Metabolic Resistance: The Protection of Enzymes against Drugs which are Tight-Binding Inhibitors by the Accumulation of Substrate

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Blockade of a metabolic pathway by interaction of a drug with a particular 'target enzyme' results in depletion of essential end-products of the pathway and accumulation of intermediates prior to the blockade. *Metabolic resistance* to a particular drug can arise if the substrate of the inhibited enzyme accumulates to levels sufficiently high to compete effectively with the inhibitor, leading to restoration of full activity of the metabolic pathway after a transitory delay.

Such resistance has recently been demonstrated *in vitro* for the interaction of the tight-binding inhibitor *N*-phosphonacetyl-L-aspartate (*P*AcAsp) with the aspartate transcarbamoylase activity of the trifunctional protein which initiates pyrimidine biosynthesis in mammals [Christopherson, R. I. and Jones, M. E. (1980) *J. Biol. Chem. 255*, 11381–11395]. Carbamoyl phosphate, the product of the carbamoyl phosphate synthetase activity of this trifunctional protein, accumulates to a sufficiently high concentration that the inhibitory effect of *P*AcAsp is effectively abolished. We have developed a theoretical model for metabolic resistance which quantitatively accounts for these experimental data. This model has been used to simulate the interaction between the following potential or proven anti-cancer drugs and their target enzyme, under conditions similar to those which would occur *in vivo*: *P*AcAsp with aspartate transcarbamoylase; various OMP analogues [the 5'-monophosphates of 6-azauridine, pyrazofurin and 1-(β-D-ribofuranosyl)-barbituric acid] with OMP decarboxylase; 5-fluorodeoxyUMP with thymidylate synthase; methotrexate with dihydrofolate reductase; and deoxycoformycin with adenosine deaminase.

The interaction of a tight-binding inhibitor with a 'target enzyme' of a pathogen or tumour cell provides a common basis for chemotherapy of infections and cancer. The selective toxicity of the drug for pathogens may arise because the target enzyme of the invading organism has different binding properties to that of the host or because that enzyme is absent from the host. In cancer chemotherapy, the selective toxicity of a tight-binding enzyme inhibitor is usually dependent upon the faster growth rate, and hence the more rapid rate of nucleic acid biosynthesis, of the tumour when compared with normal body cells. A variety of anti-cancer drugs have been developed which inhibit nucleic acid biosynthesis at particular enzymatic steps and selectively kill cancer cells at an appropriate dose because of their faster rate of growth [1].

Common forms of drug resistance arise through enhancement of drug metabolism, inhibition of drug uptake and induction or alteration of the target enzyme, but the consequences of accumulation of the substrate(s) of the inhibited enzyme have received relatively little attention. This

accumulation results in a phenomen we shall call 'metabolic resistance'. Consider the simplified pathway:

$$\xrightarrow{v_0} A \xrightarrow{E_1} B \xrightarrow{E_2} C \xrightarrow{E_3} \text{etc.} \tag{1}$$
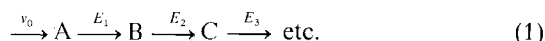
where A, B and C are sequential intermediates of the pathway and E_1, E_2 and E_3 are the enzymes catalyzing the reactions. If A is being produced at a constant rate (v_0), inhibition of E_1 by a drug (I) will result in accumulation of A and depletion of B, C and subsequent intermediates. Metabolic resistance to the drug can arise if A accumulates to levels which are sufficiently high to compete effectively with I for interaction with E_1, leading to restoration of the original flux (v_0) through the pathway after a transitory delay. For a drug which is a 'classical' competitive inhibitor (i.e. which causes inhibition only at concentrations which are considerably greater than the enzyme concentration) [2], metabolic resistance can be described by the equation:

$$A_i = A_0 (1 + I/K_i) \tag{2}$$

where A_i and A_0 are the steady-state concentrations of the substrate A in the presence and absence of the inhibitor, respectively, and K_i is the inhibition constant of I (Eqn III-3 of Segel [3]; Eqn 8 of Christopherson and Jones [4]). A_i is the new steady-state concentration of A necessary to restore the original flux through the pathway.

