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THE *IN VIVO* METABOLIC STABILITY OF DIPEPTIDE ANALOGUES OF THE QUINAZOLINE ANTIFOLATE, ICI 198583, IN MICE

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Abstract—In the search for quinazoline thymidylate synthase inhibitors that are not subject to intracellular polyglutamation, a class of dipeptide analogues of the diglutamate of 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI 198583-γ-L-glu) has been evaluated for their stability to *in vivo* hydrolysis. Replacement of the second glutamate with another amino acid, e.g. alanine, prevented polyglutamation *in vitro* but such compounds were subject to hydrolysis when injected into mice. The extent of hydrolysis was measured in plasma, liver and kidney by HPLC analysis of tissue removed from mice 1 hr after i.p. injection. The enzyme responsible for this hydrolysis is thought to be a γ-glutamyl hydrolase which hydrolyses the amide bond, releasing ICI 198583 which may then be polyglutamated. Development of stable dipeptide compounds was achieved by structural modification in two principal ways: either by replacement of the second amino acid (e.g. glutamate or alanine) with its D-enantiomer or removal of the carboxyl on the α-carbon of the second amino acid (α′-COOH). In this second approach two series of compounds were investigated. Monocarboxylate-derived dipeptides, e.g. ICI 198583-γ-L-phenylalanine or ICI 198583-γ-phenylglycine, resulted in stable compounds after removal of the α′-COOH (to give -ethylamide and -benzylamide derivatives, respectively). However, for the dicarboxylic amino acids a less clear picture emerged. Although removal of the α′-COOH from ICI198583-γ-L-glutamate to give ICI 198583-γ-γ-aminobutyric acid resulted in a stable compound, the corresponding aspartate analogue (-β-alanine) was subject to hydrolysis.

N^{10}-Propargyl-5,8-dideazafolic acid (CB3717‡) is a folate-based inhibitor of the enzyme thymidylate synthase (TS, EC 2.1.1.45) with a K_i of ~3 nM [1–4]. CB3717 was found to be an active anticancer agent in early clinical trials, but its usage was limited by renal and hepatic toxicities, thought to be mediated in part by its poor solubility at physiological, and in particular urinary pH [5]. Removal of the amino group at the 2-position of CB3717 or addition of a methyl substituent has been shown to improve solubility and L1210 growth inhibition [4, 6–8]. In particular, 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI 198583; Fig. 1) was found to have only a 3-fold less affinity for TS (K_i = 10 nM) when compared to CB3717, but was a 34-fold more active antitumour agent against L1210 cells *in vitro* than CB3717 [8]. In contrast to CB3717, which causes renal and hepatic toxicity in mice following a bolus dose of 100 mg/kg, ICI 198583 was found to be non-toxic to the liver and kidneys at a dose of 500 mg/kg [9].

Many of the intracellular reduced forms of folic acid can be linked through a γ-peptide linkage to a number of further glutamate residues. This process, catalysed by the enzyme folylpolyglutamyl synthetase (FPGS), is commonly referred to as polyglutamation [10, 11]. The polyglutamate forms of reduced folates often demonstrate increased affinity (over the monoglutamate form) for a range of enzymes utilizing them as cofactors, and are considered to be the "preferred" substrates. In addition polyglutamates of higher chain length are not readily effluxed from the cell. A number of antifolates are also substrates for FPGS and this property can have a profound effect on their activity [12–18]. Both CB3717 and ICI 198583 have similar substrate activity for FPGS (40 μM) but ICI 198583 forms polyglutamates more readily inside cells probably because, unlike CB3717, it uses the reduced-folate/methotrexate carrier for cell entry [2, 8]. Polyglutamate metabolites of CB3717 and ICI 198583 have been shown to be more potent inhibitors of TS (the target enzyme), the addition of a single glutamate (diglutamate) enhancing their potency by ~20–30-fold (K_i ~ 0.1 and 0.4 nM, respectively) [2, 8, 19, 20]. Although potency was enhanced by the addition of further glutamate residues (~100-fold), the largest increment was seen between the mono- and diglutamate. *In vitro* antitumour testing (L1210 cells) demonstrated that growth inhibition was similar for the ICI 198583 and the diglutamate form (0.1 μM IC_{50}), but the addition of further glutamates reduced the efficacy, suggesting that transport into the cell was reduced [21].

