Synthesis and biological activity of N^α-[4-[N-[(3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-N-propargylamino]phenyl-acetyl]-L-glutamic acid[★]

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2-Deamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI 198583) is a potent inhibitor of thymidylate synthase. Its analogue, N^α-[4-[N-[(3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-N-propargylamino]phenylacetyl]-L-glutamic acid, containing p-aminophenylacetic acid residue substituting p-aminobenzoic acid residue, was synthesized. The new analogue exhibited a moderately potent thymidylate synthase inhibition, of linear mixed type *vs.* the cofactor, $N^{5,10}$-methylenetetrahydrofolate. The K_i value of 0.34 μM, determined with a purified recombinant rat hepatoma enzyme, was about 30-fold higher than that reported for inhibition of thymidylate synthase from mouse leukemia L1210 cells by ICI 198583 (Hughes *et al.,* 1990, *J. Med. Chem.* **33,** 3060). Growth of mouse leukemia L5178Y cells was inhibited by the analogue (IC$_{50}$ = 1.26 mM) 180-fold weaker than by ICI 198583 (IC$_{50}$ = 6.9 μM).

Thymidine 5′-monophosphate (TMP) which, metabolised to TTP, is essential for DNA replication and repair, is formed intracellularly *de novo* in a process of the C(5) methylation of 2′-deoxyuridylate (dUMP), catalyzed by the enzyme thymidylate synthase (EC 2.1.1.45). The dUMP methylation reaction involves a concerted transfer and reduction of the one-carbon group (at the aldehyde oxidation level) of $N^{5,10}$-methylenetetrahydrofolate (CH$_2$H$_4$PteGlu), with concomitant production of thymidylate and dihydrofolate (Carreras & Santi, 1995). Inhibition of thymidylate synthase by analogues of the nucleotide sub-

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strate or pteridine cofactor is employed in anticancer, antiviral, antifungal and anti-protozoan chemotherapy (Heidelberger *et al.*, 1983; Rathod, 1997; Georgopapadakou & Walsh, 1996; Takemura & Jackman, 1997).

The cofactor analogue, 2-deamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI 198583; Fig. 1) is a potent inhibitor of thymidylate synthase (EC 2.1.1.45), a target in anticancer chemotherapy. In order to study the effect of insertion into ICI 198583 of a CH_2 spacer between the phenyl and CONH moiety on interaction with thymidylate synthase, its analogue N^α-[4-[*N*-[(3,4-dime-thyl-2-methyl-4-oxo-6-quinazolinyl)me-thyl]-*N*-propargylamino]phenylacetyl]-L-gluta-mic acid (**8**; Fig. 2), containing a *p*-amino-phenylacetic acid residue substituting the *p*-aminobenzoic acid residue, was synthesized and tested as an inhibitor of mammalian tu-mour thymidylate synthase. Cell growth inhi-bition by the new analogue was also studied.

MATERIALS AND METHODS

Chemistry

6-Bromomethyl-3,4-dihydro-2-methyl-4-oxy-quinazoline (Hughes *et al.*, 1990), 4-(4,6-di-methoxy-1,3,5-triazin-2-yl)-4-methylmorpholin-ium chloride (DMTMM) (Kunishima *et al.*, 1999) and dimethyl glutamate hydrochloride (Greenstein & Winitz, 1961) were synthesized as previously described. 4-Nitrophenylacetic acid was the Lancaster (# 2577) product. Tetrahydrofuran, dioxane, diethyl ether and triethylamine were distilled over sodium and stored over sodium wire. DMF was azeo-tropically distilled and stored, similarly as DMA, over activated (25°C) 4 Å molecular sieves. The hydrogenolysis catalyst was 10% Pd/C used at 20% of the substrate weight. Melting points were determined on a Boëtius heating block and are uncorrected. ^{1}H-NMR spectra were determined using a Varian 500 MHz spectrometer. HPLC analyses were car-

ried out using a Beckman System Gold chromatograph, a 5 μL loop, an Alltech Alltima, C_{18} 5 μm, 150 × 4.6 mm column, de-tection at 210 nm and flow rate of 1 mL/min.