A reversible, tight-binding, competitive inhibitor exerts its inhibitory effect at a concentration comparable to that of the enzyme. Allowance must therefore be made for the reduction of free inhibitor concentration which occurs as a result of its binding to the enzyme [2]. In general, it is tight-

binding inhibitors which are of therapeutic value, but no quantitative relationship equivalent to Eqn (2) has been developed for this more complex case although several authors [5,6] have acknowledged the importance of substrate accumulation in response to drug administration *in vivo*. In this paper, we present the equation which describes metabolic resistance to tight-binding inhibitors and demonstrate the use of this equation in describing published experimental data [4] for the interaction of PAcAsp with aspartate transcarbamoylase. Further, we use the model to simulate metabolic resistance for a number of potential or proven anti-cancer drugs under conditions similar to those which would occur *in vivo*.

THEORY

The general theory of reversible tight-binding inhibitors of enzyme-catalyzed reactions has been reviewed by Williams and Morrison [2] and theoretical aspects of slow, tight-binding inhibition have been presented by Morrison [7]. These workers have not considered the effects of substrate accumulation in a metabolic pathway such as that described by Eqn (1) and the relevant theory will be developed here. In such a situation where there is tight-binding inhibition, Eqn (2) still holds except that I refers to concentration of free inhibitor rather than total inhibitor (I_T). I and I_T are related by the expression:

$$I_T = I[1 + E_T/\{I + K_i(1 + A_0/K_a)\}] \qquad (3)$$

where K_a is the Michaelis constant for interaction of the substrate with the target enzyme and E_T is the total concentration of enzyme. Rearranging Eqn (2):

$$I = K_i(A_i/A_0 - 1). \qquad (4)$$

Substituting Eqn (4) into Eqn (3) and solving for A_i:

$$A_i = A_0 [Q + (Q^2 + 4 K_i E'_T)^{1/2}]/2 K_i \qquad (5a)$$

where

$$Q = K_i + I_T - E'_T \qquad (5b)$$

and

$$E'_T = E_T/(1 + A_0/K_a). \qquad (5c)$$

Eqn (5) describes metabolic resistance for a tight-binding inhibitor and permits calculation of the concentration of A necessary to restore the original flux through the metabolic pathway. The general form of the equation is that of an hyperbola and a plot of A_i versus I_T is curved at low I_T while approaching an asymptote at higher inhibitor concentrations. This asymptote is described by the equation:

$$A_i = (A_0/K_i)I_T - (A_0/K_i) (E'_T - K_i) \qquad (6)$$

which has a slope of A_0/K_i and an intercept on the abscissa at $I_T = E'_T - K_i$. For a classical inhibitor, $K_i \gg E'_T$ and Eqn (6) reduces to Eqn (2).

Eqn (5) is useful for predicting steady-state concentrations but contains no information on the time course of the approach to this steady-state. Such information is clearly relevant to chemotherapy; while metabolic resistance will eventually overcome the inhibition of an enzyme, any cellular damage which has occurred during the transition period may be irreparable. It has not been possible to derive an expression which describes the accumulation of A as a function of time by analytical integration of the appropriate differential equations. These equations were, therefore, integrated numeri-

cally using a fourth-order Runge-Kutta method [8]; further details will be given elsewhere (Duggleby and Christopherson, unpublished work). Fitting of progress curves was performed using the CRICF program [9].

