Mechanisms of acquired resistance to the quinazoline thymidylate synthase inhibitor ZD1694 (Tomudex) in one mouse and three human cell lines

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Summary: Four cell lines, the mouse L1210 leukaemia, the human WI12 lymphoblastoid and two human ovarian (CH1 and 41M) cell lines, were made resistant to ZD1694 (Tomudex) by continual exposure to incremental doses of the drug. A 500-fold increase in thymidylate synthase (TS) activity is the primary mechanism of resistance to ZD1694 in the WI12:R¹⁰⁸⁴⁴⁴ cell line, which is consequently highly cross-resistant to other folate-based TS inhibitors, including BW1843U89, LY231514 and AG337, but sensitive to antifolates with other enzyme targets. The CH1:R¹⁰⁸⁴⁴⁴ cell line is 14-fold resistant to ZD1694, largely accounted for by the 4.2-fold increase in TS activity. Cross-resistance was observed to other TS inhibitors, including fluorodeoxyuridine (FdUrd). 41M:R¹⁰⁸⁴⁴⁴ cells, when exposed to 0.1 μM [¹⁴]HDJ1694, accumulated ~20-fold less [¹⁴]H-labelled material over 24 h than the parental line. Data are consistent with this being the result of impaired transport of the drug via the reduced folate/ methotrexate carrier. Resistance was therefore observed to methotrexate but not to CB3717, a compound known to use this transport mechanism poorly. The mouse L1210:R¹⁰⁸⁴⁴⁴ cell line does not accumulate ZD1694 or Methotrexate (MTX) polyglutamates. Folyopolyglutamate synthetase substrate activity (using ZD1694 as the substrate) was decreased to ~13% of that observed in the parental line. Cross-resistance was found to those compounds known to be active through polyglutamation.

Keywords: ZD1694; thymidylate synthase; drug resistance; polyglutamation; reduced folate carrier; folyopolyglutamate synthetase

N-(5-[(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-l-glutamic acid (ZD1694; Tomudex) is a potent thymidylate synthase (TS; EC 2.1.1.45) inhibitor (Jackman et al., 1991a,b; Marsham et al., 1991) which has successfully completed European phase I (Clarke et al., 1994) and phase II clinical studies (Burris et al., 1994; Gore et al., 1994; Smith et al., 1994; Zaleberg et al., 1994). ZD1694 was designed as a non-nephrotoxic, highly potent analogue of N⁰-propargyl-5,8-dieazafoolic acid (CB3717; Jones et al., 1981) which was active in phase I/II studies, particularly in breast tumours, hepatomas and platinum-refractory ovarian cancer (Calvert et al., 1986; Clarke et al., 1993). CB3717 was withdrawn from further study because of dose-limiting nephrotoxicity, a feature overcome, in mouse models, by the synthesis of more water-soluble analogues (Jones et al., 1989; Hughes et al., 1990; Jackman et al., 1990, 1991c; Marsham et al., 1991). Many of these new compounds showed improved substrate activity for the reduced folate/methotrexate cell membrane transport carrier protein (RFC) and folyopolyglutamate synthetase (FPGS), rendering them potent as cytoxic agents (Jackman et al., 1991d). Polyglutamates of ZD1694 rapidly accumulate within the cell and studies have confirmed these to be the cytotoxic species (Jackman et al., 1991a, 1993a; Ward et al., 1992; Gibson et al., 1993). For example, a major metabolite, the tetraglutamate, is a 60-fold better TS inhibitor (Kᵣ = 1 nM) than the parent drug and is not readily effluxed from cells. In combination, these properties explain why ZD1694, although a 20-fold poorer inhibitor of isolated TS, is 500-fold more cytotoxic than CB3717.

The pyrrolopyrimidine, LY231514, is another TS inhibitor currently in phase I clinical study and shares many of the properties of ZD1694 (Grindley et al., 1992), whereas BW1843U89, a benzoquinazoline, is unusual in that it has superior affinity for the human than for the murine RFC and cannot be metabolised by FPGS beyond the diglutamate (Duch et al., 1993). The interaction of such folate-based TS inhibitors with the RFC and FPGS provides an opportunity for the design of novel agents that are not substrates for one, or both, of these proteins. Such compounds may express a different anti-tumour or toxicity profile. Intrinsic or acquired resistance due to reduced levels or altered expression of the RFC or FPGS would be expected to be overcome by such compounds. AG337 is such an example. It is a lipophilic TS inhibitor (in phase I study) which is believed to diffuse into cells and is structurally precluded from polyglutamation (Webber et al., 1993). Our own studies have identified a series of highly lipophilic benzylamide analogues of 2-desamin-2-methyl-N⁰-propargyl-5,8-dieazafoolic acid (ICI 198583) with good in vitro activity (Barker et al., 1991; Skelton et al., 1994). However, we have realised greater in vivo activity in mice with water-soluble compounds that use the RFC for cell entry but which are not substrates for FPGS. Structural modifications of ICI 198583 which produce this profile include dehydration of the 7-position of the quinazoline ring (Boyle et al., 1993) and the replacement of glutamate with either natural or unnatural amino acids (Barker et al., 1991, and manuscript in preparation) or dipeptides (Bavetsias et al., 1993; Jackman et al., 1993b).

To aid the understanding of potential resistance mechanisms to ZD1694 that may occur in man, and the development of TS inhibitors that may overcome such resistance, requires the establishment of a panel of in vitro tumour cell lines with defined resistance mechanisms. Four cell lines, the L1210 mouse leukaemia, the WI12 human lymphoblastoid and two human ovarian (CH1 and 41M) cell lines, were exposed to increasing concentrations of ZD1694 until they were established in 1–5 μM of the drug. A range of techniques was used to define the resistance mechanism(s) in each cell line. These included measurement of TS enzyme activity and protein levels, TTP and dUMP concentrations, uptake and polyglutamation of the drug, and cross-resistance studies to antifolates with known mechanisms of action. Two of the
lines (CH1 and W1L2) expressed elevated TS activity, 4- and 500-fold respectively. Resistance in the 41M cells was accounted for by an impaired reduced folate carrier and in the L1210 cells by a reduced ability to accumulate ZD1694 polyglutamates.

