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Quinazoline Antifolates with Dual Biochemical Loci of Action. Biochemical and Biological Studies Directed Towards Overcoming Methotrexate Resistance*

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Abstract—*We report experiments on two substituted quinazolines, which are inhibitors both of dihydrofolate reductase and thymidylate synthetase,* N-(p-(((2,4-*diamino-5-methyl-6-quinazolinyl)methyl)amino)benzoyl)-L-glutamic acid (*CB* 3703) *and* N-(p-(((2-*amino-4-hydroxy-6-quinazolinyl)methyl)amino)benzoyl)-L-glutamic acid (*CB* 3705). *CB* 3703 *inhibits dihydrofolate reductase* (*EC* 1.5.1.4, *DHFR*) *as effectively as methotrexate* (*MTX*) *and also inhibits thymidylate synthetase* (*EC* 2.1.1.45, *TS*). *Its toxicity to cultured* L1210 *cells is comparable with that of MTX, and is reversed by thymidine/hypoxanthine or thymidine/folinic acid combinations. It is transported via the reduced folate pathway, but reaches higher intracellular levels than MTX. CB* 3705 *also inhibits DHFR and TS. Its toxicity* in vitro *may be reversed either by folinic acid or by thymidine alone. It is probably transported neither via the folate nor via the reduced folate pathway.*

Studies of the toxicology of CB 3703 *in mice indicate that the drug is approximately* 100*-times more potent than MTX, although it has a short plasma half life. In animals bearing the* L1210 *ascites tumour a dose of* 2 mg/kg *will produce a* 64% *increase in life span.*

CB 3705 *was considerably less potent, a dose of* 480 mg/kg *being non-toxic to tumour bearing animals and producing only a minimal increase in life span.*

INTRODUCTION

METHOTREXATE (MTX) has a wide clinical application in the treatment of several malignant diseases [1] and will produce a significant number of antitumour responses in some common tumours such as carcinoma of the breast [2], bronchus [3] and head and neck [4]. Its usefulness is however, frequently limited by resistance of the tumour, which may be intrinsic or acquired, with the result that few (if any) patients with the commoner solid tumours obtain remissions of long duration from the drug. It is therefore of interest to study antifolates with properties capable of overcoming the known causes of methotrexate resistance. In experimental tumour systems, intrinsic resistance may be due to a decreased affinity of MTX for its target enzyme dihydrofolate reductase (DHFR, EC 1.5.1.4) [5]. Acquired resistance may be due to either an increased cellular content of DHFR [6–8] or to reduction in the rate of transport of the drug through the system responsible for uptake both of MTX and reduced folates [9, 10]. Both these mechanisms have also been demonstrated in human acute lymphocytic leukaemia [11, 12].

In the case of resistance attributable to an elevated DHFR activity the increased concentration of MTX necessary for cytotoxicity probably results in the accumulation of free intracellular methotrexate sufficient to inhibit the thymidylate synthetase (TS) (EC 2.1.1.45)

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[13]. Under these circumstances the latter enzyme may become the rate limiting target for the drug. However, the affinity of MTX for TS is rather low [10]. Hence the properties desired of an antifolate likely to be effective against MTX resistant cells would be: an increased affinity for DHFR; a greatly increased affinity for TS; more efficient uptake of the drug either by an increased flux through the reduced folate site or the utilisation of a separate site. Numerous quinazoline derivatives which are effective inhibitors of DHFR have been described [14–16], some of which appear to be more effective than MTX, particularly at physiological pH [16]. Some quinazoline analogues of folic acid have been described which are effective inhibitors of TS [17, 18], particularly those substituted at the N-10 position. The transport of some quinazoline derivatives into L1210 cells has been measured [19] and appears in some cases to be superior to that of MTX. Finally, antitumour effects against experimental tumours in animals have been documented [14, 16]. For these reasons a series of substituted derivatives of *N*-(*p*-(((6-quinazolinyl)-methyl)amino)benzoyl)-L-glutamic acid were synthesised and tested firstly as inhibitors of various folate metabolising enzymes and secondly as growth inhibitors in cell culture. The six compounds tested comprised three 2,4-diamino compounds with the 5-position either unsubstituted or substituted with a methyl group or a chlorine, and a similar series of three 2-amino-4-hydroxy compounds.

