Inhibition of Dihydrofolate Reductase. 3. 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(2-substituted-phenyl)-*s*-triazine Inhibition of Bovine Liver and Mouse Tumor Enzymes[1,2]

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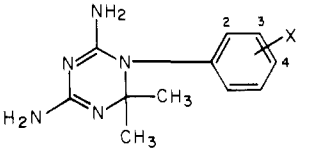
Inhibition of dihydrofolate reductase from bovine liver and murine L5178YR-C_3 tumor cells has been examined for a series of 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(2-X-phenyl)-*s*-triazines. For both enzymes all 2-X substituents cause a decrease in inhibitory activity relative to X = H, with the notable exeption of X = SH which is 7.4–12 times as active as X = H. Although there is a high correlation between the activities of these compounds vs. these two enzymes, significant deviations from this correlation for three of the triazines (X = CF_3, CH_2CN, and Cl) suggest that (a) there may exist significant differences in the two enzymes and their interactions with these triazines and (b) exploitation of such differences might allow for the selective inhibition of enzyme from tumor cells.

In the search for better anticancer drugs, the most important consideration is to find agents with greater selective toxicity for tumor cells. While there are now many molecules with well demonstrated antitumor activity, these compounds are, for the most part, highly toxic to normal cells as well as being carcinogenic. In order to circumvent the carcinogenicity problem, we have elected to study enzyme inhibition as a potential source of anticancer compounds rather than to explore DNA perturbation. We decided to study dihydrofolate reductase (DHFR) with a view to gaining a deeper understanding of the structure–selectivity relationship of various inhibitors vs. enzymes from different sources. Our hope is that differences between enzyme from normal host cells and enzyme from tumor cells can be found and exploited so that selectivity can be established at the molecular level.

DHFR from different species has been demonstrated[4] to show quite different inhibition profiles and responses for various inhibitors. In addition, DHFR isoenzymes with differing responses to the same inhibitor have been observed for certain species. Nixon and Blakley[5] have found two DHFR isozymes from a single methotrexate-resistant *Streptococcus faecium* strain; a "wild type" and a "mutant type" were isolated and shown to exhibit different responses to several inhibitors. These facts have encouraged us to believe that if any enzyme might show significant differences when enzyme from normal tissue is compared with enzyme from tumor, DHFR might do so.

Failure to uncover selectivity at the enzyme level does not necessarily mean that better antitumor drugs cannot be developed. Methotrexate, a highly potent but relatively unselective inhibitor of DHFR, is still widely used in cancer chemotherapy. Baker's antifol [I, where X = 3-Cl, 4-$OCH_2C_6H_4$-3-$CON(CH_3)_2$] is also a potent inhibitor of DHFR and is now in clinical trials.

We have been pleased to find in preliminary studies using mammalian and bacterial enzymes that the great differences in response to derivatives I are interpretable



I

via quantitative structure–activity relationships (QSAR).[2,6-9]

In the present report we compare the action of inhibitors of type I (X = 2-substituents) on DHFR from bovine liver and mouse L5178YR-C_3 tumor. Although it would have been desirable to compare enzyme from murine tumor with enzyme from normal murine cells, for matters of convenience in this initial study we have used bovine liver enzyme as a model for DHFR from normal mammalian cells. In earlier studies[8,9] of inhibitors of type I acting on mammalian DHFR, we established via QSAR that the 3-substituents of I appear to bind in a hydrophobic pocket of the enzyme. Such an area, made up of mostly apolar amino acid residues, seemed to be a poor region in which to look for highly specific interactions which might vary from isozyme to isozyme. Substituents in the 4 position of I (at least relatively small groups) also seemed to be falling in enzymatic hydrophobic space, although the evidence is not as clear as for 3-substituents.[9] Although B. R. Baker had tested a number of 2-substituted I, the substituents were mostly apolar and we were unable to distinguish any type of substituent effect (i.e., hydrophobic, electronic, etc.).

