Quinazoline Inhibitors of Thymidylate Synthase: Potent New Anticancer Agents

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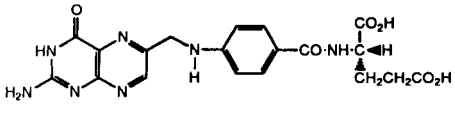
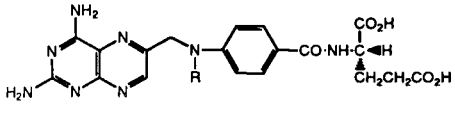
Folic Acid has, over the past forty years, formed the basis of an enormous amount of medicinal chemistry aimed at finding improved anticancer agents. This stemmed originally from the almost empirical observations that the folic acid analogues Aminopterin (**2**) and Methotrexate (**3**, MTX) showed clinical activity, particularly against the childhood leukaemias [1]. MTX has been in clinical use since the late 1940's and is still widely prescribed today. Over the past decade there has been a resurgence in the number of folic acid analogues entering



1 Folic Acid



2 R = H Aminopterin
3 R = CH_3 Methotrexate

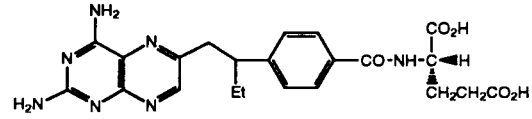
Folate Dependent Enzymes

Dihydrofolate Reductase (DHFR)	Purine & Pyrimidine Nucleotides
Thymidylate Synthase (TS)	Thymidylate (Pyrimidine Nucleotide)
Aminoimidazolecarboxamide Formyl Transferase (AICAR Transformylase)	Purine Nucleotides
Glycinamide Ribonucleotide Formyl Transferase (GAR Transformylase)	Purine Nucleotides

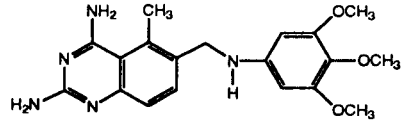
the clinic which has resulted from the knowledge that a number of key enzymic reactions in the biosynthesis of deoxyribose nucleotides depend on folate cofactors. MTX is now known to exert its antitumour activity *via* the inhibition of dihydrofolate reductase (DHFR) although this enzyme was only discovered nine years after MTX entered clinical use. More recent DHFR inhibitors that have entered the clinic are Edatrexate (**4**) [2], Trimetrexate (**5**) [3] and Piritrexim (**6**) [4]. Lometrexol (**7**) [5] is the prototype GAR transformylase inhibitor in the clinic. This presentation focuses on folate based inhibitors of Thymidylate Synthase (TS) as exemplified by CB3717 (**8**) and Zeneca ZD1694 (**9**).

In order to follow the evolution of these inhibitors it is first necessary to understand the role of TS in DNA biosynthesis and also the involvement of the folate cofactors. TS is a pivotal enzyme in pyrimidine biosynthesis

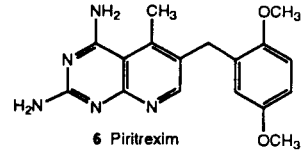
Folate Based Anticancer Agents that have been in Clinical Trial



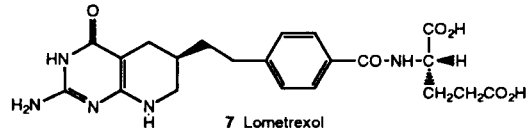
4 Edatrexate



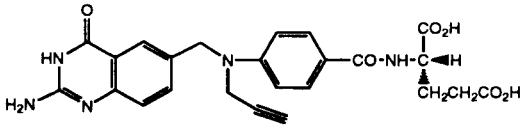
5 Trimetrexate



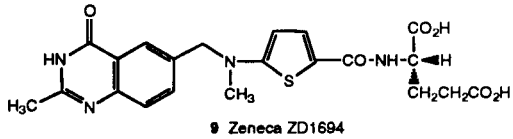
6 Piritrexim



7 Lometrexol



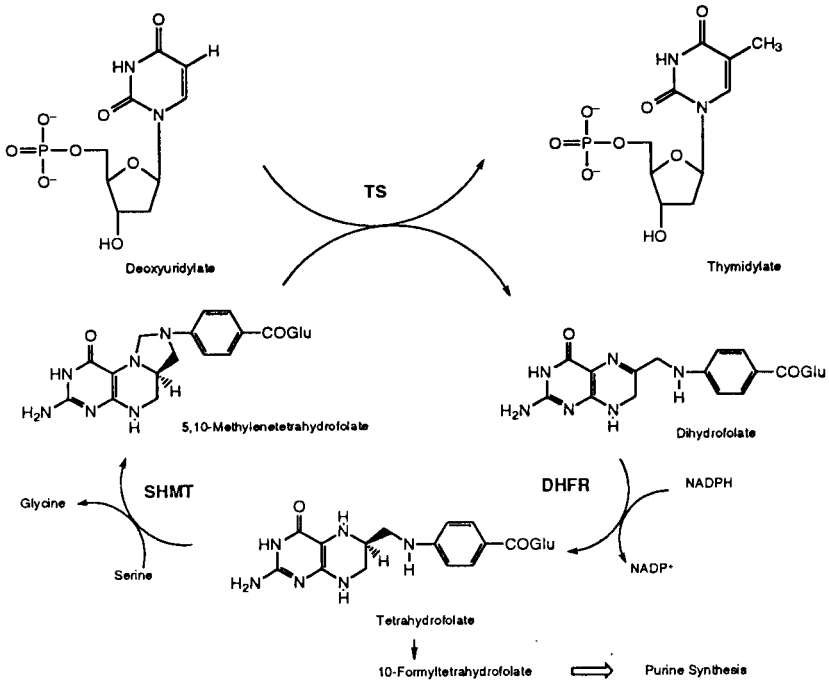
8 CB 3717



9 Zeneca ZD1694

which catalyses the conversion of deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (thymidylate, dTMP) and represents the only *de novo* source of this nucleotide required for DNA synthesis (Scheme 1). The cofactor, 5,10-methylenetetrahydrofolate (CH_2FH_4), supplies the one carbon fragment required for the reaction in a process where TS transfers the 5,10-methylene function to the 5-position of the pyrimidine ring. In this reaction the CH_2FH_4 is transformed into dihydrofolate. Regeneration of the cofactor from dihydrofolate is achieved in a cycle of reactions catalysed by the enzymes DHFR and serine hydroxymethyl transferase (SHMT). In this sequence the dihydrofolate is initially reduced by DHFR to tetrahydrofolate followed by meth-