Dimethyl N^α-(4-nitrophenylacetyl)-L-glu-tamate (3). To a stirred for 10 min solution of 4-nitrophenylacetic acid (0.724 g, 4 mmol), HCl·Glu(OMe)$_2$ (0.848 g, 4 mmol) and *N*-methylmorpholine (440 μL, 4 mmol) in THF (24 mL), DMTMM (1.218 g, 4 mmol) was added. After 3 h stirring at room temperature *N*-methylmorpholine hydrochloride was fil-tered off and H_2O (25 mL) was added to the fil-trate. The mixture was extracted with Et_2O (2 × 20 and 3 × 30 mL). The combined Et_2O extracts were washed subsequently with satu-rated Na_2CO_3 (4 × 20 mL), H_2O (2 × 20 mL), 2 M HCl (4 × 20 mL), H_2O (2 × 20 mL) and brine, dried (Na_2SO_4) and evaporated to give crude **3** as a colourless viscous oil that was crystallized from the mixture of CH_2Cl_2/hex-ane: 0.738 g (55%); mp. 86–88°C; HPLC (0.1% TFA/ACN, 30:70) t_R =1.98; purity = 94%.

Dimethyl N^α-(4-aminophenylacetyl)-L-glutamate (4; Fig. 1). A solution of **3** (dimethyl N^α-(4-nitrophenylacetyl)-L-gluta-mate; 1.36 g, 4 mmol) in MeOH (50 mL), con-taining 10% Pd/C (0.272 g) in suspension was stirred under hydrogen for 1.5 h, whereupon TLC ($CHCl_3$/MeOH, 9:1) showed the absence of the starting material. The catalyst was re-moved by filtration and the filtrate evapo-rated to dryness giving a colourless oil that crystallized on standing: 1.227 g (99%); white needles mp. 94–96°C; HPLC monitoring (0.1% TFA/ACN, 70:30) t_R=1.92; purity 98%.

Dimethyl N-$^\alpha$[4-(N-propargyl)phenyl-acetyl]-L-glutamate (5; Fig. 1). A mixture of **4** (dimethyl N^α-(4-aminophenylacetyl)-L-gluta-mate; 1.210 g, 4 mmol), $CaCO_3$ (0.6 g, 6 mmol) and propargyl bromide (0.64 mL, 8 mmol) in DMF (15 mL) was stirred in the dark at 20°C for 1.5 h with TLC monitoring ($CHCl_3$/ace-tone, 9:1). Then, the mixture was poured into chloroform (50 mL), salts were filtered off, chloroform was evaporated and ethyl acetate (100 mL) was added. The organic phase was

washed with H_2O (7 × 50 mL) and brine (50 mL), dried (over Na_2SO_4) and the solvent was removed *in vacuo*. The resulting orange oil (662 mg) was dissolved in chloroform (3 mL) and chromatographed on silica gel (60H, Merck Art. 7736) column [gel (25 g); 4 cm i.d. × 6 cm l], which was eluted under low pressure with $CHCl_3$ (50 mL) and $CHCl_3$/acetone (9:1) (350 mL). Concentration of the appropriate fraction gave a yellow oil (350 mg, 25%). HPLC monitoring (0.1% TFA/ACN, 70:30); t_R = 5.32; purity 95%.

Dimethyl N^α-[4-[N-[(3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-N-propargylamino]phenylacetyl]-L-glutamate (7; Fig. 1). A mixture of **5** (dimethyl N^α-[4-(*N*-propargyl)phenylacetyl]-L-glutamate; 0.350 g, 1 mmol), 6-bromomethyl-3,4-dihydro- 2-methyl-4-oxoquinazoline (0.430 g, 1.7 mmol) and $CaCO_3$ (0.1 g, 1 mmol) in DMA (5 ml) was stirred at 20^oC in the dark for 24 h with TLC monitoring ($CHCl_3$/acetone, 8:2). The inorganic salts were filtered off and the filtrate evaporated *in vacuo*; the resulting oil was dissolved in chloroform (5 mL) and chromatographed on silica gel (60H, Merck Art. 7736) column [gel (25 g); 4 cm i.d. × 6 cm l], then eluted under low pressure with $CHCl_3$ (50 mL) and subsequently with $CHCl_3$ /acetone mixtures: 8:2 (400 mL), 7:3 (400 mL), 1:1 (200 mL), and 3:7 (450 mL). Concentration of the appropriate fraction gave an amorphous yellow solid: 0.380 g (73%), mp. = 208–210°C, HPLC (0.1% TFA/ACN, 70 : 30); t_R = 8.97; purity 95%.