Materials and methods

Development of resistance to ZD1694

L1210 (mouse leukaemia) and W1L2 (human lymphoblastoid) cells were grown by suspension culture in RPMI-1640 (20 mM HEPES) supplemented with 2 mM L-glutamine, 10% donor horse serum (L1210) or fetal calf serum (W1L2) (ICN Flow, Thame, Oxfordshire, or Imperial Laboratories, Andover, UK) 20 µg ml⁻¹ gentamycin and 0.5 µg ml⁻¹ amphotericin as previously described (Jackman et al., 1990). The human ovarian CH1 and 41M (Hills et al., 1989) cell lines were grown as monolayers in Dulbecco’s modified Eagle medium containing 2 mM glutamine, 10% fetal calf serum (Imperial Laboratories), 50 µg ml⁻¹ gentamycin, 2.5 µg ml⁻¹ amphotericin B, 10 µg ml⁻¹ insulin, and 0.5 µg ml⁻¹ hydrocortisone in 10% carbon dioxide/90% air. Cells were exposed initially to an IC₅₀ concentration of ZD1694 until they had adapted and grew normally (~2 weeks). This was followed in doubling doses until the cells were growing normally in 1 µM (CH1), 2 µM (41M) or 5 µM (L1210 and W1L2) ZD1694. The 41M:R/D₉₀⁰⁰ ovarian cell line was successfully cloned to produce a monoclonal cell line by dilution of a cell suspension to ~1 cell ml⁻¹ and plating 200 µl per well of a 96-well microtitre plate. The CH1:R/D₉₀⁰⁰ cell line was not cloned. Resistant ovarian cell lines were banked in liquid nitrogen without drug. Once removed from liquid nitrogen they were subcultured without drug for more than 4 weeks. The L1210:R/D₉₀⁰⁰ and W1L2:R/D₉₀⁰⁰ cell lines were cloned by dilution of the cell number to 100 and 1 cells ml⁻¹ respectively and grown in 2 ml of sloopy agar (0.2%) (L1210) or 200 µl of medium in microtitre plates (W1L2). Once these monoclonal cell lines were established they were subcultured (and banked) in medium containing 5 µM ZD1694 and then grown without drug for 2 weeks before experiments. Resistance to ZD1694 was not stable for the W1L2 cells and was lost between 3 and 5 months in drug-free medium. The L1210:R/D₉₀⁰⁰ cells retained their resistance to ZD1694 for at least 1 year. Doubling times for the cells lines were L1210 (~12 h), L1210:R/D₉₀⁰⁰ (~14 h), W1L2 (~18 h), W1L2:R/D₉₀⁰⁰ (~20 h), CH1 (~18 h), CH1:R/D₉₀⁰⁰ (~18 h), 41M (~28 h) and 41M:R/D₉₀⁰⁰ (~28 h).

TS activity, TS enzyme-linked immunosorbent assay and FPGS

TS activity was measured by H² release as described previously (Jackman et al., 1986). For the measurement of CH1 ovarian tumour TS kinetic parameters, enzyme was partially purified and assayed as previously described (Jackman et al., 1990). The use of mixed diastereoisomers of 5,10-methylene tetrahydrofolate [5,10-CH2F₂H₄(6R,S)] rather than the pure 6R-enantiomer was not affected the analysis, although the Ke₅₀ values were twice the 6R value (Ward et al., 1992). The values of the kinetic parameters, Kₑ and Ke₅₀, which are the dissociation constants for I from 1E (TS.dUMP.I) and IES (TS.dUMP.5,10CH₂F₂H₄) complexes, respectively, were determined for ZD1694 and its triglutamate by multivariate non-linear regression in which measured velocities were fitted to different rate equations (Ward et al., 1992). TS protein was estimated by enzyme-linked immunosorbent assay (ELISA) using a TS antibody (Aberne et al., 1992). Pure human TS standard was kindly provided by R Ferone (Wellcome Laboratories, Research Triangle Park, NC, USA). Dihydrofolate reductase (DHFR) activity was assayed by a spectrophotometric assay (Jackman et al., 1986). FPGS assays were performed on crude cytosolic extracts as described by Jansen et al. (1990).

ZD1694 and MTX transport studies

[5־H]ZD1694 (sp. act. 10 Ci mmol⁻¹) was synthesised by Zeneca Cambridge Research Biochemicals (Billingham, UK) and purified for use as described previously (Gibson et al., 1993). 3[H,5,7,1-H(N)]MTX sodium salt was purchased from Moravek Radiochemicals (Brea, CA, USA) at a sp. act. of 15–30 Ci mmol⁻¹. L1210 cells at ~3–5 x 10⁶ ml⁻¹ were resuspended in unsupplemented RPMI or transport buffer, at a concentration of 2–4 x 10⁶ ml⁻¹. Transport buffer consisted of 107 mM sodium chloride, 20 mM HEPES, 26.2 mM sodium bicarbonate, 5.3 mM potassium chloride, 1.9 mM calcium chloride, 1 mM magnesium chloride, and 7 mM D-glucose, adjusted to pH 7.4 with sodium hydroxide (Jansen et al., 1990). After incubation with 0.1 µM [5-¹H]ZD1694 (sp. act. = 10 Ci mol⁻¹) or 0.5 µM MTX (sp. act. = 2.5 Ci mmol⁻¹), at 37°C for various times, 1 ml of the cell suspension was immediately added to 10 ml of ice-cold phosphate-buffered saline (PBS) or transport buffer and spun at 500 g for 5 min. The cells were resuspended in PBS or transport buffer, spun and then washed again. The final cell pellet was digested with 0.5 ml of 1 M sodium hydroxide, neutralised with hydrochloric acid and added to 10 ml of Ultima Gold Scintillant (Canberra Packard, Pangbourne, UK) and the radioactivity estimated by liquid scintillation counting. Kinetic parameters for MTX (Kₑ and Vₑ₅₀) were obtained by varying the MTX concentration (0.5–30 µM) and obtaining the incubation values. Values were obtained by fitting the data to the Michaelis–Menten equation [v = (Vₑ₅₀ x S)/(S + Kₑ)] by non-linear least-squares analysis (Jenrich et al., 1968). Inhibition of ³¹P MTX transport by ZD1694 was performed at 5 min (0.5 µM MTX) using a range of ZD1694 concentrations (0.5–8 µM). Data were fitted to the competitive inhibition equation [v = (Vₑ₅₀ x S)/(S + Kₑ(1 + [I]/Kₑ))] by non-linear least-squares analysis and values for Kₑ obtained. Since 41M cells are adherent to plastic, a different assay procedure was employed. Cells were grown in T25 Nunc tissue culture flasks (Gibco BRL, Paisley, UK) for several days until they reached a concentration of ~3 x 10⁶ cells (not confluent). The medium was decanted, and 2 ml of unsupplemented Dulbecco’s modified Eagle medium containing 0.1 µM [³¹P]ZD1694 was added followed by incubation at 37°C by floating the flasks on a water bath for the times indicated. The cells were washed twice with 50 ml of ice-cold PBS, digested with sodium hydroxide and counted as described above.