RESULTS AND DISCUSSION

Christopherson and Jones [4] studied the interaction *in vitro* of PAcAsp with the aspartate transcarbamoylase activity of hOro synthetase[1], the trifunctional protein which catalyzes the initial steps of pyrimidine biosynthesis in mammals. In the presence of PAcAsp, a tight-binding inhibitor of aspartate transcarbamoylase [10], Cbm-P accumulates as it is continuously produced by the carbamoyl phosphate synthetase activity of the protein. As this accumulation proceeds, Cbm-P eventually reaches a concentration where it can compete effectively with PAcAsp resulting in restoration of the original flux through the aspartate transcarbamoylase reaction. The concentrations of Cbm-P necessary to overcome the inhibition by various total concentrations of PAcAsp have been reported (Fig. 7B of [4]) and these data are reproduced here (Fig. 1b).

In the experiment reported by Christopherson and Jones [4], incubation of hOro synthetase with substrates and PAcAsp was limited to 40 min owing to the instability of carbamoyl phosphate synthetase and of Cbm-P [4]. Calculation of the time course of Cbm-P accumulation (Fig. 1a) showed that the concentration of Cbm-P at 40 min is well below the steady-state value for all but the lowest PAcAsp concentration. Thus in order to simulate the data of Christopherson and Jones [4] it was necessary to calculate a theoretical curve representing 40-min concentrations rather than steady-state concentrations. This is shown by the solid line of Fig. 1b which was obtained using a K_i of 6.2 nM for PAcAsp, a value which lies within the range reported for the mammalian enzyme [10,11]. This theoretical line is clearly in very good agreement with the data. Also shown (Fig. 1b, broken line) is the expected steady-state concentrations of Cbm-P as calculated from Eqn (5).

Fig. 1a and b illustrate two important aspects of metabolic resistance. Firstly, for a tight-binding inhibitor, a plot of A_i ([Cbm-P]) versus I_T ([PAcAsp]) has an initial portion of upward curvature which becomes linear at higher values of I_T (Fig. 1b, broken line). At low inhibitor concentrations, A_i does not increase greatly because the concentration of free inhibitor is significantly decreased by binding to the enzyme. Secondly, it is clear from Fig. 1a that at a given rate of synthesis of A (Cbm-P), it takes longer for metabolic resistance to develop at higher concentrations of inhibitor.

Having established the validity of our model for metabolic resistance in a system *in vitro*, values for the parameters of Eqn (5) were collected from the literature for the interaction between a number of tight-binding drugs and their target enzymes (Table 1). The drugs listed all have potential or proven anti-cancer activity and the values were chosen to be representative of cancer cells *in vivo*. Fig. 2 shows theoretical curves simulated for metabolic resistance, using Eqn (5) and appropriate values from Table 1 for the interaction of three OMP analogues with OMP decarboxylase. Classical competitive

