Folate-based thymidylate synthase inhibitors as anticancer drugs

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Summary

The enzyme, thymidylate synthase (TS) is considered an important target for the development of new anticancer agents. Moreover, the folate-binding site in TS is believed to offer better opportunities for the design of highly specific inhibitors than the pyrimidine (dUMP) binding site. This belief led to the design of N⁹-propargyl-5,8-dideazafolic acid (CB3717), a quinazoline-based drug which had antimembrane activity in clinical studies. Occasional, but serious nephrotoxicity led to the withdrawal of CB3717 from further clinical study. More water-soluble and non-nephrotoxic analogues were developed with an interesting diversity in biochemical profile, particularly with respect to interactions with the reduced-folate cell membrane carrier (RFC) and polyglutamate synthetase (FPGS). An example of a compound that uses both of these processes well is the quinazoline, ZD1694 (Tomudex), a drug which is about to complete phase III evaluation for colorectal cancer. High chain length polyglutamates are formed that are up to 70-fold more potent TS inhibitors than the parent drug (Ki tetr glutamate = 1 nM). Furthermore they are retained in cells/tissues for a prolonged period. A number of other novel folate-based TS inhibitors are currently in pre-clinical or clinical study. For example, LY231514 is a pyrrolopyrimidine analogue in phase I study and, although less potent as a TS inhibitor, has biochemical properties similar to ZD1694. Another compound in phase I study is the benzoquinazoline, BW1843U89 which has somewhat different properties. It is a very potent TS inhibitor (Ki = 0.09 nM) and an excellent substrate for the RFC (human) and FPGS, but polyglutamation proceeds to diglutamate only and is not accompanied by increased TS inhibition. Another highly water-soluble compound in pre-clinical development is ZD9331 which was specifically designed to use the RFC but not be a substrate for FPGS. Potent TS inhibition (Ki = 0.4 nM) was achieved through a rational programme of computerised molecular modelling of the active site of TS and a large database of structure-activity relationships. Two lipophilic compounds were designed to be devoid of interactions with either the RFC or FPGS. High resolution crystal complexes of E. coli TS were central to obtaining potent TS inhibitors and both AG337 (Ki human recombinant TS = 16 nM) and AG331 (Ki = 12 nM) are in clinical studies. This portfolio of novel compounds therefore comprehensively addresses the potential of TS as a target for cancer chemotherapy.

Key words: thymidylate synthase, antifolates

Introduction

Thymidylate synthase (TS) catalyses the methylation of dUMP to give TMP, which after metabolism to TTP is exclusively incorporated into DNA (Fig. 1). This fact alone makes TS an attractive target for the development of an anticancer agent. The co-substrate for the reaction is the reduced-folate cofactor, 5,10-methylene tetrahydrofolate (CH₂FH₂) which becomes oxidised to dihydrofolate (FH₂) during this reductive methylation reaction. Methotrexate (MTX; Fig. 2a) and 5-fluorouracil (FU; Fig. 2b) were amongst the earliest anticancer drugs developed and both partly act through inhibition of TS (Fig. 1). MTX primarily inhibits dihydrofolate reductase (DHFR), an enzyme which functions to regenerate FH₂ (produced in the TS reaction) to the fully reduced tetrahydrofolate (FH₄) form (reviewed in [1]). This in turn accepts a 1 carbon unit from a donor such as serine allowing it to function once more in 1 carbon transfer in various folate-dependent reactions such as THF and the enzymes involved in de novo purine synthesis (Fig. 1). FU is metabolised intracellularly to produce a number of anabolites. One of these, 5-fluorodeoxyuridine monophosphate (FdUMP) binds tightly to the dUMP binding site of TS [2]. However other anabolites of FU are believed, at least in some tumours and in some administration protocols, to have other effects, particularly after incorporation of the base into RNA [3, 4].

MTX and FU have each found roles in the treatment of certain tumour types, alone or in combination with other drugs. For example, MTX is still an important single agent treatment for low risk choroidal carcinoma [5], and, when combined with other drugs, for the treatment of childhood acute lymphoblastic leukemia (maintenance therapy) and non-Hodgkins lymphoma [6]. MTX and/or 5-FU, when used in combination with other agents, have a place in the treatment of the common solid tumours e.g. CMF (cyclophosphamide/MTX/5-FU) therapy for breast cancer [7]. Bolus 5-FU
as a single agent had, for many years, been the standard drug for the treatment of colorectal cancer, albeit with very limited response rates (~10%) and no marked improvement on the survival of treated patients (reviewed in [8,9]). More recently the use of an adjuvant FU + Levamisole regimen for Dukes C colorectal cancer has been associated with a marked improvement in survival [10], suggesting a potentially curative role for drugs of this type (data from studies randomised against FU alone are not available). It is thus clear that the place of these drugs in clinical protocols has established the principle that some solid tumours may be antifolate-sensitive.

