 ml scintillant. Samples were counted on the SL30 liquid scintillation spectrometer.

Total UV Absorbance

The 2mg/ml, 10mg/ml and 20mg/ml solutions were diluted 1 in 200, 1 in 1000 and 1 in 2000 respectively using 0.1M sodium hydroxide. The absorbance of these solutions at 301.5nm (CB3717 301.5nm e=26,600) was recorded in a 1cm silica cuvette on a Unicam SP500 Spectrophotometer.

Radiochemical Purity

50ul aliquots of the solution of ^{14}C-CB3717 prepared as above were analysed by high performance liquid chromatography using the separation system described in Chapter III. 1ml fractions were collected into 8ml Cocktail T scintillant and were counted. 50ul of the solution were put straight into 8ml Cocktail T to obtain a 100% value. Counting was performed for 4 minutes on the SL30 liquid scintillation spectrometer.

Determination of Long Term Exposure of Mice to ^{14}C-CB3717

An injection solution was prepared to contain 2mg/ml ^{14}C-CB3717 (5.95uCi/ml) dissolved in 0.15M sodium bicarbonate, adjusted to pH 9.0. A further two solutions were similarly prepared using cold CB3717 and a proportion of the ^{14}C-CB3717 2mg/ml solution to give final concentrations of 10mg/ml and 20mg/ml. The activity of the solutions were thus 11.9uCi/ml (2mg/ml), 11.5uCi/ml (100mg/ml) and 11.0uCi/ml (200mg/ml).

Groups of mice (3 per time point per dose) were weighed and injected intraperitoneally with the above solutions to receive respectively doses of 20mg/kg, 100mg/kg and 200mg/kg

CB3717. The mice were then maintained on a normal diet with water ad libitum until the times of sacrifice at 26.5, 74, 143, 243, 359 and 550.5 hours after treatment. At these time points, animals were anaesthetised with diethyl ether and then exsanguinated by cardiac puncture, the blood being withdrawn into syringes pre-rinsed with heparin. The plasma was subsequently separated by centrifugation and 200ul aliquots were absorbed onto Combusto-pads placed within Combusto cones (Packard Instruments Ltd). The liver and both kidneys were excised and weighed. Two portions of the liver were sectioned and reweighed and along with the kidneys, these tissues were individually wrapped in Whatman No. 1 filter paper 5.5cm and placed in Combusto cones. All the samples were then subjected to the burn procedure as described in the standardisation section.

V.3.c Experiment to Determine The Effect of Paracetamol and CB3717 on Liver Glutathione and Plasma Enzyme Levels in Mice

The following experiments were performed on separate days using freshly made drug solutions as described below.

i) Paracetamol (N-acetyl-p-aminophenol) was dissolved in 150mM sodium chloride and 1M sodium hydroxide to give a final concentration of 40mg/ml at pH 10. Treatment doses were 400mg/kg, administered intraperitoneally, and control animals were injected with 150mM sodium choride adjusted to pH 10 with 1M sodium hydroxide.

ii) CB3717 was dissolved in 150mM sodium bicarbonate adjusted to pH 9.0 with 1M sodium hydroxide to give a concentration of 10mg/ml and 30mg/ml. Treatment doses were 100mg/kg and 300mg/kg administered intraperitoneally and

control animals were injected with 150mM sodium bicarbonate at pH 9.0.

Duplicate groups of 3-4 mice per time point were injected i.p. with either the drug or control solution. Animals were sacrificed by cardiac puncture exsanguination whilst under diethyl ether anaesthesia at 4 hours (paracetamol treated) 1,3,4 and 6 hours (CB3717 100mg/kg) and 3 and 6 hours (CB3717 300mg/kg). Blood was withdrawn into a 1 ml syringe containing 20IU heparin and then was dispensed into microfuge tubes. Plasma was separated by centrifugation and stored frozen at -20°. Within one week the samples were thawed at room temperature and analysed for alanine transaminase and alkaline phosphatase levels. Plasma from the CB3717 treated animals were also analysed for drug levels using the high performance liquid chromatography technique described in detail in Chapter III section 2.d.

Glutathione Assay

The livers were excised, weighed and assayed for reduced glutathione (γ -glutamyl-cysteinyl-glycine) using a colorimetric assay adapted from Ellman (Ellman GL 1959). The tissue was homogenised with 3ml/g liver of ice cold 0.1M trishydroxymethylammonium chloride/4M urea buffer using five strokes of a teflon/glass homogeniser. 2ml aliquots were removed, mixed with 0.5ml/g liver of 0.05M sodium hydroxide and incubated at 40° for 30 minutes. The protein was then precipitated with 2ml/ml sample of 7.5% solution sulfosalicylic acid. Mixing was done in conical flasks by magnetic fleas and when the frothing subsided the contents were transferred to plastic tubes for centrifugation at 4° for 10 minutes. Duplicate 0.5ml aliquots of the supernatant

were treated with 2.5ml 0.5M phosphate buffer (pH 6.8) and 0.5ml Ellmans reagent (0.15mg/ml 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.5M phosphate buffer). After mixing, the absorbance of the samples at 412nm was read against reagent blanks. A standard curve for glutathione was prepared on each occasion using glutathione standards of 0.41, 0.82, 1.64 and 3.28 mg/ml dissolved in water. 1ml aliquots of these were treated in the same way as 1g liver before reading the absorbance values at 412nm.

V.3.d Experiment to Determine The Interaction of N-acetylcysteine and Glutathione with CB3717

CB3717 was prepared at a concentration of 200uM in 0.05M phosphate buffer pH 7.0. Glutathione and N-acetyl cysteine were prepared as standard solutions of 20mM, 2mM and 200uM in 0.05M phosphate buffer adjusted to pH 7.0 with 1M sodium hydroxide. 0.25ml aliquots of the glutathione or N-acetylcysteine solutions were added to either 0.25 ml of CB3717 solution or to 0.25ml 0.05M phosphate buffer to give mixtures of 100uM, 1mM, 10mM N-acetyl cysteine or glutathione $\pm$ 100uM CB3717. These samples were then incubated at 20° for 4 hours in the dark following which the thiol concentration of each was determined using the Ellmans assay as described previously. The 10mM solutions of N-acetylcysteine and glutathione required 1 in 10 dilution prior to this.

V.3.e Experiment to Determine The Effect of N-acetylcysteine Administration on CB3717 Induced Hepatotoxicity in Mice

CB3717 solutions were prepared as for earlier experiments at a concentration of 10mg/ml, pH 9.0. Treatment doses were 100mg/kg i.p. N-acetylcysteine was

dissolved in water and 1M sodium hydroxide to give a concentration of 50mg/ml at pH 7.0. Treatment doses were 500mg/kg i.p. (LD50 dose for mice 3725mg/kg i.p.) (Johnston R.E. Hawkins H.C. & Weikel JH 1983).

i) Mice were injected with CB3717 and N-acetylcysteine i.p. at time 0. Control groups comprised animals injected with 150mM sodium bicarbonate buffer pH 9.0, animals injected with CB3717 alone and animals injected with N-acetylcysteine alone. At 1,3,6 and 9 hours groups of three mice (treated and controls) were sacrificed under diethyl ether anesthesia. Blood sampling and liver extraction were performed and plasma enzyme levels and liver glutathione levels were determined as previously described.

ii) In another experiment test animals were injected with N-acetylcysteine, 500mg/kg, 30 minutes prior to injection with CB3717 100mg/kg then at 1,2,3,4 and 5 hours after treatment. Sacrifice occurred at 3 and 6 hours with blood and liver sampling as before.

V.3.f Experiment to Determine The Effect of Thymidine, Folinic Acid and 5-Methyltetrahydrofolate on CB3717-Induced Hepatotoxicity in Mice

CB3717 solution was prepared at a concentration of 10mg/ml as for earlier experiments. Thymidine was dissolved in water 50mg/ml. 5-methyltetrahydrofolate was prepared as 1mg/ml solution dissolved in 10mM ascorbic acid in 150mM sodium chloride. Folinic acid solution was prepared from 3mg/ml calcium leucovorin (Lederle) diluted 1 in three with 10mM ascorbic acid in 150mM sodium chloride to give a final concentration of 1mg/ml.

i) Mice were injected intraperitoneally at time 0 with CB3717 (100mg/kg). Thymidine (500mg/kg) was injected at time 0 and 4 hours, the latter time point being chosen on the basis of the pharmacokinetics of thymidine (Taylor G., personal communication). Control animals received 150mM sodium bicarbonate or CB3717 (100mg/kg) only. Animals were sacrificed at 6 hours and plasma was assayed for liver enzyme levels.

ii) Mice were injected intraperitoneally at time 0 with CB3717 (100mg/kg). 5-methyltetrahydrofolate (10mg/kg) or folinic acid (10mg/kg) was injected i.p. at time 0, or time 0,2 and 4 hours. Control animals received either 10ml/kg of the drug diluents or 5-methyltetrahydrofolate (10mg/kg) or folinic acid (10mg/kg) or CB3717 (100mg/kg) alone. Animals were sacrificed at 3 and 6 hours and plasma was assayed for liver enzyme levels.

V.3.g Experiment to Determine if CB3705 and CB3714 are Hepatotoxic in Mice

CB3705, the N^{10}-unsubstituted analogue of CB3717, was dissolved in 150mM sodium bicarbonate at a concentration of 9.2mg/ml and 27.6mg/ml (pH adjusted to 9.0 with 1M sodium hydroxide). CB3714, the N^{10}-ethyl analogue of CB3717 was similarly prepared to give a concentration of 10mg/ml and 30mg/ml. The structures of these compounds are shown in Figure V.5. The drugs were injected intraperitoneally at time 0 at doses of 92mg/kg and 276mg/kg of CB3705 and 100mg/kg and 300mg/kg of CB3714, thus using doses that were equimolar to CB3717 100mg/kg and 300mg/kg (0.21 and 0.62mmole/kg). Control animals received only 150mM sodium bicarbonate (pH 9.0). Animals were sacrificed at 3 and 6 hours by cardiac puncture exsanguination under diethyl ether

anaesthesia. Blood samples were collected and plasma separated in the manner described for previous experiments. In addition to plasma liver enzyme assays, plasma CB3705 and CB3714 levels were measured using an HPLC system similar to that used for CB3717 estimation and described in detail in Chapter III. 100uM standards of CB3705 and CB3714 were used for calibration and in the case of CB3705 the HPLC solvent mixture comprised 30% methanol/70% aqueous acetic acid (1%) instead of the 35% methanol/65% aqueous acetic acid (1%) mixture used for CB3714 and CB3717.

V.4 Results

V.4.a CB3717 Hepatotoxicity In Mice

100mg/kg CB3717 produced marked elevation of plasma alanine transaminase levels in mice with peak values being observed at 6 hours after treatment. Alkaline phosphatase levels were not affected. The results are presented in Table V.1 and Figure V.1.

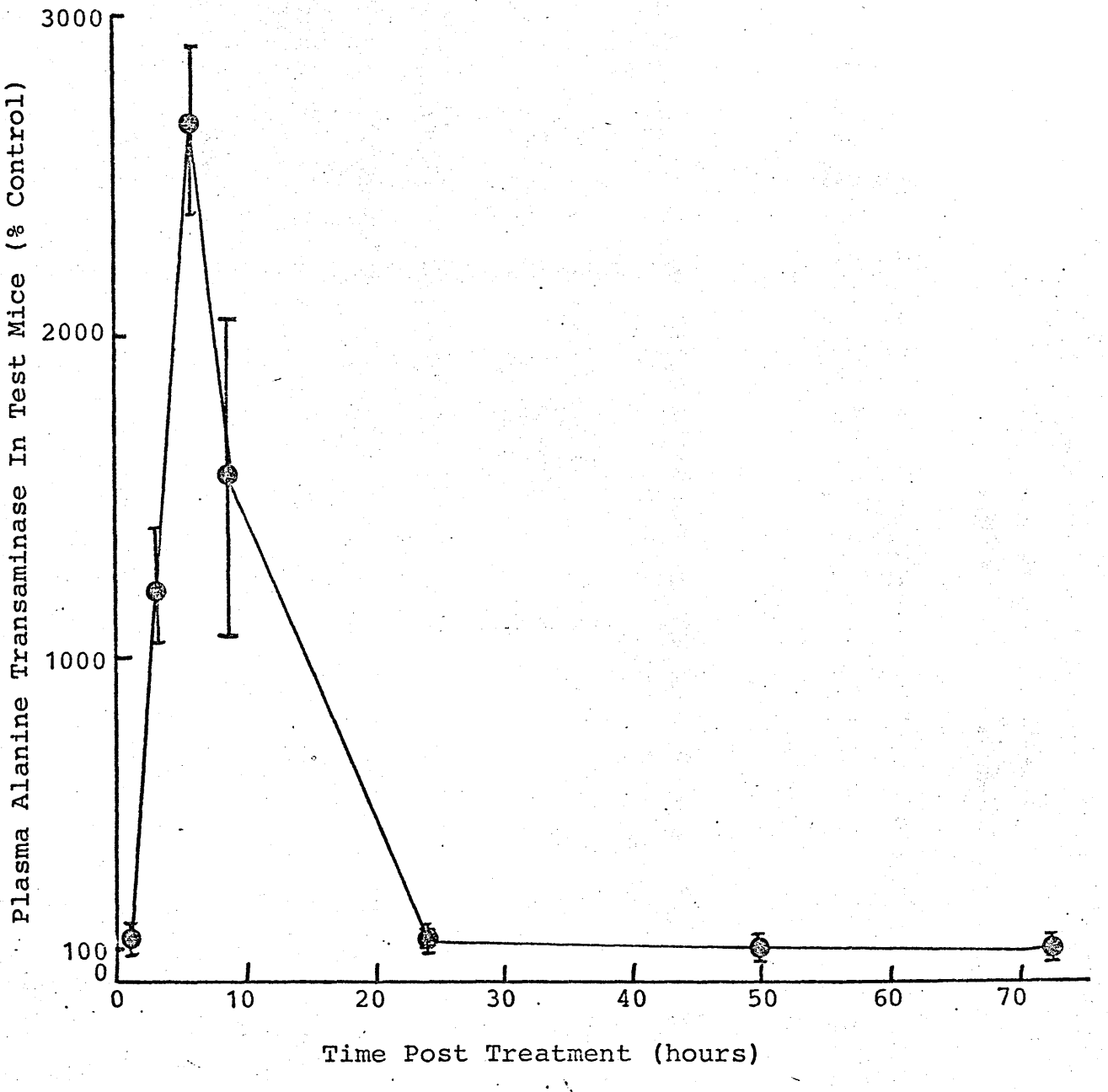
V.4.b Experiment to Determine The Long Term Exposure of Liver, Kidney and Plasma to ^{14}C-CB3717 Following i.p. Injection in Mice