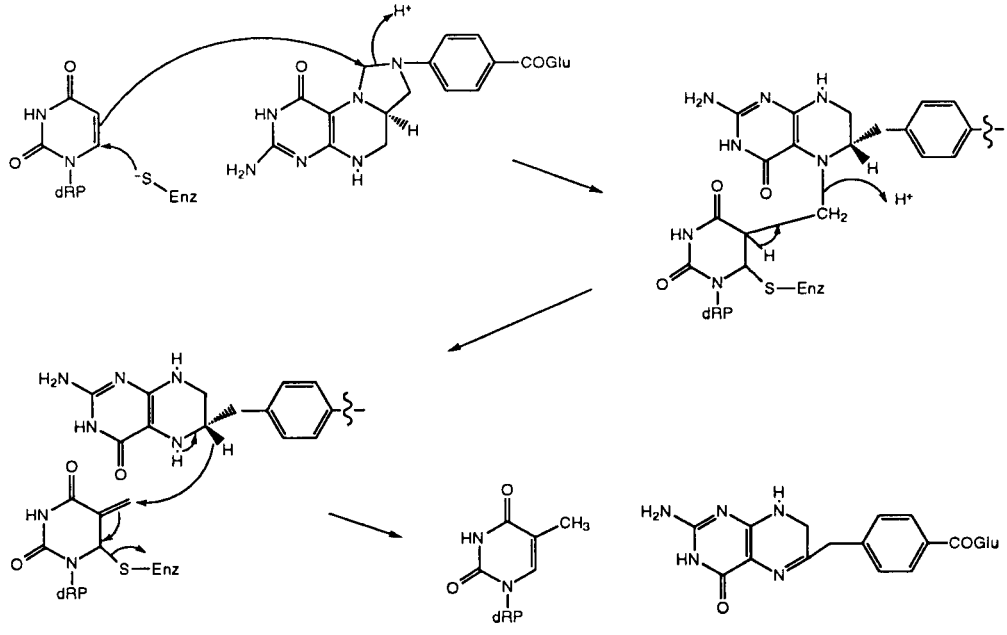
Scheme 1



ylene transfer from a molecule of serine catalysed by SHMT. The sequence of events in the transfer of CH_2 from CH_2FH_4 to dUMP is shown in Scheme 2. A key step is the Michael addition of a cysteine SH to the 6-position of the pyrimidine ring, which both anchors the substrate in the enzyme and facilitates the attack on the 5,10-CH_2 group of the cofactor.

In turning our attention to TS as a target for antifolate chemotherapy we anticipated several potential advantages over inhibitors of DHFR. Principal amongst these was that inhibition of TS should not affect purine, protein or RNA synthesis and in this respect may avoid some of the side effects seen with DHFR inhibitors such as MTX [6]. A folate based TS inhibitor was also seen as an alternative

Scheme 2

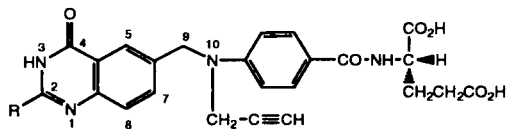


target since it would not require metabolic activation, which is required with the pyrimidine based TS inhibitor, 5-fluorouracil. It should also not have the toxicity problems of a pyrimidine due to incorporation into nucleic acids.

A traditional medicinal chemistry approach of molecular modification of the natural folate cofactor resulted in the synthesis of CB3717 (**8**) [7], the first folate based pure TS inhibitor to enter the clinic. This agent was developed in a collaboration between the Institute of Cancer Research and ICI Pharmaceuticals (Zeneca) in the early 1980's. It was shown to be an excellent inhibitor of TS from a variety of different sources (both bacterial and mammalian) and to be highly cytotoxic against a range of tumour cell lines. In the clinic it exhibited a high degree of activity against a variety of breast, ovarian and liver tumours [8-10]. However it also exhibited unpredictable liver toxicity and life threatening kidney toxicity in some patients [11,12]. This latter problem was ascribed to its lack of solubility at physiological pH since crystalline deposits of the drug were observed in the kidneys of treated mice. Because of these problems the clinical development of CB3717 was terminated.

However, because of the impressive antitumour activity of CB3717 in patients, we felt strongly that the hypothesis of a folate based TS inhibitor was still valid and we decided to attempt to refine the structure of CB3717 in a search for an analogue that would retain the same level of antitumour activity but that would be devoid of any non-TS mechanism related toxicity. Because of the kidney toxicity (which is not related to the compound's inhibition of TS) we considered that CB3717's poor solubility was a major factor. On the basis of this argument the synthesis of analogues with enhanced aqueous solubility became a primary target. In the design of more soluble analogues one of our early hypotheses was that the poor solubility of CB3717 was due to the concentration of hydrogen bond donors and acceptors in the quinazoline part of the molecule. This, we argued, was giving rise to a stabilisation of the crystal lattice. We therefore began a program of systematically removing or modifying these functions, namely the ring nitrogens at positions 1 and 3, the 4-oxo group and the 2-amino substituent. We were particularly pleased to discover that the 2-amino group was not essential for activity as a TS inhibitor even though this group is an integral part of all natural folates. Replacement of this amino group by either a hydrogen atom or a methyl group caused only a slight loss of potency against isolated TS *in vitro* but on the other hand these modifications actually gave a significant enhancement of cytotoxic potency (Table 1), illustrated by the IC_{50} values against the L1210 cell line which is a mouse leukaemia commonly used as a primary cytotoxic screen. As predicted by our hypothesis