The biochemical pharmacology of both MTX (reviewed in [1]) and FU (reviewed in [9]) has been very widely investigated and understood, and it may be argued that the vast amount of knowledge accrued is out of proportion to the clinical usefulness of either drug. However such fundamental research has led to the design of alternative administration protocols, drug combinations, modulatory and rescue agents that improved response rates and overcame resistance in particular tumour types [8,9]. Examples include the use of high-dose MTX followed by Leucovorin (LV) rescue for the treatment of osteogenic sarcomas [11] and the sequential dosing of MTX followed by FU for colorectal cancer (reviewed in [8,9]). The most notable example in recent years relating to the use of FU was the discovery that the folate cofactor for the TS reaction can be low and limiting for the formation of the stable ternary complex in which FdUMP binds (reviewed in [9]). The provision of LV to elevate the cofactor pool forms the basis for the now commonly used FU/LV clinical protocols for the treatment of advanced colorectal cancer [8,9] as well as for adjuvant therapy. However, it is probably fair to conclude that increased response rates, in the order of 25%-30%, are seldom accompanied by a significant improvement in patient survival [8,9].

Other antifolates have also found roles in cancer therapy (particularly the leukemias) but are not generally considered particularly active in solid tumours. It may be pertinent to question whether the concept of using antifolate therapy to treat the common (and usually slowly proliferating) cancers is flawed, whether we are approaching the wrong targets or whether the targets are right but we are just using inadequate drugs. Thymidylate synthase is probably one of the best examples of a target where, in a few years, these answers should be available. This is for a number of reasons. First, there has been the rational design of specific, folate-based inhibitors of TS in the last 15-20 years. Commitment to the rapid development of TS inhibitors for clinical study initially lay with the Institute of Cancer Research and their collaborators, ICI Pharmaceuticals (Zeneca Pharmaceuticals) which led to the discovery and clinical development of CB3717 (Fig. 2c) in 1979 and 1981, respectively [12-17]. Since then at least three other drug companies (usually with external academic collaborators) have been actively involved in this area. It was soon realised that the scope for novel chemical modifications was large, and that factors other than potent TS inhibition were generally important for the pharmacological activity of novel compounds. For one of the companies, Agouron Pharmaceuticals, the approach to the design of novel chemical entities was somewhat different. Their starting point was the X-ray crystal structure of bacterial TS co-crystallised with FdUMP and CB3717 [18]. These different approaches have led to an interesting diversity in chemical structures and biochemical profiles. A second reason why elucidation of the usefulness of TS as a target may be forthcoming, is the wealth of knowledge available on the mechanisms involved in novel compound activity and potential mechanisms by which cells may be, or become, resistant. Much of this has been the direct result of the large amount of fundamental research into antifolates and fluorinated pyrimidines that has been performed by academic organisations over several decades. Undoubtedly this has aided ‘fast-track’, pre-clinical development of new folate-based TS inhibitors and specific examples will be highlighted below.

CB3717: The first clinically evaluated folate-based TS inhibitor

CB 3717, the first folate-based TS inhibitor to be developed clinically, was developed following the discovery that certain 2-amino-4-hydroxy quinazoline analogues of folic acid both inhibited TS and had antitumour
activity [16]. The observations both of clinical activity and toxicity of CB3717 (Fig. 2c) are salient to the development of subsequent analogues and are outlined in the context of knowledge of TS inhibitors. The potency of CB3717 as a TS inhibitor (Ki ~3 nM) was central to its further development as it represented a major advance in the search for a specific, potent, folate-based inhibitor of this enzyme [12, 19, 20]. CB3717 was shown to have slightly better folylpolyglutamate synthetase (FPGS) substrate activity than MTX and polyglutamate metabolites were formed intracellularly [20, 21]. These polyglutamates, particularly those of higher chain length, were shown to be exquisitely potent TS inhibitors with estimated Ki values of ~30 pM [22, 23]. Furthermore, these polyglutamates were retained inside cells [21]. Later, the rate of this polyglutamation was demonstrated to be relatively slow compared with certain other quinazoline analogues, a property assigned to the poor affinity of CB3717 for the reduced-folate cell membrane carrier (RFC) and its moderate affinity for FPGS [24, 25].