Measurement of ZD1694, ICI 195835 and MTX uptake and polyglutamation

The method for analysing ZD1694 polyglutamates using an ion-pair high-performance liquid chromatography (HPLC) system has been described in detail elsewhere (Gibson et al., 1993) and was applied to all the cell lines. Cells, at ~2–3 x 10⁶ ml⁻¹, were grown for the times indicated in supplemented medium as described above for the cell culture. MTX polyglutamation in L1210 cells was estimated using a similar method and the polyglutamate standards were purchased from Schircks Labs (Jona, Switzerland). Cells were incubated with 0.5 µM [³¹P]MTX (sp. act. = 19 Ci mmol⁻¹) for 24 h. Similarly, 1 µM (benzoyl-³¹P)ICHI 195835 (sp. act. = 18 Ci mmol⁻¹) was added to L1210 cells for 24 h and ICI 195835 and its polyglutamates measured by reversed-phase chromatography as previously described (Jackman et al., 1991c).

dUMP and TTP pool measurements

L1210 or W1L2 cells (10 ml in duplicate) at a cell density of ~2–3 x 10⁶ ml⁻¹ were treated for 4 h with ZD1694 at the concentrations indicated. The cells were harvested by centrifugation and immediately extracted with cold perchloric acid (PCA) (0.5 M). Similarly, flasks containing ~3 x 10⁶ non-confluent 41M or CH1 ovarian cells were treated with ZD1694, washed once with PBS and PCA added directly to the flask. The cells were detached from the flasks by scraping
and the extract transferred to conical test tubes. Following neutralisation with potassium hydroxide (Curtin et al., 1991), the ribonucleotides were destroyed using sodium periodate. TTP pools were measured directly on aliquots of the extract using a radioimmunoassay (RIA) (Aherne et al., 1993) sensitive to ~1 pmol 10^{-4} cells. An RIA for dUTP (Paiui et al., 1989) was adapted to estimate ‘immuno-reactive’ deoxyuridine nucleotides (mainly dUMP) in the same cell extracts by utilising the cross-reactivity of the antiserum to dUDP and dUMP (Raynaud et al., 1993). The sensitivity of this assay was also ~1 pmol 10^{-4} cells. All RIAs were performed in duplicate at three dilutions of the extracts.

Cross-resistance studies

The quinazoline TS inhibitors were synthesised at Zeneca Pharmaceuticals or the Institute of Cancer Research (Jones et al., 1981; Hughes et al., 1990; Marsham et al., 1991, and in preparation; Bavetsias et al., 1993; Boyle et al., in preparation). LY231514 and AG337 were synthesised at Zeneca Pharmaceuticals. Lometrexol (DDATFH) was a gift from G Grindey, Lilly Laboratories, Indianapolis, IN, USA and BW1843U89 was a gift from R Ferone. Trimetrexate (TMQ) was a gift from The National Cancer Institute (Bethesda, MD, USA). Aminopterin, FdUrd and deoxythymidine (dThd) were purchased from Sigma (London, UK) and MTX was obtained from Nils Klass (Klausen, Denmark). Folinic acid was supplied by David Bull Laboratories (Warwick, UK).

Cells were grown in the culture conditions described above and IC_{50} values determined as previously described (Jackman et al., 1990). Each value was determined for at least five dilutions (in duplicate) of each compound. Results are given as the means ± s.d. of several experiments (three or more) or as the individual results of one or two experiments.

Results

Development of resistance

Although the four resistant cell lines were developed by establishment in either 1 μM (CH1 and 41M) or 5 μM (L1210 and W1L2) ZD1694, the actual concentration to which they were resistant varied considerably. The IC_{50} values for the former lines were 0.36 and 1.7 μM respectively, while those of the L1210 and W1L2 were >100 μM.

Thymidylate synthase activity and protein level

Enzyme activity was similar for all four parental cell lines and was significantly elevated in two of the resistant lines (Table I). The 514-fold increase in TS activity in the W1L2:R^{1094} cells over the parental cells is, however, significantly less than the >22 000-fold increase in IC_{50} for ZD1694 observed, and similarly the 4.2-fold greater activity in the CH1:R^{1094} cells is less than the 14-fold resistance to the drug. An ELISA for TS indicated that an increase in the TS protein was probably responsible for this raised TS activity, although activity was at least twice the protein level in each case. No change in dihydrofolate reductase activity was found in any of the resistant cell lines (data not shown).

