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THE BIOCHEMICAL PHARMACOLOGY OF THE THYMIDYLATE SYNTHASE INHIBITOR, 2-DESAMINO-2-METHYL-N^{10}-PROPARGYL-5,8-DIDEAZAFOLIC ACID (ICI 198583)

ANN L. JACKMAN,* DAVID R. NEWELL,† WILLIAM GIBSON, DUNCAN I. JODRELL, GORDON A. TAYLOR,† JOEL A. BISHOP, LESLIE R. HUGHES‡ and A. HILARY CALVERT†

Drug Development Section, Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey SM2 5PX; and ‡ICI Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire, U.K.

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Abstract—2-Desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI 198583) is a more water-soluble analogue of the quinazoline-based thymidylate synthase (TS) inhibitor, N^{10}-propargyl-5,8-dideazafolic acid (CB3717). A 3-fold loss in TS inhibitory activity (murine and human TS, K_i = 10 nM) was accompanied by a 40-fold increase in growth inhibitory potency against L1210 and W1L2 cells *in vitro* (IC_{50} = 0.085 and 0.05 μM, respectively) when compared with CB3717. In L1210 cells a concentrative uptake mechanism was demonstrated for [^{3}H]ICI 198583 (K_t = 2.9 μM). The L1210:1565 cell line, with an impaired ability to transport reduced folates or methotrexate (MTX), was resistant (100-fold relative to the wild-type L1210 line) to ICI 198583 (but not CB3717) and did not take up [^{3}H]ICI 198583 significantly. The measurement of folylpolyglutamate synthetase (FPGS) substrate activity demonstrated a K_m of 40 μM for ICI 198583 and a V_{max}/K_m (relative to folic acid) of 3.5. The formation of intracellular polyglutamate derivatives was demonstrated in both L1210 (mouse) and W1L2 (human) cells grown *in vitro* after exposure to 1 μM [^{3}H]ICI 198583. In L1210 cells, by 4 hr, ~50% of the intracellular ^{3}H(~1 μM) was found as polyglutamate forms of ICI 198583, principally as tri- and tetraglutamates. After 24 hr the ICI 198583 polyglutamate pool had expanded, the tetraglutamate metabolite predominanted and there was significant formation of the pentaglutamate. Upon resuspension of L1210 cells in drug free medium, ICI 198583 was largely lost from the cells but the polyglutamates were preferentially retained, after 24 hr ~70% remained. Synthetic ICI 198583 polyglutamates were shown to be up to 100-fold more potent as inhibitors of isolated TS than the parent compound. Following *in vivo* administration (500 mg/kg i.v.) ICI 198583 was cleared rapidly from the plasma of mice ($T_{1/2\beta}$ = 16 min, clearance = 42 mL/min/kg). Despite this clearance there was prolonged, dose-dependent inhibition of TS in L1210:NCI cells *in vivo*. Thus, following 500 mg/kg i.v. the flux through TS was inhibited by >80% for at least 24 hr. Administration of five doses at 5 mg/kg daily of ICI 198583 to L1210:ICR tumour-bearing mice resulted in >60% of the mice being cured, a 10-fold improvement in potency over CB3717. The maximum tolerated dose (MTD) for ICI 198583 using this schedule was >500 mg/kg/day compared with 200 mg/kg/day of CB3717. ICI 198583 is therefore a potent inhibitor of TS *in vitro* and *in vivo* with a marked improvement in therapeutic index over CB3717 in mice.

Thymidylate synthase (TS§; EC 2.1.1.45) has received considerable attention in the last decade as a target enzyme for the development of novel folate-based anticancer agents. The prototype drug, N^{10}-propargyl-5,8-dideazafolic acid (CB3717§) [1] proved to be a very potent inhibitor of TS with reported K_i values in the range of 1 to 20 nM for both murine and human enzymes [1–6]. Intracellular metabolism of CB3717 to form polyglutamate derivatives, which are ~100-fold more potent in inhibiting TS and are retained by cells, may be an important determinant of CB3717 cytotoxicity [5, 7–9].

In clinical studies CB3717 showed activity against a number of tumour types, notably breast cancer,

* To whom all correspondence should be addressed.

† Current address: Cancer Research Unit, University of Newcastle upon Tyne, Medical School, Framlington Place, Newcastle upon Tyne NE2 4HH, U.K.

§ Abbreviations: TS, thymidylate synthase (EC 2.1.1.45); DHFR, dihydrofolate reductase (EC 1.5.1.3); CB3717, N^{10}-propargyl-5,8-dideazafolic acid; desamino-CB3717, 2-desamino-N^{10}-propargyl-5,8-dideazafolic acid; ICI 198583, 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid; IC_{50}, 50% inhibitory concentration; 5,10-CH_2FH_4, $N^{5,10}$-methylene tetrahydrofolic acid; dThd, thymidine; dUrd, deoxyuridine; FPGS, folypolyglutamate synthetase; MTD, maximum tolerated dose; K_{iapp}, $K_{iapparent}$, apparent inhibition constant; MTX, methotrexate; L1210:ICR, L1210 subline carried at the Institute of Cancer Research (previously called L1210:CBRI); PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; folinic acid, N^5-formyl tetrahydrofolic acid; ICI D1694, *N*-(5-[*N*-(3,4-dihydro - 2-methyl - 4-oxoquinazolin - 6-ylmethyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid; DDATHF, 5,10-dideazetetrahydrofolic acid; glu_n, total number of glutamates in the molecule (i.e. ICI 198583 plus 1 extra glutamate = diglutamate = glu_2).

