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 (PAcAsp) 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 PAcAsp 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: PAcAsp 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 rate of growth, 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 phenomenon we shall call ‘metabolic resistance’. Consider the simplified pathway:

\[ \overset{v}{A} \overset{k_{1}}{\rightarrow} B \overset{k_{2}}{\rightarrow} C \overset{k_{3}}{\rightarrow} \text{etc.} \]

where A, B and C are sequential intermediates of the pathway and E₁, E₂ and E₃ are the enzymes catalyzing the reactions. If A is being produced at a constant rate \( v \), inhibition of E₁ 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 E₁, leading to restoration of the original flux \( \frac{v}{v_0} \) 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:

\[ A_i = A_0 \left(1 + \frac{I}{K_i}\right) \]

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 (Eqnu 3 of Segel [3]; Eqnu 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:

$$I_T = [1 + E_T/[1 + K(1 + A_0/K_0)]]$$

(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):

$$I = K_a(A_I/A_0 - 1).$$

(4)

Substituting Eqn (4) into Eqn (3) and solving for $A_I$:

$$A_I = A_0 \left[ Q + (Q^2 + 4 K_a E_T) \right]^{1/2}\/ 2 K_1$$

(5a)

where

$$Q = K_1 + I_T - E_T$$

(5b)

and

$$E_T = E_T/(1 + A_0/K_0).$$

(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:

$$A_I = \left( A_0/K_1 \right) I_T - (A_0/K_1) (E_T - K_1)$$

(6)

which has a slope of $A_0/K_1$ and an intercept on the abscissa at $I_T = E_T - K_1$. 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 numerically 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, 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. 1 b).

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. 1 a) 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. 1 b 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. 1 b, broken line) is the expected steady-state concentrations of Cbm-P as calculated from Eqn (5).

Fig. 1 a and b illustrate two important aspects of metabolic resistance. Firstly, for a tight-binding inhibitor, a plot of $A_i$ (Cbm-P) versus $I_T$ (IPAcAsp) has an initial portion of upward curvature which becomes linear at higher values of $I_T$ (Fig. 1 b, 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. 1 a 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-Dihydropolate 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 dihydroorotate.
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 $A_0$, $k_a$, $E_f$ 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 of utilisation of Cbm-P may be calculated (Eqn 1 of [2]) provided that the substrate concentration at that time ($A$) is known. Hence the net rate of substrate accumulation is given by $dA/dt = v_0 - v_t$. Values for $A_0$ are obtained by numerical integration (see Theory) using the fact that $A_0 = A_0$ when $t = 0$. The numerical values used for the simulations were: $A_0 = 0.2 \mu M$, $k_a = 22.1 \mu M$, $E_f = 0.093 \mu M$; turnover number, $3720 \text{ min}^{-1}$; and $K_a = 6.2 \text{nM}$. 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 $\mu M$, $K_a = 6.2 \text{nM}$, $E_f = 0.093 \mu M$ (indicated by arrow) and $K_a = 22.1 \mu M$. The solid line (-----) represents the expected concentration of Cbm-P after a 40-min incubation and was calculated as described in Fig. 1a. 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 $K_a$ 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 ($0.14 \mu M$) and the dotted lines are the asymptotes calculated from Eqn (6) in inhibition is shown by zUMP with no curvature visible in the plot of $A_i$ versus $I_f$ (Fig. 2a) due to its relatively high $K_i$ value of $0.51 \mu M$. For this case, the dependence of $A_i$ upon $I_f$ is adequately described by Eqn (2) (see Theory). For PMP ($K_i = 5 \text{nM}$), there is curvature up to an inhibitor concentration of approximately $0.2 \mu M$ (Fig. 2b) after which the plot is linear. BMP has a very low $K_i$ value of $8.8 \text{ pM}$ (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 ($0.14 \mu M$, 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 ($I_f$) is sufficiently great for the plot of $A_i$ versus $I_f$ to approach a linear asymptote (cf. Fig. 2). Under these conditions, $A_i$ is a linear function of $I_f$ 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 ($A_i/I_f$) and intercept ($E_f - K_i$) from Table 1 into Eqn (6). Naturally, a more accurate simulation of metabolic resistance will be obtained if values for $E_f$, $K_i$, $K_a$, $A_0$ are
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 8.8 pM (BMP), so the slope $A_0/K_i$ 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 H$_2$folate from an initial concentration of 0.03 μM (Table 1)
been listed where applicable in Table 1. The effects of this
hibitor, leading to the substrate accumulating to a level
the enzyme-inhibitor complex is not established rapidly. This
slow interaction between a tight-binding inhibitor and its
substrate of the target enzyme can accumulate should provide
an additional incentive for development of irreversible inhibi-
tors. 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 ac-
cumulation of substrate.

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 ac-
cumulation and residual active enzyme will be detailed in a
subsequent communication (Duggleby and Christopherson,
unpublished work). It should be noted that the slow disso-
ciation of an enzyme-inhibitor complex could contribute to the
totality of the drugs of Table 1 under some conditions.

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 Eqs (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 inhibi-
tors. 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 ac-
cumulation of substrate.

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