Baker's data did show that all 2-substituents greatly depress the inhibitory power of I (see Table I); hence, with little or no knowledge of the character of the enzymatic space into which 2-substituents fit, we selected the compounds in Table I because they were relatively easy to synthesize and, as a data set, these 2-substituents are reasonably orthogonal with respect to π, MR, and σ. Also, we felt it better to do the preliminary work with small substituents, as well as groups which contained some

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Table I. Inhibitory Activities of Triazines (I) vs. Bovine Liver and Murine Tumor Dihydrofolate Reductase

		log (1/C)[a]			
		bovine liver DHFR[b]		murine L5178YR-C_3	Walker 256
no.	X	pH 6.25	pH 7.20	DHFR:[b] pH 7.20	tumor DHFR[c]
1	2-CF_3	3.15 ± 0.03	3.53 ± 0.03	3.03 ± 0.08	
2	2-I	3.43 ± 0.02	3.75 ± 0.04	2.49 ± 0.05	4.62
3	2-SCH_3	3.54 ± 0.02	3.91 ± 0.06	2.48 ± 0.05	
4	2-OCH_3	3.56 ± 0.04	4.04 ± 0.03	3.11 ± 0.05	3.68
5	2-OH	3.86 ± 0.02	4.18 ± 0.04	2.92 ± 0.05	
6	2-CH_2CN	4.02 ± 0.03	4.27 ± 0.03	2.73 ± 0.05	
7	2-C_2H_5	4.02 ± 0.04	4.48 ± 0.04	3.38 ± 0.04	
8	2-NH_2	4.11 ± 0.03	4.58 ± 0.02	3.41 ± 0.05	
9	2-Cl	4.86 ± 0.02	5.19 ± 0.04	4.72 ± 0.03	4.15
10	2-CH_3	4.62 ± 0.03	5.23 ± 0.02	4.44 ± 0.04	4.00
11	2-F	4.86 ± 0.07	5.41 ± 0.05	4.39 ± 0.05	4.74
12	H	6.33 ± 0.06[d]	6.74 ± 0.05	5.78 ± 0.04	6.92
13	2-SH	7.20 ± 0.03	7.69 ± 0.02	6.86 ± 0.06	

[a] $C = K_{i(app)} = I_{50}$; uncertainty units are 95% confidence limits; see Experimental Section. [b] This study, unless otherwise noted. [c] B. R. Baker's data; see ref 9. [d] Data from ref 8.

strong hydrogen-bonding capabilities.

Results and Discussion

With one notable exception (2-SH), we found, as did Baker, that all 2-substituents lower activity considerably compared to the unsubstituted parent compound; however, with both the bovine and murine enzymes, the 2-SH compound is dramatically more active than the parent molecule (by a factor of 7.4–12). This is especially surprising, since SH is rather large and easily polarizable. MR (molar refractivity[10]) for SH is 9.2, while MR for CH_3 = 5.6, Cl = 6.0, F = 0.9, and CF_3 = 5.0; nevertheless, all of these smaller, less polarizable groups are depressing on inhibitory potency when placed in the 2 position of I. Obviously, SH has an unusual mode of binding which warrants further study. It is surprising that a group as small as F has such a pronounced affect on activity. It is hard to attribute this to anything other than an electronic effect. The variation in the physicochemical parameters of the substituents in Table I is so diverse that attempts to formulate QSAR's for these activities have so far yielded unsatisfactory results. In addition, there is the problem of both inter- and intramolecular steric effects; hence, we prefer to withhold comment on the QSAR until considerably more data on 2-substituents have been obtained.