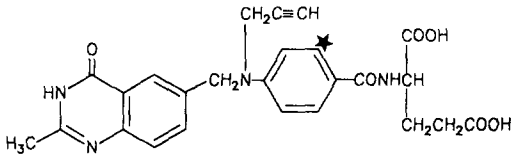

Fig. 1. The structure of 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI 198583. * Indicates the position of the ^3H in the tritiated compound.

ovarian cancer and hepatoma [10–15]. The drug was withdrawn from clinical study because of side effects, in particular dose-limiting and life-threatening kidney toxicity which was often associated with serious and unpredictable myelosuppression. The poor aqueous solubility of CB3717, particularly at the acid pH of urine, was hypothesized to be the cause of this toxicity [15, 16]. Unpredictable but self-limiting hepatotoxicity was also seen in ~50% of the patients treated. Both renal and hepatic toxicities were observed also in rodents [17]. As the poor solubility of CB3717 may be related to inter-molecular hydrogen bonding, an attempt was made to improve water solubility by synthesizing the 2-desamino analogue of CB3717 [18]. Desamino-CB3717 was indeed substantially more water soluble and, despite an 8-fold decrease in TS inhibitory activity compared with CB3717, was 10-fold more potent as an inhibitor of cell growth [18]. Desamino-CB3717 administered *in vivo* was not toxic to the liver or kidneys of rodents and retained an anti-tumour activity toward the L1210:ICR tumour; thus, a significant increase in the therapeutic index was apparent [2, 19]. In an attempt to improve still further the biological properties of this class of agent, activity at the 2-position was optimized [19–21]. In structure–activity studies, methyl was the favoured 2-substituent in terms of TS inhibition and potency against L1210 cells in culture, respresenting only a 2–3-fold loss in TS activity together with a 40-fold improvement in L1210 growth inhibitory activity compared with CB3717.

The present paper describes experimental results that confirm that 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI 198583), Fig. 1, has TS as its intracellular locus of action in both murine (L1210) and human (W1L2) cells *in vitro*. Studies have determined also those properties of ICI 198583 important for potency *in vitro*, with particular reference to transport and polyglutamation. Measurement of the pharmacokinetics and pharmacodynamics (i.e. the inhibition of TS in L1210 cells growth *in vivo*) of ICI 198583 in mice has led us to the conclusion that rapid uptake into cells and subsequent polyglutamation are important determinants of anti-tumour activity. Certain aspects of this study have been reported in a preliminary form [19, 22].

MATERIALS AND METHODS

ICI 198583 and its synthetic polyglutamates

ICI 198583 was synthesized as described previously [21]. The synthesis of the γ-polyglutamates (trifluoroacetic acid salts) has also been reported [23]. For *in vitro* studies, all compounds were dissolved in 0.15 M $NaHCO_3$ and dilutions were made in either H_2O for TS inhibition studies, 0.15 M $NaHCO_3$ for FPGS assays or RPMI1640 for cell culture (the volume added was 10% of the final volume). For *in vivo* studies, the compounds were formulated in 0.05 M $NaHCO_3$ and the pH was adjusted to 9 with 1 M NaOH.

[Benzoyl-^{3}H]ICI 198583 diethyl ester was supplied in ethanol:water (1:1 v/v) (customer synthesis, Amersham International, Amersham, U.K.) at a radiochemical concentration of 2 mCi/mL. The ^3H was only present in the 2′-benzoyl position (Fig. 1) as shown by ^3H-NMR spectrometry. Prior to use, the diethyl ester (2–4 mCi) was hydrolysed using 1 M NaOH (1:25 v/v NaOH:[^{3}H]ICI 198583 diethyl ester) for 4 hr at room temperature. The resultant solution of [^{3}H]ICI 198583 was neutralized with 1 M acetic acid (1:12 v/v acetic acid:[^{3}H]ICI 198583), reduced to ~100 μL under nitrogen and purified by HPLC using the method described below. The specific activity of the purified [^{3}H]ICI 198583, determined by UV spectrophotometry, was 19 Ci/mmole and the radiochemical purity was >99.5% (HPLC, see below). The purified [^{3}H]ICI 198583 in HPLC mobile phase was used immediately or diluted with ethanol 1:1 v/v and stored at −20°.

Enzyme assays

TS inhibition was assayed by ^3H release using partially purified enzyme preparations as described previously [2]. Enzyme sources were the L1210:C15 murine line (45-fold overproduction of TS) and the W1L2:C1 human line (200-fold overproduction of TS) [24, 25]. K_{iapp} values were obtained from kinetic analyses applicable to tight-binding inhibitors as described previously [2]. The method of analysis was a non-linear least squares fit [26] to the Goldstein equation [27]. The K_i for ICI 198583 was determined by the method of Henderson [28].

FPGS was partially purified from mouse liver and substrate activity was assayed by a charcoal adsorption method, as described by Moran *et al.* [29, 30].

Cell culture

A full description of all the cell lines and the conditions for their culture has been published recently [2]. The L1210:1565 cell line has acquired resistance to the antitumour antibiotic, CI 920 and is ~200-fold cross-resistant to MTX [31]. Evidence suggests that CI 920 penetrates L1210 cells via the reduced folate carrier mechanism and resistance is due to a very much reduced drug uptake [31].

Uptake studies with [^{3}H] ICI 198583

[^{3}H]ICI 198583 (19 Ci/mmol) with purity of >99.5% was diluted with unlabelled ICI 198583 in 0.05 M $NaHCO_3$ to a specific activity of 1 Ci/mmol (5 μM). Cells in the logarithmic growth phase (~5×10^5/mL) were centrifuged for 5 min at 600 *g* and the pellets resuspended in 160 mM HEPES buffer pH 7.4 at a concentration of 5×10^6/mL. Cells were incubated with 0.5 μM [^{3}H]ICI 198583 at

37°. At the times indicated 1 mL of cells was removed, added to 5 mL of ice-cold PBS and centrifuged immediately at 4°. The cell pellets were resuspended in 10 mL of PBS and centrifuged again. This washing procedure was repeated twice before the pellets were dissolved overnight in 0.5 mL 1 M NaOH. After neutralization with 0.51 mL of 1 M HCl, 10 mL of Ultima Gold scintillant (Canberra Packard Ltd, Pangbourne, U.K.) were added before liquid scintillation counting. The same procedure was followed for the K_t determination except that 1 mL cell suspension samples were incubated in glass 15 mL test tubes with [^{3}H]ICI 198583 at concentrations ranging between 0.1 and 6 μM for 2 min. The K_t was determined by fitting the equation, $v = (V_{max} \times S)/S + K_t)$, using a computerized non-linear regression analysis [26].