The purpose of this communication is to present a preclinical evaluation of two compounds selected for their ability to inhibit TS. The transport and rescue characteristics of these compounds have been studied in addition to their toxicity and antitumour activity in experimental animals.

MATERIALS AND METHODS

Quinazolines were synthesised and their purity established as described in the accompanying paper [20].

Reagents were purchased from BDH Ltd. (Poole, Dorset, England) or from Hopkin and Williams Ltd. (Romford, Essex, England). AnalaR grades were used where available. NADPH, dihydrofolic acid, thymidine and hypoxanthine were purchased from Sigma (London) Ltd. (London, England). Radioisotopes were purchased from The Radiochemical Centre (Amersham, Bucks, England). Folinic acid was purchased from Lederle Laboratories (Gosport, Hants, England). *S*-Adenosyl-L-methionine was purchased from Boehringer Mannheim Ltd. (Lewes, East Sussex, England).

Enzyme assays

Partially purified DHFR was prepared from rat liver by the method of Bertino and Fischer [21] and used for I_{50} determinations (see below). Purified DHFR was prepared by affinity chromatography from the rat liver extract and from L2110 cells by the previously published modification of the method of Whiteley *et al.* [5, 22]. DHFR activity was assayed spectrophotometrically by a previously published method [5] except that the buffer used was 0.15 M potassium phosphate, pH 7.0. The Michaelis–Menten constant (*Km*) for dihydrofolate was measured by use of the integrated Michaelis–Menten equation [23]. Inhibition constants (*Ki*) were calculated by a zone B analysis as described previously for MTX [5].

TS for I_{50} determinations was prepared from Yoshida ascites sarcoma. Cells (2×10^8/ml in 0.1 M Tris buffer, pH 7.5, containing 0.1 M 2-mercaptoethanol and 0.72% NaCl) were sonicated at 20 KHz for 2 min. The supernatants after centrifugation at 30,000 ***g*** for 30 min were used immediately in the assay [24].

For determination of kinetic constants TS was prepared from L1210 cells as follows. Cells at 2×10^8/ml were harvested from animals, washed in saline and homogenised with a Potter homogeniser in 10 nM Tris buffer, pH 7.4, containing 5 mM dithiothreitol, 5 mM $CaCl_2$ and 0.25 M sucrose. After centrifugation for 1 hr at 100,000 ***g***, 4 ml aliquots of the supernatant were layered on a 2.5 × 25 cm Sephadex G-200 column which was both equilibrated and eluted with 0.05 M Tris containing 5 mM dithiothreitol, pH 7.4. Fractions were assayed both for TS and phosphatase activities as described below and those containing TS only pooled and used for kinetic studies. This procedure was necessary owing to the presence of a phosphatase in the crude preparation which rapidly degraded deoxyuridylic acid (dUMP) to deoxyuridine. TS and phosphatase activities were determined simultaneously by a radioisotopic method as follows:

TS and phosphatase activities

The 0.5 ml incubation mixture contained (5-^{3}H) dUMP 23 nmole, 0.5 μCi; DL-tetrahydrofolic acid 50 nmole; formaldehyde, 322 nmole; dithiothreitol 2.5 μmole; Tris–HCl buffer (pH 7.4), 5 μmole; NaF 50 μmole and quinazoline inhibitor at the concentration stated. The reaction was started by adding 0.2 ml of the enzyme preparation (3.7 mg protein/ml) which converted approximately 0.2 nmole substrate in the 1 hr incubation period. The reaction was stopped by adding 1 ml of iced water to the mixture which was then passed down a 3 × 0.5 cm Dowex 1 chloride column which was eluted in with a further 2 ml of iced water. The total 3.5 ml effluent was mixed with 10 ml of PCS scintillant (Hopkin and Williams) and counted in an Intertechnique SL30 liquid scintillation counter. It was previously demonstrated that phosphatase activity could be detected as ^{3}H deoxyuridine in the effluent obtained from the incubation in the absence of tetrahydrofolic acid. TS activity added ^3H_2O counts to this background when tetrahydrofolate was added to the incubation mixture. This method was preferred to that of Roberts [24] since in our hands it gave better reproducibility and also allowed the activity of undesired phosphatases to be assessed. Kinetic constants were calculated by the method of Wilkinson [30] using an algorithm kindly supplied by Dr. R. C. Jackson, Indiana University School of Medicine, Indianapolis, U.S.A.