Kinetic parameters for TS isolated from sensitive and resistant CH1 ovarian tumour cells

The inhibition of human ovarian CH1 and CH1:R^{1094} TS by ZD1694 and its trituglamate (the major metabolite in this cell line) is mixed non-competitive with respect to (6R,S) 5,10-

CH_{2}FH_{4}. This is concluded from the fact that both K_{i} and K_{m} could be determined (Table II). The relatively high values of K_{m} indicate that inhibition tendency towards competitive, as previously found using L1210 TS (Jackman et al., 1991a). The kinetic parameters were not significantly different for TS isolated from the two cell lines. The K_{i} values for ZD1694 were 91 and 85 nM for CH1 and CH1:R^{1094} respectively and similar to that reported for L1210 TS (60 nM) (Jackman et al., 1991a; Ward et al., 1992). The trituglamate of ZD1694 had a K_{i} (1.6 nM) 57-fold lower than that of ZD1694 itself. This enhancement in the K_{i} was also demonstrated for the tetratuglamate of ZD1694 using L1210 TS (Jackman et al., 1991a; Ward et al., 1992).

dUMP and TTP pool measurements

None of the four resistant cell lines had endogenous TTP levels significantly different from their parental forms (Figure 1). When L1210:R^{1094} or W1L2:R^{1094} cells were either continuously subcultured (data not shown) or challenged for 4 h (after 2 weeks in drug-free medium) with 5 μM ZD1694, TTP remained at the control level. The effect of ZD1694 on TTP and deoxyuridine nucleotide pools ('dUMP') is also shown in Figure 1. Perturbations in TTP and 'dUMP' pools occurred in all four parental cell lines after exposure to ZD1694 at appropriate doses. A 4 h exposure to 50 μM ZD1694 reduced TTP and increased 'dUMP' pools in L1210:R^{1094} cells by only 15% and 12-fold respectively, indicating some inhibition of TS. This compares with a 95% reduction in TTP and a 26-fold increase in 'dUMP' with just 0.1 μM ZD1694 in the parental line. Similarly, 'dUMP' and TTP levels were unaffected in the W1L2:R^{1094} cells at 50 μM drug, although in the parental line 0.4 μM reduced TTP pools by 74% and increased 'dUMP' 24-fold. These observations are consistent with the >100 μM IC_{50} for the drug in these two resistant lines. In 41M:R^{1094} cells, both TTP and 'dUMP' pools were marginally affected by 0.1 and 1 μM drug exposure (IC_{50} =

| Table I Thymidylate synthase activity and TS protein level in ZD1694 sensitive and resistant cell lines |
|---|---|---|---|---|---|
| Cell line | IC_{50} (nM) | IC_{50} (μM) | IC_{50} (μM) | IC_{50} (μM) | IC_{50} (μM) |
| CH1 (human ovarian) | 1.5 ± 0.66 | 6.3 ± 1.3 | 4.2* | 0.24 | 0.51 | 2 |
| 41M (human ovarian) | 2.0 ± 0.64 | 2.5 ± 0.49 | 1.3** | 0.15 | 0.092 | 0.6 |
| W1L2 (human lymphoblastoid) | 1.6 ± 0.33 | 820 ± 200 | 514* | 0.052, 0.012 | 5.7 | 178 |
| L1210 (murine leukaemia) | 1.4 ± 0.21 | 2.3 ± 0.91 | 1.6** | 0.022, 0.023 | 0.014 | 0.5 |
| TS activity was measured by 'H release from [5-3H]dUMP (mean ± s.d. of at least three experiments: *P<0.05; **not significant) and TS protein levels by ELISA. |

| Table II Kinetic parameters for TS isolated from the sensitive and resistant human ovarian tumour CH1 cells |
|---|---|---|---|
| CH1 TS | CH1:R^{1094} TS |
| K_{m} (6R,S) 5,10-CH_{2}FH_{4} (μM) | 24 ± 0.8 | 22 ± 0.8 |
| K_{m} ZD1694 (nm) | 91 ± 4.2 | 85 ± 6.1 |
| K_{m} ZD1694 (nm) | 542 ± 23 | 440 ± 59 |
| K_{m} ZD1694 trigg (nm) | 1.6 ± 0.10 | 1.6 ± 0.12 |
| K_{m} ZD1694 trigg (nm) | 10 ± 0.70 | 9.1 ± 0.62 |
1.7 μM) compared with 60% and 80% reduction in TTP and 31- and 38-fold increase in dUMP in the parental line at these doses. Pool size perturbations in the CH1 and CH1: R<sup>101694</sup> lines were not so markedly different. ZD1694 at 0.1 μM for 4 h caused TTP pools to fall by 81% and 58% in the sensitive and resistant line respectively and by 88% in both lines following 1 μM ZD1694. IC<sub>50</sub>values for growth inhibition of the resistant CH1 line was 0.35 μM ZD1694. ‘dUMP’ pools mirrored the changes in TTP pools.

**Folyopolyglutamate synthetase activity**

FPGS activity was determined in the L1210 cell lines using D1694 as the substrate (3 μM). The activity in L1210 cells was 2.0 ± 0.84 pmol h<sup>-1</sup> 10<sup>-6</sup>, whereas for the L1210: R<sup>101694</sup> cells it was only 0.25 ± 0.20 pmol h<sup>-1</sup> 10<sup>-6</sup> cells. A similar difference in activity was observed using MTX as the substrate (data not shown).

**Cellular transport/uptake studies**

The 41M and L1210 sensitive and resistant lines were incubated with 0.1 μM ZD1694 and the cellular levels measured up to 20 or 30 min (Figure 2). Uptake of the drug was significantly lower in both resistant lines compared with the parental cells (~15–25%). Closer examination of the L1210 parental cells revealed that within 15 min approximately half of the intracellular drug was present as polyglutamates (data not shown). [3H]MTX transport was therefore measured in these cells, the advantage being that polyglutamate formation is relatively slow and no polyglutamates could be detected by 15 min (data not shown). There was no significant change in the affinity (K<sub>i</sub>) of MTX for the RFC, but at least a 50% reduction in the V<sub>max</sub> was observed (Table III). The K<sub>i</sub> for ZD1694 as an inhibitor of MTX transport was only marginally higher for the resistant L1210 cells. This small alteration in the RFC is not sufficient to explain the >11 000-fold resistance to ZD1694. Furthermore, very little cross-resistance was seen to MTX, suggesting that impaired transport is not a major determinant of resistance (see Table VIII). However, the rate of transport of [3H]MTX into the 41M: R<sup>101694</sup> cells was significantly reduced. At an extracellular concentration of 0.5 μM ~0.6 pmol of MTX had accumulated within 30 min in the 41M cells. This was only 0.2 pmol in the 41M: R<sup>101694</sup> cells (data not shown). In addition, there was a concomitant 36-fold cross-resistance to MTX (123-fold resistant to ZD1694) (see Table VII).