Polyglutamation studies

Cell culture and extraction of polyglutamate metabolites. Purified [^{3}H]198583 (see above) was diluted with non-radioactive ICI 198583 to give a specific activity of 10 Ci/mmol and a final concentration of 40 μM. L1210 or W1L2 cells (2 × 10 mL cultures at 3–5 × 10^5 cells/mL) were incubated with 1 μM [^{3}H]ICI 198583 for 4, 8 or 24 hr. Thymidine (dThd), 20 μM was added to prevent cell death. After incubation the cultures were counted for cell number, centrifuged, 600 *g* for 5 min at 4°, resuspended in 50 mL of ice-cold PBS, centrifuged again and the pellets were suspended in 0.5 mL H_2O. The cells were sonicated for 45 sec at 4°. Total cellular ^{3}H was estimated by taking 4 × 25 μL samples which were hydrolysed overnight at 40° in 0.5 mL 1 M NaOH, neutralized with 0.51 mL 1 M HCl and radioactivity was then determined by liquid scintillation counting. The remaining extract was boiled for 15 min and the pre-weighed glass test tubes re-weighed to estimate any volume change during sample processing. After centrifugation for 10 min at 600 *g* supernatants were removed (400 μL), 40 μL of a standard mix of ICI 198583 and the synthetic polyglutamates (each at 200 μM) were added and a 100 μL sample was analysed by HPLC as described below. Duplicate 100 μL aliquots were also counted to determine the total radioactivity applied to the HPLC column.

For efflux experiments L1210 cells were initially incubated for 24 hr with 1 μM [^{3}H]ICI 198583 as described above, followed by centrifugation and resuspension in drug-free medium also containing 20 μM dThd. Cell extracts were prepared as described above after 4 and 24 hr in drug-free medium.

HPLC analysis. ICI 198583 and its polyglutamates (glu_2–glu_5) were separated on a 10 × 0.46 cm Spherisorb 5-μm C_6 column (Phase Separation Ltd, Deeside, U.K.). The compounds were separated using 20 min linear gradient elution from 5:95 (v/v) to 15:85 acetonitrile:0.1 M sodium acetate, pH 5.0, at a flow rate of 2 mL/min, starting at the time of injection. Synthetic standards were detected by UV absorbance at 313 and 280 nm and this is illustrated in Fig. 2. [^{3}H]ICI 198583 and its polyglutamate metabolites were quantified by collection of the HPLC effluent (1 mL fractions) and liquid scintillation counting in 10 mL Emulsifier Safe scintillant (Canberra Packard Ltd, Pangbourne, U.K.).

Overall radiochemical recovery (extraction and HPLC procedures) was >70%. Each result is expressed as the mean of duplicate cultures processed and analysed separately. The results have been expressed in two ways: first the total level of ICI 198583 and its polyglutamates recovered after extraction and HPLC; secondly, as there was no evidence of preferential loss of one fraction over another or of ^{3}H not associated with ICI 198583 or its polyglutamates (data not shown), the level of each fraction is expressed as % total cellular ^{3}H recovered.

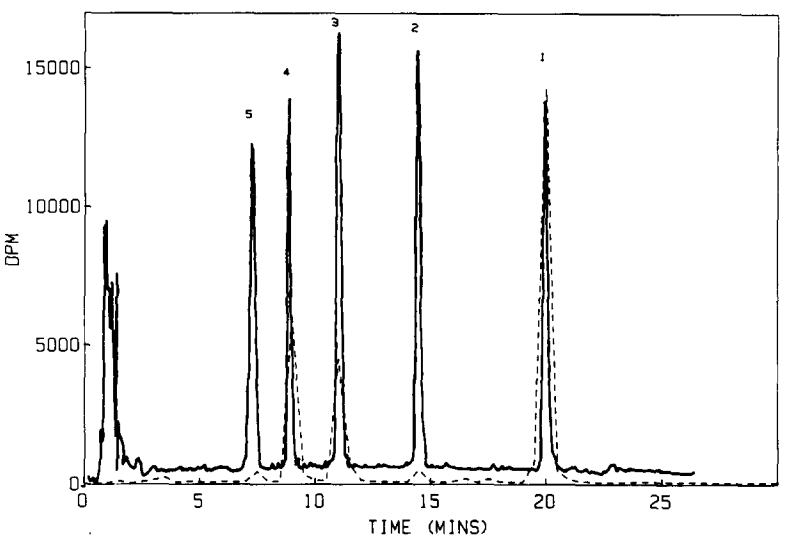

Fig. 2. HPLC separation of synthetic ICI 198583 polyglutamates (solid line). Peak 1 is ICI 198583 and peaks 2, 3, 4 and 5 are the di-, tri-, tetra- and pentaglutamates of ICI 198583, respectively (2 nmoles of each compound; 313 nm; 0.05 AUFS). The dotted line is the radiochromatogram of an extract of L1210 cells exposed to 1 μM [^{3}H]ICI 198583 for 4 hr.