Standardisation of ^{14}C-CB3717 Injection Solution

The counting efficiency for ^{14}C-hexadecane was 89.7%. Correcting the counts obtained for the diluted solutions, the total radioactivity of the doses administered are shown on Table V.2.a. The concentrations of the solutions as determined by UV absorbance at 301.5nm are shown on Table V.2.b. The analysis by HPLC found that 95.8$\pm$0.84% of the test solutions comprised the peak attributed to CB3717.

Time Post Treatment (hrs)	Control Value (IU/l) $\bar{x} \pm$ S.D.	Test Value (CB3717 Treated) $\bar{x} \pm$ S.D. (IU/l)	t test Value
1	22 ± 2	29 ± 13	0.425
3	24 ± 4	293 ± 78	0.002
6	20 ± 5	531 ± 90	0.002
9	11 ± 4	173 ± 95	0.060
24	21 ± 5	28 ± 5	0.137
48	22 ± 6	17 ± 6	-
72	24 ± 3	19 ± 2	-

TABLE V.1: Plasma Alanine Transaminase Levels In Mice Following CB3717 100mg/kg i.p.



Values shown are $\bar{x}$ ± S.D. (n=3)

FIGURE V.1: Plasma Alanine Transaminase Levels In Mice After CB3717 100mg/kg i.p. (Values Expressed As % Control)

Solution ^{14}C-CB3717 mg/ml	Dose Administered mg/kg	Total Radio-activity of dose uCi/kg
2	20	510
10	100	481
20	200	446

TABLE V.2.a: Total Radioactivity of The Doses Of ^{14}C CB3717 Administered To Mice

Solution ^{14}C-CB717 mg/ml		A°301.5nm	Calculated Concentration mM	mg/ml
2	diluted 1:200	0.550	4.1	1.96
2		0.555	4.2	2.02
10	diluted 1:2000	0.280	21.1	10.13
10		0.290	21.8	10.46
20	diluted 1:1000	1.010	37.9	18.19
20		1.050	39.5	18.96

TABLE V.2.b: Absorbance of Solutions of CB3717 at 301.5nm

Scintillation spectrometry of the collected fractions detected 91.2±1% of the total radioactivity within the CB3717 peak. The radiochemical purity of the ^{14}C-CB3717 was thus rather inadequate for the purposes of detailed distribution studies but was sufficient for determining the half life in kidney and liver.

Long Term Distribution of ^{14}C-CB3717 in Mouse Liver, Kidney and Plasma

Previous studies (D.R. Newell and M. Manteufel-Cymborowska, personal communication) had demonstrated the pronounced distribution of ^{14}C-CB3717 within the liver and kidneys and a summary of this data is shown in Figure V.2. The results of the extended study are shown in Table V.3 and represented graphically in Figure V.3. The half life in liver and kidney is approximately 16.6 and 62.5 hours respectively (calculated using a non-linear regression program) (Sampson J. 1969). Both liver and kidney concentrated the drug as the concentration within these tissues exceeded plasma CB3717 concentrations at all time points observed.

V.4.c Effect of Paracetamol and CB3717 on Liver Glutathione and Serum Enzyme Levels

i. Paracetamol

The effect of paracetamol on liver glutathione levels in mice has been well documented (Mitchell J.R. et al 1973, Davis D.C. et al 1974). Depletion is generally dose related up to 375mg/kg and a nadir is reached by 1 hour after

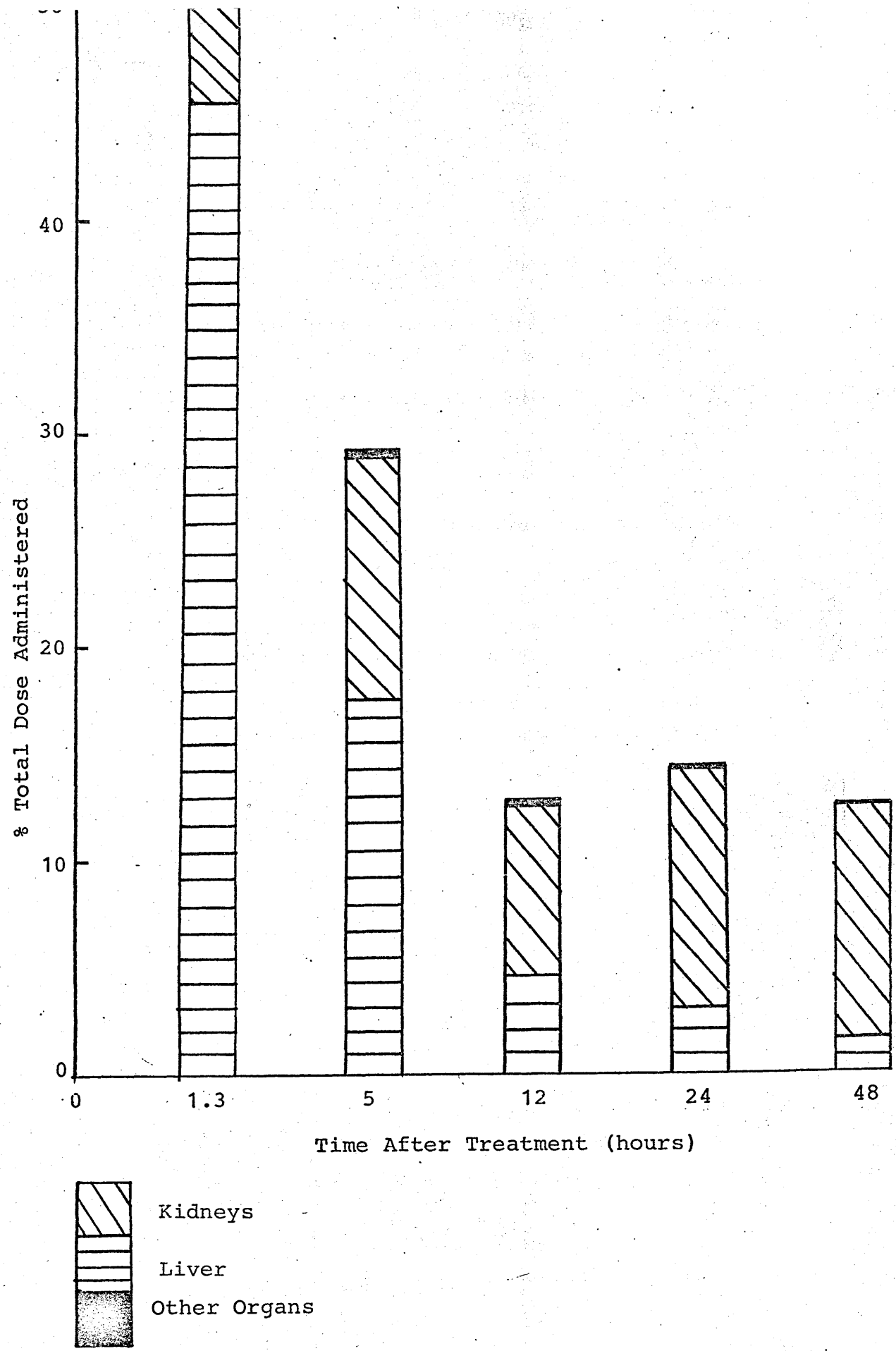


FIGURE V.2: Distribution of CB3717 In The Organs Of Mice Treated With 100mg/kg ^{14}C CB3717 i.p.
Data of D.R. Newell & M. Manteuffel Cymborowska)

Time Hours	Dose mg/kg								
	20			100			200		
	Tissue Conc^{n} (nmoles/g)			Tissue Conc^{n} (nmoles/g)			Tissue Conc^{n} (nmoles/g)		
	Liver	Plasma*	Kidney	Liver	Plasma*	Kidney	Liver	Plasma*	Kidney
26.5	9.1±1.8	0.259	5.8±0.5	175±18	2.4	1963±340	586±71	17.4	5615±653
74	2.8±1.2	-	3.5±1.6	50±14	0.77	1149±407	171±80	1.73	3553±966
143	-	-	-	16±3.7	-	1092±664	-	-	-
243	1.2±0.2	-	0.9±0.1	6.3±0.5	-	932±197	-	-	-
359	-	-	-	5.3±1.9	-	785±250	-	-	-
550.5	-	-	-	2.5±0.6	-	693±101	8.4±1.8	-	1553±201

$\bar{x}$ ± S.D. n=3 *nmoles/ml

TABLE V.3: Long Term Distribution of ^{14}C-CB3717 In Mice

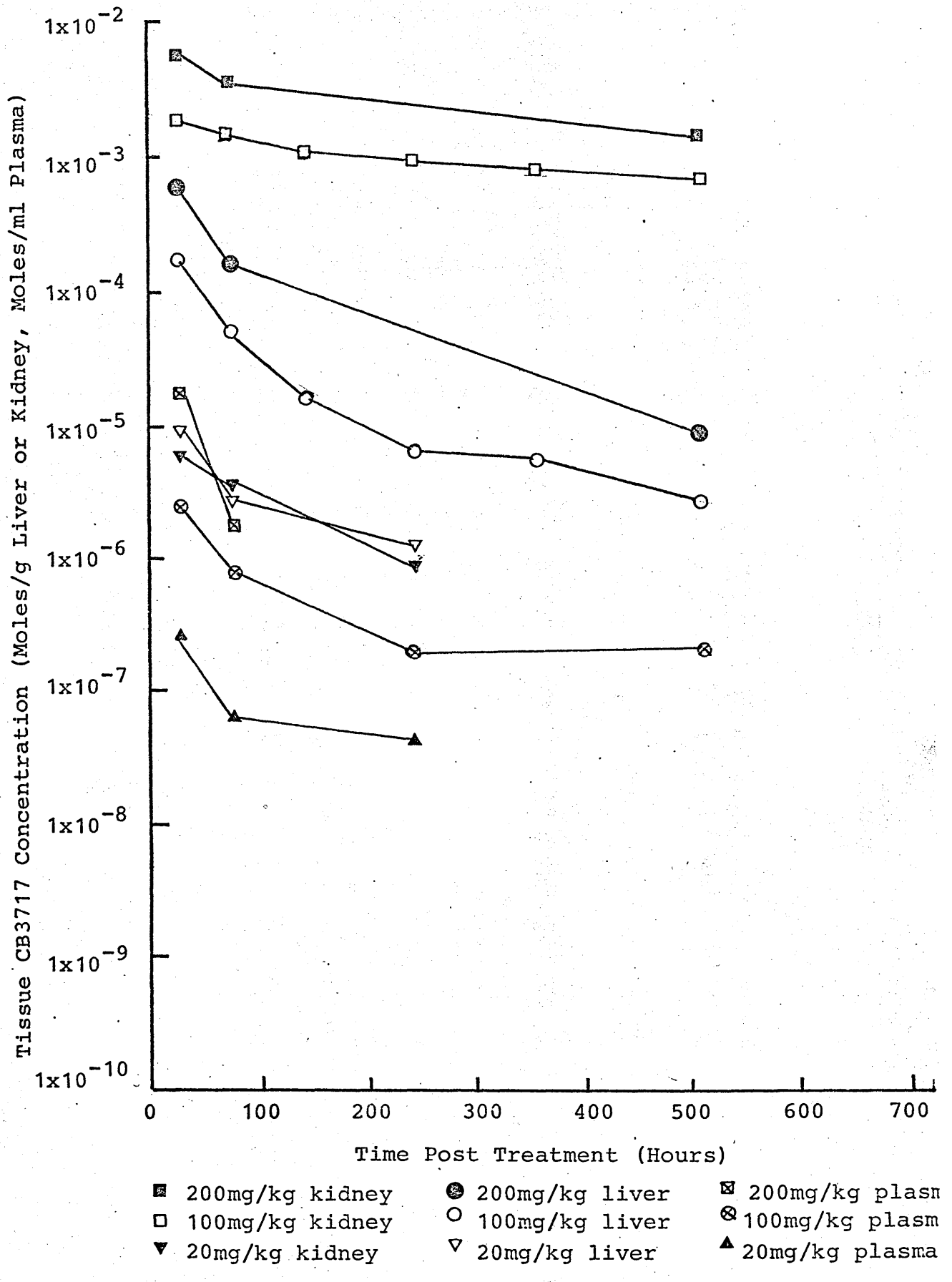


FIGURE V.3: Long Term Distribution of CB3717 In Mice Injected With 20,100 or 200mg/kg ^{14}C CB3717 i.p.

treatment with levels remaining low for several hours. A time point of 4 hours was chosen for study on this occasion. Other workers have previously reported that the depletion reflected a loss of total glutathione and not a conversion to oxidised glutathione (Mitchel J.R. et al 1973). In fact, the assay used here measured total non-protein bound thiol (SH) levels but for practical consideration this can be assumed to approximate closely glutathione levels.

