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ORIGINAL ARTICLE

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AG337, a novel lipophilic thymidylate synthase inhibitor: *in vitro* and *in vivo* preclinical studies

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Abstract 3,4-Dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)-quinazoline dihydrochloride (AG337) is a water-soluble, lipophilic inhibitor of thymidylate synthase (TS) designed using X-ray structure-based methodologies to interact at the folate cofactor binding site of the enzyme. The aim of the design program was to identify TS inhibitors with different pharmacological characteristics from classical folate analogs and, most notably, to develop non-glutamate-containing molecules which would not require facilitated transport for uptake and would not undergo intracellular polyglutamylation. One molecule which resulted from this program, AG337, inhibits purified recombinant human TS with a K_i of 11 n*M*, and displays non-competitive inhibition kinetics. It was further shown to inhibit cell growth in a panel of cell lines of murine and human origin, displaying an IC_{50} of between 0.39 μ*M* and 6.6 μ*M*. TS was suggested as the locus of action of AG337 by the ability of thymidine to antagonize cell growth inhibition and the direct demonstration of TS inhibition in whole cells using a tritium release assay. The demonstration, by flow cytometry, that AG337-treated L1210 cells were arrested in the S phase of the cell cycle was also consistent with a blockage of TS, as was the pattern of ribonucleotide and deoxyribonucleotide pool modulation in AG337-treated cells, which showed significant reduction in TTP levels. The effects of AG337 were quickly reversed on removal of the drug, suggesting, as would be expected for a lipophilic agent, that there is rapid influx and efflux from cells and no intracellular metabolism to derivatives with enhanced retention. *In vivo*, AG337 was highly active against the thymidine kinase-deficient murine L5178Y/TK$^-$ lymphoma implanted either i.p. or i.m. following i.p. or oral delivery. Prolonged dosing periods of 5 or 10 days were required for activity, and efficacy was improved with twice-daily dose administration. Dose levels of 25 mg/kg delivered i.p. twice daily for 10 days, 50 mg/kg once daily for 10 days, or 100 mg/kg once daily for 5 days elicited 100% cures against the i.p. tumor. Doses required for activity against the i.m. tumor were higher (100 mg/kg i.p. twice daily for 5 or 10 days) but demonstrated the ability of AG337 to penetrate solid tissue barriers. Oral delivery required doses of $\geq$150 mg/kg twice daily for periods of 5–10 days to produce 100% cure rates against both i.m. and i.p. implanted tumors. These results were consistent with the pharmacokinetic parameters determined in rats, for which oral bioavailability of 30–50% was determined, together with a relatively short elimination half life of 2 h. Clinical studies with AG337 are currently in progress.

Key words AG337 · Thymidylate synthase · Antifolate

Abbreviations *TS* Thymidylate synthase · *CB3717* N^{10}-propargyl-5,8-dideazafolic acid · *ZD1694* (*Tomudex*) N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-L-glutamic acid · *FPGS* folylpolyglutamyl synthase · *AG337*, 3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)-quinazoline dihydrochloride · *AG331* N^6- [4-(morpholinosulfonyl)benzyl]-N^6-methyl-2,6-diaminobenz[*cd*] indole glucuronate · *dUMP* 2′-deoxyuridine 5′-monophosphate · *DMSO* dimethyl sulfoxide · *MTT* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide · *dUdR* 2′-deoxyuridine · 5-^{3}*HdUdR* [5-^{3}H]-2′-deoxyuridine · *HPLC* high performance liquid chromatography · *TK*, thymidine kinase · $T_{1/2}$ half life · *V* volume of distribution · Cl_s systemic clearance · *AUC* area under the concentration-time profile · *ICI 198583* 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid · *DHFR* dihydrofolate reductase

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Introduction

The validity of TS (EC 2.1.1.45) as a therapeutic target has been demonstrated by the clinical activity of CB3717 [2, 4] and, more recently, of ZD1694 (Tomudex) [13]. Both agents are 'classical' antifolates in that they contain a glutamate moiety. Compounds of this type require a facilitated transport mechanism for cellular uptake and also gain potency from intracellular conversion by FPGS to polyglutamate derivatives which show enhanced TS inhibition [13, 14, 16, 22]. These derivatives also accumulate within cells, such that *in vivo* activity can be demonstrated following bolus or fractionated bolus delivery both in mice and in man. AG337 (Fig. 1) was specifically designed to display a different pharmacological profile from these types of agents with the goal of circumventing sources of intrinsic or acquired resistance to which classical antifolates might be subject. These would potentially include loci affecting transport, polyglutamylation, or the hydrolysis of polyglutamates. It would be expected that nonclassical, lipophilic inhibitors would enter cells by passive diffusion but that in the absence of significant intracellular retention, they would require prolonged administration at potentially higher dose levels than agents such as ZD1694 to elicit efficacy. These issues have been explored at Agouron Pharmaceuticals with the design of AG337 and other structurally diverse TS inhibitors, using an X-ray crystal structure-based design approach [1] founded on the knowledge of the three-dimensional structure of the TS protein. The procedure involves sequential structure determination of enzyme-ligand complexes in an iterative process to derive tight binding molecular structures which interact at the cofactor binding site of the enzyme [26]. The data presented here summarize the preclinical studies conducted on AG337 prior to its selection as a candidate for clinical study. The experimental results indicate that AG337 is indeed a potent TS inhibitor with *in vitro* and *in vivo* antitumor activity indicative of this enzyme target as its locus of action. The biochemical characteristics which distinguish it from classical TS inhibitors are borne out by its behavior *in vitro* and *in vivo* and have provided the basis for selection of an appropriate dosing regime for clinical studies with this agent [3, 21].