Pharmacokinetic studies

The pharmacokinetics of ICI 198583 were investigated in non-tumour bearing male C57BL × DBA2 F1 hybrid mice at the age of 8–10 weeks. ICI 198583 was administered at 500 mg/kg into a tail vein and groups of four mice were killed by CO_2 asphyxiation at 30, 60, 90 and 120 min, thereafter. These time points were chosen because, in pilot experiments, they were shown to describe the elimination phase for the compound. Blood was collected by direct cardiac puncture and heparinized plasma (10 I.U./mL) was prepared and stored at −20°. ICI 198583 levels were determined by HPLC with UV detection at 280 and 254 nm. ICI 198583 present in the HPLC effluent was identified by co-chromatography with synthetic compound and 280/254 nm wavelength ratios. Prior to HPLC analysis, 100 μL of mouse plasma was treated with 200 μL of methanol and precipitated protein removed by centrifugation at 1500 *g* for 15 min at 4°. Ten microlitres of the resultant supernatant were analysed and separation was achieved on a 15 × 0.46 cm Spherisorb C_6 column fitted with a Co:Pell ODS precolumn (5 × 0.21 cm, Whatman, U.K.) using an HPLC mobile phase of 59:40:1 water:methanol:glacial acetic acid (v/v/v). A standard curve was prepared in mouse plasma for the concentration range of 10 to 1000 μM and recovery was found to be complete (>95%) and linear ($r = >0.99$). A one compartment open model was fitted to the plasma ICI 198583 concentration/time data using non-linear least squares analysis [26] with a weighting function of $1/(y + \hat{y})^2$, i.e. the concentration (C) at time $t = Ae^{-\alpha t}$ where A is a concentration constant and α is the first order rate constant for the exponential decrease in ICI 198583 levels. The area under the ICI 198583 plasma concentration vs time curve (time zero to infinity) was calculated as: $AUC = A/\alpha$ and the total ICI 198583 plasma clearance was calculated as: clearance = dose/AUC. Finally, the ICI 198583 half life was calculated as: half life = $0.693/\alpha$.

Antitumour studies

The L1210:NCI tumour or the L1210:ICR tumour was routinely passaged in male DBA/2 mice. For experiments, 5×10^4 cells were injected i.p. into male C57BL × DBA2 F_1 hybrid mice and treatment (i.p.) commenced 3 days later. The body weights of individual mice were recorded daily for 2 weeks. Mice were killed if the ascitic tumour load or general condition of the mice prevented free access to food and water. Mice were classified as cured if they were healthy and free of ascites 100 days after treatment. The MTD in tumour-bearing mice was defined here as the dose resulting in >20% loss in body weight during or after treatment in those mice that eventually were classified as cured. The L1210:NCI tumour is refractory to treatment with folate-based TS inhibitors such as CB3717 or desamino-CB3717 [2] and may be representative of most murine models, where the high plasma dThd levels found in mice may compromise the activity of TS inhibitors by allowing sufficient thymidylate to be synthesized via dThd salvage [32]. The L1210:ICR tumour is a tetraploid variant line sensitive *in vivo*, for unknown reasons, to folate-based TS inhibitors [2, 32, 33]. Both lines are equally sensitive to TS inhibitors *in vitro* where thymidine levels are very low [33].

Inhibition of TS in vivo

The whole cell TS assay (rate of ^3H release from 5-[^{3}H]dUrd) was performed on L1210:NCI cells removed 2, 4, or 24 h after i.v. administration of 50, 250 or 500 mg/kg of ICI 198583 and using the method reported recently [2, 19, 34]. Briefly, this involved resuspension of the ascites cells in RPMI 1640 medium followed by incubation of the cells with 0.3 μM 5-[^{3}H]dUrd. ^3H_2O formation was measured over a 1 hr period and the rate at equilibrium was calculated by linear regression analysis. This assay is a measure of the inhibition of the flux through TS due to drug or drug-derived material which cannot efflux from the cell during the 1 hr incubation period [2].

RESULTS

Enzyme studies

ICI 198583 was found to be a potent inhibitor of L1210 TS with a K_i of 10 nM. The mechanism of inhibition is a "mixed" non-competitive type and the Henderson Plot for L1210 TS inhibition is presented in Fig. 3. The same mechanism of inhibition and K_i value were obtained using human (W1L2) enzyme (data not shown).

The synthetic polyglutamates of ICI 198583 were also tested against isolated L1210 TS. The addition of one and two extra glutamate residues resulted in a 28- and 78-fold improvement in TS inhibitory activity ($K_{i\text{app}}$), respectively. Further elongation of the polyglutamate chain up to four additional residues had little or no further effect on activity (Table 1).

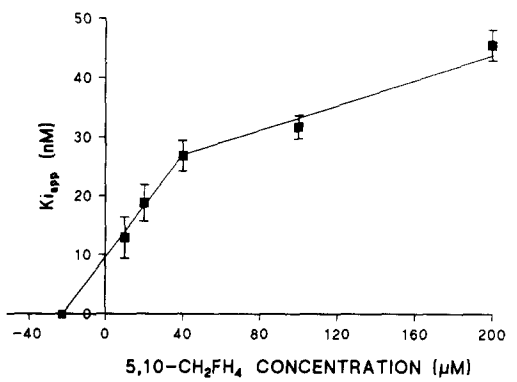

Fig. 3. A Henderson Plot describing the inhibition of L1210 TS by ICI 198583. $K_{i\text{app}}$s were determined by fitting data obtained over a range of inhibitor concentrations to the Goldstein equation [27] by a non-linear least squares regression analysis [26]. The $K_{i\text{app}}$s at the different (±)-5,10-CH_2FH_4 concentrations are plotted together with the SE. The K_i was estimated from the intercept of the curve with the ordinate (the negative value of the K_m for the substrate being an additional point to the curve on the abscissa).

Table 1. Inhibition of L1210 TS by ICI 198583 and its polyglutamate derivatives

	K_{iapp} ± SE (nM)	Fold improvement in TS inhibition
ICI 198583 (glu_1)	31 ± 1.6*	—
glu_2	1.1 ± 0.05	28
glu_3	0.40 ± 0.03	78
glu_4	0.27 ± 0.03	115
glu_5	0.40 ± 0.03	78

* K_i L1210 TS = 10 nM.