Comparing the different activities of Table I with each other via regression equations is valuable. Equation 1

$$\log (1/C)_{7.2} = 1.03\ (\pm 0.05) \log (1/C)_{6.25} + 0.29\ (\pm 0.25) \quad (1)$$

$$n = 13;\ r = 0.997;\ s = 0.102$$

compared bovine enzyme tested at pH 6.25 with enzyme tested at pH 7.2. The slope of 1 and the high correlation show that the structure–activity relationships (SAR) are independent of pH, although the bovine enzyme is, on the average, about twice as active (intercept = 0.3) at the higher pH. Triazines of type I have pK_a values of about 2.2 and 11.2[11] and, thus, will be completely monoprotonated at either pH 6.25 or 7.20. The increased log (1/C) values observed upon increasing the pH from 6.25 to 7.20 are therefore most likely the result of slight changes in the enzyme and, hence, its ability to interact with substrate, cofactor, and inhibitors due to the pH and/or buffer and ionic strength changes. Assuming that the standard deviation is largely due to experimental error provides an idea of how large a deviation from a regression equation one would need before one could say a substituent effect represents a structural difference between two enzyme preparations. It is noteworthy that the log (1/C) values at pH 6.25 and at 7.2 for inhibition of the bovine liver DHFR were obtained by two different workers.

Table II. Correlation of Activities of Triazines (I) vs. Murine Tumor and Bovine Liver Dihydrofolate Reductases

		murine L5178YR-C_3 DHFR: log (1/C)		
no.	X	obsd	calcd[a]	|Δ log (1/C)|
1	2-CF_3	3.03	2.41	0.62
2	2-I	2.49	2.65	0.16
3	2-SCH_3	2.48	2.82	0.34
4	2-OCH_3	3.11	2.96	0.15
5	2-OH	2.92	3.11	0.19
6	2-CH_2CN	2.73	3.21	0.48
7	2-C_2H_5	3.38	3.43	0.05
8	2-NH_2	3.41	3.54	0.13
9	2-Cl	4.72	4.20	0.52
10	2-CH_3	4.44	4.24	0.20
11	2-F	4.39	4.43	0.04
12	H	5.78	5.86	0.08
13	2-SH	6.86	6.89	0.03

[a] Calculated using eq 2.

Comparing bovine liver and murine tumor enzyme via eq 2, we do see some interesting effects from our substituent probes which suggest significant structural differences in these two enzymes (see Table II). While the slope of eq 2 is essentially 1, the intercept of −1.39 brings to light the fact that, on the average, the murine enzyme preparation is about 25 times less easily inhibited than the bovine. There are three substituents in Table II that do not fit eq 2 well: CF_3, CH_2CN, and Cl. If these three points are dropped and the remaining ones refit, one obtains eq 3. The deviations of CF_3, CH_2CN, and Cl from

$$\log (1/C)_{\text{murine},7.2} = 1.08\ (\pm 0.17) \log (1/C)_{\text{bovine},7.2} - 1.39\ (\pm 0.84) \quad (2)$$

$$n = 13;\ r = 0.973;\ s = 0.324$$

$$\log (1/C)_{\text{murine},7.2} = 1.11\ (\pm 0.10) \log (1/C)_{\text{bovine},7.2} - 1.61\ (\pm 0.49) \quad (3)$$

$$n = 10;\ r = 0.995;\ s = 0.161$$

eq 3 are, respectively, 0.73, 0.39, and 0.58. The next most

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Table III. Triazine Inhibitors (I)