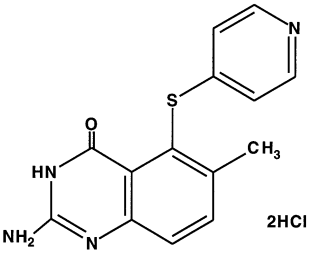

Fig. 1 Structure of 3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)-quinazoline dihydrochloride (AG337)

Materials and methods

Materials

AG337 was synthesized at Agouron as described previously [26].

Enzyme kinetics

TS activity was measured using purified recombinant human TS by a modified tritium release method [17]. Inhibition constants were determined by steady state analysis against the cofactor (6R,6S)5,10-methylene tetrahydrofolate which was generated *in situ* by reaction of tetrahydrofolate (Fluka, Ronkonkoma, N.Y.) with formaldehyde under conditions of essentially saturating [5-^{3}H] dUMP (Moravek Biochemicals, Brea, Calif.). Assays contained 50 mM TRIS-Cl pH 7.6, 10 mM dithiothreitol, 1 mM ethylenediamine-tetraacetic acid, 25 mM $MgCl_2$, 15 mM formaldehyde, 1% DMSO, 25 μM [5-^{3}H]dUMP (specific activity 2×10^8 cpm/μmol), tetrahydrofolate (10–80 μM), AG337 (0–100 nM), TS (4 nM) and 1–5 μg/ml bovine serum albumin to stabilize the enzyme. Reactions were carried out at 25°C for 6 min and quenched by the addition of charcoal then centrifuged to remove unreacted dUMP. Tritium release from the 5 position of dUMP was determined by liquid scintillation of the supernatant. The apparent K_i ($K_{i,app}$) was determined at each concentration of 5,10-methylene tetrahydrofolate by computer-assisted non-linear least squares fitting of the data to the tight binding inhibition equation of Morrison [19].

Cell culture

Cell lines were obtained from the American Type Culture Collection with the following exceptions: L1210/R6, Dr. F.M. Huennekens, Research Institute of Scripps Clinic, La Jolla, Calif.; GC_3/M(TK^-), Drs P.J. and J.A. Houghton, St. Jude Children's Research Hospital, Memphis, Tenn.; L5178Y, L5178Y/TK^-, Hazleton Laboratories, Madison, Wis.; P388/ADR, Southern Research Institute, Birmingham, Ala.; L1210/APZ, Dr. W. Klohs, Warner Lambert Parke-Davis Research Division, Ann Arbor, Mich. Cell lines were maintained without antibiotics in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, Utah) except for L1210, CCRF-CEM and L1210/R6 cells, which were passaged in 5% serum. The R6 line was further maintained in the presence of 2 μM methotrexate but was passaged in its absence a minimum of twice before use in assays. Growth inhibition studies were performed by continuous exposure to AG337 in 96-well flat bottomed microtiter plates using a modification of the colorimetric method of Mosmann [20]. Determination of viable cell number was based on the reduction of MTT (Sigma Chemical, St. Louis, Mo.) to formazan, which occurs as a result of dehydrogenase enzyme activity in the mitochondria of viable cells. The formazan product was quantitated after dissolution in DMSO by absorbance measurement at 540 nm. In each assay, the effects of eight or nine serial twofold dilutions of the test agent were tested in quadruplicate microcultures. IC_{50} values, representing the concentration of AG337 which reduced the optical density to 50% of that observed in control cultures, were derived from semi-logarithmic plots of cell growth (expressed as a percent of control) versus AG337 concentration. For protection studies designed to address the intracellular locus of activity of AG337, thymidine, hypoxanthine, and folinic acid (Sigma Chemical, St. Louis, Mo.) were present in assays at concentrations of 10, 35 and 0.5 μM, respectively.

Ribonucleotide and deoxyribonucleotide pool levels

L1210 cells (approximately 5×10^7 cells at a density of 10^6 cells/ml) were incubated for 2 h at 37°C either alone or with 10 μM AG337. Ribonucleotide profiles were determined by anion-exchange HPLC of neutralized perchloric acid extracts as described previously [18]. Briefly, cells were pelleted by centrifugation and resuspended in 0.2 ml 0.7 N perchloric acid. The acid precipitate was removed by centrifugation and the supernatant neutralized with solid $KHCO_3$. Deoxyribonucleotides were recovered from the neutralized extracts by a modification of the procedure of Garrett and Santi [9]. Aliquots (175 μl) of extracts were treated with 5 mM deoxyguanosine (10 μl) and 0.5 M $NaIO_4$ (40 μl), followed after several minutes by 4 M methylamine pH 7.5 (25 μl) and incubation at 37°C for 30 min. Excess $NaIO_4$ was reduced by a 5-min incubation at room temperature with 1 M rhamnose (20 μl). A mixture of standards containing ATP (2 mM) and 10 mM dATP, dCTP, TTP and dGTP were processed similarly to verify recovery.