Further characterisation of the transport properties of these 41M cells was impaired by methodological problems with this adherent cell line. Reproducible data could not be obtained at short incubation times necessary to perform such experiments. Changing the method to using single-cell suspensions was not possible with this cell line as ‘clumping’ occurred during the incubation.

**Polyglutamation of ZD1694, MTX and ICI 198583**

The total intracellular level of the drug in the 41M cells 24 h after exposure to 0.1 μM [3H]ZD1694 was 8.8 μM, i.e. 23-fold higher than in the resistant 41M cells (Figure 3a). The levels of the parent monoglucamate and its polyglutamate forms were all reduced in the resistant cell line. However, the predominant metabolite was the triglutamate in both cell lines. These data are consistent with a reduced rate of membrane transport of ZD1694 in the resistant cells. The intracellular pool of ZD1694 (particularly the triglutamate) could be increased by raising the extracellular concentration to 1 μM. There was relatively more parent drug in the 41M: R<sup>101694</sup> cells, which may indicate some reduction in the rate of polyglutamate formation. The uptake of 0.1 μM [3H]ZD1694
over 24 h into L1210 cells resulted in a 38-fold higher intracellular concentration of the drug, 98% of which was present as polyglutamate forms (Figure 3b). However, in L1210: R<sup>D<sub>1694</sub></sup> cells, the level was approximately equivalent to the extracellular concentration and no polyglutamates were found. Raising the extracellular level to 1 μM resulted in small, but detectable levels of polyglutamates. Resistance to ZD1694 is clearly associated with a severely reduced ability to form or maintain polyglutamates. To determine whether this was a general effect on antifolates, [3H]MTX (0.5 μM) polyglutamation in the sensitive and resistant L1210 cell lines was compared. Relative to ZD1694, MTX polyglutamation was low in L1210 cells, with only ~20% of total (5.3 μM) intracellular drug being found as polyglutamates at 24 h (di-pentaglutam). However, only parent drug (4.2 μM) was seen in the L1210: R<sup>D<sub>1694</sub></sup> cells at the same dose (data not shown). [3H]Cl (198583) (1 μM) was well metabolised to polyglutamates in L1210 cells (total intracellular drug at 24 h = ~3 μM; 75% as di-pentaglutamates, principally tetraglutamate) (Jackman et al., 1991c). Again the L1210: R<sup>D<sub>1694</sub></sup> cells were unable to form polyglutamates, with 98% of the cellular [3H]-labelled material (total = 2.6 μM) being identified as parent drug (data not shown).

After 24 h exposure to 0.1 μM [3H]ZD1694, significant intracellular accumulation was found in W1L2: R<sup>D<sub>1694</sub></sup> cells (~3 x W1L2 parental line). Most was parent drug (28%) or diglutamate (62%) (Figure 3c). In parental W1L2 cells only 1% and 2% of the drug was in these forms, the vast majority being tetra- and pentaglutamates.

The TS-overproducing cell line, CH1: R<sup>D<sub>1694</sub></sup>, did not accumulate a significantly higher level of ZD1694 when compared with parental cells. However, there was, at 4 and 24 h, a shift to a greater proportion in the mono- to triglutamate form in the resistant cells. The significance of this result is difficult to interpret (Figure 3d).

**Cross-resistance studies**

The structures of the quinazoline TS inhibitors, together with their TS inhibitory activity and FPGS substrate activity, are given in Table IV. Results indicating their use of the RFC is indicated in Figures V–VIII, and details can be found in the following publications: Jackman et al. (1991a,c,d, 1993a,b), Marshall et al. (1991), Boyle et al. (1993). Details of the other compounds are described by Duch et al. (1993), Pavlovic (1993) and Webber et al. (1993). W1L2: R<sup>D<sub>1694</sub></sup> cells were highly cross-resistant to all quinazoline, pyrrolopyrimidine (LY231514) and benzoxazinoline (BW1843U89) TS inhibitors, consistent with their high expression of TS (Table V). Lower cross-resistance was seen to the lipophilic analogue, 10, which possibly relates to a second locus of action becoming important to growth inhibition at the higher compound concentration. No cross-resistance was
Table IV Structures of quinazoline TS inhibitors

| R₁ | R₂ | Aryl | R₄ | Inhibition of L1210 TS (IC₅₀, μM) | Mouse liver FPGS (K₅₀, μM) |
|----|----|------|----|-------------------------------|---------------------------|
| CH₃ | H | CH₂ | Glu | 0.67 (K₅₀ = 60 μM) | 1.3 |
| CH₃ | CH₃ | CH₃ | Glu | 0.11 | 143 |
| CH₃ | H | CH₂CH₃ | Glu | 0.42 | 0.91 |
| CH₃ | H | CH₂CH₃ | Glu | 0.05 (K₅₀ = 10 μM) | 43 |
| CH₃ | CH₃ | CH₂CH₃ | Glu | 0.02 (K₅₀ = 3 μM) | 40 |
| CH₃ | H | CH₂CH₃ | Glu | 0.028 | Non-sub |
| CH₃ | CH₃ | CH₂CH₃ | Glu | 4.5 | 6.4 |
| CH₃ | H | CH₂CH₃ | Glu | 0.06 | Non-sub |
| CH₃ | H | CH₂CH₃ | Val | 0.0092 (K₅₀ = 0.2 μM) | Non-sub |
| CH₃ | H | CH₂CH₃ | meta-CN benzamide | 0.026 | Non-sub |

*Jackman et al. (1991a); City et al. (1994).