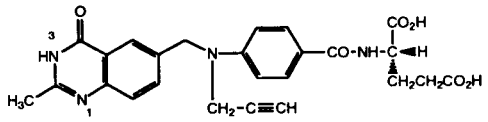
Table 1



R	TS IC_{50}, nM	L1210 IC_{50}, uM	Solubility pH 7.4 mg/ml	ALT (dose mg/kg)
NH_2	20	3.40	0.31	508 (100)
H	160	0.36	>100	16 (500)
CH_3	40	0.09	>100	13 (500) [control 15]

ALT = Alanine transaminase

these modifications at C2 gave analogues that were several orders of magnitude more soluble than the prototype CB3717. Encouraged by the fact that modification of the C2 substituent gave analogues that were still highly potent cytotoxic TS inhibitors we went on to look at further modifications to the quinazolinone. We removed in turn the nitrogens at positions 1 and 3 to give the corresponding quinolinone and isoquinolinone as well as the corresponding aromatised quinoline and isoquinoline systems. Although these molecules did show some activity as TS inhibitors their potencies were more than an order of magnitude lower than the parent quinazolinones. From this we concluded that potent TS inhibition requires both the 1-nitrogen as a hydrogen bond acceptor and the 3-NH as a hydrogen bond donor. Subsequent crystallographic studies of the enzyme-inhibitor complex [13] provided a full explanation of these empirical observations. Moreover the nature of the C2 substituent can have a fine tuning effect on the hydrogen bond donor-acceptor properties of these two ring nitrogens. For example the strongly electron withdrawing trifluoromethyl group at C2 lowers the p*K*a of the 3-NH to 5.9 which is thus ionised at *p*H 7 and the folate analogue containing this substituent is significantly less potent as a TS inhibitor. However the enzyme will tolerate a wide variety of less electron withdrawing substituents including relatively bulky ones such as phenyl. Overall the best C2 substituents to give a combination of TS inhibitory activity and cytotoxicity were the methyl and fluoromethyl groups. At this stage of the project we therefore felt that by making a relatively small change to the CB3717 molecule we had achieved our target of a TS inhibitor that was both sufficiently cytotoxic and sufficiently soluble to consider as a replacement for CB3717 in the clinic. The front runner at this time was the compound ICI 198583 (**10**) [14], which was the 2-methyl analogue of CB3717. This compound proved considerably more potent as a cytotoxic agent *in vitro*. Moreover it also possessed potent antitumour activity in our primary mouse models and validated our hypothesis



10 ICI 198583

TS 40 nM (2x less potent than CB 3717)

L1210 0.09 μM (40x more potent than CB 3717)

* In a mouse tumour model (L1210:ICR) 198583 cured 90% of mice at 5 mg/kg daily x 5 days

* ICI 198583 was not toxic to liver and kidneys of mice at 500 mg/kg iv whereas CB 3717 was very toxic at 100 mg/kg iv

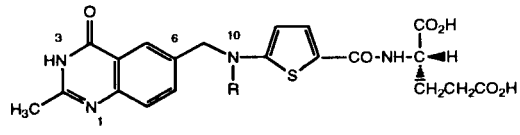
that enhancing aqueous solubility would abolish the liver and kidney toxicities [15]. What is particularly interesting here is the observation that a compound that is slightly less potent as an inhibitor of the pure TS enzyme should be 40-fold more potent as a cytotoxic agent. Biochemical studies [16] demonstrated that there are two principal reasons for this:

1. Enhanced transport into cells by an active uptake system termed the reduced folate carrier (RFC) which is the normal mechanism whereby cells take in natural folates.

2. Enhanced retention of the compound within cells *via* the formation of polyglutamated species by the enzyme folylpolyglutamate synthetase (FPGS). Again this is the normal mechanism whereby cells retain natural folates and involves the addition of one or more glutamic acid residues to the γ-position of the first glutamate.

Having optimised the quinazoline part of the molecule our attention was turned to the central *p*-aminobenzoate (PABA) moiety. Modifications here could have profound effects on the geometry of the whole molecule and thus affect interactions with key enzymes. A wide range of substituents on the PABA ring was looked at as well as replacement of the benzene ring with a variety of heterocycles. It was this latter exercise that gave rise to an extremely interesting series containing the thiophene isostere [17]. Here we looked at a range of substituents on N10 from which propargyl, ethyl and methyl have been chosen to illustrate the theme (Table 2). Although these thiophene containing compounds are actually an order of magnitude less potent than ICI 198583 against the TS enzyme they are considerably better cytotoxics. In particular the N10-methyl analogue is one of the most potent that we have seen. This N10-methyl compound which we designated Zeneca ZD1694 also had highly potent antitumour activity in our animal models and as expected it had no liver or kidney toxicity since it had the required degree of aqueous solubility. These properties of ZD1694 clearly illustrate a very important principle in designing an enzyme inhibitor as a potential drug: namely that the potency of the compound as an inhibitor of the isolated

Table 2



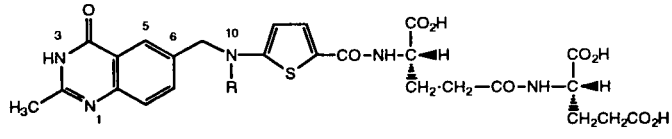
R	TS Inhibition IC_{50}, uM	Cytotoxicity IC_{50}, uM
$-CH_2-C\equiv CH$	0.44	0.06
$-CH_2CH_3$	0.58	0.016
$-CH_3$	0.65	0.0067
$-H$	25	3.00
ICI 198583	0.04	0.09

(198583 = N10-propargyl-p-aminobenzoyl analogue)

Zeneca ZD1694 R = CH_3

enzyme does not always correlate with the potency in whole cell systems either *in vitro* or in animal models. In the case of ZD1694 it was again demonstrated [18] that its very high cytotoxic potency was due to high affinities for the RFC and FPGS. The structure of the diglutamate form is shown in Table 3. The higher polyglutamates simply have additional glutamate residues linked through the γ-carboxylic acids. These polyglutamate forms have all been synthesised as standards. Studies with 5-[^{3}H]-ZD1694 in L1210 cells have shown that the compound is both very effectively taken up into the cells and predominantly metabolised intracellularly to these highly retained polyglutamate forms [18]. Moreover the higher polyglutamate forms (which contain from four to six glutamic acid residues) are of the order of 100 times more potent than the parent as inhibitors of the TS enzyme.