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[1] L-5,6-Dihydroorotate synthetase (hOro synthetase) is the trifunctional protein initiating pyrimidine biosynthesis in mammals which was formerly called multienzymatic protein pyr1-3, CAD or complex A. This protein contains the enzymatic activities carbamoyl phosphate synthetase, aspartate transcarbamoylase and dihydroorotase.
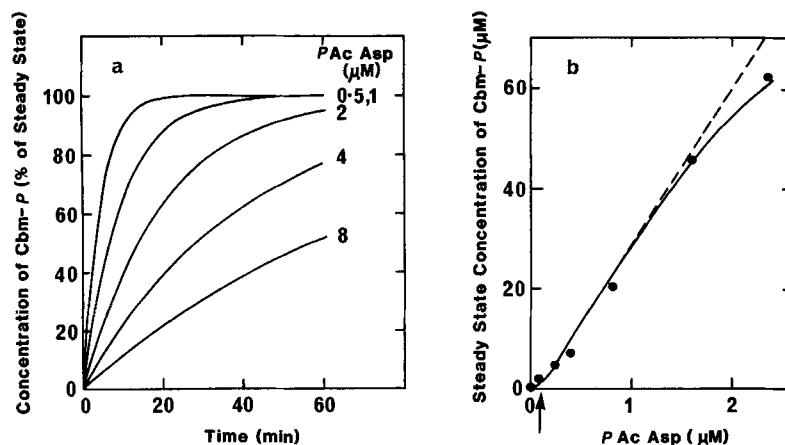
Fig. 1. *Protection of aspartate transcarbamoylase against* PAcAsp *inhibition by accumulation of Cbm-P*. (a) Simulation of the progress curve for Cbm-P accumulation at the indicated concentrations of PAcAsp. These simulations were performed as follows. Since A_0, K_a, E_T and the turnover number of aspartate transcarbamoylase are known, v_0 (Eqn 1) may be calculated. At any time (t) in the presence of PAcAsp, the rate v_t of utilisation of Cbm-P may be calculated (Eqn 1 of [2]) provided that the substrate concentration at that time (A_t) is known. Hence the net rate of substrate accumulation is given by $dA_t/dt = v_0-v_t$. Values for A_t are obtained by numerical integration (see Theory) using the fact that $A_t = A_0$ when $t = 0$. The numerical values used for the simultations were: A_0, 0.2 µM; K_a, 22.1 µM; E_T, 0.093 µM; turnover number, 3720 min^{-1}; and K_i, 6.2 nM. Ordinate values have been normalised to a value of 0 % at $t = 0$, and 100 % at $t = \infty$. (b) Concentrations of Cbm-P as a function of the total concentration of PAcAsp. The broken line (---) represents steady-state concentrations and was calculated from Eqn (5) using these parameter values: A_0 ([Cbm-P]) = 0.2 µM, $K_i = 6.2$ nM, $E_T = 0.093$ µM (indicated by arrow) and $K_a = 22.1$ µM. The solid line (——) represents the expected concentration of Cbm-P after a 40-min incubation and was calculated as described in Fig. 1 a. The experimental values are taken from [4] and are reproduced with the kind permission of the American Society of Biological Chemists. The solid line represents the best fit to the experimental data as determined by nonlinear regression using the CRICF computer program [9] and it was from this analysis that the K_i value of 6.2 nM was derived
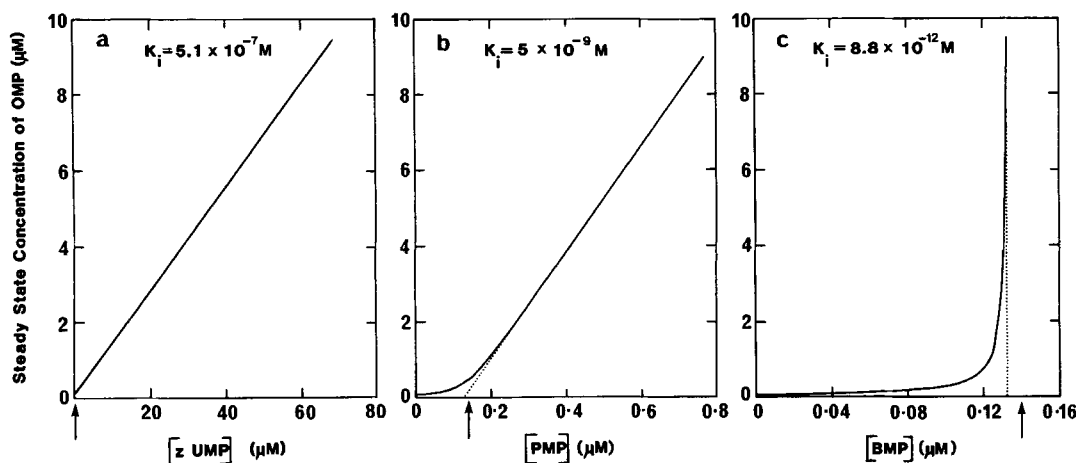