N^α-[4-[N-[(3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-N-propargylamino]-phenylacetyl]-L-glutamic acid (8; Fig. 1). The diester **7** [(dimethyl N^α-[4-[*N*-[(3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-*N*-propargylamino]phenylacetyl]-L-glutamate] (0.153 g, 0.3 mmol) was treated with the mixture of 1 M NaOH (0.660 mL) and MeOH (6 mL), to give immediately a clear yellow solution. Since the presence of starting material was shown after 2 h by TLC ($CHCl_3$/MeOH, 9:1), further three portions of 1 M NaOH (3

0.06 mL) were added during 1.5 h and the mixture was stirred for 5.5 h, MeOH was removed *in vacuo* and the residue was diluted with H_2O (5 mL). Addition of 1 M HCl (0.840 mL) to pH 3.0 gave a gelatinous slightly yellow precipitate, which was washed with H_2O (5 mL) and dried under vacuum in the presence of P_2O_5 at 40°C for 12 h. This gave 0.126 g (86%) of yellow solid mp. = 190–191°C, HPLC (0.1% TFA/ACN, 70:30): t_R=2.95; purity 92%.

Biological studies

Thymidylate synthase. Highly purified preparation of thymidylate synthases was obtained from *E. coli*-expressed rat hepatoma (Cieśla *et al.*, 1995).

Enzyme assays. The [5-^{3}H]dUMP tritium release assay, performed as previously described (Rode *et al.*, 1990), was used in inhibition studies.

Kinetic studies. To identify the type of inhibition involved, the effect of the new analogues on the dependence of reaction rate on $N^{5,10}$-methylenetetrahydrofolate concentration, in the form of Lineweaver-Burk plots, were analyzed as previously reported (Dąbrowska *et al.*, 1996) with the use of a program, based on nonlinear regression and designed for estimation of kinetic constants describing competitive (both linear and parabolic), noncompetitive, uncompetitive or mixed-type linear inhibitions. Results are presented as means ± S.E.M. or means ± % difference between the mean and each of two results, followed by the number of experiments (N) in parentheses.

RESULTS AND DISCUSSION

N^α-[4-[*N*-[(3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-*N*-propargylamino]phenylacetyl]-L-glutamic acid (**8**; Fig. 1) was synthesized (Fig. 2) and its structure was confirmed by ^{1}H NMR (Table 1).

The new analogue was found to be a moderately potent thymidylate synthase inhibitor of
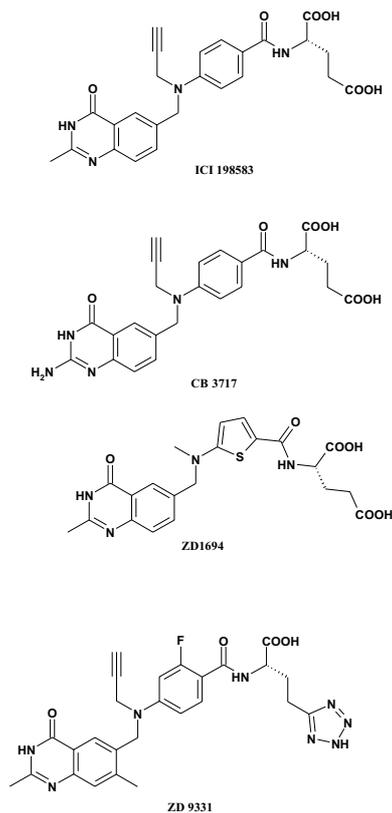
Figure 1. Structures of antifolates tested.

2-Desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolate (ICI 198583), N^{10}-propargyl-5,8-dideazafolate (CB 3717), *N*-[5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl]-L-glutamic acid (ZD 1694; Tomudex; raltitrexed) and (2S)-2-{*o*-fluoro-*p*-[*N*-(2,7-dimethyl-4-oxo-3,4-dihydroquinazolin-6-ylmethyl)-*N*-(prop-2-ynyl)amino]benzamido}-4-(tetrazol-5-yl)butyric acid (ZD 9331).

