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SHORT COMMUNICATIONS

Kinetic characteristics of ICI D1694: a quinazoline antifolate which inhibits thymidylate synthase

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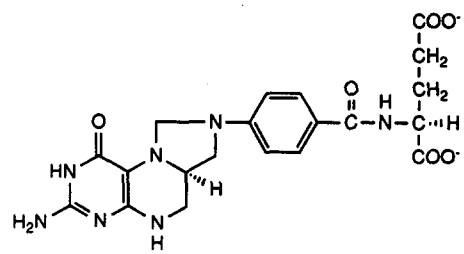
Abstract—The thymidylate synthase (TS) inhibitor ICI D1694 (*N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl)-*S*-glutamic acid) is a structural analogue of the substrate N^5,N^{10}-methylenetetrahydrofolate (5,10-CH_2FH_4) and is currently under clinical evaluation as a treatment for cancer. The compound is shown here to be a mixed non-competitive inhibitor of TS from murine leukemia (L1210) cells when 5,10-CH_2FH_4 is varied. This result suggests formation of an inactive complex between TS, 5,10-CH_2FH_4 and the inhibitor. Thus, binding to only one of the two active sites on the TS homodimer may be sufficient to prevent catalysis fully. Treatment of L1210 cells with ICI D1694 is known to cause intracellular accumulation of the tetraglutamate derivative which is shown here to have a 60-fold higher affinity for TS. The IC_{50} for inhibition of L1210 cell growth is below the K_i value of ICI D1694 for L1210 TS but above that of the tetraglutamate. The formation of polyglutamates and concentration of drug inside cells, therefore, seem to be responsible for biological activity.

Thymidylate synthase (TS*) (EC 2.1.1.45) catalyses the conversion of dUMP and N^5,N^{10}-methylenetetrahydrofolate (5,10-CH_2FH_4, Fig. 1) to TMP and dihydrofolate. The enzyme is required for *de novo* synthesis of TMP which is needed for replication of DNA. Thus, inhibition of TS represents an approach for the treatment of cancer. The quinazoline ICI D1694 (*N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl)-*S*-glutamic acid, Fig. 1) is a structural analogue of 5,10-CH_2FH_4 which inhibits TS, has well-characterized biological properties [1] and is currently under clinical study. Inside cells, folylpolyglutamyl synthetase (EC 6.3.2.17) catalyses the addition of further glutamates onto various folates and analogues including 5,10-CH_2FH_4 and ICI D1694, decreasing the rate of diffusion from the cell [1–3]. When murine leukemia (L1210) cells are treated with ICI D1694, the predominant intracellular form is tetraglutamate which carries three additional residues [1]. Polyglutamates of 5,10-CH_2FH_4 are difficult to synthesize. We have, therefore, used the monoglutamate of 5,10-CH_2FH_4 and both the monoglutamate and tetraglutamate of ICI D1694 when characterizing the kinetics of inhibition of TS from L1210 cells. We find that polyglutamation leads to an increase in affinity which is probably important for biological activity. TS is a homodimer with two active sites [4–7], but our results with both inhibitors suggest indirectly that binding at only one site could prevent catalysis.



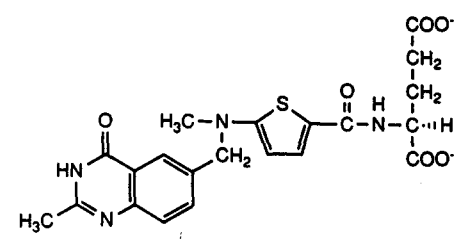


Fig. 1. Structures of the TS substrate (6*R*)-5,10-CH_2FH_4 and the inhibitor ICI D1694.

Materials and Methods

Enzyme assays. (6*R*)-5,10-CH_2FH_4 was prepared from (6*S*)-tetrahydrofolate (a generous gift from Dr R. G. Moran, University of Southern California, Los Angeles) (see Ref. 8). Drs P. R. Marsham and G. M. F. Bisset kindly synthesized ICI D1694 and its tetraglutamate [9, 10]. L1210 TS was partially purified and duplicate assays followed the release of radiolabelled protons from saturating (50 μM) [5-^{3}H]dUMP (see Ref. 2). In the presence of either inhibitor, product accumulated linearly with time for at least 60 min, indicating that the binding affinity did not increase slowly as has been reported for the quinazoline antifolate CB3717 (N^{10}-propargyl-5,8-dideazafolate) and TS from *Lactobacillus casei* [11].