Although many of the finer points of CB3717 properties were unknown at the time of its early development, it represented a major advance in this field. The problems of testing inhibitors of TS in rodent antitumour and toxicity models were beginning to be realised (see below). Nevertheless CB3717 had antitumour activity attributable to TS inhibition and it was therefore advanced to phase I clinical study. At this time the development of CB3717 became a joint venture with ICI Pharmaceuticals and a phase I study commenced in 1981 at the Royal Marsden Hospital/Institute of Cancer Research. Antitumour activity was demonstrated in this and further phase I/II studies, particularly in breast, liver and platinum refractory ovarian cancer [13, 17, 24, 26, 27]. No activity was demonstrated in colorectal cancer [28]. Transient rises in liver transaminases were noted but, more seriously, sporadic life-threatening toxicity consisting of nephrotoxicity coupled with myelosuppression was seen (unpublished data). A decision was therefore made that, although the concept of TS as an antitumour target was a good one, CB3717 itself would not be developed further. However, an ongoing biological and chemical synthetic programme at the Institute of Cancer Research had identified the poor aqueous solubility of CB3717 at acid pH as the cause of the nephrotoxicity, and had designed and synthesised a more water-soluble analogue (desamino-CB3717) devoid of this toxicity in mice [20, 29]. The discovery of desamino-CB3717 was the impetus for a new and very vigorous collaboration between the Institute of Cancer Research and ICI Pharmaceuticals which led to the synthesis and evaluation of >3,000 quinazoline analogues, most of which had TS as the intracellular locus of action. The compounds fall into four broad classes, each with distinct intracellular biochemical and pharmacological properties. These properties include different interactions with the RFC and FPGS. ZD1694 (Tomudex; in clinical development), ZD9331 (in preclinical development) and further lead compounds with different features emerged from this portfolio of compounds.
Other pharmaceutical companies (and their academic collaborators) which have actively sought folate-based TS inhibitors have now reached the stage of preclinical or clinical evaluation. These will be discussed in further detail below.

Second generation analogue design

The first compound to emerge from new analogue synthesis, which was widely investigated, was the 2-desamino-2-methyl-analogue of CB3717 (ICI 198583; MPPDF) [30]. ICI 198583 had good water-solubility, no detectable nephrotoxicity in mice and improved in vivo antitumour potency (L1210:ICR) over CB3717 and desamino-CB3717 [31]. This compound represents a class of highly active compounds displaying high affinities for the RFC and FPGS as well as for the target enzyme, TS. Results indicated that although there was no direct correlation between TS inhibition and cytotoxic potency there was a good correlation between the extent of polyglutamation and cytotoxicity [24,25]. Furthermore, the rapid polyglutamation of some analogues and the increased potency of the polyglutamate metabolites towards TS result in their cellular pharmacokinetics/dynamics being different from those of the DHFR inhibitor, MTX. Another feature of this class of compound is its activity by bolus administration in mice, not necessarily predicted from their rapid plasma clearance, but consistent with a high degree of polyglutamate formation [20,31]. Although ease of administration and cytotoxic potency were considerations that favoured the development of this class of TS inhibitor, a positive decision was made to develop a drug that took advantage of the fact that FPGS was reported to be expressed relatively highly in some tumours and believed to explain some of the selective activity of MTX in mice ([32,33] and reviewed in [1]). A more recent report by Rumberger et al. endorses this further [34].