The results shown on Table V.4 confirmed that paracetamol causes a significant decrease in liver glutathione levels (t test t=2.74, p0.029) with a mean reduction to 55±31% of control values. Alkaline phosphatase levels were not significantly different in paracetamol treated mice (t=0.4106, p=0.702) but the alanine transaminase values were elevated approximately 5 fold in one animal, fourteen fold in another and only very slightly in the third. When considered as a group the difference was not statistically significant (t test t=-1.4865, p=0.234) but clearly there was a suggestion of liver cell damage in some animals. On the basis of these data it was considered worthwhile to assess the effect of CB3717 in the same system.

ii CB3717

The decrease in liver glutathione induced by CB3717 at 100mg/kg is shown graphically in Figure V.4 with results expressed as percent controls. Considerable variation was seen but nadir values (67±7%) were found 3 hours after treatment with recovery to 100% control at 6 hours. A slight overshoot to 122±13% control values occurred at 9 hours. The pattern of plasma alanine transaminase elevation observed is also shown in Figure V.4. Plasma alkaline

		Reduced Glutathione		Alanine Transaminase		Alkaline Phosphatase	
		Conc^{n} mM	$\bar{x} \pm$ S.D.	Conc^{n} IU/l	$\bar{x} \pm$ S.D.	Conc^{n} IU/l	$\bar{x} \pm$ S.D.
Controls	1	5.9	6.9±2.1	16	14.6±2.4	155	164±10.4
	2	4.5		11.5		162	
	3	8.6		14		160	
	4	9.0		17		179	
Treated	1	2.1	3.6±1.3	26	98.7±97.9	199	170±2.5
	2	4.5		60		159	
	3	2.7		*		*	
	4	4.9		210		153	
t Test		p=0.029		p=0.234		p=0.702	

*Insufficient blood collected for assay

TABLE V.4: The Effect of Paracetamol 400mg/kg i.p. On Mouse Liver Glutathione Concentration and Plasma Liver Enzyme Levels (4 Hours Post Treatment)

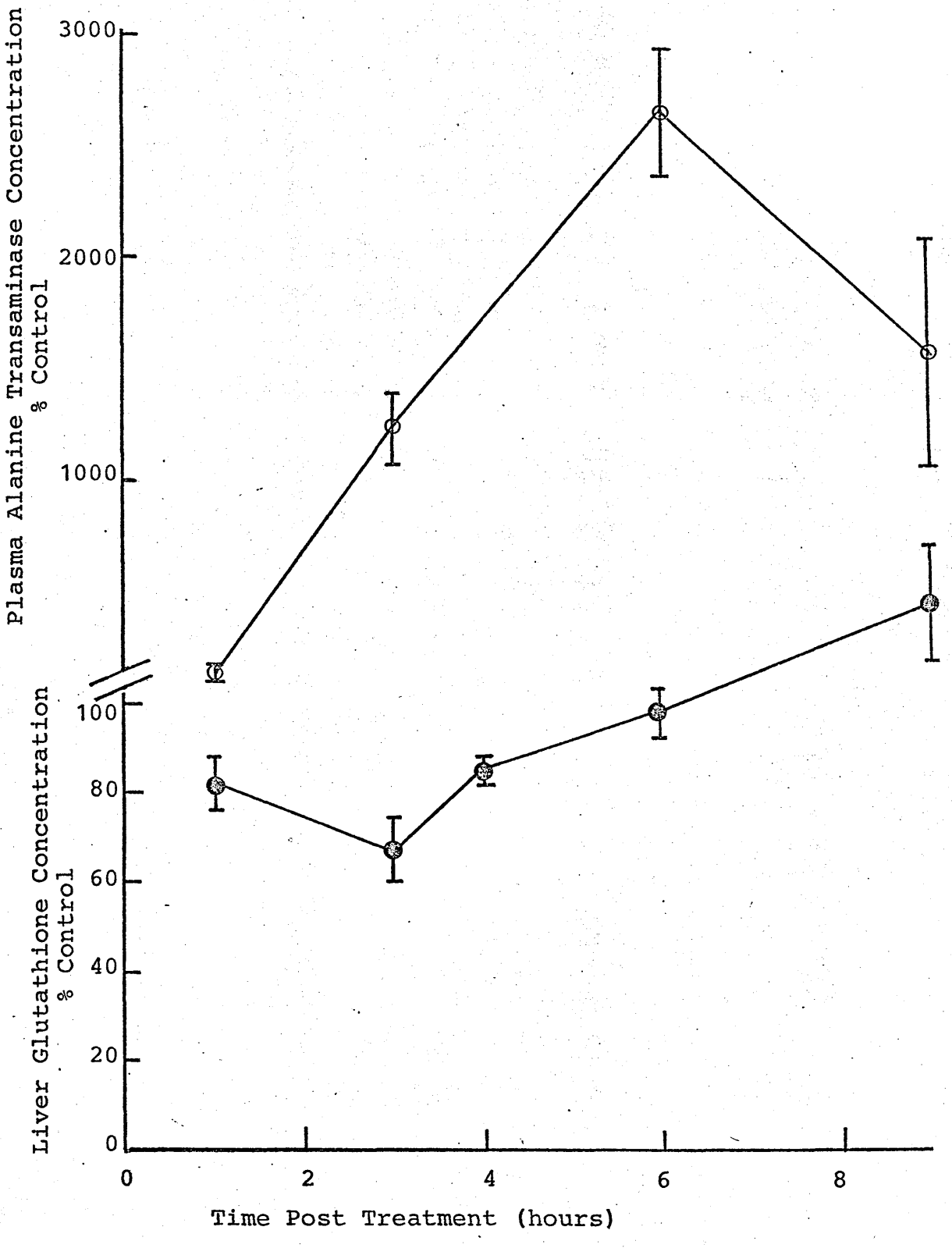


FIGURE V.4: Effect of CB3717 100mg/kg i.p. In Mice On Plasma Alanine Transaminase and Liver Glutathione levels

phosphatase levels were unaffected. A repeat experiment using both 100mg/kg and 300mg/kg CB3717 confirmed these transient abnormalities (see Table V.5) although on this occasion 100mg/kg led to only very slight reductions in liver glutathione (87±6% controls). The dose of 300mg/kg CB3717 produced larger increases in plasma transaminase levels suggesting a dose relationship to the hepatic toxicity in mice. It was noted whilst performing these experiments that a precipitate had formed in the gall bladders of mice treated with CB3717 which were sacrificed at 3-9 hours.

V.4.d Effect of Incubation of CB3717 With N-acetylcysteine and Glutathione

The absorbance at 412nm of the solutions of N-acetyl cysteine and glutathione incubated in the presence and absence of CB3717 are shown in Table V.6. The presence of CB3717 did not cause any significant loss of non-protein bound thiols. Some loss of thiols, presumably by oxidation, was evident, especially in the solutions of 0.1mM N-acetyl cysteine and 0.1mM glutathione when compared with the stronger concentrations. This was most obvious in the N-acetylcysteine solution incubated without CB3717.

V.4.e Effect of N-acetylcysteine on CB3717 Induced Hepatotoxicity

i) The results of the experiment when N-acetylcysteine was administered at a dose of 500mg/kg i.p. once only at the same time as CB3717 (100mg/kg) are shown on Table V.7 a and b. The liver thiol levels showed some variability and no statisically significant differences were found between those animals treated with CB3717 alone and those treated

Time After Treatment (hrs)	% Control				Plasma Quinazoline Conc^{n} (uM)	
	Liver GSH		Plasma ALT			
	100mg/kg	300mg/kg	100mg/kg	300mg/kg	100mg/kg	300mg/kg
3	87±6	65±13	305±41	891±284	125±3	361±14
6	106±23	78±5	1906±1157	3627±1259	72±43	353±63

Results expressed as $\bar{x} \pm$ S.D. n=3

TABLE V.5: Measurement Of Plasma Levels Of CB3717, Liver Glutathione And Plasma Alanine Transaminase Levels In Mice Treated At 100mg/kg and 300mg/kg i.p.

	Absorbance at 410nM		
	-CB3717	+CB3717	% Control
*10mM N-acetylcysteine	1.744	1.766	101
1nM N-acetylcysteine	1.632	1.626	99.6
0.1mM N-acetylcysteine	0.054	0.079	146
*10mM Glutathione	1.686	1.684	99.9
1mM Glutathione	1.632	1.620	99.3
0.1mM Glutathione	0.075	0.078	104

*Diluted 1 in 10

TABLE V.6: Effect of CB3717 Incubation with N-acetyl cysteine or Glutathione on Thiol Estimation

Time Hours	N-acetylcysteine % Control	CB3717 + N-acetylcysteine % Control	CB3717 % Control
1	108 ± 7	101 ± 9	-
3	100 ± 17	79 ± 19	94 ± 7
6	105 ± 6	116 ± 7	119 ± 3
9	91 ± 13	98 ± 15	-
	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.

TABLE V.7.a: Glutathione Levels In Liver Extracted From Mice Treated With CB3717 (100mg/kg) N-Acetylcysteine (500mg/kg) And A Combination Of CB3717 And N-acetylcysteine

Time Hours	N-acetylcysteine % Control	CB3717 + N-acetylcysteine % Control	CB3717 % Control
1	116 ± 12	83 ± 6	-
3	89 ± 24	328 ± 90	665±394
6	117 ± 32	731 ± 131	736±300
9	72 ± 9	404 ± 29	-
	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.

TABLE V.7.b: Plasma Alanine Transaminase Levels From Mice Treated With CB3717 (100mg/kg), N-acetylcysteine (500mg/kg) And A Combination Of CB3717 And N-acetylcysteine

Time (hours)	Plasma CB3717 Concentration uM	
	CB3717	CB3717 + N-acetylcysteine
3	114 ± 23	101 ± 10
6	35 ± 19	52 ± 15
	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.

TABLE V.7.c: Plasma CB3717 Concentration (uM) In Mice Treated With CB3717 (100mg/kg) ± N-acetylcysteine (500mg/kg)

with CB3717 and N-acetylcysteine at both 3 and 6 hours post treatment (t test p=0.204, p=0.269 respectively). The administration of N-acetylcysteine did not prevent the rise in plasma alanine transaminase levels caused by CB3717 and no significant difference in the enzyme elevation was noted at 3 and 6 hours between groups which received CB3717 alone or in combination with N-acetylcysteine (t test p=0.247, p=0.941 respectively). The plasma levels of CB3717 are shown in Table V.7c. N-acetylcysteine did not significantly alter CB3717 concentrations in plasma at 3 or 6 hours after treatment (t test p=0.42, p=0.27). All animals which received CB3717 (either with or without N-acetyl cysteine) were noted to have a precipitate in the gallbladder at the time of sacrifice.

ii. The results of the experiment using repeated injections of N-acetylcysteine (500mg/kg) at 30 minutes pre and 1,2,3,4 and 5 hours post CB3717 (100mg/kg) treatment are shown in Tables V.8a, b and c. The effect of multiple N-acetylcysteine injections plus CB3717 was to cause a marked depletion of liver thiol levels at 3 hours post treatment in comparison to CB3717 treatment given alone (t test, p=0.015). This effect was not sustained at 6 hours when thiol levels exceeded control values in both groups and no significant difference between groups was observed (t test p=0.627). Plasma alanine transaminase levels were similarly more elevated 3 hours after treatment in mice given the combination of N-acetylcysteine and CB3717 than in those given CB3717 alone (t test p=0.069). At 6 hours post treatment both groups showed similar enzyme elevations (t test p=0.6). CB3717 levels in plasma were higher at 3 hours in mice receiving N-acetylcysteine (t test,p=0.082). Once again all mice who received CB3717 were found to have a

Time (hours)	CB3717 + N-acetyl cysteine x6 (% control)	CB3717 (% control)
3	56±10	88±13
6	112±65	133±24
	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.

TABLE V.8.a: Glutathione Levels in Livers of Mice Treated With CB3717 (100mg/kg) ± Multiple Injections of N-acetylcysteine (6x500mg/kg i.p.)

Time (hours)	CB3717 + N-acetyl cysteine x6 (% control)	CB3717 (% control)
3	933±389	303±44
6	1509±341	1905±1158
	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.