no.	X	mp, °C[a] obsd	mp, °C[a] lit.	% yield	formula[b]
1	2-CF_3	227-228.5		25	$C_{12}H_{14}F_3N_5 \cdot HCl$
2	2-I	207.5-209	224-228[c]	69	$C_{11}H_{14}I_1N_5 \cdot HCl$
3	2-SCH_3	205-208		74	$C_{12}H_{17}N_5S_1 \cdot HCl$
4	2-OCH_3	198-201	226-229[d]	69	$C_{12}H_{17}N_5O_1 \cdot HCl$
5	2-OH	207.5-209		81	$C_{11}H_{15}N_5O_1 \cdot HCl$
6	2-CH_2CN	198-199.5		50	$C_{13}H_{16}N_6 \cdot HCl$
7	2-C_2H_5	198-199	209-211[d]	51	$C_{13}H_{19}N_5 \cdot HCl$
8	2-NH_2	277-278		85[e]	$C_{11}H_{16}N_6 \cdot HCl$
9	2-Cl	222-225[f]	217-222[g]	38	$C_{11}H_{14}Cl_1N_5 \cdot HCl$
10	2-CH_3	223-224	224-226[g]	37	$C_{12}H_{17}N_5 \cdot HCl$
11	2-F	201-204	220-225[c]	64	$C_{11}H_{14}F_1N_5 \cdot HCl$
12	H	203-206[f,h]			
13	2-SH	267-269[f]		35	$C_{11}H_{15}N_5S_1 \cdot HCl$
14	2-C_6H_5	202.5-204.5		36	$C_{17}H_{19}N_5 \cdot HCl$
15	2,3-Cl_2	211-214[f]	213-218[c]	71	$C_{11}H_{13}N_5Cl_2 \cdot HCl$
16	2,5-Cl_2	194-197	186-196[i]	19	$C_{11}H_{13}Cl_2N_5 \cdot HCl$
17	2-Cl, 5-CF_3	192.5-194.5[j]		14	$C_{12}H_{13}Cl_1F_3N_5 \cdot HCl$

[a] Recrystallized from EtOH-H_2O, unless otherwise indicated. [b] Analyzed for C and H. [c] Reference 19. [d] Reference 11. [e] Yield is from 2-nitro compound. [f] Recrystallized from H_2O. [g] Reference 20. [h] From ref 8. [i] Reference 13. [j] Recrystallized from $(CH_3)_2CO$-H_2O.

deviant group is CH_3 (0.26).

For the two enzymes studied in this report there are differences that can be brought out by means of molecular probes. It is not clear, however, whether these are due to species differences (i.e., mouse vs. cow) or to normal vs. tumor enzyme differences. In this connection, it is of interest to note that Barfknecht et al.[12] have shown that thymidylate synthetase from calf thymus is not irreversibly inhibited by 5-[(iodoacetamido)methyl]-2′-deoxyuridine 5′-phosphate, while enzyme from *ascites* tumor is. No follow-up on this has been made to show whether or not this is a species difference.

Work under way testing 3-substituted I on bovine liver and murine tumor enzyme indicates very little, if any, difference in the interaction with a series of inhibitors. This leads us to believe that the 2 position of I is a more promising position for molecular modification in the search for inhibitors with specificity against isozymes of DHFR.

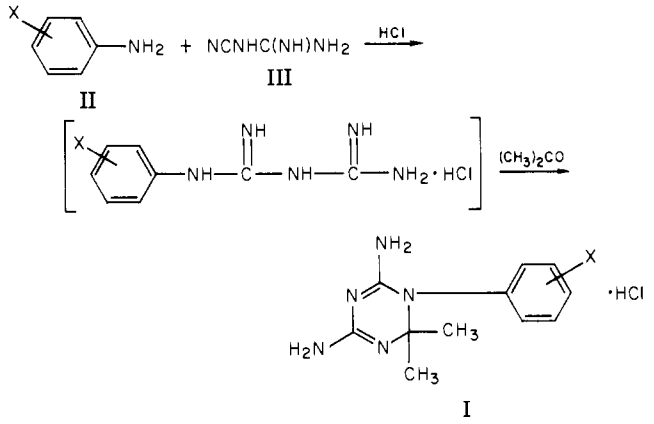
Log $(1/C)$ values are not reported for several of the inhibitors of Table III. The 2-C_6H_5 analogue was so poorly soluble and so weak as an inhibitor that its activity could not be measured. The three disubstituted compounds (2,3-Cl_2, 2,5-Cl_2, and 2-Cl, 5-CF_3) apparently are involved in such a slow DHFR/NADPH/FAH_2/inhibitor equilibrium that reliable estimation of log $(1/C)$ values for these compounds was not possible by these assay methods (see "Inhibition Assays" under Experimental Section). Further study of these inhibitors is underway.