A general theme in the synthesis and development of TS inhibitors, whatever their structural class (several of which are described later) is the elegant manner with which they can be evaluated in vitro because of the array of biological models and assays developed [25,35]. However, it is their in vivo evaluation that provides the most challenge to their development as drugs. One major problem is the high plasma thymidine (dThd) level in rodents relative to man (at least 10-fold higher) which, through the activity of the dThd salvage pathway, is able largely to circumvent any TS inhibition [36]. This accounts for the generally low activity of TS inhibitors in many mouse tumours and human tumour xenografts. Similarly their toxicity to normal proliferating tissues is low, particularly in non-chronic administration schedules, and therefore not necessarily predictive of that in man. Test systems were introduced that at least, in part, address their in vivo activity. Inhibition of TS by polyglutatable species in tumour cells that have been removed at time intervals after drug injection can serve as a pharmacodynamic measurement [20,31,37]. Thymidine kinase deficient tumours such as the L5178Y TK−/− mouse lymphoma generally respond to single bolus therapy (polyglutatable compounds) but normal proliferating tissues remain unaffected [38,39]. TK competent tumours, such as the L5178Y TK+/− [38,39], the L1210:ICR leukemia [20,31,37] or some human tumour xenografts [39,40], may be sensitive to TS inhibitors if administered for prolonged periods. This is probably, in part, the result of a fall in plasma dThd that occurs after administration of a TS inhibitor ([36] and unpublished data). Prolonged administration also gives drug-induced normal tissue toxicity. Taking these problems into account, it emerged that one compound had a better therapeutic index than the others in a short-list of active compounds (manuscript in preparation). This compound was ZD1694 (ICI D1694; Tomudex; Fig. 2d) and was selected for pre-clinical and clinical development. The model systems used in the development of the other TS inhibitors in clinical study will be dealt with under the appropriate section.

ZD1694 (Tomudex), a highly polyglutatable TS inhibitor

ZD1694 (Fig. 2d) has a Ki for isolated mouse and human TS of ~60 nM [37,41]. This 20-fold loss in inhibition compared with CB3717 is compensated for by a ~500-fold increase in cytotoxic potency [37,42]. This latter activity was prevented by co-incubation with dThd alone indicating that TS is the target enzyme for ZD1694 [37,42]. ZD1694 is internalised into cells via the RFC (Ki for the inhibition of MTX influx ~2 μM) and has a very low Km for mouse liver and human FPGS (1.3 μM) [25,37,43]. The corresponding values for CB3717 are ~40 μM for both proteins and for MTX are ~4 and ~166 μM for the RFC and FPGS, respectively [21,25]. ZD1694 is almost completely metabolised to polyglutamate forms (tetra and pentaglutamates usually predominate) in a variety of tumour cells in culture [37,44] and in certain normal tissues in mice such as liver, kidney and gut epithelium [42]. The higher polyglutamate forms (triglu and above) have Ki values for TS of ~1 nM and are not readily effluxed from the cell [37,41]. It is concluded that ZD1694 is active as the polyglutamate metabolites and indeed structural modifications that enhance TS inhibition but largely prevent polyglutamation (for example C7 methylation), give compounds that are~100-fold less potent as antitumour agents [24]. Furthermore a mechanism of acquired resistance to ZD1694 is defective polyglutamation (at least partly due to decreased FPGS activity) which illustrates the importance of this metabolising enzyme for drug activity [45]. The consequence of the formation of slowly effluxable polyglutamates is that short incubation periods with ZD1694...
(e.g., 4 h) will give a high level of cytotoxicity which is not seen with analogues that cannot be polyglutamated [25]. Indeed TTP pools in tumour cells deplete rapidly during incubation with the drug and recover very slowly after resuspension of cells into drug-free medium [46].

After a single i.p. bolus injection of ZD1694 to mice, rapid clearance of the drug from the plasma occurs (t1/2 of ~30 min) [47]. A third, much slower phase of elimination is then measured, giving a persistently low, but possibly significant, drug level. Some of the antitumour and toxicological properties of ZD1694 are to be found in the literature and will be the subject of further communications so that only a very brief summary is given here [39, 42, 47–49]. The problems of testing TS inhibitors in rodents (high dThd) means that very little useful data can be gained as to their therapeutical index and the dose and schedule applicable to humans. Nevertheless pharmacodynamic measurements, such as inhibition of TS in tumour cells in vivo, and the use of a TK deficient tumour (L5178Y TK–) demonstrated that a single bolus injection of ~10 mg/kg was very active, consistent with the formation of highly retained polyglutamates [37, 39, 50]. Repeat daily dosing in TK competent tumour-bearing mice gave some antitumour activity (including human tumour xenografts) and normal tissue toxicity (mainly gastrointestinal) [37, 39, 40, 48]. Co-administration of dThd prevented the antitumour activity (L1210:ICR) and toxicity of ZD1694 which confirmed that, in vivo, TS was the antitumour locus of action and toxicity was mechanism related [37]. Consistent with this was the fact that the non-mechanism related nephrotoxicity associated with the original compound, CB3717, was not observed [39, 48]. Antitumour activity in mice was also prevented by co-administration of LV which was explained by a series of in vitro experiments demonstrating competition for cellular uptake and polyglutamation [24, 37].