Methionine synthetase

Methionine synthetase (B_{12} dependent 5-methyl-tetrahydrofolate:homocysteine methyl transferase, EC 2.1.1.13) activities were measured on the 30,000 ***g*** supernatant of a homogenate of rat liver made in 0.15 M pH 7.0 potassium phosphate buffer. The method used was a modification of that of Weissbach [25]. The 1 ml reaction mixture contained cyanocobalamin, 0.1 μmole; *S*-adenosyl-L-methionine 0.1 μmole; homocysteine 1.25 μmole; 2-mercaptoethanol 20 μmole; (methyl ^{14}C) 5-methyltetrahydrofolate 0.25 μmole, 0.1 μCi; quinazoline inhibitor 0.1 μmole; potassium phosphate buffer containing 37.5 μmole phosphate adjusted to pH 7.0. The reaction was incubated at 37°C for 1 hr and stopped by addition of 1 ml of ice-cold water. The whole mixture was passed down a 3 × 0.5 cm Dowex 1 chloride column which was then washed through with 2 ml iced water and counted as above.

Formyl tetrahydrofolate synthetase

Formyl tetrahydrofolate synthetase (EC 6.3.4.3) activity was assayed in a preparation prepared from Yoshida cells by the method of Rabinowitz and Pricer [26]. Cells at a concentration of 10^8/ml in 0.05 M potassium phosphate buffer containing 1% ascorbic acid at pH 6.0 were sonicated for 2 min at 20 KHz. The 20,000 ***g*** supernatant was used for the assay.

Serine hydroxymethyl transferase

Serine hydroxymethyl transferase (EC 2.1.2.1) assays were performed by the method of Scrimgeour *et al.* [27]. The enzyme source was the 30,000 ***g*** supernatant of a 10% homogenate of rat liver in 0.05 M potassium phosphate, pH 7.5.

Cell culture

Cultures of L1210 cells were grown in RPMI 1640 medium (Gibco Bio-Cult) with added L-glutamine (2 mM final concentration) and 10% horse serum (Flow Laboratories), but without antibiotics. Log phase cells were diluted to approximately 10^5 cells/ml prior to each experiment, the additives being pre-sterilised by Millipore filtration. Cell counts were performed after 24 and 48 hr incubation at 37°C using an improved Neubauer haemocytometer.

Transport studies were performed by a previously described method [10] except when the uptake of quinazolines was measured. As no labelled quinazoline compounds were available the concentration of drug in the cell pellet was measured by DHFR inhibition. The pellet was suspended in 2 ml of potassium phosphate buffer at pH 7.0, sonicated at 20 KHz for 30 sec, boiled for 10 min and then centrifuged at 1000 g for 10 min. The resultant supernatant was assayed for quinazoline content by a method identical to that described for methotrexate by Bertino and Fischer [21]. This method was previously validated by adding known amounts of quinazoline to suspensions of untreated cells prior to sonication, and was also used to measure plasma levels of CB 3703.

Animal experiments were performed on 9–12-week-old female (DBA2 × C57B1) F_1 hybrid mice maintained on Laboratory Diet 1 (Spratts Ltd., London, England) and water *ad libitum*. The L1210 leukaemia was routinely passaged in ascites form in male DBA2 mice by injection of 5×10^4 tumour cells i.p. For experiments using tumour bearing animals the

same amount of tumour was injected 3 days prior to treatment. All drug injections were given intraperitoneally in 0.3 ml of an isotonic solution.

RESULTS

Enzyme inhibition effects in vitro

Details of the enzyme inhibitory effects of the compounds and their toxicity in cell culture are shown in Table 1. The I_{50} value is defined as the final concentration in the assay system necessary to reduce the reaction rate to 50% of the uninhibited rate. Dihydrofolate reductase assays were performed using the partially purified rat liver preparation, and the other enzyme assays were as detailed in the Methods. It is clear that all the compounds are inhibitors of dihydrofolate reductase, although compounds in the 2,4-diamino series are much more potent than those in the 2-amino-4-hydroxy series. The 2,4-diamino compounds are also more toxic in cell culture, where the I_{50} is defined as the concentration necessary to reduce the cell count to 50% of control after 48 hr incubation. Two compounds (CB 3703 and 3705) also cause significant inhibition of thymidylate synthetase.