Separations were performed on a Partisil-10 SAX column (Whatman International, Maidstone, UK). Ribonucleotide extracts (50 μl) were eluted with a linear gradient of 5 mM $NH_4H_2PO_4$ (pH 2.5) to 0.5 M $NH_4H_2PO_4$ (pH 4.8) over 40 min with detection at 254 nm. Retention times were verified in control runs with standards. Deoxynucleotides were separated on the same column with isocratic elution using a mobile phase of 5 mM $NH_4H_2PO_4$ pH 2.8: 0.5 M $NH_4H_2PO_4$ pH 4.8 (4:6) and detection at 284 and 270 nm.

Cell cycle kinetics

Cell cycle distributions were determined by flow cytometric analysis from the DNA histogram of nuclei stained with propidium iodide. Exponentially growing cultures of L1210 cells were treated for 20 h with concentrations of either 0.7 μM or 3.5 μM AG337 and the cell cycle kinetics compared to those of untreated cultures. For analysis, cells were fixed in 95% ethanol precooled to -20°C, washed in phosphate-buffered saline containing 5% heat-inactivated fetal calf serum and treated with 20 μg/ml propidium iodide containing 1 mg/ml RNase (Sigma Chemical, St. Louis, Mo.).

Whole cell TS assays

TS activity was measured by a tritium release assay [28] in which cells are incubated with [5-^{3}H]deoxyuridine which is converted to the TS substrate, dUMP, following cellular uptake. Enzyme activity is quantitated as tritium released from [5-^{3}H]dUMP during the TS-catalyzed reaction. Each reaction mixture contained 30 μM dUdR (Sigma Chemical, St. Louis, Mo.), 5 μCi/ml 5-^{3}HdUdR (Moravek Biochemicals, Brea, Calif.) and 8×10^5 cells. Reactions were carried out at 37°C and 5% CO_2 in the presence and absence of AG337 and were initiated by the addition of cells. They were stopped after varying incubation times by the addition of 0.4 ml ice-cold 1 M perchloric acid and processed as described previously [28].

Antitumor studies

The thymidine kinase deficient L5178Y/TK$^-$ tumor was maintained by passage in DBA/2 mice. For experiments, either 1×10^6 cells (i.p.) or 5×10^6 cells (i.m.) were injected into B6D2F$_1$ hybrid mice. For i.m. studies, the tumor was implanted in the gastrocnemius muscle. The day of tumor inoculation was designated day 0, and treatment was initiated on day 1, 3 or 5 for 5–10 days. AG337 was dissolved in 5% dextrose and administered by i.p. or oral dosing. Antitumor effects were evaluated in animals bearing i.p. tumors by increase in life span compared to controls which received vehicle alone and for which the mean survival time was 26.3 days after tumor inoculation. Animals surviving to day 100 were designated cured. For the i.m. implanted tumor, the diameter of the leg at the site of inoculation was measured daily and animals were sacrificed at a fixed end point diameter of 14 mm, which represents an approximately 2.5-fold increase in size. Animals were considered cured if the leg diameter at day 35 was less than or equal to the leg diameter on day 0. Thymidine kinase-replete L1210 and P388 tumors were implanted i.p. at 1×10^6 cells per mouse (day 0) and treatment was initiated on day 3.

Pharmacokinetics

Studies were conducted in male Sprague-Dawley rats with jugular vein cannulated and exteriorized (Hilltop Lab Animals, Scottdale, Pa.). After i.v. or oral administration of AG337, blood samples (200 μl) were withdrawn from the cannula and plasma separated by centrifugation. Plasma samples (100 μl) were extracted with 1 ml acetonitrile for 2 min, centrifuged at 2000 rpm for 5 min and the supernatant evaporated at 40°C under nitrogen. Dried samples were reconstituted with 300 μl mobile phase and AG337 levels quantitated by HPLC. Separation was effected using a C_{18} column (4.6 × 150 mm, 5 μm) and isocratic elution with 0.05 M sodium phosphate buffer, pH 4.0 and acetonitrile (80:20); detection was at a wavelength of 230 nm. Plasma AG337 concentration-time data were fitted to multi-exponential equations using nonlinear regression analysis. Pharmacokinetic parameters, as follows, were calculated from the parameter estimates of the y-axis intercepts and rate constants: absorption, distribution, and elimination half lifes ($T_{1/2-ka}$, $T_{1/2-\alpha}$, $T_{1/2-\beta}$, respectively), volumes of distribution (V_c, V_β), and systemic clearance (Cl_s). Absolute oral bioavailability was calculated as $[AUC_{oral} \div AUC_{iv}] \times 100$ using model-independent values for AUC.

Results

Enzyme inhibition

The inhibition of TS by AG337 was examined over a range of 5,10-methylene tetrahydrofolate concentrations from 10 to 80 μM. The $K_{i,app}$ was independent of cofactor concentration over this range and the kinetics of inhibition were therefore non-competitive. A value of 11 ± 2 nM (mean of 20 determinations) was determined for the non-competitive $K_i(K_{i,nc})$ using tight binding kinetic analyses. The existence of a mixed non-competitive inhibition pattern [where competitive K_i $(K_{i,c}) \approx K_{i,nc}$] was not investigated because it required the use of cofactor concentrations below the reliability limit of the assay. In addition, the tight binding nature of the interaction between AG337 and TS precluded the use of replotted primary inhibition data to determine $K_{i,c}$.