TABLE V.8.b: Plasma Alanine Transaminase Levels in Mice Treated with CB3717 (100mg/kg) ± Multiple Injections of N-acetylcysteine (6x500mg/kg i.p.)

	Plasma CB3717 Concentration	
Time (hours)	CB3717 + N-acetyl cysteine x6 (% control)	CB3717 (% control)
3	151±23	118±9
6	71±43	-
	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.

TABLE V.8.c: Plasma CB3717 Concentration (uM) in Mice Treated With CB3717 (100mg/kg) ± Multiple Injections of N-acetylcysteine (6x500mg/kg i.p.)

precipitate in the gall bladder at sacrifice and the mice who received repeated i.p. injections of N-acetylcysteine had an accumulation of fluid in the peritoneal cavity and were hypokinetic with ruffled coats after the fourth injection.

V.4.f The Effect of Thymidine, Folinic Acid and 5-methyltetrahydrofolate on CB3717 Induced Hepatotoxicity

The results are displayed in Table V.9a and b. None of these agents ameliorated CB3717-induced alanine transaminase elevation in mice. Indeed 5-methyltetrahydrofolate and CB3717 caused significantly higher enzyme elevations than CB3717 alone at both 3 and 6 hours post treatment and on single and multiple injections.

V.4.g Comparison of Plasma Drug Levels and Alanine Transaminase Levels In Mice After Equimolar Doses of The Quinazolines CB3705, CB3714 and CB3717

The structures of these different quinazoline molecules are shown in Figure V.5 and their effect on plasma alanine transaminase levels is detailed in Table V.10. A precipitate was observed in the gall bladders of mice treated with CB3714 and CB3717 but not in those treated with CB3705. Neither CB3705 or CB3714 caused any significant elevation of plasma alanine transaminase levels at 3 or 6 hours after treatment. The plasma levels of these two drugs were also markedly lower than CB3717 levels at comparable time points.

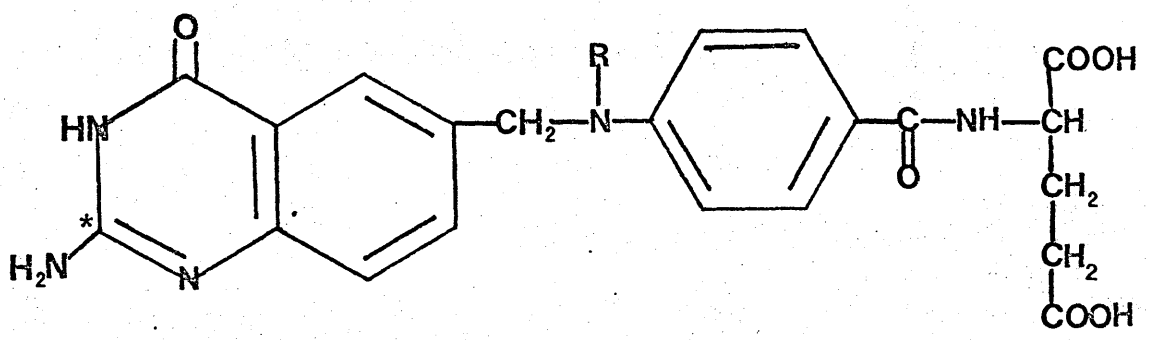
Time (hours)	Thymidine (% Control)	Thymidine + CB3717 (% Control)	CB3717 (%Control)
6	114±10	1381±654	1905±1157
	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.

TABLE V.9.a: Plasma Alanine Transaminase Levels In Mice Treated With Thymidine (500mg/kg x 2) and CB3717 (100mg/kg) ± Thymidine

Time (hours)	5-methyl tetrahydro folate 10mg/kg (% control)	Folinic acid 10mg/kg (% control)	CB3717 + 5-methyl tetrahydrofolate 10mg/kg x1 (% control)	CB3717 + Folinic Acid 10mg/kg x1 (% control)	CB3717 + 5-methyl tetrahydrofolate 10mg/kg x3 (% control)	CB3717 + folinic acid x3 (10mg/kg)	CB3717 100mg/kg
3	103±10	164±36	779±122 ($p=0.003$)	568±262 ($p=0.156$)	-	-	279±45
6	80±13	111±28	884±188 ($p=0.007$)	502±224 ($p=0.253$)	868±283 ($p=0.035$)	442±211 $p=0.399$	314±116
	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.

p values from t test (comparison with CB3717 alone)

TABLE V.9.b: Plasma Alanine Transaminase Levels In Mice Treated With 5-methyltetrahydrofolate (10mg/kg) Folinic Acid (10mg/kg) and CB3717 (100mg/kg) ± 5-methyltetrahydrofolate or Folinic Acid



N_{10} SUBSTITUENT R	COMPOUND
H	CB3705
CH_2-CH_3 (ETHYL)	CB3714
$CH_2-C\equiv CH$ (PROPARGYL)	CB3717

* Position of ^{14}C Label

FIGURE V.5: Structures of CB3705, CB3714 and CB3717

CB3705

Time Hours	ALT (% control) ~100mg/kg	~300mg/kg	Plasma Conc (uM) 100mg/kg	300mg/kg
3	94±14	-	15±2	-
6	140±36	132±21	1.8±0.3	15±9

CB3714

Time Hours	ALT (% control) ~100mg/kg	300mg/kg	Plasma Conc (uM) 100mg/kg	300mg/kg
3	78±13	140±32	6±3	55±25
6	80±16	189±87	<1	<2

CB3717

Time Hours	ALT (% control) ~100mg/kg	~300mg/kg	Plasma Conc (uM) 100mg/kg	300mg/kg
3	305±41	361±15	125±3	361±15
6	1905±1157	3627±1260	72±42	352±13
	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.

TABLE V.10: Comparison of Plasma Levels of CB3705, CB3714 and CB3717 in Mice After Equimolar Doses and The Effect on Plasma Alanine Transaminase Levels

V.5 Discussion

Reversible hepatic toxicity, with a transient increase of plasma alanine transaminase levels, was demonstrated in mice treated with therapeutic doses of CB3717. As mentioned earlier, although the time course of enzyme elevation differed from that in man (see Chapter II), for reasons which remain unclear, it was deemed worthwhile to investigate the problem in such an experimental system and to seek possible modulating factors.

The prolonged tissue distribution study of CB3717 in mice revealed the long exposure time of the kidneys and liver to the drug at fairly high concentrations. After treatment at 200mg/kg (which is a therapeutic dose for mice) 8.4nmoles/g and 1553nmoles/g of drug were present in liver and kidney tissue 23 days after treatment. One explanation for this in the kidneys could be related to drug precipitation and hence reduced renal elimination. By comparison, early studies of the persistence of methotrexate in normal mouse tissues using non toxic doses (1-3mg/kg i.p. on alternate days x 1-3 or 1mg/kg i.v. on alternate days x3) reported that parent drug was present in the kidneys and liver 21 days after treatment and as long as 8 months in the liver in one instance (Fountain J.R. et al 1953). Charache also found persistence of methotrexate in the liver for more than 100 days in man and mouse (Charache S.R. Condit P.T. & Humphreys S.R. 1960). Baugh and co-workers subsequently established that methotrexate followed the biosynthetic routes of folic acid and was to some extent converted to poly-δ -glutamyl derivatives (Baugh C.M. Krumdieck C.L. & Nair M.G. 1973).

Other groups have confirmed the presence of methotrexate polyglutamates in various tissues of mice, rats and men (Whitehead V.M. Perrault M.M. & Stelcner S. 1975, Gewirtz D.A. et al 1979, Poser R.G. Sirotnak F.M. & Chello P.L. 1981, Krakower G.R., Nylen P.A. & Kamen B.A. 1982). The enzyme responsible, folyl polyglutamate synthetase (FPGS), has been partially purified from rat liver (McGuire J.J. et al 1980) and recently CB3717 was found to be a substrate for the enzyme (Moran R., personal communication) with a Km of approximately 50uM. It seems likely that CB3717 is converted to polyglutamate forms in the liver and kidney which might delay its elimination from these tissues. Additionally at high concentrations (>100uM) CB3717 inhibited FPGS (Moran R., personal communication). If this is the case, in vivo, presumably FPGS activity on natural folates might be perturbed. The importance of this to the hepatotoxic properties of CB3717 is uncertain but interestingly the polyglutamate forms of CB3717 have also been shown to be more potent inhibitors of thymidylate synthetase than the monoglutamate (in permeabilised cells), with stoichiometric inhibition occurring with the tri, tetra and pentaglutamate forms (Kisliuk R.L. et al 1984). It seems possible that within the liver an interaction of CB3717 with FPGS could be relevant to its toxicity to this tissue.

The series of studies on liver glutathione levels after CB3717 treatment are difficult to interpret. The decrease of liver thiol levels measured 3 hours after a dose of 100mg/kg CB3717 varied from 67±7% control to 94±7% controls when all the experiments are considered. Recovery to values ≥100% control values by 6 hours was observed in all cases except when the higher dose of 300mg/kg CB3717 was used (liver thiols 65±13% controls at 3 hours, 78±5% at 6 hours).

In contrast, paracetamol can induce depletion of liver glutathione in mice at hepatotoxic doses (>375mg/kg) to values of ~25% normal (Mitchell et al 1973) which may not recover to normal even by 10 hours (Peterson F.J. et al 1983). There is a species difference in susceptibility to paracetamol toxicity which correlates with the degree of glutathione depletion it causes, the mouse and hamster being much more sensitive than rats or guinea pigs (Davis D.C. et al 1974). However, the variable effect of CB3717 on mouse liver thiol levels, despite more consistent elevations of alanine transaminase levels, implies that this transitory perturbation may be caused by and not causative of, the hepatic injury. In support of this, other drugs have been noted to cause slight liver glutathione depletion in mice including cocaine (25-30% reduction at 1 hour) propoxyphene (20-30% at 3-6 hours) and morphine (50% at 3 hours) (Thompson M.L. Shuster L. & Shaw K. 1979, James R.C. et al 1982), but there was no correlation between this effect and the observed rise in plasma alanine transaminase values caused by these narcotics. It may be that these compounds and CB3717 produce liver damage in the presence of adequate, though decreased, liver glutathione. Perhaps caution should be exercised in the co-administration of known glutathione-depleting agents with other hepatotoxic drugs in order to avoid additive effects.

The inability of N-acetylcysteine to prevent CB3717 hepatotoxicity is consistent with the above conclusions. Once again, considerable variation was seen in the liver glutathione levels of animals treated once only with N-acetyl cysteine but there was no appreciable effect on the slight CB3717-induced depletion of glutathione at 3 hours. The more marked depletion of liver thiols observed in mice treated with multiple intraperitoneal injections of N-

acetylcysteine plus CB3717 compared with those receiving CB3717 only, is difficult to explain. It may be related to the reported ability of large doses of N-acetylcysteine administered intraperitoneally to cause GSH depletion in rats (Vina J. et al 1980). These workers found that 1.0g/kg N-acetylcysteine given i.p. to rats depleted liver GSH to 42% control values by 4 hours. Recovery did not occur till 24 hours later. Further work by the same group confirmed this finding but reported that oral N-acetylcysteine did not cause GSH depletion (Estrela J.M. et al 1983). Their explanation for this is that if large doses of cyteine or N-acetylcysteine enter the blood plasma, they may be oxidised very rapidly. This can generate hydroxyl and thiyl radicals which cause GSH depletion. Oral administration, ensures maintenance of the amino acids in a reduced form with slow release into the small intestine and slow absorption. However, i.v. administration of N-acetylcysteine to severe paracetamol overdose patients remains the treatment of choice in man (Prescott L.F. 1983) which seem incompatible with this logic.

Recently some doubts have been expressed concerning the proposed mechanism of paracetamol-induced hepatotoxicity. Cimetidine, an H_2 antagonist, binds to cytrochrome P_{450} and can inhibit hepatic drug metabolism. In mice, concomitant administration of cimetidine 100mg/kg and paracetamol (350mg/kg) protected the animals from paracetamol-induced hepatic injury, but did not significantly affect plasma pharmacokinetics of paracetamol, prevent depletion of hepatic glutathione or alter in vivo covalent binding of paracetamol to hepatic proteins (Peterson F.J. et al 1983). The subject of drug-associated depletion of liver glutathione and the role of thiol donating agents as protection is thus complex and probably not entirely

relevant to CB3717 hepatotoxicity.

Reversal of the antitumour effect of CB3717 in L1210 tumour bearing mice can be achieved by administering thymidine (Jackman A.L. et al 1984). However, no improvement in the hepatic effects of CB3717 occurred when thymidine was tried as an antidote, which strongly suggests that inhibition of thymidylate synthetase is not the hepatotoxic lesion. Furthermore, any effect of CB3717 on other enzymes of the folate pathway i.e. dihydrofolate reductase, should have been reversed by the provision of folinic acid or 5-methyltetrahydrofolate but neither of these agents were able to counteract the liver toxicity. This refutes the hypothesis that the antifolate properties of CB3717 are culpable.