Antifolates reliant on polyglutamation for activity including ICI 198583 and its diglutamate metabolite

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‡ Abbreviations: TS, thymidylate synthase; ICI 198583, 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid; CB3717, N^{10}-propargyl-5,8-dideazafolic acid; FPGS, folylpolyglutamyl synthetase; ACN, acetonitrile; GABA, γ-aminobutyric acid.

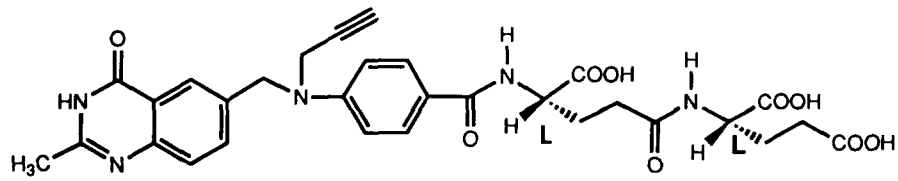


Fig. 1. ICI 198583-γ-L-glu.

have poor activity against cells with low or altered FPGS activity [21–24]. Therefore, as part of a drug development programme aimed at synthesizing TS inhibitors which are non-substrates for FPGS, and consequently active in such resistant lines, a series of modified ICI 198583-γ-diglutamate analogues (Fig. 1) was made. From structure–activity relationship studies a number of γ-linked di- and tri-carboxylate dipeptide analogues with potent activity against TS, and good *in vitro* growth inhibitory activity, were identified [21, 25]. Many of these compounds lacked a terminal-COOH on the second amino acid (e.g. alanine) and therefore were structurally precluded from polyglutamation. Others retained the terminal COOH, e.g. aspartate and adipate, but were apparently not polyglutamated as demonstrated by the retention of activity in a cell line [21] unable to polyglutamate antifolates (data not shown).

Since the novel dipeptide analogues could be susceptible, *in vivo*, to hydrolytic enzymes such as γ-glutamyl hydrolase (EC 3.4.22.12) [26], resulting in formation of ICI 198583, which could then be polyglutamated, their potential benefits could be lost. Therefore, novel dipeptide analogues have been tested in an *in vivo* assay to assess the extent of hydrolysis to their parent monoglutamate form, e.g. ICI 198583 (or its D-enantiomer where appropriate). Plasma, liver and kidney tissue samples were analysed by HPLC for both the dipeptide and its monoglutamate forms. Stability was assessed at a single time (1 hr). Rapid elimination ($T_{1/2\beta} \sim 15$ min) of the 2-methyl quinazoline antifolates and their dipeptide analogues meant that later time points reduced the sensitivity of the determination.

MATERIALS AND METHODS

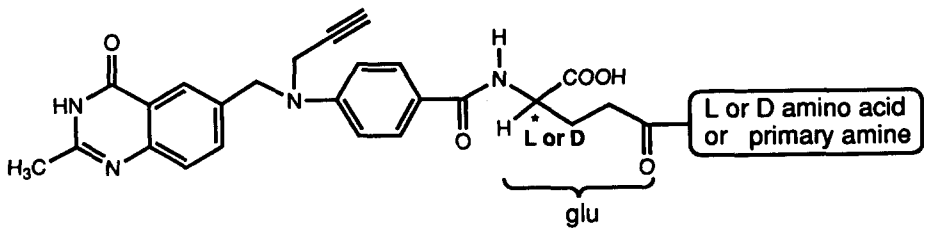
Dipeptide analogues of ICI 198583 were synthesized as trifluoroacetic acid salts at the Institute of Cancer Research [25] and supplied as powders. Compounds were dissolved in 0.05 M $NaHCO_3$ with the pH adjusted to between 9.0 and 9.5 using NaOH. Injection solutions were prepared to allow administration at a constant volume of 10 mL/kg (10 mg/mL). Injection solutions were protected from the light and kept frozen until used, when they were thawed at room temperature. All other chemicals were analytical grade where available and obtained from standard suppliers.