Fig. 2. *Simulated substrate protection of OMP decarboxylase against OMP analogues by accumulation of OMP.* (a) zUMP; (b) PMP; (c) BMP. The expected steady-state concentrations of OMP were calculated using Eqn (5) with appropriate values from Table 1. The arrows indicate the enzyme concentration (0.14 µM) and the dotted lines are the asymptotes calculated from Eqn (6)

inhibition is shown by zUMP with no curvature visible in the plot of A_i versus I_T (Fig. 2a) due to its relatively high K_i value of 0.51 µM. For this case, the dependence of A_i upon I_T is adequately described by Eqn (2) (see Theory). For PMP ($K_i = 5$ nM), there is curvature up to an inhibitor concentration of approximately 0.2 µM (Fig. 2b) after which the plot is linear. BMP has a very low K_i value of 8.8 pM (Table 1) and the plot for metabolic resistance (Fig. 2c) has an initial near-horizontal region followed by a very steep rise beyond the equivalent concentration of OMP decarboxylase (0.14 µM, Table 1). Fig. 2c demonstrates a potential difficulty in using truly tight-binding inhibitors as anti-cancer drugs. Too little inhibitor has virtually no effect but once the concentration of inhibitor exceeds the equivalence point for the concentration of the active sites of the target enzyme, there is an abrupt and very potent inhibitory effect. Thus, for a selective toxicity to

cancer cells relative to normal cells to be achieved, the cellular concentration of inhibitor must be very closely controlled.

Metabolic resistance may be considered more easily in quantitative terms if it is assumed that the total cellular concentration of the inhibitor (I_T) is sufficiently great for the plot of A_i versus I_T to approach a linear asymptote (cf. Fig. 2). Under these conditions, A_i is a linear function of I_T and the calculated slopes and intercepts on the abscissa of the asymptote for each drug—target-enzyme interaction are included in Table 1. These slopes and intercepts have predictive value: if the cellular concentration of the inhibitor is known, the concentration of substrate (A_i) necessary to overcome the blockade can be calculated by substituting the values for the slope (A_0/A_i) and intercept ($E_T - K_i$), from Table 1 into Eqn (6). Naturally, a more accurate simulation of metabolic resistance will be obtained if values for E_T, K_i, K_a, A_0 are

Table 1. *Appropriate parameters specifying drug—target-enzyme interactions* in vivo

The cellular concentration of subunits (E_T) was calculated from: $E_T = 25 \times$ (specific activity of crude extract)/(specific activity of pure protein) × (subunit molecular weight). The factor of 25 comes from the assumption that the intracellular concentration of soluble protein is 25 µg/µl cytosol. The values of K_a represent the Michaelis constant in the case of the single-substrate reactions catalysed by OMP decarboxylase and adenosine deaminase. The remaining enzymes catalyse two-substrate reactions and K_a is the apparent Michaelis constant at physiological concentrations of the cosubstrate. Intracellular concentrations of substrates were calculated using published data and an estimate of 1 µl for the intracellular volume of 10^6 mammalian cells