As our objective was to study compounds which could inhibit both the above enzymes (DHFR, TS), these two compounds were selected for further study. Inhibitory effects were noted on the other folate metabolising enzymes studied, but these were much less marked. For example, the *Ki* of CB 3703 measured on methionine synthetase was 0.36 mM.

Table 2 shows the *Ki* values for CB 3703 and 3705 on DHFR and TS. Inhibition of DHFR was assumed to be competitive for the purpose of calculating the *Ki* (see Methods, ref. [5]). Inhibition of thymidylate synthetase by CB 3703 was found to be non-competitive with respect to methylene tetrahydrofolate while the inhibition by CB 3705 was competitive.

Protection of cultured L1210 *cells from drug toxicity*

The results for CB 3703 are shown in Fig. 1, where a slight protection could be achieved with thymidine alone, and a partial protection with folinic acid. A combination of folinic acid and thymidine improved on either protectant individually but complete protection was only achieved with the purine/thymidine combination. In contrast, protection from CB 3705 toxicity could be achieved with either thymidine or folinic acid alone (Fig. 2). Hypoxanthine alone did not protect cells from the toxicity of either drug.

Table 1. *Enzyme inhibitory properties of quinazoline antifolates*

X	Y	CB No.	L1210 cell culture I_{50} (μM)	Dihydrofolate reductase I_{50} (nM)	Thymidylate synthetase I_{50} (μM)	Inhibition at 100 μM (%) Methionine synthetase	FH_4 formylase	Serine hydroxymethyl transferase
NH_2	H	3702	0.02	6.5	100	21	27	10
NH_2	CH_3	3703	0.019	6.8	0.25	27	29	12
NH_2	Cl	3704	0.021	6.7	4.3	30	20	22
OH	H	3705	3.4	250	0.29	1	24	37
OH	CH_3	3706	2.7	1100	22	1	21	46
OH	Cl	3707	4.2	630	5.5	0	20	37

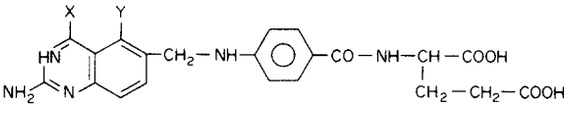

Table 2. Ki *values for* CB 3703 *and* CB 3705

No.	Substituents	DHFR	TS
CB 3703	2,4-Diamino-5-methyl	0.6 pM*	0.47 μM
CB 3705	2-Amino-4-hydroxy	0.35 nM†	67 nM

*Purified enzyme from L1210.
†Purified enzyme from rat liver.

Transport characteristics

The ability of quinazoline to influence the uptake either of tritiated methotrexate or of tritiated folic acid into L1210 cells was mea-

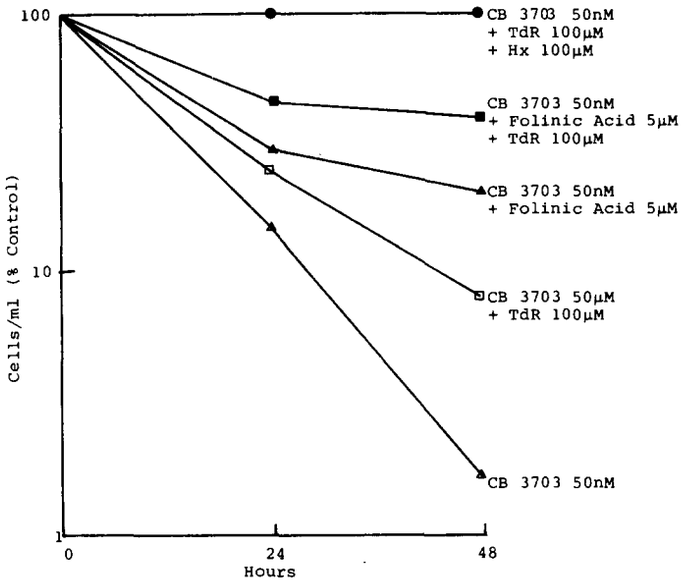

Fig. 1. Protection of L1210 *cells in culture from CB* 3703 *toxicity. All additions to cultures were made simultaneously at* 0 hr. *Each curve is expressed as the percentage of a control containing all the same additions except the drug CB* 3703.