Experiments were performed using C57/DBA2 F1 hybrid or DBA2 male mice between 6 and 10 weeks old [National Medical Research Institute (NMRI), London, U.K.]. Hydrolysis of ICI 198583-γ-linked diglutamate was evaluated in both strains and the same result was obtained. The compounds were given at 100 mg/kg by intraperitoneal (i.p.) injection (actual free acid concentration varied between 73 and 97 mg/kg). Mice were killed by CO_2 asphyxiation and exsanguinated by open cardiac puncture, 60 min after compound administration. All results are adjusted for the concentration of free acid. Blood was placed in heparinized microfuge tubes and centrifuged immediately. Plasma was separated and frozen for subsequent analysis. At the same time the liver/gall bladder and kidneys were removed. The organs were thoroughly washed with de-ionized water and frozen for subsequent quantification of their compound content.

Plasma samples were thawed at room temperature. Extraction was performed by the addition of 100 μL of acetonitrile (ACN) to 100 μL of plasma and centrifugation at 1500 *g* at 4° for 20 min. The supernatant was diluted 1:4 in 0.05 M $NaHCO_3$ and analysed by HPLC (see below). Following weighing, tissue samples were soaked in 0.1 M Tris pH 10 (9 mL/g tissue wet wt) for 1 hr and then homogenized in a Teflon–glass homogenizer. Drugs were extracted by the addition of 0.5 mL of ACN to 0.5 mL of homogenate. Following centrifugation and dilution as described above, the supernatant was analysed by HPLC.

HPLC system. All HPLC analyses were performed on Waters Associates (Northwich, U.K.) chromatography equipment. Data acquisition was carried out using a Kontron MT2 data module (Kontron Instruments, St Albans, U.K.). Separation was performed on a Spherisorb 5 μm C6 (Phase Separation Ltd, Deeside, U.K.) column (15 × 0.46 cm) with a CO:Pell ODS precolumn (6.5 × 0.21 cm, Whatman Ltd, Maidstone, U.K.). Samples were eluted using either an isocratic or linear gradient system at a flow rate of 1.5–2 mL/min. The mobile phase consisted of ACN in 0.1 M CH_3COONa (pH 5.0). Isocratic systems were used for R = L-glu, gly, D-glu (12.5% ACN), γ-aminobutyric acid (GABA), β-ala (13.8% ACN). Gradient systems were required for R = L-ala, D-ala, PheGly, benzylamide, ethylamide (10–25% ACN over 15 min) and R = L-asp, L-Phe, D-Phe (5–30% ACN over 15 min). Gradient systems were also used for the D-glu-D-glu and D-glu-L-glu compounds (5% ACN, 0.085 M CH_3COONa to 20% ACN, 0.1 M CH_3COONa pH 5.0 over 15 min). All solvents were filtered prior to use and degassed. Samples (100 μL) were applied to the column. Standard concentrations of the analogue and ICI 198583 (the

Table 1. The *in vivo* stability of dipeptide analogues



Dipeptide	Plasma concentration (μM)		Liver content (nmol/g tissue)*	
	Dipeptide	ICI 198583 or D-glu equivalent	Dipeptide	ICI 198583 or D-glu equivalent
-L-glu-L-glu	27 ± 5	14 ± 2	56 ± 28	1421 ± 461
-D-glu-L-glu	50 ± 66	5 ± 4	615 ± 142	63 ± 20
-D-glu-D-glu	15 ± 4	ND	539 ± 73	ND
-L-glu-D-glu	34 ± 6	ND	881 ± 66	ND
-L-glu-GABA	20 ± 5.6	ND	125 ± 39	ND
-L-glu-gly	26 ± 12	4.2 ± 0.4	28 ± 12	342 ± 103
-L-glu-L-asp	38 ± 16	3.1 ± 1.4	861 ± 220	98 ± 25
-L-glu-β-ala	25 ± 9.9	2.6 ± 1.3	47 ± 12	181 ± 40
-L-glu-L-ala	19 ± 12	3.3 ± 0.3	135 ± 108	115 ± 28
-L-glu-D-ala	33 ± 8	ND	159 ± 22	ND
-L-glu-ethylamide	7.7 ± 4.5	ND	132 ± 52	ND
-L-glu-L-phe	4.5 ± 1	4 ± 0.9	43 ± 20	48 ± 10
-L-glu-D-phe	29 ± 28	ND	77 ± 43	ND
-L-glu-phegly	15 ± 15	2 ± 0.6	49 ± 3	63 ± 25
-L-glu-benzylamide	20 ± 5	ND	54, 81	ND