Figure 2. Synthesis of N^{α}-[4-[*N*-[(3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-*N*-propargyl-amino]phenylacetyl]-L-glutamic acid (8).

linear mixed type *vs.* the cofactor, $N^{5,10}$-methylenetetrahydrofolate. The K_i value, determined with the purified recombinant rat hepatoma enzyme (Table 2), was 9-fold higher than that describing inhibition of the same enzyme by ICI 198583 (Table 2) and about 30-fold higher than that reported for L1210 thymidylate synthase inhibition by ICI 198583 (Hughes *et al.*, 1990). On the other hand, the analogue was only 3-fold less potent than (2S)-2-{*o*-fluoro-*p*-[*N*-(2,7-dimethyl-4-oxo-3,4-dihydroquinazolin-6-ylmethyl)-*N*-(prop-2-ynyl)amino]benzamido}-4-(tetrazol-5-yl)butyric acid (ZD 9331; Table 2). The newly synthesized analogue inhibited growth of mouse leukemia L5178Y cells 180-fold weaker than ICI 198583 (Table 2). However, it should be
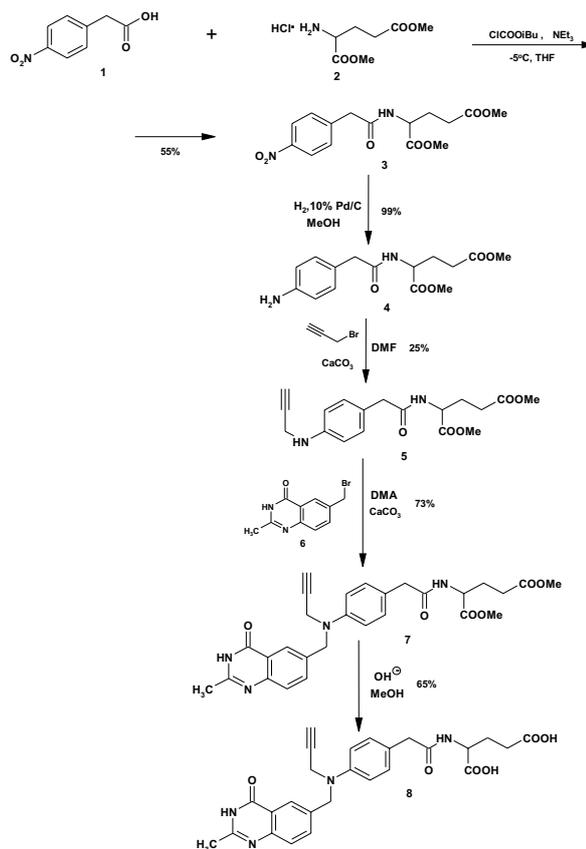
noticed that L5178Y cells used in our experiments were 2–20-fold less sensitive than L1210 cells to growth inhibition by several known antifolates (Table 1; cf. Fig. 1), including ICI 198583, N^{10}-propargyl-5,8-dideazafolate (CB 3717), *N*-[5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl]-L-glutamic acid (ZD 1694; Tomudex; raltitrexed) and (2S)-2-{*o*-fluoro-*p*-[*N*-(2,7-dimethyl-4-oxo-3,4-dihydroquinazolin-6-ylmethyl)-*N*-(prop-2-ynyl)amino]benzamido}-4-(tetrazol-5-yl)butyric acid (ZD 9331).

Substitution of the *p*-aminobenzoic acid residue in ICI 198583 by *p*-aminophenylacetic acid residue resulted in a weaker inhibition of both cell growth and thymidylate synthase activity. Since inhibition of cell growth was influenced to a higher extent than inhibition of the enzyme, this may indicate a possible inter-