The study of two related quinazoline molecules was enlightening. Neither the N^{10} unsubstituted molecule CB3705, or the N^{10} ethyl compound, CB3714, caused any elevation of plasma alanine transaminase levels. CB3714, however, is a fairly potent inhibitor of TS with an I_{50} for the L. Casei enzymes of 27nM and 38nM respectively (Calvert A.H. et al 1980b) and this lends support to the belief that TS inhibition per se is not a hepatotoxic event. Further, despite approximately equimolar doses of CB3705, CB3714 and CB3717 being given, the plasma concentrations of both CB3705 and CB3714 were very much lower than CB3717 at 3 and 6 hours after treatment. One explanation for this could be that the damage to hepatocytes caused by CB3717 may reduce its rate of clearance in the bile. Whatever the reason for the differing plasma pharmacokinetics of these quinazolines, the evidence points to the N^{10} propargyl group of CB3717 as a key feature in its hepatotoxicity. A few other compounds with triple carbon bond substituents have been cited as

hepatotoxins including pargyline hydrochloride (Lowe M.C. 1978) and danazol (Pearson K. & Zimmerman H.J. 1980). The toxicity is thought to be mediated by metabolic activation of the acetylenic substituent which causes a measurable decrease of hepatic microsomal cytochrome P_{450} (White I.H. 1978). Although metabolites were not detected in human and mouse plasma or urine (see Chapter III and Newell D.R. et al 1982) it is conceivable that small amounts of a reactive metabolite are formed by interaction of the N^{10} propargyl group with cytochrome P_{450}. If this were the case, phenobarbitone pretreatment (a hepatic microsomal enzyme inducer) might worsen CB3717 toxicity. Alternatively, cimetidine, which binds to hepatic cytochrome P_{450} and inhibits hepatic drug metabolism, should help prevent the toxicity as has been recently reported for paracetamol and cocaine (Peterson F.J. et al 1983). Clearly both these studies need to be performed.

The nature and significance of the gall bladder precipitate observed in mice treated with CB3717 and CB3714 requires exploration, particularly as only one drug is apparently hepatotoxic. Some preliminary studies by D.R. Newell (personal communication) performed on rats (who also develop a precipitate in the common bile duct and elevations of plasma ALT levels after CB3717 treatment) indicate that the precipitate is not cholesterol. It is possible that one, or a mixture, of the bile acids may be responsible.

In summary, these investigations of CB3717-induced hepatitis in mice have made an important contribution to the definition of those features of the drug which are most likely to play a role in its clinical liver toxicity. In addition, some areas for further study have been indicated with the hope that future effort will provide a more precise

explanation for this phenomenon.

CHAPTER VI

EXPERIMENTAL RESISTANCE TO CB3717

CHAPTER VI

EXPERIMENTAL RESISTANCE TO CB3717

VI.1 Introduction

The development of resistance to cytotoxic drugs is a common and frustrating problem in the treatment of clinical cancer. Many workers have explored the nature of such resistance by experimental systems with resistant tumour cell lines using both *in vivo* and *in vitro* models (reviewed by Selby P. 1984). Although these studies cannot reflect accurately the situation in patients many useful indications of the adaptive measures involved at a cellular level have been established. A well known and frequently cited example of such work concerns the dihydrofolate reductase inhibitor, methotrexate. Several mechanisms of resistance to this antimetabolite have been described which include an increase in levels of dihydrofolate reductase activity (Fischer G.A. 1961, Friedkin M. *et al* 1962, Schrecker A.W. *et al* 1971), a change in the cellular transport of methotrexate (Fischer G.A. 1962, Sirotnak F.M. Kurita S. & Hutchinson D.J. 1968, Harrap K.R. *et al* 1971) and kinetically altered dihydrofolate reductase (Jackson R.C. Hart L.I. & Harrap K.R. 1976, Jackson R.C. & Niethammer D. 1977). One or more of these changes may be present in cells which acquire MTX resistance (Niethammer D. & Jackson R.C. 1975). Further investigation of cells with elevated dihydrofolate reductase activity has implicated amplification of the DHFR gene as the basis for such an effect (reviewed by Bertino J.R. *et al* 1981). Indeed, Schimke and coworkers concluded from their work with the murine sarcoma cell line S-180 that "the prolonged administration of a single drug in ever increasing concentrations which is retained in the environment is

precisely that form of administration most likely to result in amplification of genes in a stable state, thereby importing stable resistance". (Schimke R.T. et al 1978). In one leukaemic patient receiving MTX therapy, DHFR gene amplification was found when resistance to MTX emerged (Horns R.C. Dower W.J. & Schimke R.T. 1983).

In the preclinical studies, CB3717 was toxic to mouse L1210 leukaemia cells grown in continuous suspension culture with an ID_{50} concentration at 48 hours of 5uM (Jones T.R. et al 1981). Resistant cells were raised by exposure of these L1210 cells to incremental concentrations of CB3717, in culture medium containing negligible amounts of salvageable thymidine ($<10^{-7}$). Approximately 100 fold resistance developed over 2 years with the emergence of L1210 cells refractory to the presence of CB3717 at a concentration of 500uM. (The poor solubility of CB3717 at neutral pH prohibited the preparation of solutions in excess of 25mM which were diluted 1 in 50 in the growth medium to attain the final concentration of 500uM.) The subsequent isolation of several monoclonal lines and their characterisation, is described in this chapter and has afforded some further insight into the properties of CB3717 with the elucidation of at least one possible mechanism of clinical resistance. Additionally, some experiments to determine cross-resistance with other antifolate and fluoropyrimidine compounds produced interesting results, which are also described. This work was performed in collaboration with A.L. Jackman as indicated.

VI.2 Materials

VI.2.a Chemicals

Reagent chemicals (analar grades when available) were obtained from BDH Ltd (Poole, Dorset) and Hopkin and Williams Ltd. (Romford, Essex). Nicotinamide adenine dinucleotide phosphate, (reduced-NADPH), dihydrofolate (FH_2), deoxyuridine monophosphate (dUMP), 5,10-methylenetetrahydrofolate (5,10-CH_2FH_4) were supplied by Sigma Chemicals Co. (London). ^{3}H-dUMP was supplied by Amersham International PLC.

VI.2.b Drugs

The quinazoline compounds CB3703, and CB3705 were prepared by Dr. T.R. Jones (JonesT.R. 1980). CB3717 was supplied by ICI Pharmaceuticals Division (Alderley Park, Macclesfield, Cheshire). Methotrexate by Nils Klaas Klausen (Denmark). 5-fluorouracil and 5-fluorodeoxyuridine by Sigma Chemical Co. (London).

VI.2.c Tumour Cells and Media

Mouse L1210 leukaemia cells and the resistant line (induced by culture of the sensitive L1210 cells in incremental sublethal doses of CB3717) were grown continuously in suspension culture in RPMI 1640 medium containing 20mM Hepes buffer and 10% donor horse serum (mycoplasma and bacteriophage tested - Flow Labs) which was supplemented by 2mM glutamine. Resistant cells were passaged in the presence of 500uM CB3717 in addition.

VI.3 Methods

VI.3.a Cell Culture

Solutions of CB3717 for use in tissue culture were prepared by dissolving the diacid powder in 10ml deionised water with a few drops of 1N sodium hydroxide to a concentration of 25mM drug. One drop of phenol red indicator was then added, as a pH indicator, and the solution was sterilised by filtration through millipore Millex 0.22um filters. The pH was adjusted to neutral (orange/red) by the dropwise addition of sterile 1N hydrochloric acid using a sterile pipette. Solutions were stored at $4^{o}C$ and used within 1 month.

L1210 cells and L1210 cells resistant to CB3717 (L1210R3717) were diluted in fresh complete medium to a concentration of $5x10^{4}$ cells/ml. 10ml subcultures were used routinely in $25cm^{2}$ flasks. 0.2ml of the 25mM CB3717 solution (prepared as above) was added to the 10ml medium for the resistant cell line (final concentration 500uM). Cells were counted in an improved Neubauer haemocytometer. Incubations took place at $37^{o}C$.

VI.3.b Colony Formation of The L1210 Cells Resistant to CB3717 500uM

L1210 resistant cells were serially diluted in complete medium containing 500uM CB3717 to achieve a concentration of 40 cells/ml on day 1. As a lag phase prior to logarithmic growth is usual when cells are diluted to such low concentrations, on day 2 cells were assumed to be still approximately at a concentration range of 40/ml. Further dilutions of the cells were made to provide final concentrations of 6.5, 10, 15, 20 and 25 cells/ml in total

volumes of 25ml (24ml medium). Just prior to plating, 1ml of warm 4% agar solution was rapidly injected into the flasks (to provide final volume 25ml) which were vigorously shaken. 4x5ml of this mixture was then plated onto small petri dishes using a sterile technique. The petri dishes were stacked in a sandwich box, the sides of which were moistened by wads of dampened absorbent paper and the lid of which was sealed with tape. The box was left in the incubator at 37°C for fifteen days. After this time colonies were observed in all the dilutions, just visible to the naked eye, and fifteen were removed. This was achieved using a sterile pipette and a gentle suction force to lift individual colonies from the agar and place them in 10ml plastic tubes containing 1ml of complete tissue culture medium. Dilution with a further 4ml medium took place the next day and after one week 7 of the colonies had given rise to satisfactory cell growths (C3,C5,C6,C7,C10,C14,C15). These monoclonal resistant cell lines were subcultured twice weekly thereafter in the presence of CB3717 (500uM) and one cell line, C15, was additionally passaged in the absence of drug for 9 months. It should be noted that prior to the successful colonisation of the resistant cells three earlier attempts using cell dilutions ranging from 2-12 cells/ml had been unsuccessful.

VI.3.c Growth Characteristics of L1210 Cells and 3 Mono-Clonal L1210 Lines Resistant to CB3717

L1210 cells and the monoclonal resistant lines C3, C10, and C15, in suspension culture were counted using a modified Neubauer haemocytometer and were then diluted to a concentration of $5x10^4$ cells/ml in complete medium. The resistant cells had been grown in the absence of CB3717 for one week prior to the experiment but dilutions were made in

the presence and absence of CB3717 (500uM) for comparison. Triplicate 5ml cultures were set up in 25cm^2 flasks and the cells were counted 4.5, 28, 46.5 and 72 hours afterwards with incubation at 37^{o}C in the interim.

VI.3.d Estimation of Thymidylate Synthetase Activity
(Performed with A.L. Jackman)

The monoclonal resistant L1210 cell lines were subcultured in the absence of CB3717 for 14 days prior to preparation for thymidylate synthetase assay. Bulk cultures 35-100ml) of the resistant cells and sensitive L1210 cells were prepared at a concentration of 7x10^4/ml and on the following day these were counted accurately and a precise measurement of their volumes was made. The cells were centrifuged at ~170g for 5 minutes in a pre-cooled centrifuge (4^o), the supernatant was decanted off and the cell pellets were washed in ice cold saline and respun. 3ml of buffer (0.05M potassium phosphate/0.01M mercaptoethanol pH 7.4) was added to each of the remaining pellets which were sonicated. Centrifugation at 50,000g for 1 hour in a pre-cooled centrifuge (4^o) was performed and the supernatant was retained for thymidylate synthetase estimation. Thymidylate synthetase was assayed by a modification of the method of Calvert (Calvert A.H. et al 1980a) which essentially involves counting the tritiated water by-product of the reaction between ^{3}H-dUMP and 5,10-methylene tetrahydrofolate (unutilised ^{3}H-dUMP being retained on a Dowex chloride column). Counting was performed on an SL30 Intertechnique liquid scintillation counter. The 0.5ml incubation mixture contained 100uM ^{3}H dUMP (0.225uCi) and 220uM (±)-L-5,10-CH_2FH_4 (made by dissolving 7mg (±)-L-FH_4 in 5ml of 0.1M dithiothreitol with the addition of formaldehyde (20mM) and 0.1ml of 1N NaOH). The reaction was started by

the addition of 0.25ml of the enzyme preparation and incubation was for one hour at 37°C. Under these conditions less than 2% of the substrate was converted to product. The reaction was stopped by the addition of 1ml iced water and the whole mixture was then run down a 3x0.5cm Dowex 1 chloride column. Further elution with 2ml iced water gave a total effluent of 3.5ml which was mixed with 10ml of PCS scintillant and counted. The experiment was repeated nine months later using C15 resistant cells grown in the absence of CB3717.

VI.3.e Purification of Thymidylate Synthetase From C15 and L1210 Cells With Estimation of Kinetic Constants
(Performed with A.L. Jackman)

Bulk cultures of L1210 and C15 cell lines were used to prepare cell lysates as described above (C15 cells grown out of CB3717 for 1 month prior to experiment). Purification was achieved by salt precipitation followed by a single affinity chromatographic stage that was dependent on reversible ternary complex formation with the biospecific ligand, CB3714 (N-(4((2-amino-4-hydroxy-6-quinazolinyl) methyl)ethylamino)benzoyl-L-glutamic acid) (Jackman A.L. et al 1984). The fractions containing enzyme were pooled and the thymidylate synthetase activity was estimated using variable concentrations of substrate. CB3717 was made up as a 1mM solution in 0.1M sodium hydroxide and diluted in water and was introduced to the assay mixture in different concentrations in order to calculate the I_{50} for the purified thymidylate synthetase enzymes. Kinetic constants were calculated by the method of Wilkinson using an algorithm supplied by Dr. R.C. Jackson (Wilkinson G.N. 1961).