Table 3



Diglutamate of Zeneca ZD1694

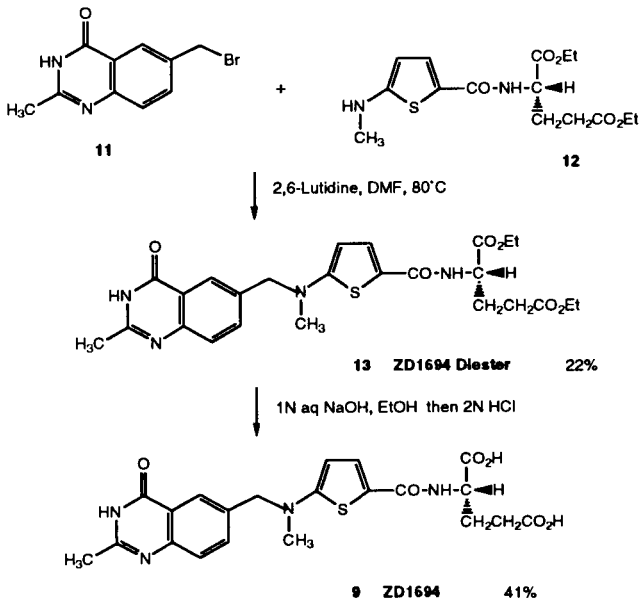
* 4 hour exposure of cells to 5-[^{3}H]-ZD1694 - Ratio of Intracellular to Extracellular label was 15:1

* 17% as triglutamate, 70% as tetraglutamate, 8% as pentaglutamate

	TS, $K_i$app, nM	Fold improvement in TS Inhibition
ZD1694	418	1
Glu_2	24	17
Glu_3	7.5	56
Glu_4	4.7	89
Glu_5	3.8	110
Glu_6	3.7	112

In a Phase I clinical trial ZD1694 has been shown to be free of liver and kidney toxicity at the maximum tolerated dose. The dose limiting toxicities were all TS mechanism related, namely nausea, vomiting, diarrhoea and myelosuppression. A Phase II clinical trial is now underway in a variety of tumour types.

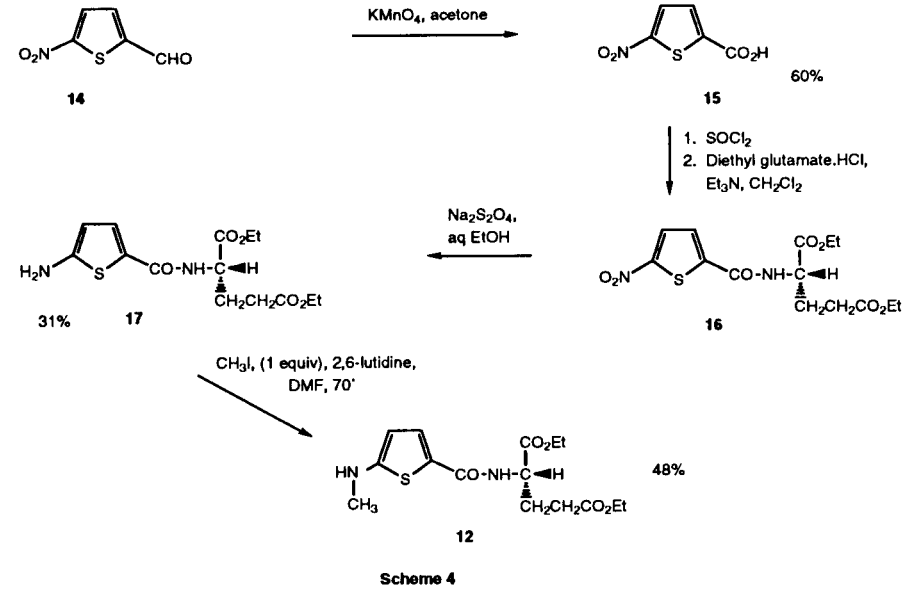
Original Research Route to Zeneca ZD1694