Table V Activity of antifolates against W1L2 and WIL2:R₅₃ D₁₆₉₄ human lymphoblastoid cells

| Locus | RFC | FPGS | Inhibition of W1L2 cell growth (IC₅₀, μM) | Resistance factor |
|-------|-----|------|-------------------------------------------|------------------|
| (IC1 D1694) | TS | + | 0.0064 ± 0.0031 | >100 (n = 2) | >22,000* |
| 2 | TS | + | 0.70, 0.75 | >100 | >140 |
| 3 | TS | + | 0.0029, 0.011 | >100 | 14,000 |
| 4 (IC1 198583) | TS | + | 0.058 ± 0.0074 | >100 (n = 2) | >1700* |
| 5 (CB3717) | TS | + | 2.6 ± 0.29 | >100 (n = 2) | >38 |
| 6 | TS | + | 0.15 ± 0.047 | >100 (n = 2) | >350* |
| 7 | TS | + | 0.031 ± 0.023 | >100 | >3000 |
| 8 | TS | + | 0.42, 0.35 | >100 | >260 |
| 9 | TS | + | 0.17, 0.093 | 63 | 630 |
| 10 | TS | + | 0.086, 0.084 | 11, 18 | 190 |
| 1 | TS | + | 0.029, 0.056 | >100 (n = 2) | >2400 |
| BW1843U89 | TS | + | 0.0023, 0.0022 | 3.4, 4.2 | 1700 |
| AG337 | TS | + | 0.78 ± 0.04 | >10 | >58 |
| FdUrd | TS | + | 0.0054 ± 0.0013 | 0.0078 ± 0.0069 | 1.4** |
| FdUrd + 5 μM LV | TS | + | 0.0015 | 0.95 | 630 |
| Trimethetrexate | DHFR | - | 0.01 ± 0.018 | 0.023 ± 0.021 | 1.3** |
| Methotrexate | DHFR | + | 0.0094 ± 0.0018 | 0.0026, 0.0021 | 0.26* |
| Lometrexol | GARTF | + | 0.033 ± 0.011 | 0.074, 0.045 | 1.8 |

*Determined by inhibition of [H]MTX transport and/or activity against resistant cell lines with an impaired RFC (Jackman et al., 1991a,c,d, 1993a,b; Marsham et al., 1991; Boyle et al., 1993; Duch et al., 1993; Pavlovic et al., 1993; Webber et al., 1993). The significance of the difference between IC₅₀ values for the S and R cell lines is:

*P<0.05; **Not significant.

Table VI Activity of antifolates against human ovarian CH1 and CH1:R₅₃ D₁₆₉₄ cells

| Compound | Locus | RFC | FPGS | Inhibition of CH1 cell growth (IC₅₀, μM) | Resistance factor |
|----------|------|-----|------|-------------------------------------------|------------------|
| (IC1 D1694) | TS | + | + | 0.025 ± 0.011 | 0.36 ± 0.12 | 14* |
| 4 (IC1 198583) | TS | + | + | 0.34 ± 0.17 | 4.7, 7.0 | 17* |
| 5 (CB3717) | TS | - | + | 7.0 ± 1.8 | 55, 91 | 10* |
| 6 | TS | + | - | 7.9 ± 4.7 | 167 ± 97 | 21* |
| 7 | TS | + | - | 61 | ~250 | ~7 |
| 9 | TS | + | - | 1.3 ± 0.26 | 9.5 ± 3.9 | 7.3* |
| LW231514 | TS | + | + | 3.0 ± 3.0 | 67 ± 2.9 | 22* |
| BW1843U89 | TS | + | + | 0.0012 ± 0.0006 | 0.0084 ± 0.0025 | 7* |
| AG337 | TS | - | - | 9.3 ± 1.2 | 22 ± 6.2 | 2.4* |
| FdUrd | TS | - | - | 0.14 ± 0.025 | 0.41 ± 0.23 | 9.3* |
| Trimethetrexate | DHFR | - | - | 0.0066, 0.0065 | 0.0073, 0.094 | 1.5 |
| Methotrexate | DHFR | + | + | 0.017, 0.019 | 0.018, 0.0043 | 1.7 |
| Lometrexol | GARTF | + | - | 0.042 ± 0.055 | 0.48 ± 0.2 | 1.1** |

*P<0.05; **Not significant.
observed to the pyrimidine-based TS inhibitor 5-fluorodeoxyuridine (FdUrd) unless folinic acid (leucovorin, LV) was given. The addition of DTd (as well as LV) rendered FdUrd poorly active in either cell line (IC₅₀ ~65 μM). Inhibitors of DHFR (MTX and trimetrexate) and glycaminide ribotide transformylase (GARTF) (Lomotrexol) retained activity in the ZD1694-resistant W1L2 cells.

Lower levels of resistance to ZD1694 and other TS inhibitors, including FdUrd, were seen in the CH1:R₁₀⁷⁸⁸⁴ cells (2- to 22-fold) (Table VI). The 2.4 cross-resistance to AG337 may relate to its poor activity against the parental line (9.3 μM) with the possibility of another locus becoming significant at the higher concentration used against the resistant cells. No significant cross-resistance was seen to inhibitors of other folate-dependent enzymes, e.g. MTX, trimetrexate or Lomotrexol.

The pattern of cross-resistance to the antifolates was very different in the two other cell lines, and the results are crucial in explaining the mechanisms of resistance involved. This is because the data separating transport from an FPGS defect were not definitive (see above). The 41M:R₁₀⁷⁸⁸⁴ cells were only cross-resistant to compounds (whatever the target enzyme) known to use the RFC, although the degree of cross-resistance varied widely (6- to 264-fold) (Table VII).