The clinical evaluation of ZD1694 began in Europe in February 1991, recruitment being principally at the Royal Marsden Hospital/Institute of Cancer Research, one of the institutes of the co-discovers of the drug [17, 49, 51]. The starting dose was 0.1 mg/m² (15 min i.v. infusion) every three weeks, which was predicted to be a safe starting dose from studies in dogs (data of Zeneca Pharmaceuticals). Dose escalation culminated up to 3.5 mg/m² and included 61 patients with a range of solid tumours [51]. Dose-limiting toxicities were malaise, gastrointestinal and haematological (leucopenia or thrombocytopenia). Other toxicities which were encountered included reversible rises in liver transaminases, skin rash and anorexia. Three objective partial responses were seen (2.6 and 3 mg/m²) in previously treated patients with ovarian and breast cancer and adenocarcinoma of unknown origin [51]. Clearance from the plasma was triphasic and the half lives for the beta and gamma phases were ~2 and 75 h, respectively [51]. No relationship was observed between various pharmacokinetic parameters and response or toxicity. A dose of 3 mg/m² was recommended as the phase II dose. Another phase I was performed by the NCI in the U.S.A. (using the same protocol but in a mainly pre-treated colorectal patient population (76%)), the results of which led to their recommendation of a phase II dose of 4 mg/m² [52]. However the first phase II studies were performed at 3 mg/m² and the general experience seen in the phase I was reproduced in these multicentred trials. Objective responses (CR and PR) were seen at interim analysis in breast (25%), platinum resistant ovarian (8.5%), non-small cell lung (10%), and pancreatic carcinomas (14%) [53]. The most exciting response rate was seen in colorectal cancer where, in a study of 176 patients with advanced disease, 26% (95% CI. 19%–33%) had objective responses which included 4 complete and 41 partial responses [54]. A further 30% of patients had minor responses. The median time to progression was 18 weeks and median survival was 42 weeks. Grade III and IV toxicities included asthenia (12%), nausea and vomiting (11%), diarhoea (10%) and leucopenia (6%). Overall, toxicity was considered acceptable and manageable. The colorectal response rate is comparable to many of the reported FU/LV studies (23%–30%). These facts, taken together with the relative ease of ZD1694 administration, encouraged the initiation of the European phase III study in 1993 (3 mg/m², 15 min infusion every three weeks), randomised against FU and LV given as 5 daily i.v. bolus injections (425 mg/m² FU, 20 mg/m² LV) repeated weeks 4, 8 and then 5 weekly (Mayo Regimen). The first results of this study (439 patients) at a median follow-up of 5.3 months are now published [55]. 20% of all patients receiving ZD1694, compared with 13% receiving FU/LV, had objective partial or complete responses (p = 0.059; odds ratio 1.7). In addition, a further 9.4% of patients receiving ZD1694 had a 40%–50% reduction in measurable lesion size (3% in FU/LV group). There was less grade III and IV leucopenia and mucositis in the ZD1694 arm of the study (p < 0.001). Asymptomatic and reversible rises in transaminases were seen more frequently in patients receiving ZD1694. Overall the authors concluded that ZD1694 compares favourably with FU/LV, with relatively good antitumour activity and an acceptable toxicity profile.

LY231514, a pyrrolopyrimidine folate-based TS inhibitor

LY231514, a pyrrolopyrimidine folate-based TS inhibitor (Fig. 2e) serendipitously arose from a chemical programme at Princeton University synthesising analogues of DDAHTF (Lometrexol), an inhibitor of GAR transformylase [56]. LY231514, further developed by Eli Lilly, is a relatively poor inhibitor of isolated recombinant human TS (Ki ~ 0.34 μM) but is highly cytotoxic to cultured CCRF-CEM human or mouse leuke-
compounds, new crystal complexes led to potent TS inhibitors with in vitro growth inhibitory potency similar to CB3717 (~1 μM). AG337 (compound 21 in [18]) is a quinazoline structure linked through C5 to a 4-pyridylthio moiety (Fig. 2f) that was designed to access a hydrophobic cavity in the active site of the enzyme that normally associates with the para-aminobenzoyl portion of CB3717 or of the natural substrate, CH$_2$FH$_2$. The reported Ki for *E. coli* TS is 49 nM and for recombinant human TS 16 nM. Curative antitumour activity was observed in the mouse i.m. or i.p. L5178Y TK− lymphoma (i.p. or oral drug administration) and significant growth delay was seen against the human GC$_2$M/TK− colon tumour xenograft [61].