Parameter	Unit	Value for drug—target-enzyme combinations				
		PAcAsp—aspartate-trans-carbamoylase	OMP-analogue—OMP-decarboxylase	FdUMP—thymidylate-synthase	methotrexate—dihydrofolate-reductase	deoxycoformycin—adenosine deaminase
Specific activity of crude cell-free extract	pmol min⁻¹ (µg protein)⁻¹	18.1 [4]	2.21 [12]	0.195 [13]	0.867 [14]	160 [15]
Specific activity of pure protein	pmol min⁻¹ (µg protein)⁻¹	18600 [4]	7800 [16]	383 [13]	3800 [14]	486000 [15]
Molecular weight of subunit containing one active site		200000 [17]	51500 [16]	38500 [13]	21000 [14]	44000 [15]
Cellular concentration of subunits	µM	0.12	0.14	0.33	0.27	0.19
K_i	M	6.2×10^{-9}ᵃ	5.1×10^{-7} [18] (zUMP) 5.0×10^{-9} [19] (PMP) 8.8×10^{-12} [18] (BMP)	1.1×10^{-8} [6]	5.3×10^{-12} [5]	9.2×10^{-11} [15]
Apparent K_a for substrate	µM	22.1ᵇ (Cbm-P)	1.2 [20] (OMP)	2.8 [6] (dUMP)	0.16 [5] (H₂ folate)	48 [15] (Ado)
Initial concentration of substrate	µM	0.9 [21] (Cbm-P)	0.07 [22] (OMP)	5 [23] (dUMP)	0.03 [24] (H₂ folate)	<0.07 [25] (Ado)
Half-time for dissociation of EI complex	min	–	198 [18] (BMP)	23.1 [6]	50.2 [5]	2310 [26]
Asymptote of Eqn (5): Slope (A_0/K_i) of Eqn (6)		145	0.137 (zUMP) 14.0 (PMP) 7955 (BMP)	455	5660	761
Intercept on I_T abscissa ($E'_T - K_i$) of Eqn (6)	µM	0.109	−0.38ᶜ (zUMP) 0.127 (PMP) 0.132 (BMP)	0.107	0.227	0.190

ᵃ This value lies within the range of values reported in the literature [10,11] and was determined as described in Fig. 1 b.

ᵇ For the trifunctional protein hOro synthetase, the local concentration of Cbm-P at the aspartate transcarbamoylase site is 2.2-fold higher than that prevailing in the bulk solvent [4]. Hence the apparent K_a for Cbm-P of 48.7 µM is effectively decreased to a value of 48.7/2.2 = 22.1 µM.

ᶜ For zUMP, Eqn (6) approximates to Eqn (2) for which the intercept on the abscissa is −0.51 µM. The difference between this value and that calculated from Eqn (6) is a measure of the extent of the approximation. This difference is insignificant in comparison with the concentrations of zUMP shown in Fig. 2.

obtained for the particular system of interest rather than from Table 1.

Considering the values in Table 1 defining the asymptote, zUMP behaves as a classical competitive inhibitor with a linear plot for metabolic resistance passing through A_0 (Eqn 2, Fig. 2a). As the K_i decreases from 0.51 µM for zUMP to 5nM (PMP) and 8.8pM (BMP), so the slope A_0/K_a increases (Table 1). The slope value of 7955 for BMP indicates that much larger quantities of OMP must accumulate for metabolic resistance to operate when compared to an equivalent amount of zUMP with a slope value of 0.137 (Table 1). The intercept on the abscissa ($E'_T - K_i$, Table 1) is primarily a function of E'_T for a tight-binding inhibitor; E'_T is the concentration of free enzyme present in the uninhibited system (Eqn 5c) and is directly proportional to the total amount of the target enzyme (E_T). It is clear that cells with a higher concentration of enzyme will have their curve for metabolic resistance shifted to the right and mutant cell lines resistant to methotrexate and PAcAsp have been isolated which overproduce dihydrofolate reductase [14] and hOro synthetase [10] by 150-fold and more than 100-fold, respectively. Fig. 3 shows the effect of a 100-fold increase in hOro

synthetase upon the plot for metabolic resistance to PAcAsp. The intercepts of the asymptotes to the curves on the abscissa are 0.109 µM for normal cells and 11.5 µM for the mutant cells with a 100-fold greater concentration of the target enzyme. The slopes of the two asymptotes (A_0/K_i) are identical because the K_i for PAcAsp is unchanged for the overproduced enzyme [10] and the initial steady-state concentration of Cbm-P (A_0) is primarily a function of the ratio of carbamoyl phosphate synthetase activity to aspartate transcarbamoylase (Eqn 4 of [4]) which is unchanged for this trifunctional protein. Fig. 3 shows that the mutant cells would need to accumulate far less Cbm-P to overcome the effects of a particular PAcAsp concentration.