Inhibition of cell growth and locus of action

The activity of AG337 against ten murine and human cell lines is shown in Table 1. Activity was most potent against the L1210 line (mean $IC_{50} = 0.39$ μM). The LoVo human adenocarcinoma line was the least sensitive ($IC_{50} = 6.6$ μM), with the remaining cell lines

Table 1 Growth inhibitory activity of 3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)-quinazoline dihydrochloride (AG337) against a panel of murine and human tumor cell lines *in vitro*. Each IC_{50} was estimated during continuous exposure to AG337. Values given are the mean of results from a minimum of seven experimental determinations

Cell line	Origin	$IC_{50} \pm$ S.D. (μM)
L1210	Mouse, lymphocytic leukemia	0.39 ± 0.14
CCRF-CEM	Human, lymphoblastic leukemia	1.27 ± 0.33
CCRF-SB	Human, lymphoblastic leukemia	2.00 ± 0.37
P388	Mouse, leukemia	2.72 ± 1.46
L5178Y	Mouse, lymphoma	2.62 ± 0.85
L5178Y/TK$^-$	Mouse, thymidine kinase-deficient L5178Y lymphoma	0.74 ± 0.16
Lewis Lung	Mouse, carcinoma	0.60 ± 0.22
LoVo	Human, colon adenocarcinoma	6.60 ± 1.22
L1210/R6	Mouse, leukemia. Dihydrofolate reductase overproducing strain	0.97 ± 0.19
GC_3/M(TK$^-$)	Human, thymidine kinase-deficient colon adenocarcinoma	1.54 ± 0.33

exhibiting IC_{50} values of less than 3 μM. The ability of thymidine to prevent the growth inhibitory effects of AG337 was explored to determine whether TS was indeed the intracellular locus of activity of AG337. The addition of 10 μM thymidine to L1210 cultures treated with AG337 significantly ablated growth inhibitory effects. In a series of 18 experiments, the IC_{50} for AG337 measured in the presence of thymidine was between 29.5-fold and 103-fold (mean 68-fold) higher than that measured without the addition of exogenous nucleoside, suggesting that the activity of AG337 is primarily due to its potent inhibition of TS. TS-related effects were further investigated by a comparison of the sensitivity of a TK-deficient (L5178Y/TK$^-$) and a TK-replete (L5178Y) cell line to AG337. The TK-deficient cells showed a 3.5-fold enhancement in cytotoxicity compared to the TK$^+$ line (Table 1), consistent with an inability to utilize salvaged thymidine to circumvent TS inhibition.

The possibility that AG337 may exert a secondary effect on other folate-dependent enzyme systems was also investigated using L1210 cells treated with AG337 in the presence of the following reversal agents which would implicate such activity: folinic acid, hypoxanthine, and combinations of thymidine with either folinic acid or hypoxanthine. The results of these experiments are shown in Table 2. While the addition of exogenous 10 μM thymidine produced a greater than 50-fold reversal of growth inhibition, 0.5 μM folinic acid or 35 μM hypoxanthine produced no significant effects. Both agents produced a greater effect, however, when co-administered with thymidine and the IC_{50} for AG337 was increased 2- to 3-fold over that observed with thymidine alone.

Table 2 The effect of thymidine, folinic acid and hypoxanthine on the growth-inhibitory properties of AG337 in L1210 cells. L1210 cells were exposed to a range of AG337 concentrations for 72 h either alone or in the presence of 10 μM thymidine (*dThd*), 5 μM folinic acid (*CF*), 35 μM hypoxanthine (*HX*), or combinations of dThd with CF or HX at the same concentrations. Mean IC_{50} values calculated for each experimental condition are from a minimum of eight experiments

Condition	$IC_{50} \pm$ SD (μM)	Fold increase in IC_{50}
AG337	0.45 ± 0.28	—
AG337 + dThd	24.56 ± 6.31	54.58
AG337 + CF	0.86 ± 0.29	1.91
AG337 + HX	0.50 ± 0.26	1.11
AG337 + dThd + CF	$42 - > 50$	$93.3 - > 111$
AG337 + dThd + HX	$42 - > 50$	$93.3 - > 111$

The absence of any effect of AG337 on dihydrofolate reductase activity was confirmed by examining the growth inhibitory effects of AG337 toward a variant L1210 line (L1210/R6) which overexpresses the enzyme. While this cell line was more than 2000-fold resistant to methotrexate, an antifolate inhibitor of DHFR (IC_{50} L1210 = 0.0033 μM, IC_{50} L1210/R6 = 7.3 μM), it was less than 2.5-fold resistant to AG337 (Table 1, IC_{50} L1210 = 0.39 μM, IC_{50} L1210/R6 = 0.97 μM)).

