Hydrophobic Interactions between the 5-Alkyl Group of 2,4-Diamino-6-methylpyrimidines and Dihydrofolate Reductase†

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ABSTRACT: The inhibition of purified dihydrofolate reductase from cultured Sarcoma 180 cells by 5-substituted derivatives of 2,4-diamino-6-methylpyrimidine has been studied. The 5 substitution involved the following groups: methyl, ethyl, propyl, *tert*-butyl, pentyl, hexyl, heptyl, octyl, decyl, cyclohexyl, and adamantyl. Except for 2,4-diamino-5,6-dimethyl- and 2,4-diamino-5-ethyl-6-methylpyrimidines, which did not inhibit the enzyme at concentrations of 1.0 and 0.8 mM, respectively, all other compounds were competitive inhibitors with respect to dihydrofolate. Their inhibition constants were found to vary between 0.006 and 7.5 μM. The partition of these compounds between aqueous buffer solution and *n*-heptane was also studied. The standard free energy for the transfer of each compound from the organic to the aqueous phase was calculated as a measure of their hydrophobicity and a correlation with molar volumes has been established. The hydrophobicity increases approximately proportionally with increasing molar volume. The cyclohexyl derivative does not differ in this respect from the compounds having an aliphatic chain at carbon 5 and the adamantyl derivative seems to differ only slightly. A correlation between the affinity of the analogs to the enzyme and their hydrophobicity was established. Hydrophobic binding to the enzyme was observed with the compounds which are substituted with a chain of five or more carbon atoms. In the case of smaller substituents van der Waals forces are postulated to be mainly responsible for binding to the enzyme. Both, cyclohexyl- and adamantyl-substituted pyrimidines have considerably higher affinity for the enzyme than compounds of similar hydrophobicity but having straight hydrocarbon chains at carbon 5. It is suggested that this is due to the rigidity of the structure of the cyclohexyl and adamantyl derivatives as contrasted with the conformational freedom of the straight-chain hydrocarbons.

Several 2,4-diamino-6-methylpyrimidines having saturated straight-chain, alkyl, *tert*-butyl, cyclohexyl, or adamantyl groups in the 5 position were synthesized in this laboratory as potential antifolic agents (Jonak *et al.*, 1971, 1972). All of these compounds proved to be moderate to good growth inhibitors of mammary adenocarcinoma cells (TA3) in culture. Except for 2,4-diamino-6-methyl-5-propylpyrimidine, which was a poor growth inhibitor, there was no clear relationship between the length of the alkyl chain and the inhibitory potency. The concentration for half-maximal inhibition (ID_{50}) of cell growth varied from 1.1 to 3.0 μM. However, compounds with bulky substituents at carbon 5, like cyclohexyl (C_6) or adamantyl (C_{10}), were much better inhibitors than the corresponding compounds with straight chains of 6 or 10 carbon atoms, respectively.

Recently, a study of the effect of these compounds on the inhibition of purified dihydrofolate reductase isolated from cultured Sarcoma 180 cells (strain AT 3000) has been undertaken. Hydrophobic bonding between lipophilic substituents at carbon 5 of diaminopyrimidines and dihydrofolate reductase was postulated earlier by Baker and his coworkers (Baker *et al.*, 1965; Baker and Ho, 1965, 1966). Hydrophobic bonds result from the increase of entropy which accompanies the removal of a water layer separating nonpolar molecules. Theoretical considerations indicate that partition of such molecules between aqueous and organic solvents should reflect their hydrophobicity. Indeed, a relationship between the free energy of partition and the strength of hydrophobic binding has been demonstrated in the case of aromatic hydrocarbons as inhibitors of α-chymotrypsin (Wildnauer and Canady, 1966) and acetylcholine analogs as inhibitors of acetycholinesterase (Belleau and Lacasse, 1964).

The present study describes the correlation between the affinity of 5-alkyldiaminopyrimidines for dihydrofolate reductase with their hydrophobicity, as determined from the partition coefficients of these compounds between aqueous and organic solvents. A preliminary report of this study has been presented (Zakrzewski and Ho, 1972).