The L1210:R₁₀⁷⁸⁸⁴ cell line produced cross-resistance results that can all be explained as a consequence of defective polyglutamation (Table VIII). High cross-resistance was observed to the thiazole derivative of ZD1694 (3) and to 7, both very good FPGS substrates (Kₐ = 1.0 and 64 μM respectively) (Jackman et al., 1991d). The degree of cross-resistance to other analogues was lower, and was again consistent with their reduced potential to form intracellular polyglutamates. Thus, IC₅₀ 198583 with a relatively high Kₐ for FPGS (40 μM) is less extensively polyglutamated in L1210 cells than ZD1694 (Jackman et al., 1991c). No significant cross-resistance was seen to all antifolates known to be non-substrates for FPGS and that do not use the RFC, i.e. 10, AG337 and trimetrexate. Similarly no cross-resistance was observed to FdUrd. Non-polyglutamatable compounds that use the RFC (e.g. 6, 8 and 9 and other unpublished examples) have cross-resistance values (2-3) significantly greater than 1, consistent with the small transport defect in the resistant line described above. A very low level of cross-resistance was observed to CB3717, MTX and aminopterin (2- to 3-fold). However a higher level of resistance (15-fold) was demonstrated to MTX if the drug exposure period was limited to 6 h (data not shown). Indirect evidence supports polyglutamation of natural folates occurring normally in the L1210:R₁₀⁷⁸⁸⁴ cells. The addition of 5-methylfolinic acid to cell cultures containing FdUrd resulted in a significant enhancement in growth inhibition (~2.5-fold) in both lines (data not shown). This is a well-recognised synergistic combination since stable ternary complex formation is promoted by elevating the 5,10-CH₂FH₄ pool with leucovorin. Similarly, when leucovorin (5 μM) was used to protect against the activity of the DHFR inhibitor, trimetrexate, both sensitive and resistant L1210 cells were protected to the same degree (~20-fold increase in IC₅₀). Since leucovorin is metabolised to natural folate polyglutamates, this provides circumstantial evidence for natural folate polyglutamation in L1210:R₁₀⁷⁸⁸⁴ cells.
Acquired resistance to ZD1694
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Discussion
Continuous exposure of four cell lines to incremental increases in the concentration of ZD1694 led to each line acquiring at least one of three mechanisms of resistance: TS overproduction, alteration in the RFC or defective polyglutamation. There was always one primary mechanism of resistance, although a second mechanism (either independent or a consequence of the first) was also observed in each cell line.

Although the cell lines were adapted to grow in 1–5 μM ZD1694, two of the resistant cell lines (WL12 and L1210) were highly resistant, even when challenged with 100 μM ZD1694. Consistent with TS inhibition, all parental cell lines had depletion of TTP and elevation of dUMP pools following incubation for 4 h with growth-inhibitory concentrations of ZD1694. These pools were either not, or minimally, disturbed when L1210 or WL12 resistant cells were exposed to 50 μM ZD1694, demonstrating that TS operates relatively normally under these conditions. This would be expected from the high level of resistance described above. The two resistant ovarian lines, particularly the CH1:R^{D884} line, showed disturbances in pool size at much lower concentrations consistent with their lower levels of resistance.

Two of the human cell lines, WL12 and CH1, had significantly elevated TS activity (500- and 4-fold respectively). TS protein levels were similarly increased when determined by ELISA (Table 1). Immunoblotting demonstrated a similar increase, and this was accompanied by a 100- and 2- to 3-fold increase in TS gene copy number and mRNA respectively (Freamantle et al., 1993). The human ovarian cell line, CH1, has a low level (14-fold) of ZD1694 resistance, and here the resistance is generally consistent with the small elevation in TS activity (levels). No change in the TS inhibition kinetic parameters (ZD1694 or its triglutamate) was observed. As expected, cross-resistance was seen to all TS inhibitors, including FdUrd, but not to inhibitors of DHFR (MTX) or GARTF (Lometrexol). Mechanism of cellular uptake was not a factor that correlated with cross-resistance, and indeed intracellular ZD1694 levels were not significantly different in the sensitive and resistant cells. However at 24 h, a consistent shift in the distribution of the polyglutamate forms to shorter chain length was apparent in the resistant line, which may contribute to the resistance seen.

The human WL12 resistant cell line was highly resistant to ZD1694 (IC₅₀ > 100 μM) even though it had been adapted to grow in just 5 μM drug. Contrary to what we expected, the resistant cells accumulated significantly higher levels of intracellular drug than the sensitive cells. However, the majority was present as either the parent drug or diglutamate, and this is most readily explained by the 500-fold increased level of TS protein. The high level of TS may reduce ‘free’ drug levels by binding the mono- to triglutamate, preventing the addition of further glutamate residues by FPGS. The relatively weaker binding to TS of the mono- and diglutamate compared with the higher polyglutamates must allow polyglutamation to proceed as far as the triglutamate, but the slower dissociation of this form from TS may prevent further polyglutamate chain elongation by reducing substrate availability. This phenomenon has also been observed in another TS-overproducing line (WL12:C1) with resistance to ICI 198583. Here there was a 30-fold higher intracellular concentration of drug with 97% in the monoglutamate form compared with 9% in the parental cells (O’Connor et al., 1992). The lower level of cross-resistance to quinazoline TS inhibitors that are not substrates for FPGS, e.g. 9 and 10, is therefore expected.

Sensitivity to the DHFR inhibitors (MTX and trimetrexate) and the GARTF inhibitor (Lometrexol) is completely consistent with the abundance of L1210 cells with WIL2:R^{D884} cells. Sensitivity to FdUrd is explained by sequestration of the 5,10-CH₂FH₄ pool into the ternary complex with TS (in large excess) and FdUMP, resulting in inhibition of TS and other folate-dependent enzymes through substrate depletion. This is why supplying the cell with folinic acid, which reinstates de novo purine and TMP synthesis by replenishment of the reduced folate pool, allows resistance to FdUrd to be revealed. We have previously recorded this phenomenon in other cell lines with amplified TS genes (Jackman et al., 1986; O’Connor et al., 1992).