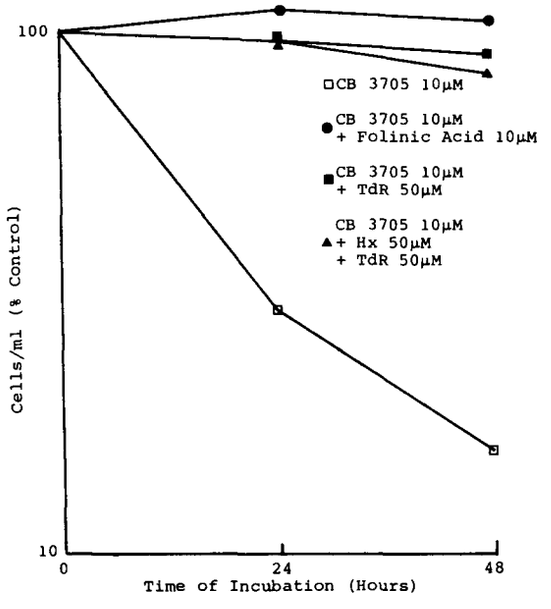

Fig. 2. Protection of L1210 *cells in culture from CB* 3705 *toxicity. All additions to cultures were made simultaneously at* 0 hr. *Each curve is expressed as the percentage of a control containing all the same additions except the drug CB* 3705.

sured. The results for CB 3703 are shown in Fig. 3, where a marked reduction in MTX uptake was observed, and a slight reduction in folic acid uptake, suggesting that CB 3703 and MTX may share a common uptake pathway. The effect of CB 3705 was assessed under the same conditions and the results are shown in Fig. 4. Little effect was seen on the uptake of MTX, 5-methyltetrahydrofolate or folic acid, suggesting that CB 3705 does not utilise either the folate or reduced folate transport mechanism.

The transport of CB 3703 into L1210 cells was also studied directly, assaying the cell pellets by DHFR inhibition (see Methods). The effect of MTX on the uptake of CB 3703 could not be measured by this method since the MTX itself will inhibit DHFR. However, the effect of folic acid could be measured. The time course of transport of CB 3703 into L1210 cells is shown in Fig. 5, where the transport of MTX measured in identical conditions is shown for comparison. Notable features are: firstly that CB 3703 achieves approximately ten-fold higher intracellular concentrations than MTX and has a higher non-specific binding at time 0; secondly, CB 3703 concentrations have not reached a plateau at 40 min, in contrast to MTX which is essentially unchanged after 10 min. The uptake of 4 μM CB 3703 in medium containing 4 μM folic acid was measured at 20 min and was identical to the uptake in the absence of folic acid, suggesting that the folic acid transport pathway is not utilised by CB 3703.

Toxicity in animals

This was established for a single i.p. dose in both tumour and non-tumour bearing animals. The results are shown in Table 3: CB 3703 proved to be extremely toxic. The LD_{50} was between 2 and 4 mg/kg in contrast

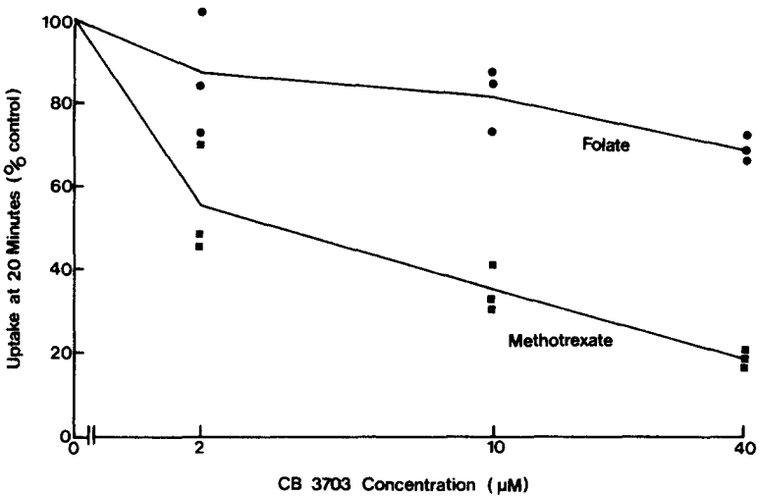

Figure 3. *The effect of CB* 3703 *on the uptake of tritiated MTX and tritiated folic acid into* L1210 *cells.* (●) *Folic acid,* (■) *MTX. Each point represents the mean of triplicates from a separate experiment. MTX concentration in the medium was* 4 μM, 1 μCi/ml. *Folic acid concentration was* 4 μM, 0.5 μCi/ml.