Identification of mechanism of inhibition. Initial velocities were analysed by unweighted non-linear fitting of 12 different rate equations (see Ref. 12). Substrate and

* Abbreviations: TS, thymidylate synthase; 5,10-CH_2FH_4, N^5,N^{10}-methylenetetrahydrofolate; E, enzyme; S, substrate, I, inhibitor; K_i and K_{ies}, apparent dissociation constants of I from EI and IES complexes, respectively; TS inhibitors: ICI D1694, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl)-*S*-glutamic acid; CB3717, N^{10}-propargyl-5,8-dideazafolate; CB3804, 2-desamino CB3717; ICI 198583, 2-desamino-2-methyl CB3717.

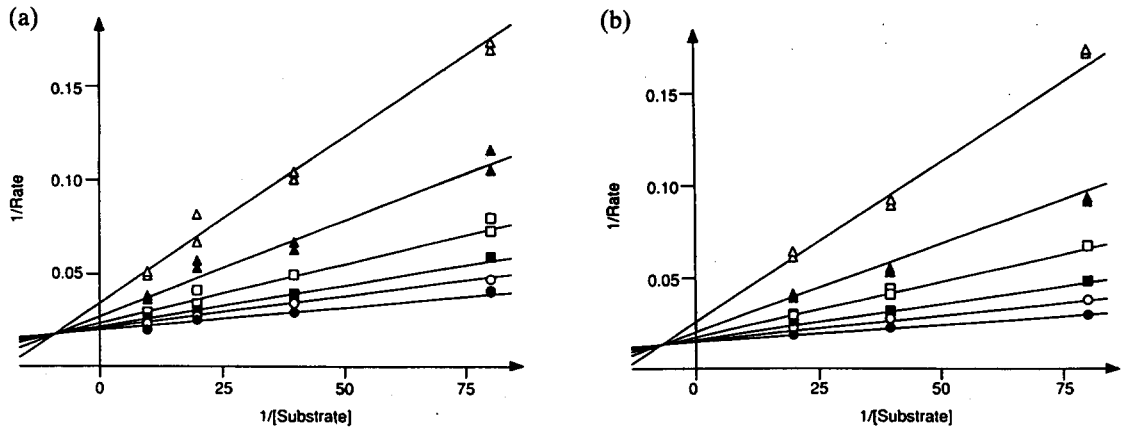


Fig. 2. Lineweaver–Burk plots for inhibition of L1210 TS. Rates were measured and analysed as described in Materials and Methods. The lines were calculated assuming mixed inhibition and the parameter values given in Table 1. The unit for 1/Rate is the inverse of thousands of dpm per assay, and for 1/[Substrate] is the inverse of 5,10-CH_2FH_4 concentration in mM. Some data have been omitted for clarity. The full range of conditions used is described in the legend to Table 1. (a) ICI D1694 at (●) 0 nM; (○) 25 nM; (■) 50 nM: (□) 100 nM; (▲) 200 nM; (△) 400 nM. (b) ICI D1694 tetraglutamate at (●) 0 nM: (○) 0.5 nM; (■) 1 nM; (□) 2 nM; (▲) 4 nM; (△) 8 nM.

inhibitor were varied in the same fit and then an F-test was used to compare the residual sum of squares in order to identify the most suitable mechanism. In each case, the selected equation gave reasonable parameter values and SEs, and the residual differences between observed and calculated rates were small and followed a random distribution.

Results and Discussion

Both ICI D1694 and the tetraglutamate lead to mixed non-competitive inhibition, indicating that they can bind either before or after 5,10-CH_2FH_4. The dissociation constants before and after association with substrate are K_i and K_{ies}, respectively. The apparent formation of IES complexes suggests that two binding sites function on each TS dimer but, conversely, fitting to two site models fails to detect ES_2 or EI_2 complexes. Analysis of kinetic data, however, cannot distinguish between (i) a mechanism where each active site in ES_2 complexes has similar values for K_m and V_{max}, and (ii) a mechanism where the second subunit in ES complexes has little affinity for substrate. Published data strongly favour the second alternative (see below). Although mixed inhibition best describes the rates, the mechanism tends towards competitive because K_i is $<K_{ies}$ (Table 1). Lineweaver–Burk plots show that mixed inhibition gives an accurate description of the rates for ICI D1694 and the tetraglutamate (Fig. 2). (The lines would intersect on the ordinate for competitive inhibition.) Polyglutamation increases potency around 60-fold, but does not change the mechanism because K_i and K_{ies} are altered by comparable factors (Table 1). Similar results are obtained when the pure (6*R*)-5,10-CH_2FH_4 is replaced by (6*R*,*S*)-5,10-CH_2FH_4 (Table 1). The value of K_m is doubled but there is no significant change in K_i or K_{ies} for either inhibitor. These results do not detect binding of the unnatural (6*S*)-isomer to L1210 TS, agreeing with studies on the enzyme from *L. casei* and *Streptococcus faecalis* [13, 14]. Thus, our results can be compared with those obtained previously using (6*R*,*S*)-5,10-CH_2FH_4. For example, polyglutamation also increases potency for several other quinazolines including CB3804 (2-desamino CB3717) and ICI 198583 (2-desamino-2-methyl CB3717) [2, 3].