For a cell to gain resistance to a tight-binding inhibitor, metabolic resistance is a much simpler mechanism than overproduction of the target enzyme by gene duplication. However, for all cases of metabolic resistance, there must be an upper limit beyond which the substrate cannot accumulate. High concentrations of Cbm-P and OMP may be enzymatically degraded in some types of mammalian cells [4,20], limiting the effectiveness of metabolic resistance. Accumulation of H₂folate from an initial concentration of 0.03 µM (Table 1)
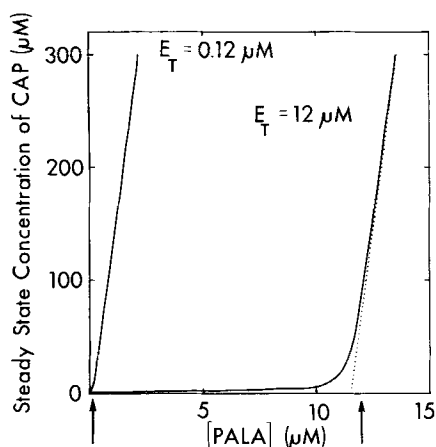
Fig. 3. *Simulated metabolic resistance to the inhibition of aspartate trans-carbamoylase by PAcAsp*. Theoretical curves were calculated from Eqn (5) using parameter values from Table 1 with $E_T = 0.12\,\mu$M for normal cells and $E_T = 12\,\mu$M for PAcAsp-resistant cells (indicated by arrows)

in response to methotrexate may be limited, at least in the short term, because the total concentration of the folate pool is only 5.5 μM [23]. When massive accumulation of the substrate is not possible, a more complex mechanism for resistance to the inhibitor, such as overproduction of the target enzyme, would then be expected to operate. This latter process has been shown to occur for the enzymes aspartate trans-carbamoylase, (hOro synthetase) [10], dihydrofolate reductase [14] and OMP decarboxylase (UMP synthase[2]) [27].

Many of the inhibitors of Table 1 are slow, tight-binding inhibitors; that is, the equilibrium between free enzyme and the enzyme-inhibitor complex is not established rapidly. This may be because only a very low concentration of inhibitor is required to produce inhibition [28]. Alternatively, the tight-binding inhibitor may resemble the transition state for the reaction and the mechanism involves the formation of a loose initial encounter complex which slowly isomerizes to a form in which the inhibitor is more tightly bound [29]. Half-times for dissociation of these enzyme-inhibitor complexes have been listed where applicable in Table 1. The effects of this slow interaction between a tight-binding inhibitor and its target enzyme upon the progress curves for substrate accumulation and residual active enzyme will be detailed in a subsequent communication (Duggleby and Christopherson, unpublished work). It should be noted that the slow dissociation of an enzyme-inhibitor complex could contribute to the potency of the drugs of Table 1 under some conditions. Once such enzyme-inhibitor complexes are formed, the accumulation of substrate will not readily displace the inhibitor, leading to the substrate accumulating to a level greater than that required to restore the uninhibited rate for freely reversible inhibition. The deoxycoformycin—adenosine-deaminase complex has an exceptionally long half-time for dissociation of 2310 min (Table 1) and preliminary simulations of this system have demonstrated an 'overshoot' in the accumulation of adenosine.

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[2] UMP synthase is the bifunctional protein found in mammals which contains orotate phosphoribosyltransferase and OMP decarboxylase

In this paper we have provided a theoretical basis for what is probably a major difficulty in the use of reversible, tight-binding inhibitors as chemotherapeutic agents. We hope that use of the data in Table 1 in conjunction with Eqns (5) and (6) will provide an additional avenue for understanding resistance to such drugs. The fact that metabolic resistance to reversible tight-binding inhibitors can occur whenever the substrate of the target enzyme can accumulate should provide an additional incentive for development of irreversible inhibitors. Such inhibitors could incorporate some of the structural features of tight-binding inhibitors but, as a result of formation of a covalent or coordination bond at the active site of the enzyme, would not be readily removed by accumulation of substrate.