VI.3.f Estimation of Dihydrofolate Reductase Activity

L1210 cells and three monoclonal CB3717 resistant strains (C3, C10 & C15) were prepared as 150ml bulk cultures, CB3717 having been omitted from the culture medium of the resistant cells for 2 weeks prior to the experiment. Whilst in a logarithmic growth phase the cells were counted and then centrifuged at 170g for 5 minutes. The supernatant was discarded. The cell pellet was resuspended in 10ml of 0.15M potassium phosphate buffer at pH 7.0 and was sonicated at 20KHz for 10 seconds using a MST ultrasonic disintegrator. These preparations were then centrifuged at 35,000g in a pre-cooled centrifuge (4^{o}) for 1 hour following which the supernatant was decanted and dialysed overnight against 0.15M potassium phosphate buffer pH 7.0. Dihydrofolate reductase was assayed spectrophotometrically (Osborn M.J. & Huenneken F.M. 1958). Dihydrofolate was dissolved in 0.25M mercaptoethanol with the minimum of 0.1N sodium hydroxide required to bring it into solution (1mg/ml). NADPH was dissolved in water (2mg/ml). 100ul NADPH, and 200 ul of the enzyme preparation from different cell lines were mixed in a cuvette, the total volume being made up to 1.8ml using 0.15M potassium phosphate buffer. A reference sample was also prepared omitting the enzyme. The change in absorbance at 340nm was followed using a Cary model 16K recording spectrophotometer. 100ul of the dihydrofolate was added to each cuvette and the rate of change of absorbance at 340nm was determined again. The "baseline" activity seen before the addition of FH_2 was minimal and was attributed to the oxidation of NADPH by reactions other than the reduction of FH_2. This rate was subtracted from the rate of the final reaction to give an accurate measure of dihydrofolate reductase activity (Jackson R.C. et al 1976). Results were calculated assuming

a molar extinction change at 340nm for the reaction of 1.22 x $10^4 cm^{-1}$. The experiment was repeated on several occasions with the different cell lines including the C15 resistant cells which had been grown in the absence of CB3717 for 9 months.

VI.3.g Cross-Resistance Studies With L1210 and C15 Cells Resistant to CB3717

C15 cells were grown in the absence of CB3717 for 2 weeks prior to each series of experiments. Bulk cultures of the L1210 and C15 cells were prepared at a concentration of $5x10^4$ cells/ml. Drugs for use in tissue culture were dissolved in de-ionised water, diluted to the required range of concentrations and sterilised by filtration through millipore Millex 0.22um filters. The drug solutions were 100 times the final concentration as 0.1ml of drug solution was added to 10ml of cells in complete tissue culture medium within $25cm^2$ flasks. Triplicate cultures were used with control flasks receiving 0.1ml sterile water in place of drug. Incubation at 37^oC was interrupted only by cell counting at 24 and 48 hours and the results were expressed in terms of control cultures.

The compounds tested were methotrexate (DHFR inhibitor), 5-fluorouracil (5-FU), 5-fluororo-2-deoxyuridine (FUdR) and two derivatives of the N-(p-(((6-quinazolinyl) methyl)amino)benzoyl)-L-glutamic acid series, CB3703 (2,4-diamino-5-methyl) and CB3705 (2-amino-4-hydroxy). Both 5-FU and FUdR are classed as thymidylate synthetase inhibitors by virtue of their metabolism to 5-fluoro-2-deoxyuridine monopohosphate (FdUMP) which is a TS inhibitor (Heidelberger C. 1982). Other mechanisms are probably involved in the cytotoxicity of 5-fluorouracil which is discussed in Chapter

I (Friedman M.A. & Sadee W. 1978). CB3703 and CB3705 are inhibitors of both thymidylate synthetase and dihydrofolate reductase, the Ki for L1210 TS being 470nM and 67nM respectively and the Ki for rat liver DHFR being 0.6pM and 350pM respectively (Calvert A.H. et al 1980).

VI.4 Results

VI.4.a Characteristics of L1210 Cells and 3 Monoclonal Cell Lines Resistant to CB3717 (In The Presence and Absence of CB3717 500uM)

The resistant cells appeared morphologically similar to the parent L1210 line although some irregularity of contour and variability in cell size was apparent. The doubling time of the resistant strains was approximately twice that of the sensitive L1210 cells and the presence of CB3717 did not affect them significantly as detailed below.

Cell Line	Approx. Doubling Time (Hours)
L1210	12.5
C3-	22.5
C3+	24.5
C10-	24.0
C10+	24.5
C15-	24.0
C15+	26.5

+ indicates grown in the presence of CB3717
- indicates grown in the absence of CB3717

When C15 cells were re-exposed to CB3717 500uM after 9 months passage in the absence of drug, no growth inhibition occurred proving maintenance of resistance over this period.

VI.4.b Thymidylate Synthetase Activity in L1210 Cells Resistant and Sensitive to CB3717

The activity of thymidylate synthetase in the different cell lines is shown in Table VI.1.a expressed as nmoles/10^9 cells/hour. All the resistant cell lines showed increased thymidylate synthetase activity (10-40 fold). Table VI.1.b demonstrates the sustained increase in activity in the C15 cells grown in the absence of CB3717 for 9 months.

VI.4.c Purified Thymidylate Synthetase From L1210 and C15 Cells:- Kinetic Constants and I_{50} CB3717

The kinetic constants and I_{50} CB3717 for thymidylate synthetase purified from the sensitive and resistant L1210 cells are shown in Table VI.2. The results suggest that the kinetics of the enzymes from these sources is identical.

VI.4.d Dihydrofolate Reductase Activity In L1210 and Resistant L1210 Cells

The dihydrofolate reductase activity of the different cell lines is shown in Table VI.3 expressed as International Units/10^9 cells. (1International Unit = 1umole product transformed/min). DHFR activity was increased by 4.8-10.5 fold in CB3717 resistant lines and in the C15 strain grown in the absence of drug for 9 months the increased DHFR activity was maintained.

Cell Line	Thymidylate synthetase Activity nmoles product/ 10^9 cells/hour	Increased Activity (fold)
L1210	0.201	-
C3	7.594	38
C5	5.763	29
C6	2.315	11.5
C7	5.187	26
C10	2.054	10
C14	2.748	14
C15	3.558	18

TABLE VI.1.a: Thymidylate Synthetase Activity in L1210 Cells Resistant and Sensitive to CB3717

Cell Line	Thymidylate synthetase Activity nmoles product/ 10^9 cells/hour	Increased Activity (fold)
L1210	0.07	-
C15-	2.10	30

TABLE VI.1.b: Thymidylate Synthetase Activity In L1210 And C15 Cells Grown In The Absence Of CB3717 For 9 Months

Cell Line	Km for dUMP (uM)	Km for 5,10 $-CH_2FH_4$ (uM)	I_{50} for CB3717 (nM)
L1210 Sensitive	2.4±0.5	17.9±1.6	14
C15 (L1210 Resistant)	3.8±0.62	18.16±4.8	14
	$\bar{x}$ ± S.D.	$\bar{x}$ ± S.D.	

TABLE VI.2: Thymidylate Synthetase Kinetic Constants and Inhibition by CB3717

Cell Line	Enzyme Activity IU/10^9 cells		Increased Activity (fold)
L1210	0.08±(0.019)	(n=3)	
C3	0.38	(n=1)	4.8
C10	0.58±0.14	(n=2)	7.3
C15	0.73±0.16	(n=2)	9.1
C15-*	0.84±0.07	(n=2)	10.5

$\bar{x}$ ± S.E.

*C15 grown in absence of CB3717 for 9 months

TABLE VI.3: Dihydrofolate Reductase Activity In L1210 Cells Resistant And Sensitive To CB3717

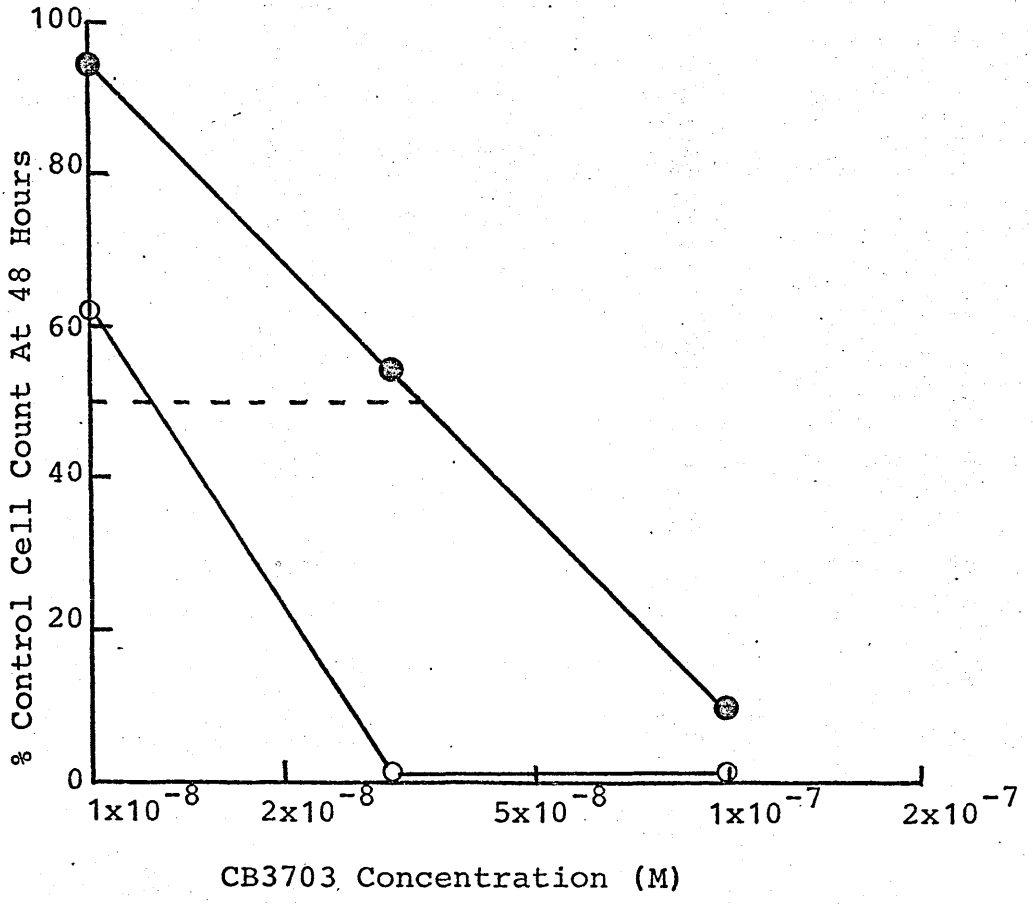
VI.4.e Cross Resistance Studies

Examples of the dose response curves of the L1210 and C15 cells to CB3703, methotrexate, 5-fluorouracil and fluorodexoyridine are shown on Figures VI.1,2,3 and 4 with a summary of the calculated ID_{50}'s shown on Table VI.4.

VI.5 Discussion

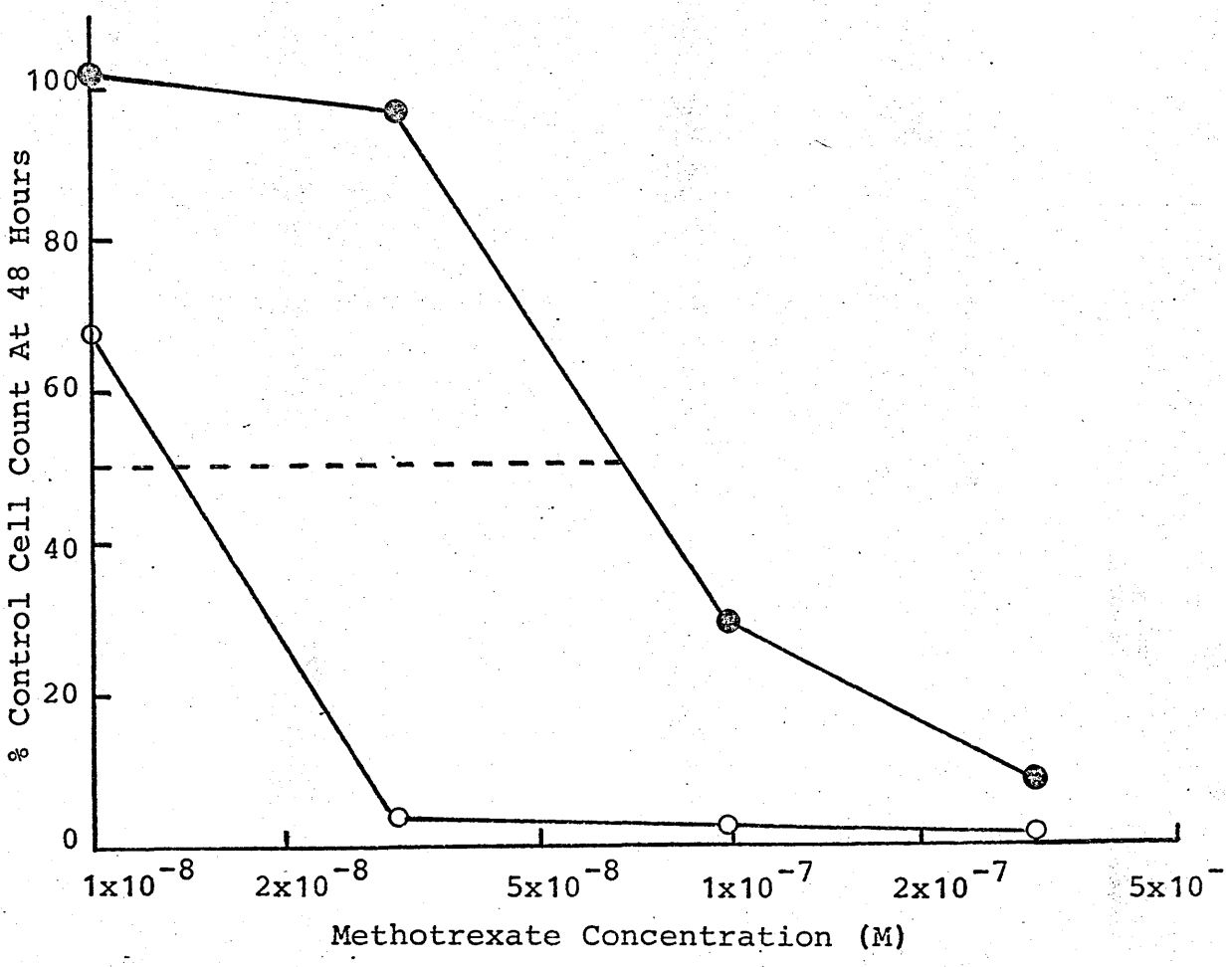
The most striking difference between the sensitive and resistant L1210 cells was the increase in thymidylate synthetase activity apparent in the seven surviving monoclonal lines (10-40 fold). Additionally, three of the lines (C3, C10 & C15) showed a slight elevation of dihydrofolate reductase activity (5-10 fold). In the C15 line these changes in enzyme activity were found to be stable over a nine month period when CB3717 was withheld from the growth medium. This accords with the maintained 100 fold resistance to CB3717 found on rechallenge with the drug. Furthermore the kinetic data attained for thymidylate synthetase purified >2000 fold (Jackman A.L. *et al* 1984) from C15 and L1210 cells suggested that the enzymes were identical.