TS was partially purified from L1210:C15 (murine) cells. The ^3H release assay (1 hr incubation) was as described in Materials and Methods. The (±)5,10-CH_2FH_4 concentration used for the K_{iapp} determinations was 200 μM.

The substrate activity of ICI 198583 for FPGS was measured using enzyme prepared from mouse liver. A K_m of 40 μM was determined (Table 2), a value identical to that of CB3717 [29]. Furthermore, the first order rate constant (V_{max}/K_m) was 3.5 relative to that of folic acid, marginally higher than the published value of 2.3 for CB3717 [29]. The synthetic diglutamate of ICI 198583 (glu_2) had a 4-fold lower K_m for FPGS and a 5-fold greater V_{max}/K_m.

Growth inhibition studies

ICI 198583 was tested against four cell lines *in vitro*, two of which exhibit resistance to MTX and are useful in determining the cellular pharmacology of antifolates. The L1210 and W1L2 lines were sensitive to ICI 198583 with IC_{50} values of 0.09 and 0.05 μM, respectively (Table 3). For both lines, at a concentration equal to 10 × IC_{50}, ICI 198583 did not inhibit cell growth in the presence of 10 μM dThd, the salvage precursor for thymidylate (data not shown). ICI 198583 retained good activity against the MTX-resistant, DHFR overproducing line, L1210:R7A (Table 3). The resistant cell line, L1210:1565, which has a greatly impaired ability to transport reduced folates and is consequently resistant to MTX, was also resistant to ICI 198583 (~100-fold) (Table 3).

Uptake studies with [^3H] ICI 198583

In 160 mM HEPES buffer the uptake of 0.5 μM [^3H]ICI 198583 into L1210 cells resulted in an intracellular plateau at ~3 μM by 10–15 min (Fig. 4). No uptake was observed at 4° (data not shown). The K_i (determined at 2 min) was found to be 2.93 ± 0.32 μM. In contrast, the L1210:1565 cell line did not take up a significant amount of labelled compound when incubated with 0.5 μM for 30 min (Fig. 4).

Polyglutamation studies

Table 4 shows the degree of metabolism of ICI 198583 to polyglutamate derivatives in L1210 cells after 4, 8 and 24 hr of incubation with 1 μM of the ^3H compound. At 4 hr half of the cellular ^3H was

Table 2. The substrate activity of CB3717, ICI 198583 and ICI 198583 diglutamate for mouse liver FPGS

	K_m (μM)	V_{max}	V_{max}/K_m*
CB3717†	(N = 2) 40 ± 2.0	0.88 ± 0.01	2.26 ± 0.04
ICI 198583‡	(N = 2) 40.1 ± 3.9	1.16 ± 0.26	3.50 ± 0.2
ICI 198583 glu_2	(N = 1) 9.9 ± 1.7	1.42 ± 0.06	16.8 ± 1.2

* Relative to folic acid used as an internal control in each experiment.

† From Ref. 29.

‡ Data provided by Dr. R. G. Moran, Cancer Research Laboratory, University of Southern California Cancer Centre, Los Angeles.

FPGS was partially purified from mouse liver and incubated for 1 hr with substrates 1 mM [^3H]glutamic acid and 5 mM ATP, and the product was isolated by charcoal absorption as described by Moran and coworkers [29, 30]. Results are expressed as the means of two experiments.

Table 3. Inhibition of cell growth *in vitro* by ICI 198583

	Drug concentration required for 50% inhibitiion of cell growth (μM)					
	L1210	L1210:R7A	Relative resistance	L1210:1565	Relative resistance	W1L2
ICI 198583	0.085 ± 0.081	0.39 ± 0.07	4.6	7.7, 8.5	96	0.056 ± 0.004
CB3717*	3.5 ± 0.4	41 ± 6	12	4.0, 3.2	1	2.6 ± 0.3

* Data previously published in Ref. 2.

The L1210:R7A line has acquired resistance (600-fold) to MTX (elevated DHFR); the L1210:1565 line has acquired resistance to CI-920 and is cross-resistant to MTX (200-fold) because of deficient reduced folate transport. IC_{50} values were derived from data determined at five or more concentrations (each in duplicate). These results are the means and SD (N = 3–5) or the individual results of two experiments. Relative resistance = IC_{50} for the resistant line/IC_{50} for the wild-type L1210 cell line.

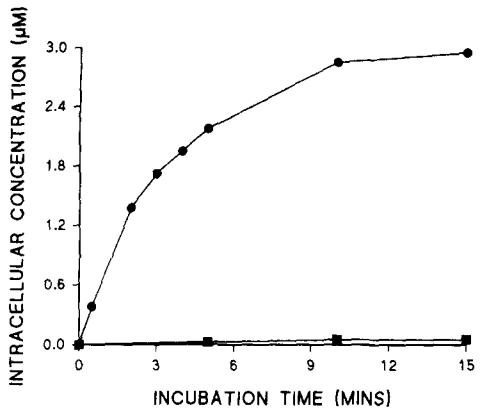

Fig. 4. The uptake of 0.5 μM [^{3}H]ICI 198583 into L1210 (●) and L1210:1565 (■) cells. Cells were incubated in 150 mM HEPES buffer, pH 7.4 for up to 15 min and the cellular ^{3}H measured as described in Materials and Methods. The L1210:1565 line has a greatly reduced ability to transport reduced folates or MTX into the cells.

still found as monoglutamate (glu_1) and the remainder as polyglutamates, principally the tri- and tetraglutamates (glu_3 and glu_4). After this time there was an increase in the total level of polyglutamates formed and a shift in the distribution of polyglutamates so that glu_4 predominated with significant formation of glu_5. The human W1L2 cell line had very little detectable glu_1 at 24 hr and a greater proportion of the cellular ^{3}H as polyglutamates, again both glu_4 and glu_5 predominanting.