In parallel studies (Table 3), the locus of action of AG337 was investigated more directly by measurement of ribonucleotide and deoxyribonucleotide pool modulation in L1210 cells treated with AG337. The results were also indicative of TS-directed effects. A significant reduction in cellular TTP pools was observed within 2 h of treatment with AG337, while cellular ribonucleotide synthesis was not significantly affected. Flow cytometric analyses were also undertaken with AG337 using L1210 cells (Table 4). Treatment for 20 h with either 0.7 μM or 3.5 μM AG337 increased the S phase fraction from 40% to approximately 70%. A similar result was obtained in a single experiment conducted with CCRF-CEM cells (data not shown) for which treatment with 3.5 μM AG337 increased the S phase fraction from 48% to 80%. These results are also consistent with intracellular TS inhibition.

In vitro inhibition of TS in whole cells

TS activity was measured directly in AG337-treated L1210 cells by estimating ^3H release in the presence of 5-^{3}HdUdR (Fig. 2). Continuous exposure to 3.5 μM AG337 during a 6-h incubation period resulted in essentially complete inhibition of tritium release. Experiments were also conducted over 21 h with the same result. However, the inhibition was not sustained after the drug was removed. Figure 2 shows the result of suspending L1210 cells in drug-free medium after a

Table 3 Effect of AG337 on ribonucleotide and deoxynucleoside triphosphate levels of L1210 cells

Treatment[a]	Number of experiments	Nanomoles/10^9 cells			
		UTP	CTP	ATP	GTP
Control	3	860 ± 10	311 ± 4	2403 ± 50	449 ± 21
AG337	4	813 ± 41	310 ± 20	2648 ± 128	464 ± 26
		TTP	dCTP	dATP	dGTP
Control	3	18.4 ± 2.5	42.5 ± 7.7	15.8 ± 2.3	4.70 ± 1.44
AG337	3	11.1 ± 3.3[b]	37.6 ± 2.8	13.4 ± 2.2	2.31 ± 1.04

[a] AG337 treatment was 10 μM for 2 h
[b] Significant difference from control cells at $P = 0.05$ (Student's *t*-test)

Table 4 Cell cycle kinetics of L1210 cells treated with AG337. Cells were treated with AG337 for 20 h prior to staining with propidium iodide and estimation of cell cycle distribution. Results represent the mean of three experiments

AG337 (μM)	Cell cycle distribution (mean% ± SD)		
	G_0/G_1	S	G_2/M
0	44.0 ± 5.3	40.0 ± 9.2	15.7 ± 6.4
0.7	21.3 ± 11.9	71.3 ± 15.9	7.7 ± 4.6
3.5	16.7 ± 9.6	69.3 ± 8.4	12.3 ± 9.2

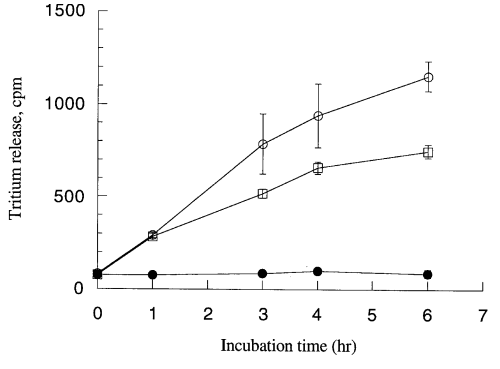

Fig. 2 Thymidylate synthase (TS) activity in L1210 cells *in vitro* following continuous or transient exposure to AG337. TS activity was measured in whole cells as ^3H released from [5-^{3}H]-2′-deoxyuridine. Cells (8×10^5) were incubated at 37°C for the indicated times with radiolabel alone (○), in the presence of 3.5 μM AG337 (●), or after a 17-h preincubation with 3.5 μM AG337 (□) followed by suspension in drug-free medium

17-h pretreatment with AG337. TS activity, as measured by ^3H release, was restored to ≥ 60% of control levels when incubations with 5-^{3}HdUdR were conducted after AG337 had been removed. This result indicates, as expected, that intracellular retention of AG337 is negligible, due to its rapid efflux from cells. A similar conclusion was drawn from *in vitro* studies which compared the IC_{50} for AG337 resulting from continuous (120-h) versus transient (6-h) exposure to this agent. The IC_{50} value for CCRF-SB cells was increased 5.7-fold by transient exposure (from 3.3 ± 1.7 μM to 18.7 ± 10.5 μM), and the IC_{50} for CCRF-CEM cells was 27-fold greater (0.75 ± 0.25 versus 24.0 ± 19.1 μM). Therefore, although the extent of intracellular retention of AG337 was variable, it was low, especially when compared to classical antifolates.