AG331 (Fig. 2g; compound 27 in [62]) is structurally unrelated to the folate cofactor and interesting because more conservative studies into structure-activity relationships would be unlikely to reveal such a compound. The Ki for *E. coli* TS is 1800 nM and for human recombinant TS is 2 nM [62]. No explanation is provided as to why these values are so different for the TS of different sources. Tumour cell growth inhibition (IC$_{50}$) falls in the 0.5−1 μM range. AG331 may have a second locus in some hepatoma cell lines which is hypothesised to be due to metabolism to another drug species [63]. Antitumour activity was observed against a mouse L5178Y TK− variant implanted i.p. in mice [64].

Clinical studies are in progress for both drugs. A pharmacokinetic and pharmacodynamic study of AG337 (dihydrochloride form) given as a 24-h continuous i.v. infusion (every three weeks) has been reported [65]. Rapid plasma clearance ($t_{1/2}$, <2 h) was observed once the infusion ceased. A dose of 1.35 g/m$^2$ was reached with no antiproliferative toxicity being observed [65]. However, plasma deoxyuridine (dUrd) was monitored and found to rise during the infusion (result of the elevation in dUMP that occurs when TS is inhibited) but normalising once the infusion was stopped. The rise was not dose-related above 900 mg/m$^2$. Antitumour studies in vitro and in vivo suggested that >24 h exposure was required to induce a significant cytotoxic effect [66].

Five days of chronic dosing (75 mg/m$^2$ 4 hourly) gave a median growth delay of 14 days against the Hela Bu25TK− human cervical cell line implanted in nude mice [66], while it was inactive when given over a shorter time period. This evidence led to the conduct of a phase I study of 5-day continuous i.v. infusion. A maximum tolerated dose of 1130 mg/m$^2$/day has been established with dose limiting toxicities of myelosuppression and mucositis [67]. The dose recommended for phase II studies is 1000 mg/m$^2$/day.

Preclinical studies with AG331 indicated that the plasma half life was considerably longer in dogs (13 h) than in rodents (2−4 h) [68]. This was confirmed as ~20 h in humans in the phase I study. Eighteen patients received AG331 (glucuronate salt) at doses escalated between 12.5 and 225 mg/m$^2$ (10 min i.v. infu-
sion) with side-effects being observed at >130 mg/m² (moderate nausea and vomiting) [68]. At the highest dose given some mild flushing was observed which is believed to be due to histamine release. A similar effect was observed preclinically. A phase I study involving a 5-day continuous infusion has now been reported (25–800 mg/m²/day) [69]. Elevated liver function tests were evident by day 3 but were not dose-limiting. Pharmacokinetics suggested saturable clearance. Phase II studies are planned.