Materials and Methods

Origin of the Compounds. The syntheses of 2,4-diamino-6-methylpyrimidines substituted at carbon 5 with adamantyl, cyclohexy, or straight hydrocarbon chains of 5 to 10 carbon atoms have been described previously (Jonak *et al.*, 1971, 1972). 2,4-Diamino-5,6-dimethylpyrimidine and 2,4-diamino-5-propyl-6-methylpyrimidine were synthesized by the method of Falco *et al.* (1951). 2,4-Diamino-5-ethyl-6-methylpyrimidine and 2,4-diamino-5-(*tert*-butyl)-6-methylpyrimidine are new compounds and were prepared essentially by the method used for synthesizing the methyl and propyl derivatives, *i.e.*, by condensing ethylaceto(2-ethyl)acetate or ethylaceto(*tert*-butyl)acetate, respectively, with guanidine and conversion of the 2-amino-4-hydroxypyrimidines to their 2,4-diamino derivatives by chlorination and amination at the four position. The structures were confirmed by elemental analysis and infrared spectroscopy.

Studies with Dihydrofolate Reductase from Sarcoma 180 AT/3000. The origin of the cells, preparation of the enzyme and spectrophotometric assay for dihydrofolate reductase were described previously (Ho *et al.*, 1972). The enzyme inhibition analysis was based on the transformation of the Lineweaver and Burk equations for high substrate concentration

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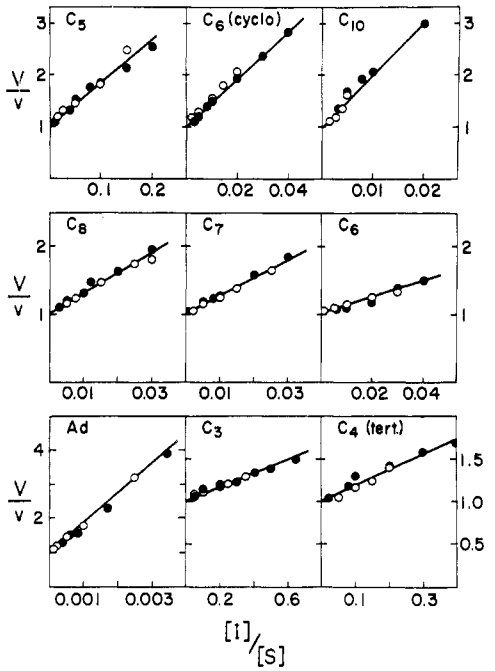


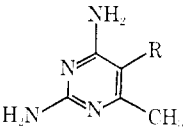
FIGURE 1: Inhibition analysis of 5-substituted 2,4-diamino-6-methylpyrimidines. V = maximal reaction velocity, v = initial velocity of the inhibited reaction, [I] and [S] inhibitor and substrate concentrations, respectively. Assay system: the enzyme activity was determined by measurement at 34° of the decrease in absorbance at 340 mμ in a Cary 14 spectrophotometer. Each cuvette contained in a total volume of 1 ml, 5 μmol of citrate buffer (pH 6.0), 0.1 μmol of NADPH, 0.1 μmol of dihydrofolate (●) or 0.2 μmol of dihydrofolate (○) the enzyme and, if required, the inhibitor. The concentration range of the inhibitor used in each experiment can be calculated from [I]/[S] values.

as described elsewhere (Ho *et al.*, 1972). In this method V/v values (maximal reaction velocity/velocity of inhibited reaction) are plotted *vs.* [I]/[S] (concentration of the inhibitor/concentration of the substrate) for two substrate concentrations. If a single line is obtained the inhibition is competitive and the slope = K_m/K_i. From K_i values standard free energies of the dissociation ($\Delta F_d°$) of the enzyme–inhibitor complex were calculated by the equation: $-\Delta F_d° = 2.3RT \log K_i$.