Table 1. ^{1}H NMR analysis of N^{α}-[4-[*N*-[(3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-*N*-propargylamino]phenylacetyl]-L-glutamic acid (8) structure
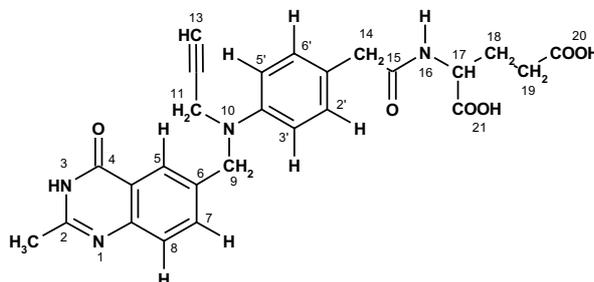
^{1}H Group	S ^{1}H	Peak multiplicities	δ [ppm]	J [Hz]
2 (-CH$_3$)	3H	s	2.332	–
3 (-NH-)	1H	s	12.173	–
5 (-H)	1H	s	7.981	–
7 (-H)	1H	d	7.694	8.24
8 (-H)	1H	d	7.536	7.33
9 (-CH$_2$-)	2H	s	4.613	–
11 (-CH$_2$)	2H	s	4.165	–
13 (CH≡C-)	1H	t	3.336	–
3′, 5′ (-H)	2H	d	6.768	8.54
2′, 6′ (-H)	2H	d	7.071	8.55
14 (-CH$_2$-)	2H	s	3.408	–
16 (-NHCO-)	1H	d	8.265	7.63
17 (-CH$^{\alpha}$-)	1H	m	4.181	–
18 ($^{\beta}$CH$_2$-)	2H	m	1.862	
19 ($^{\gamma}$CH$_2$-)	2H	t	2.273	
20, 21 (-COOH)	2H	bs	12.421	

ference of the applied substitution with cellular uptake and/or polyglutamylation.

It should be mentioned that phenylacetyl analogues of folate (Roberts & Shealy, 1973) and aminopterin (Montgomery *et al.*, 1979) have been synthesized and tested against dihydrofolate reductase and cell growth. A CH$_2$ spacer inserted between the phenyl and CONH moiety caused a marked decrease in affinity of the enzyme for the new analogues, as compared with the parent compounds, but was much more detrimental to the potential of either phenylacetyl analogue to influence cell growth. In accord with the latter, the present results concerning the corresponding ana-

logue of ICI 198583, pointed also to a much stronger influence of the structural alteration on cell growth than on the enzyme.

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Table 2. Inhibition of purified recombinant rat hepatoma thymidylate synthase and cell growth by the new analogue N^α-[4-[N-[(3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-N-propargylamino]phenylacetyl]-L-glutamic acid (8), parent 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolate (ICI 198583) and selected antifolates: N^{10}-propargyl-5,8-dideazafolate (CB 3717), N-[5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl]-L-glutamic acid (ZD 1694; Tomudex; raltitrexed) and (2S)-2-{o-fluoro-p-[N-(2,7-dimethyl-4-oxo-3,4-dihydroquinazolin-6-ylmethyl)-N-(prop-2-ynyl)amino]benzamido}-4-(tetrazol-5-yl)butyric acid (ZD 9331).

Results are presented as means ± S.E.M., or ±% difference between the mean and each of two results, followed by the number of experiments (N) in parentheses. Type of inhibition determined with $CH_2H_4PteGlu$ as a variable substrate.

Compound	Thymidylate synthase inhibition		Cell growth inhibition IC_{50} (µM)	
	K_i (µM)	Type of inhibition	L5178Y cells	L1210 cells
8 (ICI 198583 analogue)	0.34 ± 0.07 (3)	Mixed[a]	1263 ± 4% (2)	
ICI 198583	0.039 ± 0.01 (3)	Comp.[b]	6.9 ± 7% (2)	0.15[c]
CB 3717	0.004 ± 0.009% (2)	Mixed[a]	9.05 ± 1.24 (3)	5.0[d]
ZD 1694	0.029 ± 0.008 (3)[e]	Comp.[b]	0.189 ± 0.008 (3)	0.0088[d]
ZD 9331	0.13 ± 12% (2)[f]	Comp.[b]	0.268 ± 0.5% (2)	0.02[c]

[a]Mixed-type linear; [b]competitive; [c]Harrap, 1995; [d]Jackman *et al.*, 1995; [e]Sotelo-Mundo *et. al.*, 1999; [f]Rode *et al.*, 2000; purified regenerating rat liver thymidylate synthase was used.

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