**BW1843U89, a benzoquinazoline in clinical study**

The Wellcome Research Laboratories have developed this benzoquinazoline TS inhibitor for clinical study. Their drug development programme originally concentrated on the synthesis of lipophilic benzoquinazolines [70] lacking the para-aminobenzoyl glutamate chain of the natural folates, or certain antifolates such as CB3717 or ZD1694. Poor water-solubility and low activity of such analogues led to the re-introduction of this side chain, which in the case of BW1843U89 (Fig. 2h), is an isoindolinone modified glutamate. This compound is the most potent TS inhibitor described in the literature (Ki for recombinant human TS = 90 pM) [71, 72]. Furthermore, although BW1843U89 has good potency against murine cells e.g. L1210 leukemia (IC50 = 66 nM), it has sub-nanomolar activity against a number of human cell lines. This difference is explained by its unusually high affinity (Kt ~0.3 µM) for, and rate of cellular internalisation by, the human cell membrane RFC [71]. Further features of this drug also make it biochemically distinct from many other folate-based TS inhibitors. For example, BW1843U89 has a very high affinity for FPGS (Km = 0.4 µM; hog liver enzyme) and a pseudo first order rate constant higher than that of ZD1694, suggesting that metabolism to polyglutamates should be extremely rapid [71]. However, because the FPGS substrate activity of the diglutamate is very poor, further polyglutamate chain elongation does not occur to a significant extent in either an isolated FPGS assay or in intact cells [73]. In identical systems, ZD1694 was extensively metabolised to high chain length polyglutamates (tetra and pentaglutamates). It is these higher polyglutamates of ZD1694 that are believed to be selectively retained inside cells. Furthermore, and again in contrast with ZD1694 or LY231514, the synthetic polyglutamates of BW1843U89 were not found to be substantially improved inhibitors of isolated TS [71]. The contribution that polyglutamate (diglutamate) formation makes to the cytotoxicity of BW1843U89 is unclear but is probably less than that for ZD1694 and LY231514. A MTX-resistant CCRF-CEM cell line (R30dm) with only 1% of the parental cell line FPGS activity displayed resistance to ZD1694, but not notably to MTX or BW1843U89, under continuous exposure conditions [74]. Taken together these data suggest that, at least under continuous exposure conditions, polyglutamation of BW1843U89 may not be of significance. Humphreys et al., using the same resistant CEM cell line, demonstrated a very high level of cross-resistance to ZD1694 and MTX (>100-fold) under short-exposure conditions (6 h) [75]. A lower but nonetheless high degree of resistance (90-fold) was observed to BW1843U89 suggesting that polyglutamation is important for its activity due to retention of the drug intracellularly.

In vivo antitumour studies were performed using the human colon carcinoma GC3-TK− cell line implanted sub-cutaneously or under the renal capsule and tumour regression was seen [71]. Mice bearing human tumour xenografts were treated with dThd phosphorylase to lower the plasma level and allow activity to be revealed [76]. However the difference in BW1843U89 transport between mouse and human cells further complicates the assessment of its toxicity to normal tissues. However, this has not defeated the BW group and the compound has proceeded through dog toxicology to phase I clinical study.

**ZD9331, a water-soluble non-polyglutamatable TS inhibitor in pre-clinical development**

ZD9331 (Fig. 2i) is a compound in pre-clinical development designed and synthesised by the Institute of Cancer Research and Zeneca Pharmaceuticals collaborators [77-81]. There were very specific requirements in terms of the desired biological profile which included water-solubility, potent TS inhibition, cellular uptake via the RFC and non-substrate activity for FPGS. It was argued that a compound active without metabolism to polyglutamates would exhibit a different spectrum of antitumour activity compared with ZD1694, particularly against tumours expressing low levels of FPGS. Antitumour potency was to be achieved by the design of a very potent TS inhibitor that could penetrate cells via the RFC. The latter was also reasoned to give a degree of compound selectivity and was based on observations by Sirotnak et al. which suggested that MTX and its analogues may have some tumour selectivity in mice due to differences in RFC characteristics between gastrointestinal and tumour tissue [82]. The design of a potent compound was partly reliant on a large database of structure-activity relationships, and partly using a computerised molecular model of the active site of 'humanised' L. casei TS. The latter suggested that compounds should be made that extended into a region of the active site that normally interacted with the α-carboxyl of the second glutamate of a polyglutamate chain [77, 78]. Several interesting series of compounds emerged, the most promising one being a series of glutamate modified compounds with the acid mimic, tetrzole, replacing the γ-carboxyl [77]. The lead compound was ZD9331 with a Ki for isolated mouse or human TS of 0.4 nM and activity against
several cell lines approaching that of ZD1694 e.g. 7.3 nM IC$_{50}$ against the human W1L2 cell line (ZD 1694 = 4.6 nM) [79,80]. Furthermore ZD9331 was shown to be highly selective for TS, as the cytotoxic effect of concentrations up to 100 µM were still completely prevented by the co-administration of dThd. The Km for the RFC is ~2 µM which, taken together with data showing poor activity against a cell line deficient in this transport protein, indicates that ZD9331 uses the RFC for cell entry which, in turn, contributes to the cytotoxic potency required [79,80]. Importantly, ZD9331 was shown to retain activity in a ZD1694-resistant cell line with a polyglutamated defect [79,80].