Determination of Partition Coefficients (K_p). Saturated solutions of the compounds to be tested were prepared by shaking the solids mechanically (Gyrotory shaker, New Brunswick Scientific Co.) with 30 ml of 50 mM sodium citrate buffer (pH 6) at a constant temperature (26, 36, 46, or 56°). The choice of buffer was dictated by the desire to maintain conditions similar to those used in the enzyme inhibition analysis. After 1 hr the solutions were filtered to remove undissolved material and absorbance at 276 mμ was determined in a Zeiss spectrophotometer. An aliquot of the filtrate (2.5 ml) was transfered into a 125-ml erlenmeyer flask and 20–100 ml of *n*-heptane was added. The flasks were than stoppered and shaken for 3 hr as above. It had been determined in preliminary experiments that equilibration of the solute between the two phases occurred after about 2 hr. The phases were separated in a separatory funnel and the absorbance of the aqueous phase at 276 mμ was again determined. All the determinations were performed in duplicate and the partition coefficient (K_p) was calculated from

$$\frac{A_a V_{org}}{(A_b - A_a)V_{aq}} = K_p$$

TABLE I: Partition Coefficients of Diaminopyrimidines and Their Affinities to Dihydrofolate Reductase.



R	K_p[b]	$\Delta F°_p$ (kcal/mol)[c]	K_i[d] (μM)	$\Delta F°_d$ (kcal/mol)[e]
CH_3	4900 ± 390	−5.24		
C_2H_5	1800 ± 200	−4.88		
C_3H_7	720 ± 71	−4.06	7.5	+7.3
C_4H_9 (ter)[a]	410 ± 55	−3.71	3.2	+7.8
C_6H_{11} (cyclo)	270 ± 63	−3.45	0.13	+9.8
C_5H_{11}	240 ± 48	−3.40	0.72	+8.8
C_6H_{13}	75 ± 10	−2.67	0.48	+9.0
$C_{10}H_{15}$ (Ad)	17 ± 1.5	−1.75	0.006	+11.7
C_7H_{15}	13 ± 1	−1.58	0.22	+9.5
C_8H_{16}	3.9 ± 0.45	−0.84	0.19	+9.6
$C_{10}H_{21}$	1.5 ± 0.22	−0.26	0.06	+10.2

[a] ter = tertiary, cyclo = cyclohexyl, Ad = adamantyl. [b] K_p = partition coefficient (36°). [c] $\Delta F°_p$ = free energy of partition. [d] K_i = inhibition constant (34°). [e] $\Delta F°_d$ = free energy of dissociation of enzyme–inhibitor complex.

where A_a = absorbance after partition, A_b = absorbance before partition, V_{org} = volume of organic phase, V_{aq} = volume of aqueous phase.

K_p as determined by this equation represents the equilibrium constant for the transfer of a compound from an organic to an aqueous phase. This is important to keep in mind when the energetics of this process are considered. Corresponding standard free energies ($\Delta F°_p$) were calculated by the equation: $-\Delta F°_p = 2.3RT \log K_p$.

Results and Discussion

The effect of the compounds listed in Table I on purified dihydrofolate reductase was studied at two concentrations of dihydrofolate at 34°. The plots of V/v *vs.* [I]/[S] are presented in Figure 1. Since the V/v values fall along a single line for two different substrate concentrations the inhibition is in each case competitive. The calculated K_i values are presented in Table I. Since 2,4-diamino-5,6-dimethyl- and 2,4-diamino-5-ethyl-6-methylpyrimidine did not inhibit the enzyme at the limit of their solubility the energetics of binding of these two compounds to dihydrofolate reductase will not be discussed at this time.

In Table I the compounds tested are arranged in the order of increasing hydrophobicity (decrease in the negative value of $\Delta F°_p$). Hydrophobicity increases with increasing alkyl-chain length. Compounds with bulky substituents (cyclohexyl and adamantyl) behave in this respect like pentyl and heptyl derivatives. The affinity for dihydrofolate reductase follows basically the same pattern as hydrophobicity, *i.e.*, the affinity increases (the positive free energy of dissociation ($\Delta F°_d$) increases) with increasing chain length. Cyclohexyl and adamantyl derivatives, however, do not conform to this pattern, their affinities to the enzyme being unusually high. To obtain

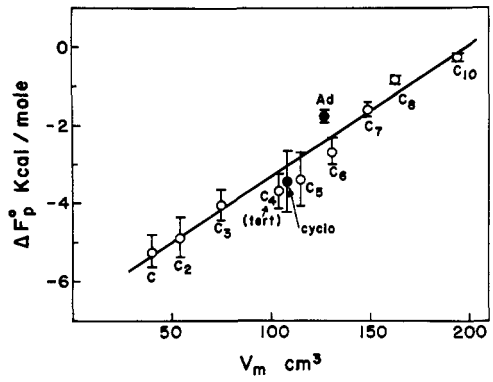


FIGURE 2: Relationship of the free energy of partition (ΔF°_{p}) to the molar volume (V_m) of the substituents at carbon 5 of the pyrimidines. Vertical lines with cross bars indicate standard deviation.