Our experiments were conducted using 5,10-CH_2FH_4 monoglutamate, whereas polyglutamates predominate inside cells. Polyglutamation of the substrate is likely to reduce the K_m value but may have no effect on K_i and K_{ies} because these values relate to zero and saturating substrate, respectively. The predictions are, however, tentative because polyglutamation may lead to complex effects which could arise from a change in the sequence of substrate addition (see Ref. 5).

The K_i is the minimum IC_{50} value against isolated enzyme which occurs when the substrate concentration is $\ll K_m$. ICI D1694 has an $IC_{50} = 7$ nM for inhibition of L1210 cell

Table 1. Kinetic parameters for inhibition of TS from L1210 cells

Inhibitor	5,10-CH_2FH_4	K_m (μM)	K_i (nM)	K_{ies} (nM)
ICI D1694	6*R*	12 ± 1	59 ± 6	630 ± 100
ICI D1694	6*R*,*S*	27 ± 1	62 ± 5	960 ± 180
ICI D1694-E_4	6*R*	12 ± 1	0.94 ± 0.06	12 ± 2
ICI D1694-E_4	6*R*,*S*	26 ± 1	1.01 ± 0.05	15 ± 3

Rates were measured and analysed as described in Materials and Methods. Each experiment gave similar values for V_{max} (≃ 0.06 nmol/min/mg protein). Best fit values are quoted ± SE. (6*R*)-5,10-CH_2FH_4 was used at 12.5–100 μM, and (6*R*,*S*)-5,10-CH_2FH_4 was employed at twice these levels. ICI D1694 was at 12.5–1,600 nM, and the tetraglutamate (ICI D1694-E_4) was at 0.25–32 nM.

growth [1]. This value is higher than the K_i for the tetraglutamate (which predominates in these cells [1]) but well below that of the parent compound (Table 1). Our results with isolated TS are, therefore, consistent with cellular studies which suggest that the formation of polyglutamates and concentration inside cells are responsible for biological activity [1]. The importance of these factors in addition to potency against isolated L1210 TS is seen by comparing various inhibitors. The K_i values for CB3717, ICI 198583, CB3804 and ICI D1694 are 3, 10, 27 and 60 nM, respectively, whereas the corresponding IC_{50} values for inhibition of L1210 cell growth are 3,500, 90, 360 and 7 nM [1–3]. The high efficacy of ICI D1694 against cells is associated with uptake via the reduced folate carrier and ability to act as a substrate for folylpolyglutamyl synthetase [1].

At high concentrations of (6*R*,*S*)-5,10-CH_2FH_4, IC_{50} values against isolated L1210 TS for CB3717, CB3804 and ICI 198583 rise to plateau levels [2, 3]. This observation implies mixed non-competitive inhibition (with $K_i < K_{ies}$) and suggests that several quinazoline antifolates form EI and IES complexes. These inhibitors are all structural analogues of 5,10-CH_2FH_4 and so seem likely to occupy the substrate binding sites of L1210 TS, especially since X-ray crystallography indicates that CB3717 occupies both active sites in TS from *Escherichia coli* [6, 7]. Furthermore, the primary structure of TS is similar for man, mouse, *E. coli* and *L. casei* [4], implying that relationships between structure and function are conserved. The two subunits of *L. casei* TS exhibit large differences in binding affinity for nucleotides and folate analogues (see Ref. 5). Similar behaviour by L1210 TS would not only explain why K_{ies} is $>K_i$, but also the failure to detect ES_2 and EI_2 complexes. Ligand binding experiments using homogeneous L1210 TS would probably demonstrate occurrence of these complexes. Furthermore, it is possible that very high levels of 5,10-CH_2FH_4 could displace I from IES complexes.

Lack of catalysis in the putative IES complexes suggests that association with inhibitor at one active site is communicated across the subunit interface and so prevents turnover at both sites. This hypothesis is consistent with the observation that treatment of L1210 TS with CB3717-pentaglutamate prevents catalysis, but not binding of 5-fluoro-2′-deoxyuridylate [15]. Chemical modification studies using *L. casei* TS indicate that only one active site is accessible on the free enzyme, and then binding of 5-fluoro-2′-deoxyuridylate and 5,10-CH_2FH_4 opens the second site [5, 16]. In *E. coli* TS, each dUMP binding site contains two conserved Arg side-chains from the other subunit [4, 6, 7]. Interaction of these residues with dUMP at one site may affect kinetics at the second subunit.

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