One of the features of ZD9331 that was considered important for its development was the lack of prolonged TS inhibition (due to drug efflux) once extracellular drug was removed [79,80]. It was believed that the combined properties of rapid plasma clearance and bi-directional transport across the cell membrane would allow for a high level of control over the length of time TS is inhibited in vivo if given by infusion protocols of different lengths. This may have certain advantages over ZD1694 in terms of toxicity control. Lack of drug-retention, not universal to non-FPGS substrates, was confirmed by the measurement of TS activity, TTP and dUMP pools after drug-treated cells in culture were resuspended in drug-free medium. All three normalised within 4 h of cell resuspension, even at high multiples of the IC$_{50}$ for growth inhibition [79,46]. Consistent with these observations is the lack of ZD9331-induced cytotoxicity after a short drug-exposure period. As predicted, the requirement for prolonged drug-exposure to inhibit TS and exert an antitumour effect meant that ZD9331 was more active in mice if given by continuous infusion. Hence a dose of 3 mg/kg infused for 1 day cured mice bearing the L5178Y TK– tumour whereas 50 mg/kg was necessary in a single bolus regimen [81]. As expected, the high level of dThd in mice meant that longer infusions were required to give normal proliferating tissue toxicity and activity against thymidine kinase competent tumours [81]. The spectrum of antitumour activity against a panel of human tumour xenografts is even wider than that of ZD1694 and the dose-limiting toxicity in mice is probably haematological rather than the gut toxicity seen for ZD1694 [81]. Further studies should define the combined properties of rapid plasma clearance and bi-directional transport to optimise toxicity control. Lack of drug-retention, not universal to non-FPGS substrates, was confirmed by the measurement of TS activity, TTP and dUMP pools after drug-treated cells in culture were resuspended in drug-free medium. All three normalised within 4 h of cell resuspension, even at high multiples of the IC$_{50}$ for growth inhibition [79,46]. Consistent with these observations is the lack of ZD9331-induced cytotoxicity after a short drug-exposure period. As predicted, the requirement for prolonged drug-exposure to inhibit TS and exert an antitumour effect meant that ZD9331 was more active in mice if given by continuous infusion. Hence a dose of 3 mg/kg infused for 1 day cured mice bearing the L5178Y TK– tumour whereas 50 mg/kg was necessary in a single bolus regimen [81]. As expected, the high level of dThd in mice meant that longer infusions were required to give normal proliferating tissue toxicity and activity against thymidine kinase competent tumours [81]. The spectrum of antitumour activity against a panel of human tumour xenografts is even wider than that of ZD1694 and the dose-limiting toxicity in mice is probably haematological rather than the gut toxicity seen for ZD1694 [81]. Further studies should define optimal administration protocols for the forthcoming clinical study (1996).

Conclusions

Novel drugs have been developed which inhibit TS by interacting with the folate-binding site. Their Ki values for the inhibition of isolated TS range from 0.09 to 340 nM. The Ki does not directly reflect their cytotoxic potency because of their dissimilar patterns of activity for cellular processes such as cell membrane transport and metabolism to polyglutamate forms. Thus, these compounds give quite different spectra of activity against cell lines which display a variety of antifolate resistance mechanisms. This should imply that they will have different spectra against human cancers. These biochemical differences, when combined with other features of the drugs such as differing physical properties (e.g. water-soluble versus lipophilic) and pharmacokinetic differences, are likely to further separate their activities and toxicities in humans. The first results of the completed phase III study of ZD1694 randomised against FU/LV for the treatment of colorectal cancer appear to endorse TS as a target for new drug development. Whether ZD1694, or one of the other drugs described above (currently less advanced in their development), will significantly improve the treatment of colorectal or other types of cancer, remains to be seen. A number of issues are being, or are likely to be, addressed in the laboratory which may optimise drug activity in humans. For example, various toxicity rescue protocols are currently being evaluated in animals treated with ZD1694 (LV or dThd) and BW1843U89 (folic acid) [83,84].

Thus, in the next few years the portfolio of drugs targeted at TS, including LV modulated FU, should give us the answer to ‘is TS the right enzyme?’ and may help to endorse, or otherwise, the role of anti-metabolites in cancer therapy. Failure to get at least one of the new folate-based TS inhibitors registered as a drug is likely to result in a decline in interest in further drug development in this area. However, the results emerging from the most advanced clinical studies (ZD1694), suggest that this will not be the case and it is tempting to predict that thymidylate synthase will be considered an excellent target, and one or more of the new compounds described above will be considered the right drug(s).

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