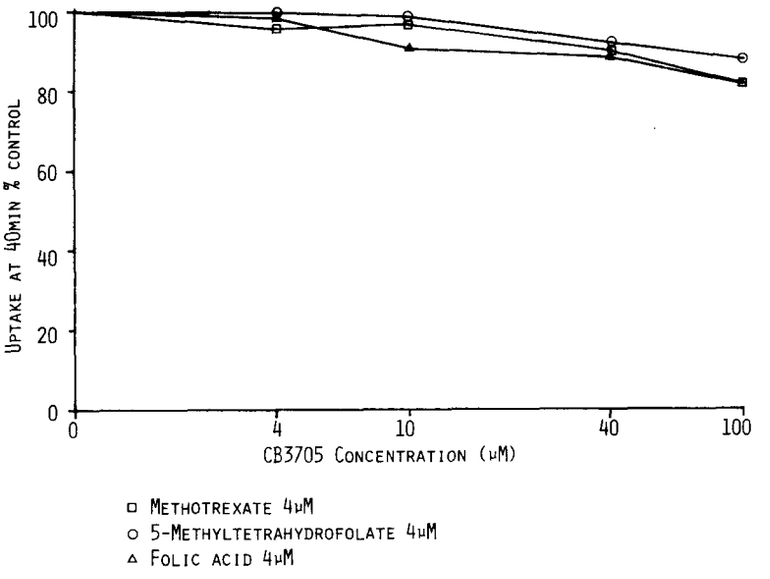

Fig. 4. *The effect of CB* 3705 *on the uptake of tritiated MTX, tritiated folic acid or* ^{14}C *methyltetrahydrofolate into* L1210 *cells. Concentrations were: MTX* 4 μM, 1 μCi/ml; *folic acid* 4 μM, 0.5 μCi/ml; *methyltetrahydrofolate* 4 μM, 0.1 μCi/ml.

to methotrexate where it was around 200 mg/kg [31]. Drug induced deaths were characterised by weight loss and gastrointestinal toxicity and occurred at 5–6 days after treatment, these features being similar to those seen with MTX. CB 3705 did not cause toxicity at the highest dose used, 480 mg/kg.

Plasma pharmacokinetics in mice

Plasma levels of CB 3703 injected with a single i.p. dose of 10 mg/kg are shown in Fig. 6. Plasma decay was approximately exponential over the first 8 hr with a half life of 1.54 hr. Plasma levels at 24 hr were barely detectable, being approximately 0.05 μM.

Antitumour effects of CB 3703 *in animals*

The survival curves of animals treated with single i.p. injections of CB 3703 are shown in Fig. 7. The median survival time of untreated controls was 14 days, and for treated animals it was 19 and 23 days for the 1 mg/kg and 2 mg/kg doses, respectively. Hence the increase in life span from a 1 mg/kg dose was