Gene amplification leading to increased TS expression as a mechanism of resistance was not observed in the WIL2 lines, perhaps because the cell lines have been maintained in a medium that is folate-deficient. The introduction of new antifolates, targeted towards TS, into the clinic may establish whether this will be a common resistance mechanism in man.

The second human ovarian 41M line (~100-fold resistant to ZD1694) appears to have a primary defect, which is reduced drug uptake via the RFC. A consequence of lower intracellular level of the parent drug is a substantially reduced concentration of the more active intracellular polyglutamates. Thus, the cross-resistance seen to antifolates known to use the RFC, including those with targets other than TS, was observed. CB717 uses this carrier poorly (as determined in L1210 cells) (Jackman et al., 1993) while the lipophilic compounds AG337 (Webber et al., 1993) and trimetrexate (Fry and Jackson, 1986) do not use it at all, and of course FdUrd uses a nucleoside transport mechanism. All of these compounds retained activity in the resistant cells. The very high cross-resistance seen to BW1843U89 may relate to the excellent affinity this compound has for the human RFC (Duch et al., 1993).

Transport defects have repeatedly been reported as a mechanism of resistance to MTX (Fry and Jackson, 1986). Defective transport is also commonly associated with other resistance mechanisms, including a reduced ability to form or maintain polyglutamates of MTX or Lometrexol (Cowan and Jolivet, 1984; Assaraf et al., 1992; Koizumi and Allegra, 1992; Rhee et al., 1993).

The importance of polyglutamation in the action of antifolates such as MTX and Lometrexol has led to the recent description of several cell lines with intrinsic or acquired resistance due to a reduced accumulation of polyglutamate species (Pizzorno et al., 1989; McIntosh et al., 1993; Van der Laan et al., 1991; Li et al., 1993; Pavlovic et al., 1993). Considering the importance of polyglutamate formation for the cytotoxicity of ZD1694 (Jackman et al., 1991a, 1993a), it is not surprising that one of our cell lines, L1210:R^{D884}, proved to be defective in this manner. The greatly reduced ability to form (or maintain) intracellular ZD1694 polyglutamates in this resistant line also extends to ICI 198583 and MTX as determined by radiolabelled drug studies. This reduction in polyglutamation potential is most likely accounted for by the ~90% reduction in FPGS activity (using MTX or ZD1694 as substrates). Deletion of FPGS has been shown to be a lethal mutation (McBurney and Whitmore, 1974), but reports of lower expression of FPGS or altered activity for antifolates (MTX and Lometrexol) has been described (Pizzorno et al., 1988; McCluskey et al., 1991; Pavlovic et al., 1993). It has also been shown that decreased FPGS activity does not necessarily reduce the ability of the cell to polyglutamate sufficient natural folates for normal cellular function (Pizzorno et al., 1988; Pavlovic et al., 1993). In other cell lines with decreased ability to polyglutamate MTX the actual mechanisms have not been fully elucidated (Pizzorno et al., 1988, 1989; Assaraf et al., 1992). Increased γ-glutamylhydrolase activity as a mechanism of antifolate resistance is increasingly being reported (Li et al., 1993; Rhee et al., 1993). However, in studies not reported here, this activity was rarely detectable in both sensitive and resistant lines with alteration in FPGS level being the limiting factor. The very low activity of γ-glutamylhydrolase in L1210 cells grown in vitro, as opposed to in mice, has been described by Samuels et al. (1986). Thus, this enzyme may play a greater role in maintaining polyglutamate homeostasis in mice and may be important in differential tissue sensitivity.
to ZD1694, and its elevation could be a potential resistance mechanism.

The results of the cross-resistance studies support a polyglutamation defect, as only TS inhibitors, active through polyglutamation, had significantly reduced activity against the L1210 resistant cells. Some apparent anomalies do exist but can be explained including the low level of cross-resistance to CB3717 (3-fold) or to the pteridine-based DHFR inhibitors, MTX and aminopterin (2- to 3-fold). CB3717 does form polyglutamates in L1210 cells, but relatively slowly. For example, after incubation with equitoxic concentrations of ZD1694 (0.1 μM) and CB3717 (50 μM) for 4 or 6 h respectively, 98% of D1694 and only 15% of CB3717 was measured as polyglutamates (Sikora et al., 1988; Jackman et al., 1991a). CB3717’s lower dependence on polyglutamation for activity, in addition to the slightly lower cross-resistance expected from a compound that does not use the RFC (small transport defect, see above), probably explains the low cross-resistance observed. A lower requirement for polyglutamation may partially explain the good activity of MTX and aminopterin in the resistant cells (MTX is poorly polyglutamated at low intracellular concentrations). Furthermore, the polyglutamates of DHFR inhibitors, unlike the quinazoline TS inhibitors, are not significant substrates as inhibitors of their target enzyme (Szeto et al., 1979; Chabner et al., 1985) than the parent monoglutamate, and the importance of their formation lies in their drug retentive properties (Galivan, 1980; Jolivet and Chabner, 1983). Thus, under continuous drug exposure conditions, the lack of polyglutamate formation is not particularly deleterious, however under short exposure conditions (6 h), when drug retention is important, MTX was ~15-fold less active in the resistant than in the L1210 parental line. Similarly, the TS inhibitor BW1843U89 has similar activity against TS in either the parent monoglutamate or diglutamate form (Duch et al., 1993) and may therefore account for the low level of cross-resistance to this agent.

The acquisition of these four ZD1694-resistant cell lines has confirmed the importance of TS, the RFC and polyglutamation for the activity of the drug. Clearly, it is likely that certain tumours may intrinsically possess or acquire these phenotypes. However, as more antifolates are developed for clinical study with differing target enzymes, transport and metabolic properties, it will be possible to overcome these resistance mechanisms. This panel of resistant cell lines has aided the development of quinazoline TS inhibitors that are not metabolised by FPGS but still require cellular uptake via the RFC and are currently being used to identify compounds whose activity is independent of either process.

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