In vivo antitumor activity

Thymidine kinase-replete rodent tumor models are typically refractory to TS inhibitors due to high plasma thymidine levels (approximately tenfold greater than those in humans), which are high enough to allow the circumvention of TS inhibition by thymidine salvage. This has been well-documented for other TS antifolates [12, 13] for which antitumor activity was optimal in the tetraploid L1210:ICR cell line, which is particularly sensitive to TS inhibition due to its increased dependence on DNA biosynthesis. AG337 similarly showed low activity against TK-replete tumors. When tested against the P388 leukemia implanted i.p., 10 days of i.p. treatment with AG337 at a dose level of 100 mg/kg twice daily produced an average 33% increase in life span. Similarly, in tests with the diploid L1210 tumor, delivery of 200 mg/kg once daily i.p. gave a mean 27% increased life span in comparison to controls. The approach subsequently adopted to evaluate the *in vivo* activity of AG337 more fully was to employ a TK^- variant of the L5178Y murine lymphoma (L5178Y/TK^-), which is sensitive to TS inhibition because it lacks the kinase required to phosphorylate salvaged thymidine. The effects of AG337 treatment in mice bearing this tumor implanted either i.p. or i.m. are summarized in Table 5. AG337 elicited 100% cures following i.p. administration against tumors implanted at either site. As would be expected, the doses required were lower for the i.p./i.p. model (50 mg/kg once daily × 10, 100 mg/kg once daily × 5, or 25 mg/kg twice daily × 10) than for the i.p./i.m. model (100 mg/kg twice daily × 5 or × 10), although the effect of once-daily dose administration on the i.m. implanted tumor was not tested. These dose levels were non-toxic to the animals, as evidenced by transient weight loss of ≤ 6.4%. Higher dose levels produced more significant weight losses. For examp-le, 5 days of treatment with 150 or

Table 5 Antitumor activity of AG337 against the L5178Y/TK$^-$ lymphoma implanted i.p. or i.m. Tumor was inoculated on day 0 at 1×10^6 (i.p.) or 5×10^6 (i.m.) cells per mouse. Treatment was initiated on day 1 or day 3 for the indicated periods. (*ILS* increase in life span relative to tumor-bearing untreated control animals)

AG337 dose (mg/kg)	Tumor site	Route of dosing	Regimen	Incidence of cures[a]	Group mean ILS (%)	Group mean maximum weight loss (%)	Weight loss nadir (day)
25	i.p.	i.p.	Once daily, days 1–10	5/6	> 290	2.2	3
50				6/6	> 344	3.1	3
100				6/6	> 344	4.5	3
25	i.p.	i.p.	Once daily, days 1–5	0/6	76	None	3
50				4/6	> 178	6.5	3
100				6/6	> 261	4.5	3
25	i.p.	i.p.	Twice daily, days 1–10	6/6	> 258	None	—
50				5/6	> 217	2.9	2
100				6/6	> 258	6.4	3
50	i.p.	p.o.	Once daily, days 1–10	1/6	> 85.4	None	—
100				3/6	> 176.5	0.9	5
200				6/6	> 274.5	1.3	5
50	i.m.	i.p.	Twice daily, days 1–10	2/6	—[b]	2.9	3
100				6/6	—	5.2	3
100	i.m.	i.p.	Twice daily, days 3–7	6/6	—	5.2	5
150				6/6	—	8.9	7
200				5/6[c]	—	16.6	9
150	i.m.	p.o.	Twice daily, days 1–8	6/6	—	6.4	5
200				6/6	—	9.6	5
200	i.m.	p.o.	Twice daily, days 3–7	6/6	—	4.3	6

[a] For i.p. tumor, cure defined as survival to day 100. For i.m. model, cure defined as leg diameter on day 35 ≤ leg diameter on day 0
[b] Increase in life span was not monitored for i.m. implanted tumor
[c] One non tumor-related death occurred on day 16

200 mg/kg AG337 twice daily produced mean 8.9% and 16.6% weight loss, respectively, but once-daily treatment with 200 mg/kg per day for 10 days was not toxic (1.3% weight loss). AG337 was also orally active against tumors implanted either i.p. or i.m., demonstrating its ability to penetrate tissue barriers. Cure rates of 100% were observed against the i.p. implanted tumor after treatment with 200 mg/kg AG337 once daily for 10 days, and against the i.m. implanted tumor after AG337 administration at 200 mg/kg twice daily for 5 days (days 3–7) or 150 mg/kg twice daily on a 1 to 8-day regime. The effect of once-daily oral dosing against i.m. implanted tumor was not examined.

Pharmacokinetics

Pharmacokinetics of AG337 in the rat after i.v. administration (Fig. 3) were best described by a two-compartment model and were dose independent at levels from 25 to 100 mg/kg. An elimination half life of approximately 2 h was determined. The volume of distribution (1.36 l/kg) was greater than whole body water, as would be expected for a lipophilic agent, and systemic clearance was approximately 0.53 l/kg. Oral bioavailability of AG337 was also demonstrable in the rat (Fig. 4), and

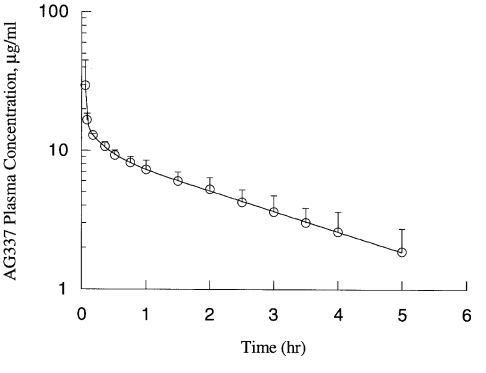

Fig. 3 Plasma levels of AG337 in the rat following i.v. dosing at 25 mg/kg. AG337 levels in plasma were estimated by reversed phase HPLC. Data shown are the mean results from four animals. Pharmacokinetic parameters derived after fitting data to a two-compartment model were as follows: $T_{1/2-\alpha} = 0.042$ h, $T_{1/2-\beta} = 1.98$ h, $AUC_{0-\infty} = 107.5$ µM*h, $Cl_s = 0.53$ l/kg, $V_c = 0.42$ l/kg, $V_\beta = 1.36$ l/kg. ($T_{1/2-\alpha}$ distribution half life, $T_{1/2\beta}$ elimination half life, $AUC_{0-\infty}$ area under the concentration-time profile, Cl_s systemic clearance, V_c, V_β volumes of distribution)