Mice were administered with 100 mg/kg of compound (trifluoroacetic acid salt) i.p. and killed 1 hr later, and blood, liver and kidneys were removed for quantitation by HPLC of the parent drug and any ICI 198583 present. Lower limit of detection in plasma ~0.2 μM and in liver ~2 nmol/g tissue.

Values are means and SD of five mice.

ND, not detected.

* Corrected for % of free drug given.

potential catabolite) were prepared in 0.05 M $NaHCO_3$ (100 μg/mL) and in mouse plasma (100 μM) for use as external standards. Detection was by UV absorbance at 280 and 313 nm and calibration curves were linear. Extraction from plasma and tissues was >95%. The overall lower detection limit of the assay was ~0.2 μM for plasma and ~2 nmol/g for liver and kidney tissue (all results refer to the free acid).

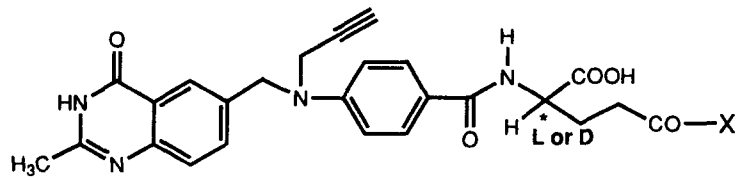
In vitro *assays of TS inhibition and* L1210 *growth inhibition.* TS inhibition was assayed by ^3H release from [5-^{3}H]dUMP using partially purified TS from a TS overproducing line (L1210:C15) as described previously [4]. The 6*R*,*S*-5,10-methylene tetrahydrofolate concentration was 200 μM and ICI 198583 has an IC_{50} of 60 nM under the conditions of the assay which corresponds to a K_i of 10 nM. The method used for measuring L1210 growth inhibition is found in the same publication [4].

RESULTS

In vivo *stability of dipeptide analogues*

The assay system described in this paper was able to detect hydrolysis of dipeptide analogues of ICI 198583 *in vivo*. ICI 198583-L-glu was clearly metabolized to ICI 198583. Sixty minutes after compound administration the ratio of diglutamate:monoglutamate was 2:1 in the plasma and 1:25 in the liver (Table 1). At this time levels of ICI 198583 in the kidneys were similar to those in the plasma (data not shown). Replacement of the second glutamate by -L-aspartate did not result in a stable compound as significant amounts of ICI 198583 were found in both the plasma and liver 60 min after drug administration. In contrast, replacement of the second glutamate by its enantiomer (D-glutamate) prevented hydrolysis, within the limits of detection, and no ICI 198583 was detected in the plasma (<1% of dipeptide concentration), liver (<6%) or kidneys. To confirm this observation compound stability was examined over an extended time course (5 min to 4 hr) and again no ICI 198583 could be detected at any point examined (data not shown). However, replacement of the first glutamate with the D-enantiomer did not bestow stability upon the compound with the ICI 198583 D-enantiomeric form being found in both the plasma and liver (Table 1).