The cumulative evidence therefore points to increased production of thymidylate synthetase as the mechanism of resistance to CB3717 possibly secondary to gene amplification. Proof of this hypothesis requires isolation of the TS gene and direct analysis of the CB3717 resistant L1210 DNA in comparison with the sensitive parental line DNA. Such a project is being undertaken by other workers who also have subjected samples with equally active thymidylate synthetase activity from L1210 and C15 cells to



	ID_{50}
● C15	3.3×10^{-8}
○ L1210	1.25×10^{-8}

FIGURE VI.1: Dose Response Curves For CB3703 Of L1210 And C15 Cells



	ID_{50}
● C15	6.7×10^{-8} M
○ L1210	1.35×10^{-8}M

FIGURE VI.2: Dose Response Curves For Methotrexate Of L1210 And C15 Cells

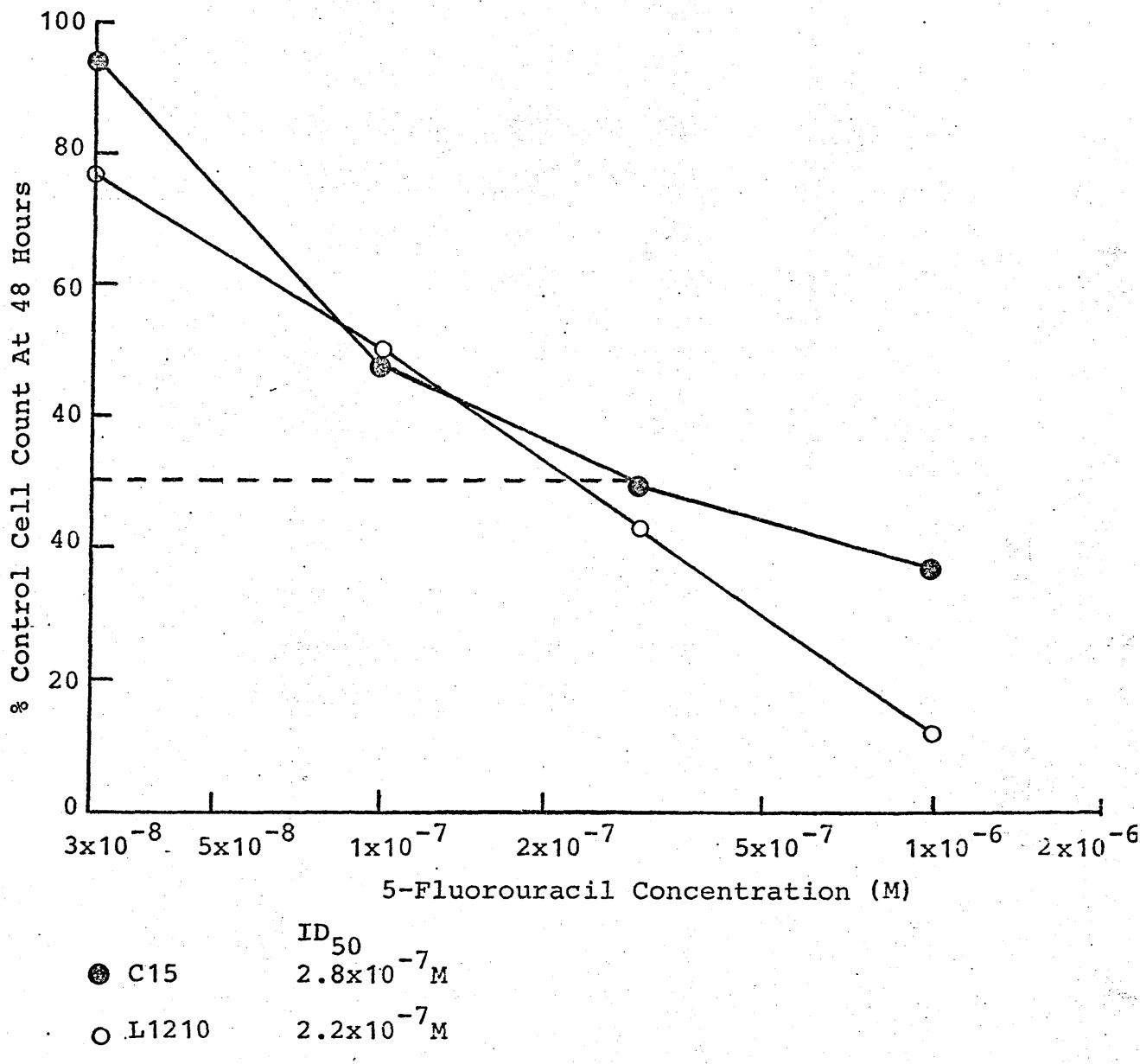


FIGURE VI.3: Dose Response Curves For 5-Fluorouracil Of L1210 and C15 Cells

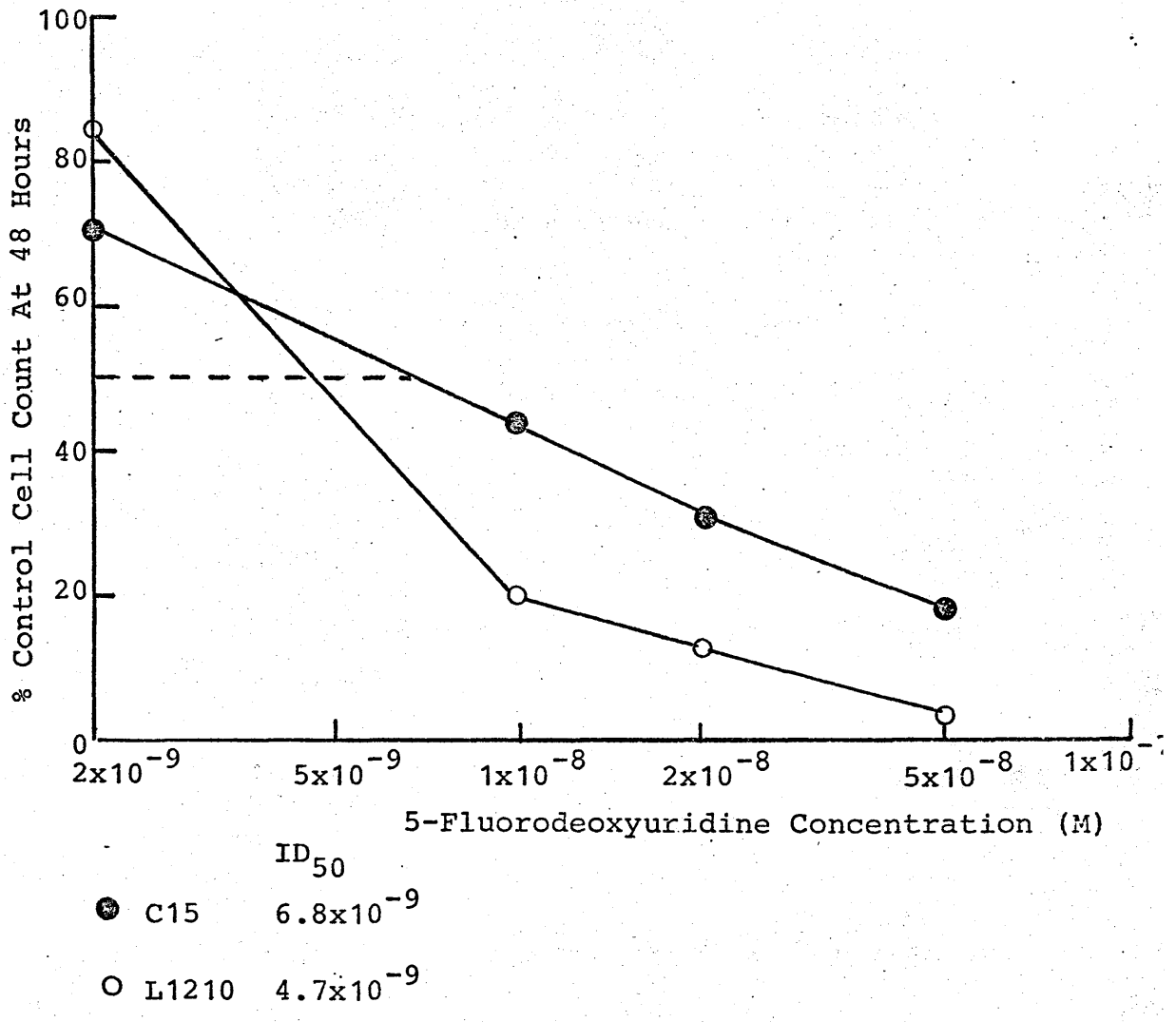


FIGURE VI.4: Dose Response Curves For 5-Fluorodeoxyuridine Of L1210 And C15 Cells

Drug	L1210 ID_{50} (uM)	C15 ID_{50} (uM)	C15 / L1210
CB3703	0.0125	0.033	2.6
CB3705	5	500	100
MTX	0.135 , 0.22 ($\bar{x}$ 0.178)	0.67, 0.75 ($\bar{x}$ 0.71)	4
5FU	0.22, 0.54 ($\bar{x}$ 0.38)	0.26, 0.28 ($\bar{x}$ 0.27)	0.7
FUdR	0.0047, 0.0018 ($\bar{x}$ 0.00365)	0.0068, 0.0047 ($\bar{x}$ 0.00545)	1.4

TABLE VI.4: Summary of ID_{50} Data in L1210 Sensitive and Resistant Strains (at 48hrs)

sodium dodecyl sulphate polyacrylamide gel electrophoresis using a silver stain to detect protein bands. In both preparations faint bands were visible around the 35-40,000 molecular weight range although many other contaminating bands were visible in the L1210 preparation (Little P. & Willison K.R. personal communication). This gives some indication of the relatively increased proportion of thymidylate synthetase (with respect to other protein) present in C15 as opposed to L1210 cells. Their findings also concord with Dunlap and coworkers who isolated TS from Lactobacillus Casei and found it to have a molecular weight of approximately 70,000 comprising two sub units of approximately 35,000 each (Dunlap R.B. Harding N.G.L. and Huennekens F.M. 1971). Evidence for the overproduction of the TS enzyme by the C15 line has now been confirmed by measurement of the total enzyme by a ^{3}H dUMP binding assay (36pmoles/mg protein) (Jackman A.L. personal communication).

Some recent data on DHFR gene amplification suggest that stable mutations are more likely to be associated with expanded coding regions on large chromosomes rather than small paired chromosomal elements called "double minute chromosomes" (Kaufman R.J. Brown P.L. & Schimke R.T. 1979, Curt G.A. et al 1983). This is because double minute chromosomes lack centromeres, do not segregate during cell division and are readily lost. The daughter cells receiving the extra DNA fragments have no survival advantage in the absence of the selecting agent and the mutation is readily lost. If amplification of the TS gene is responsible for CB3717 resistance in the C15 strain (which is stable in the absence of drug for 9 months) the above hypothesis would place the amplified region on a large chromosome. Interestingly, karyotype analysis of these cells has failed to show any double minute chromosomes or any obvious

amplification (P. Little, personal communication).