was greater in fasted animals (53% relative to i.v.) than in fed animals (22%). Oral absorption was slower in fed animals ($T_{1/2-ka} = 0.3$ h) than in fasted rats ($T_{1/2-ka} = 0.06$ h), the C_{max} was almost fivefold higher (30.2 µM versus 6.3 µM) and elimination ($T_{1/2-\beta}$) was

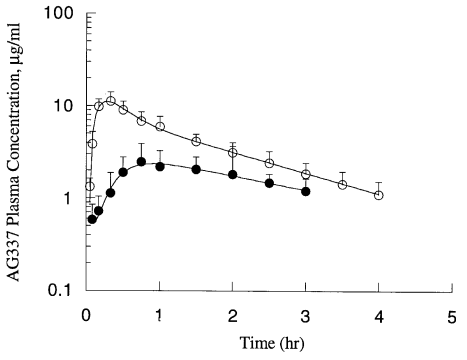

Fig. 4 Oral administration of 25 mg/kg AG337 in the rat. Plasma levels of AG337 were estimated by reversed phase HPLC after oral administration of 25 mg/kg AG337 to fasted (○) or unfasted (●) rats (four per group). Oral bioavailability relative to i.v. was 53% and 22% for fasted rats and unfasted rats, respectively

faster in the fasted state (0.9 h versus 1.6 h). Similar studies conducted in mice gave an elimination half life after oral administration of 2.92 ± 0.9 h and oral bioavailability relative to i.p. delivery was 62–90%.

Discussion

AG337 is representative of a new class of antifolate inhibitors of TS with pharmacological characteristics which differ significantly from other agents currently under preclinical or clinical evaluation. The absence of a glutamate moiety, together with the lipophilicity of AG337 (logP = 1.85), permits cellular uptake independent of the folate or reduced folate carrier systems, and also means that the drug is not metabolized to forms which show high intracellular retention. Given the proven validity of TS as a target for antitumor therapy [2, 4, 13], the clinical evaluation of AG337 together with that of glutamate-containing inhibitors promises to clarify the potential role of these various factors in relation to both *in vivo* efficacy and toxicities. The spectrum of available agents now spans those which are dependent on both specific transport and polyglutamylation for effect, exemplified by ZD1694 [13], those which use the reduced folate carrier for uptake but are not polyglutamylated, such as the dipeptide analogs of ICI 198583 diglutamate [15], and those which use neither system, which include AG337, and a second, structurally dissimilar inhibitor developed at Agouron, AG331 [25], which is also currently undergoing Phase I clinical evaluation. Agents such as the 3-methyl-substituted benzoquinazoline folate analog, 1843U89 [6], differ more subtly from agents such as ZD1694 in that the role of polyglutamylation apparently lies in assuring intracellular retention, since polyglutamylated forms do not show enhanced inhibition of TS. AG337 and AG331, despite their lipophilicity, are formulated as water soluble salts and therefore do not display the solubility limitations which have hampered the development of other lipophilic TS inhibitors [6].

AG337 is a potent inhibitor of its target, and a K_i of 11 nM was measured against purified human recombinant TS. This value approximates the potency reported for glutamate-containing antifolates against mouse TS, such as CB3717 [11], its desamino analog ICI-198583 [12], and ZD1694 [13], but is less than the activity of the benzoquinazoline 1843U89 (K_i 90 pM). However, under physiological conditions, these agents are metabolized to polyglutamate forms, which are, with the exception of the benzoquinazolines, up to 100-fold more potent. Thus AG337 is less active than the glutamate-containing inhibitors, at least when physiologically relevant derivatives are compared. Despite the fact that AG337 is a cofactor analog, the inhibition kinetics were non-competitive with respect to 5,10-methylene tetrahydrofolate over a broad cofactor concentration range, a characteristic which is shared by classical antifolate TS inhibitors, which typically exhibit mixed or non-competitive inhibition patterns [5, 12]. Patterns of differential binding of substrates and antifolate analogs to the two TS subunits have been shown to be a major factor in influencing inhibition kinetics [5]. Ligand-induced negative cooperativity of substrate binding results in differing affinities of the two TS active sites for either the folate cofactor or cofactor analogs. If the sites with strong and weak cofactor binding characteristics are termed the primary and secondary sites, respectively, then AG337 inhibition kinetics will depend on the relative affinity of the inhibitor for these two sites. The competitive inhibition pattern observed is consistent with AG337 affinity for the primary site being weaker than for the secondary site (i.e. $K_{i,c} \gg K_{i,nc}$). However, it remains possible that the inhibition kinetics observed were influenced by the range of folate cofactor concentrations (≥ 10 μM) over which determinations were made. At lower levels of 5,10-methylene tetrahydrofolate, mixed non-competitive kinetics where $K_{i,c} \approx 11$ nM might be observed, although, due to the technical limitations of the assay, these experiments could not be performed. However, non-competitive inhibition is probably the mode of inhibition observed under physiological conditions, since intracellular 5,10-methylene tetrahydrofolate concentrations are in the micromolar range. Levels of approximately 5 μM have been reported for resting cells [10] and concentrations would be expected to exceed this level under conditions of cellular proliferation.