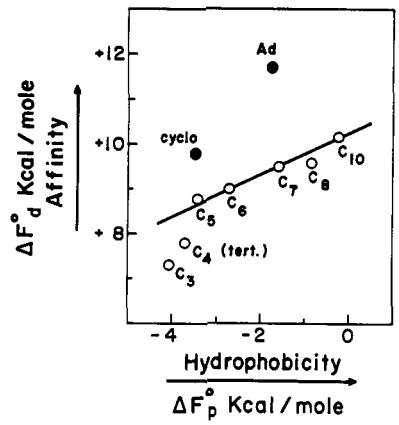


FIGURE 3: Relationship of the free energy of dissociation of the enzyme–inhibitor complex ΔF°_{d} to the free energy of partition ΔF°_{p} of some 2,4-diaminopyrimidines.

a more quantitative relationship between the energy required for the transfer of an analog from the organic to the aqueous phase and the size of the analog, ΔF°_{p} values were plotted *vs.* molar volume (V_m) of the substituents (Figure 2). The molar volumes were obtained by dividing the molecular weight by the density of the substituent. For most substituents, which are liquids at room temperature, the densities at 20° are reported in the literature. For methane, ethane and propane the densities are reported at −164, −108, and −45°, respectively, whereas adamantane is a solid at room temperature. Thus, the comparison of their molar volumes with those of the other hydrocarbons is not quite accurate. Figure 2 indicates that there is an approximately proportional increase in hydrophobicity with increasing molar volume of the analogs. The slope of the line represents the average increase of hydrophobicity per increase of molar volume of the substituent. The value of 35 cal/mol per cm^3 or an average of 550–600 cal/mol per CH_2 group has been found. This is close to the maximal strength of hydrophobic bonding estimated by Nemethy and Scheraga (1962). The cyclohexyl derivative does not differ in this respect from straight-chain hydrocarbons. The adamantyl derivative, however, has a slightly higher hydrophobicity than would be expected from its volume. Whether this difference is real or a result of the inaccuracies discussed above is not certain.

In Figure 3, the free energies of dissociation (ΔF°_{d}) of the enzyme–inhibitor complexes were plotted against the free energies of partition (ΔF°_{p}). For straight-chain hydrocarbons with 5 carbons or more the affinity of the analogs to the enzyme (increasing ΔF°_{d}) increased proportionally with the increase in hydrophobicity. This strongly indicates that hydrophobic bonds participate in the binding of these inhibitors to dihydrofolate reductase. The slope of the line is 0.5 indicating that only half of the hydrophobic energy available is utilized for binding. This may be due to the fact that a specific conformation of the alkyl chain is required for its binding to the enzyme. In contrast the affinity to the enzyme between propyl and pentyl derivatives increases in average 2.3 times more than hydrophobicity indicating that other forces in addition to hydrophobicity contribute significantly to the complex formation with the enzyme. Since the average increase in hydrophobicity between propyl and pentyl derivatives is 330 cal/mol per CH_2 group (Table I), it follows that the nonhydrophobic contribution to the binding for one CH_2 group would amount to 430 cal. Such an amount can be accounted for by van der Waals forces (Baker, 1967). It is of interest that both the cyclohexyl and adamantyl derivatives do not conform to the pattern of the other hydrocarbon derivatives of similar size. Their unusually strong binding to dihydrofolate reductase may be explained by the rigidity of the structure of these compounds which is in sharp contrast to the flexibility of straight-chain hydrocarbons.

This work provided evidence for hydrophobic interactions between 2,4-diamino-6-methylpyrimidines substituted at position 5 with alkyl chains of five or more carbon atoms. Shorter chains increase the affinity of 2,4-diaminopyrimidines for dihydrofolate reductase probably due to van der Waals interactions. Pyrimidines with rigid substituents at carbon 5 have higher affinity for the reductase than those with hydrocarbon chains of comparable molar volume.

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