The four fold resistance of C15 cells to methotrexate and the ~3 fold resistance to CB3703 (compared with L1210) may be attributed to the increased dihydrofolate reductase activity observed in this strain. Why this developed was not clear although one possibility was that in the presence of increased thymidylate synthetase, the cytotoxic locus of CB3717 moved to DHFR (Ki for rat liver enzyme 14nM, Jones T.R. et al 1981) and induced an increase in the levels of enzyme activity. Other workers have isolated a TS overproducing (up to 5 fold) 5-FUdR-resistant mouse fibroblast line which they also found to have slight (2 fold) elevation of DHFR, in comparison with the parent line. They postulated that an elevated DHFR level may provide a selective advantage to cells with increased TS activity (Rossana C. Rao L.G. & Johnson L.F. 1982). Their argument was that the possibility of increased TMP synthesis may cause a decrease in tetrahydrofolate pools and subsequent reduction in rates of purine synthesis, with all its implications. Cells with overproduction of DHFR would be able to overcome this problem. In the case of the L1210 resistant lines, a similar hypothesis for the small increase in DHFR activity could be put forward. As with TS, the change in DHFR activity was maintained in C15 cells grown out of drug for 9 months. Comparison of the kinetic properties of purified DHFR from C15 and L1210 cells would help indicate whether the increased activity reflected overproduction or altered turnover number.

CB3705, and CB3703, were reported as being inhibitors of both DHFR and TS. The Ki of CB3705 for TS is 67nM making it a considerably less potent inhibitor than CB3717 (Ki for L1210 TS ~4nM) (Jackman A.L. et al 1984). In

previous tissue culture experiments thymidine alone was capable of reversing toxicity from CB3705 in L1210 cells (Calvert A.H. et al 1980) implying that TS inhibition was the cytotoxic event. It was therefore not surprising that C15 cells with increased TS activity were resistant (100 fold) to CB3705.

The lack of resistance of C15 cells to 5-FU and FUdR was not anticipated but several possible reasons for this can be offered in explanation. FUdR is phosphorylated by the enzyme thymidine kinase to the nucleotide 5-fluoro-2'-deoxyuridine monophosphate (FdUMP) which is a powerful competitive inhibitor of TS (Reyes P. and Heidelberger C. 1965). 5-FU may also be metabolised to FdUMP via several pathways (reviewed by Heidelberger C. 1982) one of which involves prior conversion to FUdR. Increased activity of thymidine kinase in the C15 line might produce higher levels of FdUMP capable of inhibiting the increased TS. However, measurement of thymidine kinase by Dr. S.E. Barrie, using a recently developed method (Barrie S.E. et al 1984) proved that this was not the case as TK activity in C15 and L1210 cells was similar on two occasions (L1210 58nmoles/hour/10^7 cells, C15 72 nmoles/hour/10^7 cells and L1210 118nmoles/hr/10^7 cells, C15 90nmoles/hr/10^7 cells) (personal communication). A decrease of activity in the enzymes which catabolise 5-FU and its intermediates and of the phosphatases involved in the breakdown of FdUMP might also be responsible for the retained sensitivity of C15 to these fluoropyrimidines (reviewed by Ardalan D. et al 1980) and altered drug transport mechanisms must also be considered. Furthermore, in the case of 5FU, incorporation into RNA may account for its cytotoxicity (Martin D.S. 1978, Roobol C., De Dobbeleer G.B.E. & Bernheim J.L. 1984) in which case C15 and L1210 cells would be equally sensitive. Thus the

observation of non-cross resistance to fluoropyrimidines of a TS overproducing cell line provides plenty of scope for future investigation.

A decreased ability to transport CB3717 would, of course, be one other way in which the L1210 resistant cell line acquired resistance. Preliminary studies with ^{3}H CB3717 found no significant difference between the resistant and sensitive cell lines in the intracellular accumulation of drug which seems to occur very slowly by a different process from that of the reduced folate cofactors and MTX (Jackman AL, personal communication).

Hepatoma cells resistant to FUdR have been reported previously as showing 6-10 fold elevated levels of TS (Priest D.G., Ledford B.E. & Doig M.T. 1980) and alteration of TS in FUdR resistant Ehrlich ascites carcinoma cells has also been described (Jastreboff M.M. Kedzierska D. & Rode W. 1983). TS overproduction in 5-FUdR resistant mouse fibroblasts (up to 50 fold) was unstable with a half life of three weeks (Rossana C. et al 1982). The data presented in this chapter have established that the probable mode of resistance developed in L1210 cells to the quinazoline folate analogue, CB3717 is a stable overproduction of thymidylate synthetase (10-40 fold) and slightly increased activity of DHFR (5-10 fold). It is not inconceivable that such cellular changes could also be responsible for resistance in the clinic.

CHAPTER VII

SUMMARY AND FUTURE CONSIDERATIONS

CHAPTER VII

VII SUMMARY AND FUTURE CONSIDERATIONS

The concept of "a cure for cancer" is one romanticised by the media and press but one which few, if any, clinicians and scientists would uphold, being cognisant of the multiplicity of the forms and clinical presentations which are encompassed by the term "cancer". Combined modality treatment and multiple drug regimens have made some headway against certain tumours, providing grounds for cautious optimism. However, solid tumours still provide a formidable hurdle and a tremendous incentive for the development of new cytotoxic drugs.

One such agent, CB3717, evolved from the desire to produce an antifolate with different properties from, and lack of cross-resistance with, methotrexate, which is the classical agent within this group of antimetabolites. Thymidylate synthetase (TS) was sought as its cytotoxic locus instead of dihydrofolate reductase (DHFR), with the hope of avoiding antipurine effects. Additionally, it was hoped that the folate analogue structure of CB3717, based on a quinazoline ring, would avoid the problems that befall pyrimidine analogue TS inhibitors which require metabolic activation and are prey to myriad catabolic, inactivating enzymes. In Chapter I these ideas were reviewed in detail along with the biochemical evidence that CB3717 had achieved such goals in vitro which included characterisation of the inhibition kinetics against purfied TS, cellular nucleotide and folate pool measurements (pre and post exposure to CB3717) and reversal of cytotoxicity by the delayed administration of thymidine. The therapeutic effect of CB3717 against a variety of mouse tumours coupled with its

unique biochemical properties was sufficiently persuasive to warrant its clinical evaluation. Chapter I surveys both this data and the ensuing toxicological assessment in mice, where renal toxicity, due to drug precipitation in the renal tubule, was paramount. The results of studies of thymidine "salvage" in rodent tissues now suggest that the use of mouse antitumour models may be inappropriate for this type of agent (Jackman a.L. et al 1984).

The Phase I clinical trial involved some 99 patients and was the subject of Chapter II. The reason for the large numbers was primarily because of the frequent occurrence of mild hepatic toxicity which was apparent even at the starting dose. Eighty percent of patients showed a variable rise in plasma alanine transaminase levels and malaise was experienced by a similar proportion so that an association of these toxicities seemed justifiable. Altering the schedule of administration from a 1 hour to a 12 hour infusion or prescribing prednisolone for one week after treatment were two ways in which amelioration was sought but with no effect, except modest improvement in well-being in some of the steroid-treated patients. A dose relationship, within the rather narrow dose range studied, (140-600mg/m^2) could not be established. Repeated courses (>4-5) were followed by a return to (or near to) normal enzyme levels in 13 patients.

Renal toxicity, as portended by the mouse studies, was dose limiting at 500-600mg/m^2 and slight to moderate reductions in glomerular filtration rates were observed in a few patients at doses between 200-400mg/m^2. Other toxicities included occasional myelotoxicity and skin rashes which were not clearly dose related. The responses seen in ovary, breast, lung and mesothelioma were a rewarding

adjunct providing promise for Phase II evaluation at the relatively well tolerated dose of 400mg/m^2.

The pharmacokinetic evaluation of CB3717 in 17 patients was described in Chapter III and it was established that there was linearity of dose with the peak plasma concentration of drug achieved, biphasic decay of plasma drug levels, approximately 30% drug excreted in the urine in the 24 hours after treatment and extensive plasma protein binding of drug (~97%). Metabolites were not detected in plasma or urine but parent drug was still present in renal tissue from a patient who died 8 days after treatment, lending support to the belief that renal toxicity in man may also be related to drug precipitation in the acid environment of renal tubules. Hepatic toxicity, as measured by increases in alanine transaminase levels, failed to show any correlation with the pharmacokinetic parameters defined, with the exception of peak plasma drug levels (11 patients). It was felt this required confirmation in a larger series.

Chapters IV and V dealt with the investigations into the possible mechanisms of CB3717 clinical toxicity, in particular to the skin and liver. Disturbance of histamine catabolism, which is a feature of some other antifolates which cause dermatological and central nervous system toxicity, was not thought to be responsible for CB3717 induced rashes after its lack of inhibitory effect against histamine methyltransferase and diamine oxidase was demonstrated. The ascertainment of fluctuations in plasma phenylalanine levels in patients shortly after CB3717 treatment was of interest and invited speculation as to its probable cause (inhibition of dihydropteridine reductase) and possible implications (alterations of neurotransmitter levels).

Clinical hepatotoxicity needed a suitable parallel within an animal species. The mouse provided this, once it was established that transient elevation of plasma alanine transaminase levels did occur, after a therapeutic dose of CB3717, although over a shorter time period. Unlike paracetamol, CB3717 did not cause consistent or marked depletion of liver glutathione levels and toxicity could not be prevented by N-acetylcysteine administration. Other antidotes, directed at reversal of the effects of thymidylate synthetase or dihydrofolate reductase inhibition, were tried, including thymidine, folinic acid and 5-methyltetrahydrofolate. None was successful. However, the discovery that analogues of CB3717 (CB3705 and CB3714) differing only in their N^{10} substituent (hydrogen or ethyl groups respectively) were not hepatotoxic to mice at equimolar doses suggested that there may be a link between the propargyl group at the N^{10} position of CB3717 and liver toxicity.

In the final experimental chapter, the development of resistance to CB3717 was discussed in the context of an L1210 tissue culture cell line which had acquired >100 fold resistance by exposure of a sensitive cell line to incremental concentrations of drug. The characterisation of monoclonal derivatives from this line provided further evidence for TS being the cytotoxic locus of CB3717 with the finding of up to 40 fold increased TS activity as their predominant feature. The kinetic constants for TS purified from sensitive and resistant cells were identical and make overproduction of TS the most plausible explanation for the increased activity. The mutation was stable for up to 9 months in one clone and lack of significant cross-resistance to methotrexate, 5-fluorouracil and 5-fluorodeoxyuridine was

demonstrated.

Establishing a novel drug such as CB3717 in the forefront of cytotoxic chemotherapy regimens presents considerable problems. Evaluation of its antitumour efficacy, as a single agent, against different tumour types in Phase II trials is but one of these. Patients considered suitable for these studies have usually become resistant to the most effective treatment known for their disease and new agents are frequently at the end of a queue of alternatives. Evaluation of any drug under these circumstances is false but it is considered to be the only ethical way in which it may be done.

In the case of CB3717 Phase II trials are well under way at three centres (Newcastle, Glasgow and The Royal Marsden Hospital) and some preliminary results are available. At the Royal Marsden Hosptial responses in breast carcinoma look promising (2 PR, 2MR out of 11) (Calvert A.H. Macaulay V., personal communication) whilst in ovarian cancer results have been disappointing (1 PR, 4 MR our of 17, Harland S.J., personal communication). This may reflect the fact that some breast cancer patients are now being entered having received no previous cytotoxic therapy whereas all the ovarian cancer patients had received previous high dose cisplatinum or carboplatin and secondary responses after such treatment are unusual (Sessa C. et al 1983). Indeed information from Newcastle suggests that CB3717 may be more active when used in less heavily pre-treated ovarian cancer patients (A.L. Harris, personal communication). The overall picture emerging from all the centres concerned is that CB3717 is showing antitumour effects in a number of patients with "solid" tumours. It is probably an important observation since, with the exceptions

of methotrexate and 5-fluorouracil, antimetabolites have in general only showed activity in leukaemia.

The introduction of alternative schedules of administration, requiring further Phase I type evaluation, may provide a means of minimising renal and hepatic toxicity. A once weekly low dose infusion or hydration and alkalinisation regimens are obvious candidates. Complementary to this would be the measurement of plasma nucleoside levels (in particular thymidine) and intracellular nucleotide pools before and after treatment to assess the ability of CB3717 to inhibit TS in man. Refinement of the complex assays involved has been achieved (Taylor G.A., personal communication) such that this sort of study, previously performed in mice whose circulating deoxypyrimidine levels are considerably higher (Jackman A.L. 1984) may now be contemplated in man.

The toxicity of some anticancer drugs may be extremely unpleasant and dangerous but if the antitumour effect is good enough this may be tolerated. Cisplatinum provides an example of this. However new analogues devoid of renal toxicity have been developed, some of which may well supersede this useful agent. Similarly it may be that an analogue of CB3717 will emerge with improved solubility at acid pH and lack of hepatotoxicity. It is also reasonable to believe that such a group of compounds would be devoid of carcinogenic potential as seems to be the case with methotrexate (Bailin P.L. et al 1975, Rustin G.J.S. et al 1983).

Wherever its future lies, it is apparent that CB3717 has emerged as a compound of great interest in experimental and clinical cancer. Perhaps further work will establish it not as a superior, but as a peer, of methotrexate

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