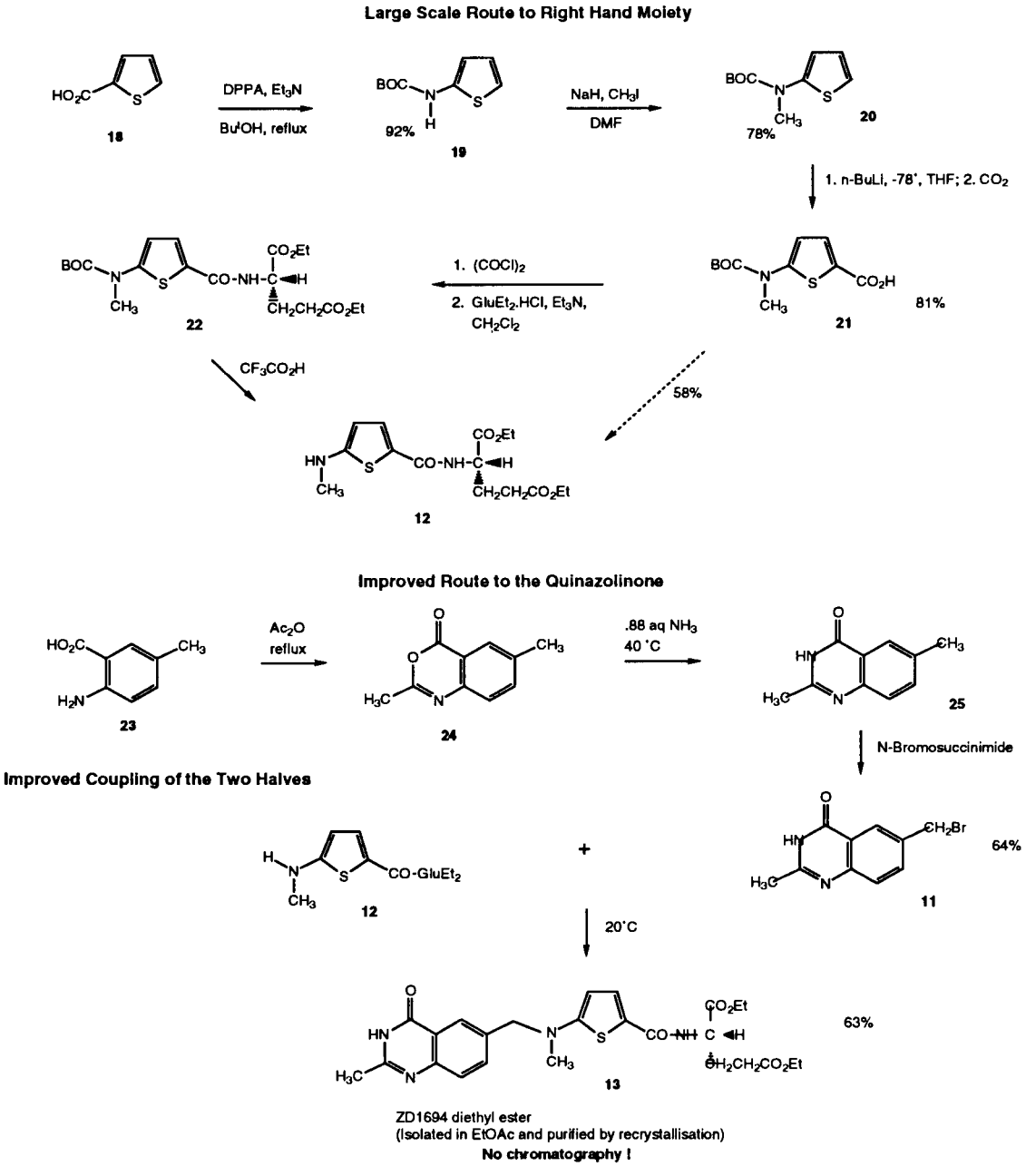
Scheme 3

The overall strategy for the synthesis of ZD1694 is shown in Scheme 3. This involves the coupling of the bromomethyl quinazolinone **11** with the methylaminothiophene derivative **12** to give **13**, the diethyl ester of ZD1694. In the original research route this coupling step was achieved by simply heating the two halves together in dimethylformamide in the presence of 2,6-lutidine as an acid scavenger. The amine right hand half **12** was originally made by the route shown in Scheme 4 in which 5-nitrothiophenecarboxaldehyde was oxidised with potassium permanganate to the carboxylic acid **15**. This was coupled *via* its acid chloride to diethyl glutamate. The resulting nitro compound **16** was reduced to the amine **17** with sodium dithionite in aqueous ethanol in 31% yield. Treatment of **17** with methyl iodide in the presence of 2,6-lutidine provided the right hand moiety **12**, but the purification of this intermediate was complicated by the presence of the *N,N*-dimethyl derivative as well as unreacted **17**, requiring careful chromatography before coupling to **11**. In order to prepare larger amounts of ZD1694 for development purposes an improved route was required. We subsequently devised a much more efficient route to **12** which utilised the conversion of thiophene-2-carboxylic acid *via* a diphenylphosphoryl azide mediated Curtius reaction to the BOC-protected amine **19**. The BOC protecting group had the advantage that it permitted the specific monomethylation of the nitrogen atom which eventually became N10 of ZD1694. This was achieved by formation of the sodium salt and treatment of this with iodomethane. The 5-position of the thiophene ring was then readily lithiated under fairly undemanding conditions and the simple addition of crushed solid CO_2 to the reaction mixture gave the acid **21**. Coupling of **21** *via* its acid chloride to diethyl glutamate and removal of the BOC

Original Research Route to the Right Hand Moiety



Scheme 4

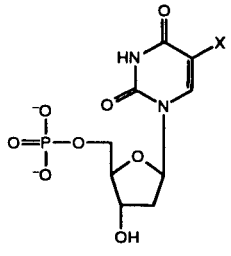


Scheme 5

group by a brief treatment with trifluoroacetic acid gave **12** in a very satisfactory yield of 58% over the last two stages. Also in devising a large scale route an improved synthesis of the quinazolinone moiety was sought as the literature procedure that we originally followed [19] involved heating the methyl anthranilic acid **23** with thioacetamide, a process that was not desirable on a multikilogram scale. The route in current use involves heating **23** with acetic anhydride to give the benzoxazinone **24** which is warmed to 40° with aqueous ammonia. The bromomethyl derivative **11** is currently coupled to the amine at 20°. Although this required a longer reaction time these conditions are milder than the original ones and also give the product diester **13** in the much improved yield of 63%. This is an isolated yield which requires no chromatography, only a simple recrystallisation.