The results of efflux experiments are shown in Table 5. L1210 cells that had been incubated for 24 hr with 1 μM ICI 198583 and resuspended in drug-free medium for a further 4 or 24 hr lost a significant amount of ^{3}H. Four hours after resuspension a small loss was accounted for primarily by a decrease in the level of glu_1 with the total polyglutamate pool being unchanged. Small changes in the level of each polyglutamate can be accounted for by an equilibration within the polyglutamate pool (towards the higher polyglutamates). After 24 hr the ~40% loss in cellular ^{3}H could only be attributed in part to a big loss of glu_1 as ~30% of the polyglutamate pool was also lost by this time. However, polyglutamates of greater chain length (glu_4–>glu_5) now accounted for ~90% of the cellular ^{3}H.

Pharmacokinetics

Over the time period 30–120 min following an i.v. bolus dose of 500 mg/kg ICI 198583, plasma ICI 198583 levels declined exponentially with a half life of 16 min. Data for the half life, AUC and clearance of ICI 198583 are given in Table 6 and compared to those previously published [17, 19] for CB3717. This comparison shows that ICI 198583 total plasma clearance is substantially more rapid than that of CB3717.

In vivo *tumour studies*

Anti-tumour activity. ICI 198583 was curative to

Table 4. The uptake and polyglutamation of 1 μM [^{3}H]ICI 198583 in mouse (L1210) and human (W1L2) cells *in vitro*

Cell line	Incubation time (hr)	*Expt. No.	Total cellular ^{3}H (μM)	Total glu_1 recovered† (μM)	Total polyglutamates recovered† (glu_2–glu_n (μM)	Cellular level of ICI 198583 and its polyglutamates (% total cellular ^{3}H recovered)					
						glu_1	glu_2	glu_3	glu_4	glu_5	$>glu_5$
L1210	4	1	2.3	1.1	0.93	54	1	19	24	1	1
		2	1.1	0.54	0.39	58	2	19	20	0.7	0
L1210	8	1	2.3	0.80	1.3	39	1	14	40	6	0.4
L1210	24	1	2.2	0.56	1.4	28	0.4	8	49	13	1
		2	4.5	0.80	2.5	24	1	5	46	22	1
W1L2	24	1	2.1	0.12	1.4	8	1	13	36	39	1
		2	4.1	0.25	2.6	9	1	14	46	29	0

* Each experiment is the mean of duplicate cultures taken through separate extraction and HPLC procedures.
† Overall recovery of ^{3}H through extraction and HPLC procedures was 70–88%.
L1210 and W1L2 cells were exposed to 1 μM [^{3}H]ICI 198583 for the times indicated. After extraction of ICI 198583 derived material the samples were analysed by HPLC (see Materials and Methods).

Table 5. Cellular levels of [^{3}H]ICI 198583 and its polyglutamates in L1210 cells after resuspension in drug-free medium

Time after resuspension in drug-free medium (hr)	Expt. No.*	Total cellular ^{3}H (μM)	[^{3}H]ICI 198583 and its polyglutamates recovered† (% of that seen before resuspension in drug-free medium in parentheses)		Cellular levels of ICI 198583 and its polyglutamates (% of total cellular ^{3}H recovered)					
			Total monoglutamate (glu_1) (μM)‡	Total polyglutamates (glu_2–glu_n) (μM)‡	glu_1	glu_2	glu_3	glu_4	glu_5	$>glu_5$
0	1	2.2	0.56	1.4	28	0.4	8	49	13	1
	2	4.5	0.80	2.5	24	1	5	46	22	1
4	1	1.9	0.12 (21)	1.5 (107)	7	0.4	10	56	23	2.5
	2	3.0	0.10 (13)	2.3 (91)	4	1.5	5	50	34	6
24	1	1.2	0.08 (14)	0.9 (64)	8	0.8	10	51	27	4
	2	2.7	0.12 (15)	1.9 (76)	6	0.5	4	40	41	10

* Each experiment is the mean of duplicate cultures taken through separate extraction and HPLC procedures.
† Overall recovery of ^{3}H through extraction and HPLC procedures was 74–88%.
‡ Corrected for dilution out by cell division.
L1210 cells were exposed to 1 μM [^{3}H]ICI 198583 for 24 hr and then resuspended in drug-free medium for the times indicated. Methods as in Table 4.

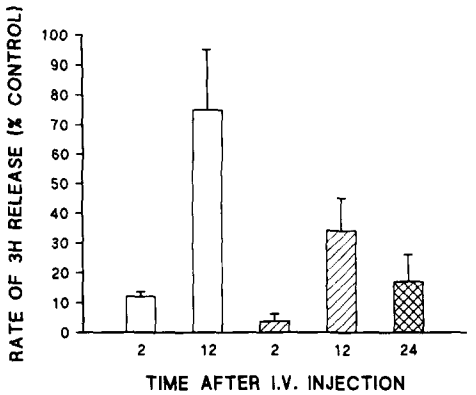

Fig. 5. The inhibition of TS in ascitic L1210:NCI cells *in vivo* after i.v. administration of ICI 198583: (□) 50 mg/kg, (▨) 250 mg/kg, (■) 500 mg/kg. ICI 198583 was injected via the tail vein into mice bearing the L1210:NCI ascitic tumour. At the times indicated the peritoneal contents were aspirated into RPMI 1640 medium, centrifuged, and resuspended to a concentration of 10^6/mL in medium at 37°. 5-[^{3}H]dUrd was added at a concentration of 0.3 μM (2–5 μCi/mL), 400 μL aliquots were removed at various times over a 90 min period and the ^3H_2O was separated and measured as described previously [2, 19, 34]. The results are given as means with SD (N = 3–7).

mice bearing the L1210:ICR tumour at 5 mg/kg on a five times daily schedule (Table 7). This represents an ~10-fold improvement in potency over CB3717 in the same tumour [2]. A marked improvement in therapeutic index was apparent as the MTD for ICI 198583 was >500 mg/kg five times daily (10% body weight loss at this dose) compared with 100–200 mg/kg five times daily for CB3717. No significant activity was observed with ICI 198583 against the L1210:NCI tumour at 500 mg/kg seven times daily.