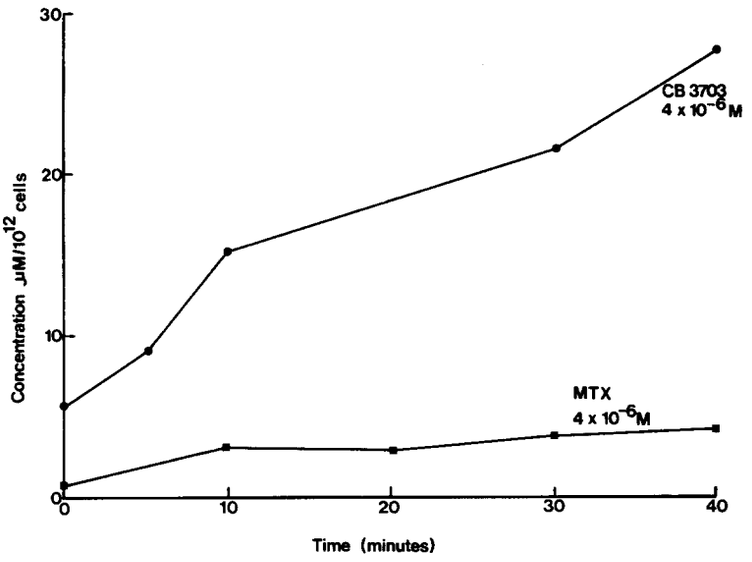

Fig. 5. Transport of CB 3703 and MTX into L1210 *cells* in vitro. *Log phase cells were incubated in the presence of drug and the levels estimated as described (see Methods, ref.* [10]). (●) *CB* 3703, 4 μM *extracellular concentration;* (■) *MTX,* 4 μM *extracellular concentration.*

Table 3. *Toxicity of* CB 3703 *in mice*

Dose (mg/kg)	Toxic deaths/No. in group	
	Normal animals	Tumour bearing animals*
1	5/11	0/5
2	1/10	0/5
4	10/10	3/5
8	10/10	5/5

*Only drug induced deaths occurring before 10 days are included in this column.

36% and from a 2 mg/kg dose it was 64%. At higher doses of drug toxic deaths occurred earlier than the tumour deaths in untreated controls. A marginal (10%) increase occurred in the median survival time of animals treated with CB 3705 at a non toxic dose of 480 mg/kg. The lack of sufficient compound has so far precluded the use of a higher dose.

DISCUSSION

The hypothesis based on enzyme inhibition data that CB 3703 has a dual locus of action is also supported by the cell culture experiments. For example, if CB 3703 inhibited only dihydrofolate reductase, then this effect would be circumvented by addition of folinic acid (as is the case with MTX). Inhibition of TS would be circumvented by addition of thymidine. In our experiments, both folinic acid and thymidine were required to give growth rates approaching the control. This would suggest that both enzyme loci were affected. Further, the fact that complete reversal of toxicity is achieved only by a purine/thymidine combination suggests that the drug may have a weaker effect on *de novo* synthesis of purines which is independent of its effect on either DHFR or TS. In other cell culture experiments (not shown here) it was found that a high level of folinic acid in the medium (50 μM) would protect the cells from CB 3703 toxicity. However, as it is likely that CB 3703 shares the transport system with MTX, and therefore also with folinic acid [28], it is probable that this 1000-fold higher level of folinic acid would prevent transport of the drug into the cells, rather than reversing the intracellular biochemical defect. Further, a concentration of 0.5 μM folinic acid will totally reverse an equally toxic level of methotrexate in the same system (unpublished observations).

The ability of folinic acid alone to protect cells from CB 3705 may be due to the increased intracellular methylene tetrahydrofolate pool overcoming the competitive inhibition of TS. As the *Ki* of CB 3705 for DHFR is much higher than that of CB 3703, it is possible that there was still sufficient flux through this pathway to allow *de novo* purine synthesis to continue when an exogenous thymidine supply was provided, thus allowing protection of the cells by thymidine alone.