At this stage in the project we had moved from a hypothesis to a clinical agent by using a traditional medi-

cinal chemistry approach. The potent cytotoxicity of ZD1694 depends on the intracellular formation of highly retained polyglutamated forms. Although this should give rise to a successful antitumour agent in the clinic we were aware of the fact that tumour cells can acquire resistance to folate based antimetabolites by losing the ability to polyglutamate them [20]. Because of this we wanted to complement ZD1694 by an agent that did not have the potential for this resistance mechanism. Thus our next target was to find analogues that were sufficiently potent TS inhibitors in the monoglutamate form that they would be highly cytotoxic as such without requiring any intracellular polyglutamation. In order to achieve this we felt that we needed a better understanding of how the inhibitor interacts with the TS enzyme. We therefore began molecular modelling studies based on information that was available to us on TS and its complexes with substrate and inhibitor. TS has been obtained from a variety of different



26 dUMP X = H
27 F-dUMP X = F

species from bacteria to man. In each case the enzyme has been shown to be a dimer of two identical subunits, each of which is capable of carrying out the transformation of dUMP to dTMP. The primary amino acid sequences have been obtained for TS from these sources and they show a remarkable degree of conservation between TS from widely different species. The crystal structure has been obtained for TS from *Lactobacillus casei* and, more recently, two groups have obtained crystal structures of the ternary complex of *Escherichia coli* TS with a pyrimidine nucleotide (either dUMP [13] or F-dUMP [21]) and CB3717. The co-ordinates for the ternary complex with dUMP, made available to us by the Stroud group, were the starting point for our modelling studies. Based on these co-ordinates a model was constructed using the known primary sequence for human TS. Figure 1 shows the dimeric structure of the "humanised" ternary complex viewed so that one can see the cleft in one subunit into which fit the pyrimidine substrate and the antifolate TS inhibitor. Figure 2 focuses in on this cleft to show the residues in the helical region that are important for binding the glutamate moiety. The crystal structure provided a number of important pieces of information. Firstly that the benzene ring of the quinazolinone and the pyrimidine

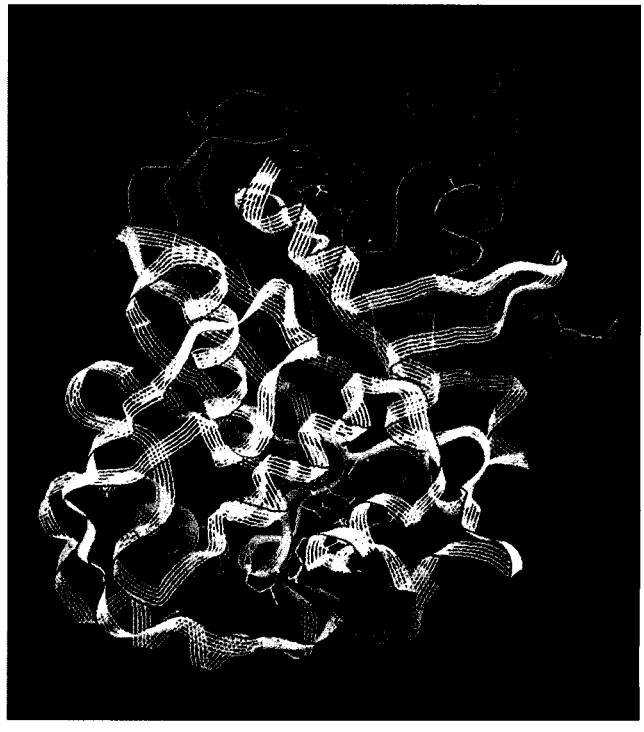


Figure 1

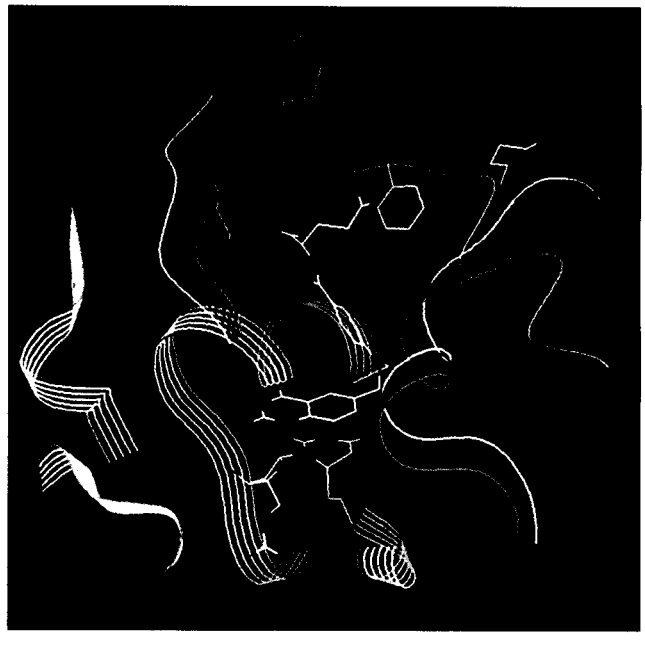


Figure 2

ring stack close together with the rings parallel and these two heterocyclic systems are held by a number of specific

hydrogen bonds to residues in the enzyme. Secondly it showed us that CB3717 binds in a partially folded conformation with the PABA ring inclined at an angle of 65° to the quinazolinone. Thirdly the α-carboxylic acid of the glutamate is bound through two water molecules (shown as red asterisks) to Lys-50. The crystal structure of the ternary complex of *E. coli* TS with a polyglutamated form of CB3717 [22] has shown that the first glutamate residue binds in the same mode as in the monoglutamate and that the carboxylic acids of the subsequent glutamate residues interact electrostatically with basic residues on the surface of the enzyme. We have modelled this for the diglutamate of CB3717 (Figure 3). We have recently obtained from

Figure 3

Professor Stroud's group the crystal structure of the ternary complex with ZD1694 [23]. This has shown us that the geometry about the PABA portion of the molecule has a profound effect on the binding of the glutamate residue for when we replace PABA by a thiophene ring it is the γ-carboxylic acid that binds through waters to Lys-50. The "humanised" form of this complex is shown in Figure 4. However the intriguing question of whether the polyglutamates of ZD1694 adopt the normal binding mode is so far unanswered. The potencies of these polyglutamates suggest that this would be the case but a crystal structure of a TS-ZD1694 polyglutamate complex is necessary to answer this question unequivocally.