TS inhibition in the L1210:NCI tumour. The flux through TS was measured in ascitic L1210:NCI cells as the rate of ^{3}H release from 5-[^{3}H]dUrd (Fig. 5). In cells removed 2 hr after a single bolus i.v. injection of ICI 198583 TS was substantially inhibited following both 50 and 250 mg/kg. At 12 hr after administration TS remained inhibited by more than 50% following 250 mg/kg. Following 500 mg/kg of ICI 198583 i.v. TS was inhibited by more than 80%, even after 24 hr. Thus ICI 198583 produced a pronounced and prolonged inhibition of TS *in vivo* at doses which do not produce acute (500 mg/kg) or chronic (250 mg/kg twice a week for 3 weeks) toxicity (see Ref. 19 and unpublished results).

DISCUSSION

The aim of these studies was to define which properties of the TS inhibitor, ICI 198583, are important for its antitumour potency both *in vitro* and *in vivo*. The replacement of the 2-amino moiety of CB3717 with a methyl group to give 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI 198583) had little effect on TS inhibitory activity or substrate activity for FPGS. The 40-fold improvement in growth inhibitory potency seen with ICI 198583

Table 6. The pharmacokinetics of ICI 198583 and CB3717

Drug	Pharmacokinetic parameters		
	AUC (μM × hr)	Clearance (mL/min/kg)	$T_{1/2\beta}$ (min)
ICI 198583 (500 mg/kg)	393	42	16 ± 1
CB3717*	592	6	94 ± 5
(100 mg/kg)	772	5	81 ± 9

The compounds were administered as an i.v. bolus injection via the tail vein and animals were killed 30–120 min thereafter. Compounds were analysed by HPLC and pharmacokinetic parameters calculated as described in Materials and Methods.
* Data for CB3717 are from Refs 17 and 19.

Table 7. Antitumour activity of ICI 198583 *in vivo* (L1210:ICR and L1210:NCI)

Tumour variant	Number of days treated	Daily dose (mg/kg)	No. cured/no. treated
L1210:ICR	5	1	0/7
L1210:ICR	5	5	4/7
L1210:ICR	5	10	4/7
L1210:ICR	5	50	6/7
L1210:NCI	7	500	0/8*

* This dose was associated with weight loss (~10% at day 10).
Mean day of death of seven control mice was 11 (range 9 to 12 days) for those bearing the L1210:NCI tumour and 13 (range 12 to 17 days) for those bearing the L1210:ICR tumour.

over CB3717 in the L1210 and W1L2 cell lines is similar to that reported elsewhere for other cell lines [35, 36]. This marked enhancement in potency is not explained by a change in the intracellular locus of action as the activity of ICI 198583 is prevented by co-administration of thymidine alone, the salvage precursor for thymidylate synthesis. Although it has been reported previously that ICI 198583 is only a weak inhibitor of isolated DHFR [21], the present study confirms that this enzyme is not the intracellular target for this compound. Thus, comparable activity was seen against wild-type L1210 cells and the MTX-resistant L1210:R7A subline which overproduces DHFR. A low level of cross-resistance to TS inhibitors, such as CB 3717 and ICI 198583, is often seen in the L1210:R7A cell line and suggestions for possible explanations have been proposed recently [2]. Further evidence for the specificity of ICI 198583 for TS comes from the derivation of a W1L2 cell line with acquired resistance to ICI 198583 (W1L2:C1) [25]. The W1L2:C1 cell line over-produces TS due to gene amplification being the mechanism of resistance and is cross-resistant to other folate-based TS inhibitors but not to inhibitors of DHFR such as MTX or trimetrexate [25]. Since there appears little doubt that ICI 198583, like CB3717, acts via inhibition of TS, the increased cytotoxic potency must be a result of some other property of the compound. Thus, studies were extended to investigate the cellular uptake of ICI 198583 and its metabolism to polyglutamate derivatives.

The studies of the transport of ICI 198583 into cells showed concentrative, saturable uptake which is consistent with an active mechanism. The fact that the L1210:1565 cell line that does not transport reduced folates or MTX [31] was unable to take up [^{3}H]ICI 198583 suggests that the compound can use the reduced folate/MTX transport system in L1210 cells grown in culture. Since the L1210:1565 cell line was significantly resistant (100-fold relative to the L1210 line) to ICI 198583 but not to CB3717, it was concluded that this mechanism of drug uptake is important for the cytotoxicity of ICI 198583 in L1210 cells *in vitro*. This is in agreement with the results of others [35] where, in a hepatoma cell line (H35) 100-fold resistant to MTX due to a transport defect, cross-resistance to ICI 198583 but not to CB3717 was seen. Further experiments by these authors demonstrated that ICI 198583, but not CB3717, effectively inhibited [^{3}H]MTX and folinic acid uptake in H35 hepatoma cells. Similarly, Jansen *et al.* [36] provide considerable evidence that ICI 198583 utilises the reduced folate/MTX transport mechanism in CCRF-CEM cells. However, it was also demonstrated that another route for uptake of ICI 198583 and CB3717 is the membrane associated folate-binding protein (FBP). The physiological significance of these uptake mechanisms is still not certain.