The results of *in vitro* studies conducted so far with AG337 indicate that its primary intracellular locus of action is TS. Its ability to inhibit cell growth is ablated by thymidine alone, AG337-treated L1210 cells showed a G_1/S phase cell cycle block, and TS inhibition was demonstrated in whole cells using a tritium release assay. Unlike many other quinazoline antifolates, AG337 does not inhibit DHFR, although TS is believed to be the primary locus of action of these other

agents [12] despite their dual activity. While folinic acid competitively inhibits the growth inhibition of ZD1694 [13], this is believed to be due to antagonistic effects on its uptake and polyglutamylation. The observation that folinic acid alone did not affect AG337 growth inhibitory effects, even when concentrations of up to 10 μM were employed (data not shown), was therefore consistent with its independence of either system. Hypoxanthine alone was also without effect, however, both folinic acid and hypoxanthine potentiated the rescue effects of thymidine in AG337-treated L1210 cells. The mechanisms underlying these effects have not been investigated further, although it remains possible that a secondary locus of action for AG337 may exist when TS inhibition is circumvented, as in the presence of excess thymidine. The fact that thymidine levels are low *in vivo* suggests, however, that such conditions may have little physiological relevance. Additional data which supported TS as the locus of action of AG337 were also obtained. Cellular TTP pools in L1210 cells treated with AG337 were reduced to 40% of control values while ribonucleotide pool level changes were slight. The observed reduction in dGTP pool levels was not statistically significant due to the high degree of variability in both treated and control cells. However, a secondary effect on dGTP levels following TTP depletion has been linked to lowered ribonucleotide reductase activity and effects on GDP reductase [7, 24].

The observation that AG337 displays negligible intracellular retention was predicted based on its lipophilicity and its inability to undergo polyglutamylation, the latter being central to the cellular retention of classical antifolates. Continuous exposure to AG337 is therefore required to maintain TS inhibition due to rapid equilibration between the extracellular and intracellular compartments. Repeat dosing regimens were therefore critical to the demonstration of *in vivo* efficacy with AG337, especially in light of its relatively short elimination half life (approximately 2 h) as determined in rats and mice. Under these conditions, AG337 displayed a high incidence of 100% cures against the L5178Y/TK$^-$ tumor *in vivo*. The dose levels of AG337 required were higher than those required for efficacy with ZD1694 [13], which was tested against the tetraploid L1210:ICR tumor, but compared favorably with dose levels required for CB3717 and desamino-CB3717 [12]. The difference in *in vitro* potency between AG337 and 1843U89 (K_i 11 nM versus 90 pM) seems also not to be sustained *in vivo*. Activity of the latter agent in TK$^-$ human xenograft models required dose levels of 100 mg/kg twice daily for 5 or 10 days [6], regimes which also produced 100% cure rates in the L5178Y/TK$^-$ system for AG337 (Table 5). With the exception of ZD1694, therefore, the potent TS inhibitory activity coupled with favorable intracellular retention exhibited by the majority of classical TS antifolates studied to date, has not resulted in *in vivo* activity in animal models which is any greater than for AG337. In addition, it is possible that lower dose levels of AG337 may be effective if administered more frequently than twice a day or if delivered by continuous infusion, the dosing regime which would be predicted as optimal for an agent of this type. Delivery of AG337 by continuous i.v. infusion was attempted in mice during the course of these studies but could only be sustained for limited periods due to local venous damage. The fact that AG337 is orally bioavailable in mice, rats and dogs [27] and shows antitumor activity by this route of administration in mice is a positive feature for circumventing potential difficulties associated with maintaining prolonged exposure to this agent in a clinical setting.

Antitumor selectivity remains one of the key issues to be addressed by comparison of the activity of lipophilic agents such as AG337 with classical antifolates. High rates of membrane transport and polyglutamylation of classical antifolates by transformed cells are believed to contribute to antitumor selectivity [8, 23], although they are also potential sources of resistance. Prolonged intracellular retention may also lead to increased toxicity in normal tissues, therefore, the rapid cellular efflux and plasma clearance of agents such as AG337 may be advantageous in allowing more direct control of exposure by manipulation of delivery schedule. The rapid reversibility of AG337-induced effects may then reduce the potential for toxicity provided, of course, that antitumor selectivity is maintained.

The comparative evaluation of agents such as AG337 with classical TS antifolates thus has the potential to delineate some of the key factors associated with the antitumor activity of the various classes of TS inhibitors. AG337 itself is also a promising new antitumor agent since its potent TS inhibitory activity is coupled with *in vivo* activity and oral bioavailability. The pharmacological characteristics of AG337 suggest that continuous i.v. infusion (or repeated oral dosing) will be optimal for the demonstration of clinical activity, therefore 5-day and 10-day continuous i.v. infusion Phase I studies are currently in progress [21] with this agent.

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