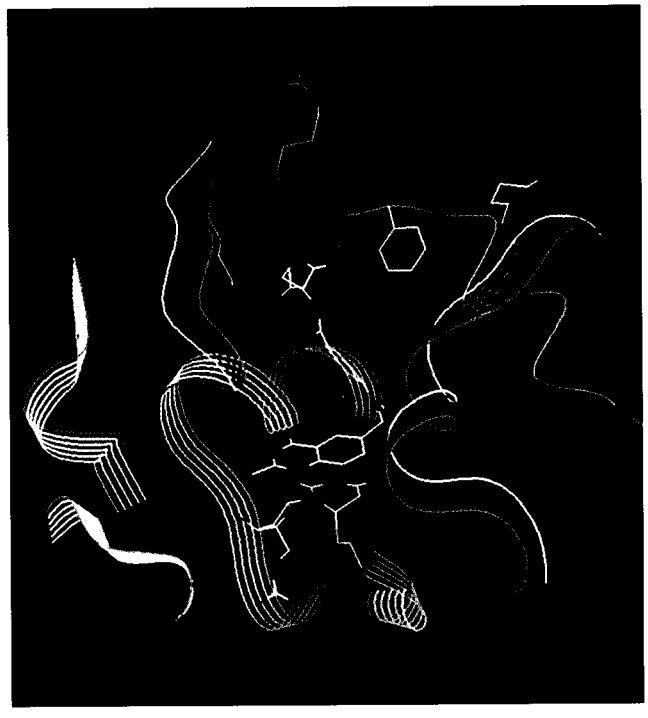


Figure 4

From these structural studies we therefore had a considerable amount of information about how these inhibitors bind to the enzyme and, in particular, information on the preferred conformation of the various parts of the molecule for optimum binding. In our modelling studies we looked at the effect of substituents in various parts of the molecule on the overall conformation. Substitution in the 7-position looked particularly promising. Molecular mechanics (SCANOPT) and semi-empirical quantum mechanical energy (AMPAC) calculations indicated that for a molecule with a substituent larger than hydrogen in the 7-position the preferred lowest energy conformation (see Figure 5) was precisely that for optimum binding to the enzyme. We therefore prepared a range of 7-substitut-

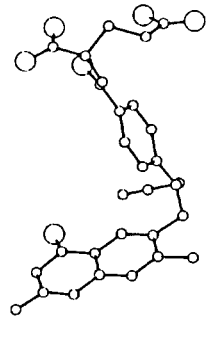
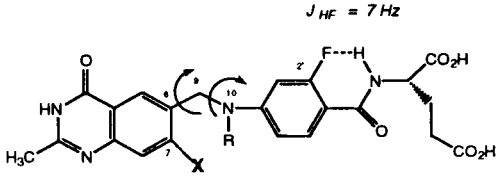


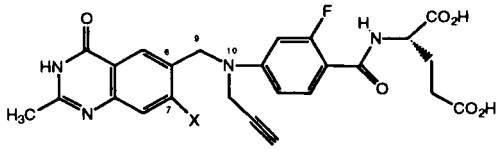
Figure 5

ed molecules of the general structure **28**. It was decided to include a 2'-fluoro-substituent in **28** as our earlier studies [24] had shown that this substituent gives a 2- to 3-fold enhancement of TS inhibition and cytotoxic potency in the PABA series. It is presumed here that the hydrogen bond between the fluorine and the glutamate NH (seen as a coupling of 7 Hz in the NMR spectrum) stabilises the preferred almost planar conformation in this region of the inhibitor molecule.



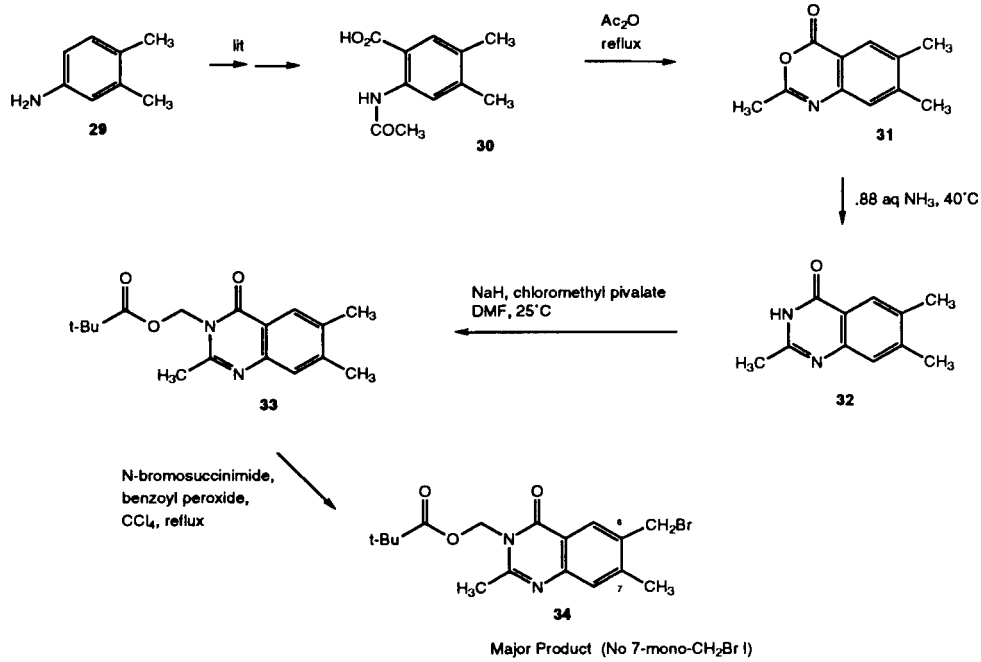
2'-F gives 2- to 3-fold enhancement of TS inhibition and cytotoxic potency

Target Molecules:

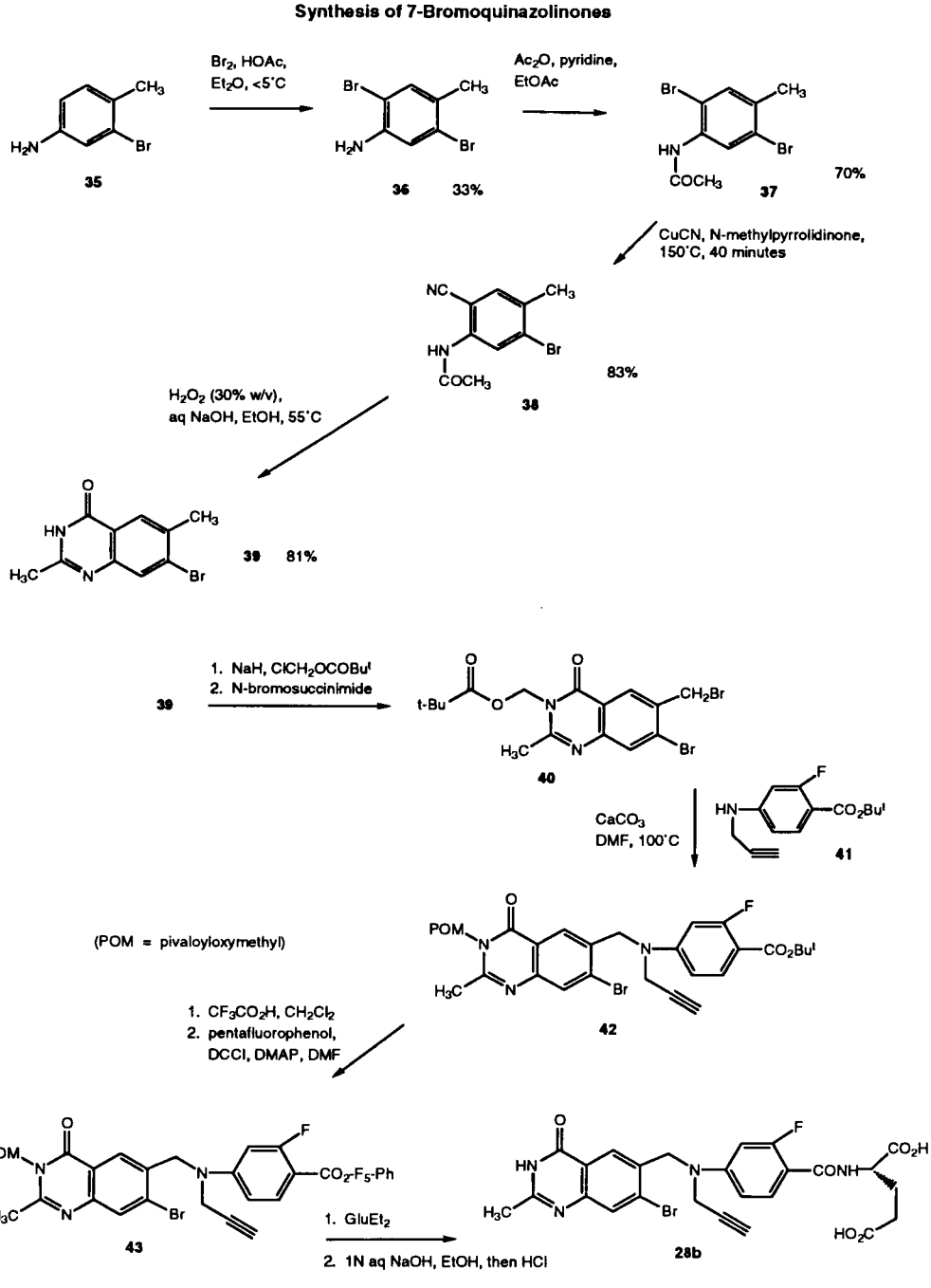


28
28a X = CH_3
28b X = Br

The synthesis of these 7-substituted-2'-fluoro analogues **28** followed the same basic strategy that was used for the corresponding 7-unsubstituted parents [24]. The novel chemistry here involved the construction of the 7-substituted-6-bromomethylquinazolinones that were required for coupling to the right hand aminomethylbenzoylglutamate esters. The methods used are illustrated for the 7-methyl (**28a**) and 7-bromo (**28b**) analogues. The acetylated dimethylanthranilic acid **30** was cyclised in two steps to the quinazolinone **32** (Scheme 6). In order to enhance the solubility of this quinazolinone and facilitate further chemistry the 3-NH function was protected as the pivaloyloxymethyl (POM) derivative **33**. Bromination (NBS) of **33** occured smoothly to give **34** as the sole monobromo product although minor amounts of dibromo impurities were formed. These could be readily removed by chromatography. The synthesis of the 7-bromo analogue **28b** is shown in Scheme 7. Bromination of 2-bromo-4-aminotoluene gave the dibromide **36** which was acetylated to **37**. Treatment of **37** with cuprous cyanide in *N*-methylpyrrolidinone under carefully controlled conditions gave the nitrile **38** which underwent cyclisation to the 7-bromo-6-methylquinazolinone **39** in high yield. Another important feature of the synthesis of **28b** is the intermediate pentafluorophenyl ester **43**. The formation of this active ester facilitated the purification of the system following the coupling of the bromomethyl **40** and amino **41** components and also gave a rapid and clean amide formation with diethyl glutamate.



Scheme 6



Scheme 7

Of the 7-substituted analogues **28** prepared, the 7-methyl, 7-ethyl, 7-bromo and 7-chloro derivatives gave enhanced inhibition of TS compared to the 7-H parent. On the other hand only the 7-methyl substitution **28a** gave improved cytotoxicity. However this cytotoxicity was shown to result from the monoglutamate form alone as this compound has been shown to be a nonsubstrate for FPGS. Electron withdrawing groups at C7, exemplified by CN and CF_3 gave poorer inhibition of TS and a corresponding drop in cytotoxic potency. This probably reflects a disruption of the stacking interaction of the quinazolinone and dUMP in the ternary complex.

In conclusion we have used a traditional medicinal chemical approach to develop a promising new anticancer

agent, Zeneca ZD1694, which is a potent inhibitor of TS in vivo due to the intracellular formation of polyglutamated forms. The application of crystal structure studies and molecular modelling has permitted the design of a new generation of TS inhibitors, exemplified by **28a** (ZM214888), that show potent in vivo antitumour activity as the parent monoglutamate form and may thus overcome resistance in cancers that cannot form polyglutamates of folic acid analogues.

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