HPLC analysis of extracts from L1210 cells treated with 1 μM [^{3}H]ICI 198583 demonstrated extensive metabolism to polyglutamates such that after 24 hr over half of the intracellular ^{3}H was found

as polyglutamate metabolites; predominantly the tetraglutamate (glu_4) and to a lesser extent the pentaglutamate (glu_5) metabolite. Although some triglutamate (glu_3) was apparent, particularly at 4 hr, diglutamate (glu_2) was barely detectable at any time point. There may be several explanations for this observation including (a) efflux of the shorter chain length polyglutamates and (b) rapid metabolism to longer chain length polyglutamates. Evidence for the latter comes from FPGS substrate activity for the ICI 198583 glu_2 derivative where the 10-fold increase in the first-order rate constant suggests that glu_2 would be metabolized rapidly to glu_3 at low intracellular levels. Unfortunately, it has not yet been possible to characterize the other polyglutamate derivatives with respect to FPGS activity because of an apparently dramatic decrease in the V_{max}. This decrease in V_{max} has been documented previously as occurring for polyglutamates of tetrahydrofolate [37]. By comparison to similar experiments with [^{3}H]CB3717 [8], we can conclude that at extracellular concentrations equal to 10 times their IC_{50} values (1 μM for ICI 198583 and 50 μM for CB3717) similar concentrations of cellular ^{3}H were observed (2–6 μM) at 24 hr with a similar proportion of the cellular drug-derived material found as polyglutamates. The higher polyglutamates (glu_4 and glu_5) account for the largest proportion of CB3717 polyglutamates at 24 hr although in the ICI 198583-treated cells the glu_4 predominated.

Efflux experiments demonstrated that after resuspension in drug-free medium for 24 hr, ~85% of the monoglutamated compound was lost from the cells with a small loss in total polyglutamates. Thus, after 24 hr the cells had preferentially retained the polyglutamates (~1 μM polyglutamates). A similar retention of polyglutamates was also observed in CB3717-treated L1210 cells [8]. Given that polyglutamates are very much more potent inhibitors of TS, the formation and retention of polyglutamates must be major determinants of the biological activity of the compound, particularly after removal of extracellular drug. The polyglutamates thus provide a pool of potent metabolites that should be capable of prolonged inhibition of TS, even upon synthesis of new enzyme. Taken together the TS, FPGS, transport and polyglutamation data indicate that the improved potency of ICI 198583 *in vitro* is due to improved uptake into cells and that the mechanism responsible for uptake is the reduced folate transport protein.

In vivo evidence for sustained TS inhibition was obtained from the measurement of the pharmacodynamics of ICI 198583 in mice. The rate of ^{3}H release from 5-[^{3}H]dUrd in ascitic L1210:NCI cells removed after an i.v. bolus injection of ICI 198583 provided a method of measuring inhibition of TS in whole cells. Although fairly high doses of ICI 198583 were required to inhibit TS by >80% at 2 hr (50 mg/kg) and at 24 hr (500 mg/kg) the results compare favourably with CB3717 where significant inhibition was not achieved at 2 hr even at the MTD of 200 mg/kg [2]. Prolonged inhibition of TS after ICI 198583 administration occurs despite tumour growth and synthesis of new enzyme since the L1210:NCI tumour was found to be refractory to ICI 198583. The observation of continued TS inhibition 24 hr after ICI 198583 administration is consistent with inhibition being due to polyglutamate forms since this assay only measures non-effluxable forms of TS inhibitors. Furthermore, as ICI 198583 was shown to be cleared very rapidly from plasma ($T_{1/2}$ = 16 min), uptake and drug retention by metabolism to polyglutamates are probably essential requirements for activity following bolus administration.

ICI 198583 was found to be substantially more potent than CB3717 against the L1210:ICR tumour. The minimum curative daily dose of 5 mg/kg was 1% of the dose that gave an indication of some toxicity. In contrast, the minimum curative dose for CB3717 is 50 mg/kg which is close to the toxic dose range for this compound (100–200 mg/kg) [2]. These data indicate a notable improvement in therapeutic index of ICI 198583 over CB3717 which can be attributed to both improved antitumour potency and a reduction in normal tissue toxicity. It has been reported previously that, unlike CB3717, ICI 198583 is not acutely toxic to the liver or kidneys of mice when given as a single bolus injection (500 mg/kg) [19]. Similarly, chronic ICI 198583 administration, 250 mg/kg twice weekly for 3 weeks, is also not toxic to the kidneys (unpublished results). The lack of toxicity of ICI 198583 is again in contrast with CB3717, a compound seriously limited by toxicity to the liver and kidneys [15–17].

In comparison to CB3717, ICI 198583 undergoes rapid plasma clearance and it is envisaged that this is also a reflection of the lack of renal and hepatic toxicity seen with ICI 198583 [19]. Thus, the liver and kidney toxicity of CB3717 limits biliary and urinary excretion, respectively. Limited excretion in turn reduces CB3717 plasma clearance. In the case of ICI 198583 there is no damage to the liver or the kidneys and thus plasma clearance is rapid. The rapid plasma clearance of ICI 198583 has also been demonstrated in the rat where clearance was found to be 64 mL/min/m^2 [38]. The reduced toxicity, and hence improved clearance, of ICI 198583 over CB3717 is probably due to the improved water solubility of ICI 198583 (1000-fold more soluble than CB3717 at pH 6 [22]) which is a common feature of the 2-desamino analogues of CB3717.

In summary, ICI 198583 is a potent inhibitor of TS with improved cytotoxic potency when compared to CB3717 and desamino-CB3717. Unlike CB3717, ICI 198583 is transported effectively via the reduced folate/MTX carrier in L1210 cells *in vitro*, a feature of ICI 198583 that allows for rapid uptake followed by subsequent metabolism to polyglutamate forms. The potency of ICI 198583 polyglutamates for TS, together with their cellular retention, presumably accounts for the prolonged inhibiion of TS *in vivo* despite rapid clearance of the compound from plasma. The improved potency and toxicity profile of ICI 198583 over CB3717 makes ICI 198583 a significant compound for the study of the biochemical pharmacology of folate-based TS inhibitors.

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