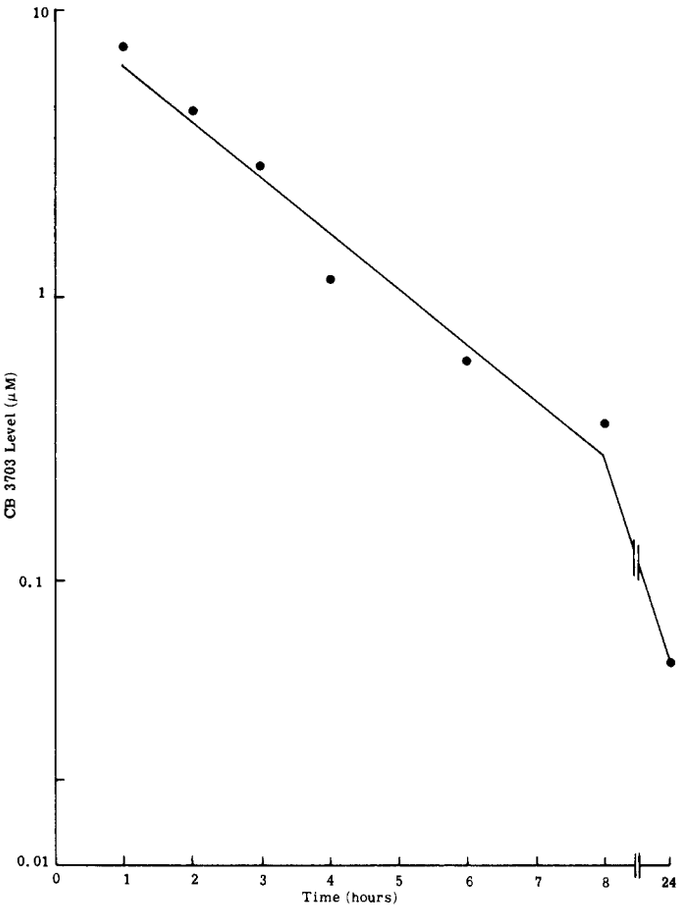

Fig. 6. Plasma levels (μM) of CB 3703 in mice given 10 mg/kg as a single i.p. dose. Two mice were anaesthetised under ether for each point. They were exsanguinated from the orbit and the blood pooled. The plasma was separated and assayed immediately (see Methods) without storage.

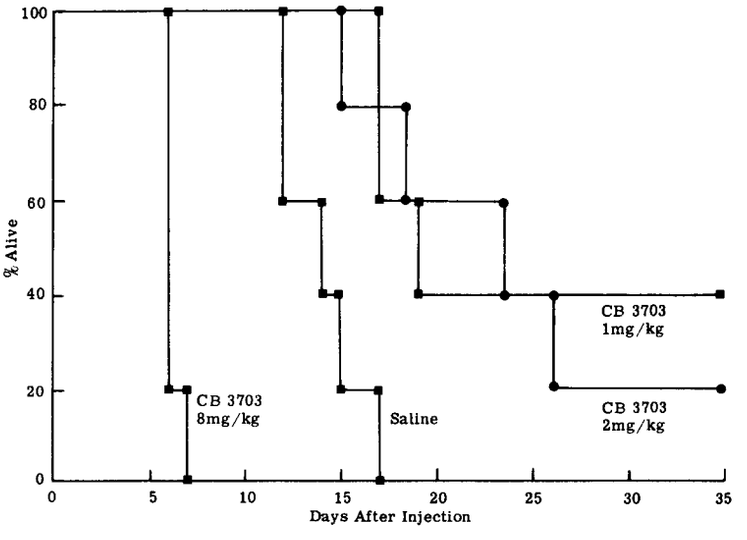

Fig. 7. Survival curves of groups of five L1210 tumour-bearing mice injected i.p. with a single dose of CB 3703.

The plasma half life of CB 3703 (1.54 hr) seen in mice is shorter than that of a comparable dose of MTX (2 hr) [29]. This may be due to a rapid localisation of the drug in the intracellular compartment, which would be consistent both with the observations on the transport in cultured cells and with the high toxicity in the whole animal. High intracellular levels of quinazolines have previously been reported and attributed to a reduced efflux of the drug [19].

These quinazolines were designed to possess enhanced activity against MTX resistant tumours. We are at present evaluating this possibility having shown that *in vivo* CB 3703 has a similar antitumour effect to that of MTX against the L1210 tumour. Preliminary results using an L1210 mutant resistant to MTX by virtue of an increased intracellular DHFR content suggest that cross resistance to CB 3703 is considerable while that to CB 3705 is much reduced.

In conclusion, the quinazolines as a class of compounds clearly possess remarkably different properties to MTX. The binding of CB 3703 to DHFR is at least as good as MTX and may be better, particularly at physiological pH [16]. In addition both quinazolines have at least one other biochemical locus. The pharmacology, toxicology, transport characteristics and *in vitro* rescue requirements suggest that certain quinazoline derivatives may possess therapeutic advantages over MTX, particularly in situations where resistance to MTX is a problem. The administration of small amounts of a more potent compound could conceivably retain benefits, but considerably reduce the problems associated with conventional high dose methotrexate therapy [1]. For these reasons, quinazoline derivatives deserve active study to identify the most promising compounds, and to investigate their optimal utilisation.

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