Table 2. The *in vitro* activity of dipeptide analogues



Dipeptide	X	Stable to hydrolysis in mice	L1210 TS IC_{50} (nM)	L1210 IC_{50} (μM) (at 48 hr)
-L-glu (ICI 198583)	OH	—	56*	0.1
-D-glu	OH	—	260	2.0
-L-glu-L-glu	NHCH(COOH')CH_2CH_2COOH	No	2	0.12
-D-glu-L-glu	NHCH(COOH')CH_2CH_2COOH	No	36	2.9
-D-glu-D-glu	NHCH(COOH')CH_2CH_2COOH	Yes	26	1.3
-L-glu-D-glu	NHCH(COOH')CH_2CH_2COOH	Yes	5	0.22
-L-glu-GABA	$NHCH_2CH_2CH_2COOH$	Yes	14	0.4
-L-glu-gly	$NHCH_2COOH'$	No	11	0.11
-L-glu-L-asp	NHCH(COOH')CH_2COOH	No	10	2.4
-L-glu-β-ala	$NHCH_2CH_2COOH$	No	14	0.92
-L-glu-L-ala	NHCH(COOH')CH_3	No	18	0.56
-L-glu-D-ala	NHCH(COOH')CH_3	Yes	12	0.56
-L-glu-ethylamide	$NHCH_2CH_3$	Yes	70	5.2
-L-glu-L-phe	NHCH(COOH')CH_2Ph	No	18	10.0
-L-glu-D-phe	NHCH(COOH')CH_2Ph	Yes	30	2.2
-L-glu-phegly	NHCH(COOH')-Ph	No	14	0.6
-L-glu-benzylamide	$NHCH_2Ph$	Yes	170	22

* K_i = 10 nM.

Hydrolysis was also seen when monocarboxyl amino acids (i.e. L-alanine, L-phenylalanine and glycine) replaced the second glutamate of the dipeptide. However, when the D-enantiomers were incorporated, as the terminal amino acid, the compounds were stable to hydrolysis (Table 1).

The effects on stability of a carboxyl group on the α-carbon of the second amino acid (defined here as α'-COOH) was investigated by comparing the ICI 198583-γ-L-glu, -L-asp, -L-ala and -L-phegly dipeptides with their structural variants in which the α'-COOH was removed (L-glu-GABA, L-glu-β-ala, L-glu-ethylamide and L-glu-benzylamide, respectively). For three of the structural pairs, removal of the α'-COOH appeared to prevent hydrolysis (Table 1). However, removal of the α'-COOH from ICI 198583-γ-L-asp to give ICI 198583-γ-β-ala did not result in a stable compound. Two possible explanations exist for the observation that there is an apparent requirement for a carboxyl (not necessarily an α') in close proximity to the amide bond for hydrolysis to occur. Either there is a direct interaction of the carboxyl with the γ-hydrolase or the carboxyl is required for activation of the amide before hydrolysis can occur.

The data in Table 2 show that all the stable compounds are potent inhibitors of TS and although less active than ICI 198583, the majority have significant activity against the L1210 cell line. The ICI 198583-γ-D-glu analogue is the most active stable dipeptide as measured by inhibition of isolated TS and of the growth of L1210 cells. Furthermore an L1210 variant, defective in its ability to form polyglutamate metabolites of antifolates, demonstrated no significant resistance to this compound (18-fold resistant to ICI 198583-γ-L-glu (data not shown)).

DISCUSSION

It has been shown that analogues of the γ-linked diglutamate metabolite of ICI 198583 are susceptible, *in vivo*, to enzymes which hydrolyse the γ-glutamyl bond. An *in vivo* assay was developed to establish, unambiguously, stability of these novel analogues. The enzyme involved is assumed to be a γ-glutamyl hydrolase and the major site for this activity is possibly the liver. Further experiments are aimed at confirming these hypotheses. A series of compounds have been synthesized and certain structural features have been identified which apparently protect them from the hydrolase activity. It appears that: (i) a D-configuration in the second amino acid in the dipeptide protects from hydrolysis (Table 1); (ii) removal of the α'-COOH from glutamate (but not aspartate) or from monocarboxylic acids such as alanine also protects from hydrolysis. Despite these modifications, a number of the stable compounds

remain active antitumour agents *in vitro* in the L1210 48 hr exposure assay (Table 2).

In summary, we have described a simple *in vivo* assay, which has been used to distinguish some structural features in dipeptide analogues of ICI 198583 diglutamate which make these analogues stable to hydrolysis of the γ-glutamyl peptide bond. Since these studies were performed in mice, localization of the enzyme (or enzymes), responsible for hydrolysis has not been defined. However, this assay has been used to guide the synthesis of novel TS inhibitors which have been shown to be stable to *in vivo* hydrolysis. The potential of such compounds as antitumour agents is currently being assessed in mouse experimental systems.

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