REFERENCES

1. Montgomery, J. A. (1979) *Methods Cancer Res. 16*, 3−41.
2. Williams, J. W. & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437−467.
3. Segel, I. H. (1975) *Enzyme Kinetics*, p. 104, John Wiley & Sons, New York.
4. Christopherson, R. I. & Jones, M. E. (1980) *J. Biol. Chem. 255*, 11381−11395.
5. Jackson, R. C., Niethammer, D. & Hart, L. I. (1977) *Arch. Biochem. Biophys. 182*, 646−656.
6. Lockshin, A. & Danenberg, P. V. (1981) *Biochem. Pharmacol. 30*, 247−257.
7. Morrison, J. F. (1982) *Trends Biochem. Sci. 7*, 102−105.
8. McCracken, D. D. & Dorn, W. S. (1964) *Numerical Methods and FORTRAN Programming*, John Wiley & Sons, New York.
9. Chandler, J. P., Hill, D. E. & Spivey, H. O. (1972) *Comput. Biomed. Res. 5*, 515−534.
10. Kempe, T. D., Swyryd, E. A., Bruist, M. & Stark, G. R. (1976) *Cell, 9*, 541−550.
11. Hoogenraad, N. J. (1974) *Arch. Biochem. Biophys. 161*, 76−82.
12. Jones, M. E. (1980) *Annu. Rev. Biochem. 49*, 253−279.
13. Rode, W., Scanlon, K. J., Hynes, J. & Bertino, J. R. (1979) *J. Biol. Chem. 254*, 11538−11543.
14. Alt, F. W., Kellems, R. E. & Schimke, R. T. (1976) *J. Biol. Chem. 251*, 3063−3074.
15. Wiginton, D. A., Coleman, M. S. & Hutton, J. J. (1981) *Biochem. J. 195*, 389−397.
16. McClard, R. W., Black, M. J., Livingstone, L. R. & Jones, M. E. (1980) *Biochemistry, 19*, 4699−4706.
17. Coleman, P. F., Suttle, D. P. & Stark, G. R. (1977) *J. Biol. Chem. 252*, 6379−6385.
18. Levine, H. L., Brody, R. S. & Westheimer, F. H. (1980) *Biochemistry, 19*, 4993−4999.
19. Dix, O. E., Lehman, C. P., Jakubowski, A., Moyer, J. D. & Handschumacher, R. E. (1979) *Cancer Res. 39*, 4485−4490.
20. Traut, T. W. (1980) *Arch. Biochem. Biophys. 200*, 590−594.
21. Huisman, W. H. & Becker, M. A. (1980) *Anal. Biochem. 101*, 160−165.
22. Hitchings, G. H. (1973) *Adv. Enzyme Regul. 12*, 121−129.
23. Moran, R. G., Spears, C. P. & Heidelberger, C. (1979) *Proc. Natl Acad. Sci. USA, 76*, 1456−1460.
24. Moran, R. G., Werkheiser, W. C. & Zakrzewski, S. F. (1976) *J. Biol. Chem. 251*, 3569−3575.
25. Hirschhorn, R., Roegner, V., Rubinstein, A. & Papageorgiou, P. (1980) *J. Clin. Invest. 65*, 768−771.
26. Frieden, C., Kurz, L. C. & Gilbert, H. R. (1980) *Biochemistry, 19*, 5303−5309.
27. Suttle, D. P. & Stark, G. R. (1979) *J. Biol. Chem. 254*, 4602−4607.
28. Cha, S. (1975) *Biochem. Pharmacol. 24*, 2177−2185.
29. Williams, J. W., Morrison, J. F. & Duggleby, R. G. (1979) *Biochemistry, 18*, 2567−2573.

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