Experimental Section

Synthesis of Triazine Inhibitors. The 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(X-phenyl)-*s*-triazine hydrochloride salts (I) were prepared by the three-component synthetic method of Modest[13] (as in our previous study[8]) from the appropriate X-substituted anilines (II), dicyandiamide (III), acetone and HCl; see Scheme I and Table III. The necessary anilines (II) were obtained commercially. 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(2-aminophenyl)-*s*-triazine hydrochloride salt was obtained from the corresponding nitro compound by catalytic hydrogenation (Parr hydrogenation apparatus) using 5% Pd/C in methanol at room temperature.

Melting points were determined on a Buchi melting point apparatus and are uncorrected. Microanalyses were performed by C.F. Geiger, Ontario, Calif., or Galbraith Laboratories, Inc., Knoxville, Tenn., and are within ±0.4% of the theoretical values.

Scheme I



I

Inhibition Assays. Assays were performed as described in our previous studies:[2,8] 1.40×10^{-5} M dihydrofolic acid (FAH_2) and 1.00×10^{-4} M NADPH in the final assay solution at 25 °C using either 100 mM phosphate buffer, pH 6.25, and 50 mM in 2-mercaptoethanol or 50 mM Tris-HCl buffer, pH 7.20, 150 mM KCl and 50 mM in 2-mercaptoethanol. Bovine liver DHFR was obtained from Sigma Chemical Co., as previously described.[8] The murine tumor DHFR (from the mouse L5178YR-C_3 clone, a gene-amplified lymphoblastoid tumor cell line)[14] was stored initially at −20 °C at a concentration of 2.65 mg/mL in 1 M Tris-HCl buffer, pH 8.5, 1 mM FAH_2, 14.4 mM 2-mercaptoethanol. A vial containing 100 μL of this solution was unfrozen and quickly diluted at 0–5 °C with 50 mM Tris-HCl buffer, pH 7.5, 150 mM KCl, 14 mM 2-mercaptoethanol to 3.00 mL; vials containing 100 μL of this diluted DHFR solution were stored at −20 °C. For assaying, one of these vials was thawed and then stored at 0–5 °C until used up (usually the same day). With a 2-cm cell path length, sufficient enzyme was used such that the final assay solution with no inhibitor present would give a 0.05–0.10 or 0.025–0.05 change in absorbance/minute for the bovine and murine enzymes, respectively. All inhibitor samples were dissolved in and diluted with the appropriate assay buffer.

As discussed in our previous study[2] and in a number of earlier papers,[15-17] some inhibitors of DHFR act by a mechanism for

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which equilibrium between enzyme and subtrate is rapidly attained, while equilibrium between the enzyme and inhibitor (tightly or weakly binding) is slow. All of the compounds in this study (for bovine enzyme at both pH values and for murine enzyme at pH 7.20) were assayed at at least eight inhibitor concentrations (each in quadruplicate) by assay procedure 1 of ref 2: i.e., the reaction was initiated by mixing a solution of inhibitor (if any) and FAH_2 with a solution of enzyme which had been preincubated with NADPH. With this assay procedure, initial reaction velocities were found to decrease as the reaction proceeds, leveling off at a constant velocity within about 5–30 s. It was assumed that this observed effect was a result of the slow (relative to the time scale of the assay) attainment of a final equilibrium between enzyme, FAH_2, NADPH, and inhibitor. V_i (reaction velocities in presence of I) were then taken once this equilibrium had been established. V_0 (initial reaction velocity in absence of inhibitor) was linear from the start of the assay. All compounds were also assayed (for both enzymes at pH 7.20) at at least one concentration (each in quadruplicate) by assay procedure 2 of ref 2: i.e., the reaction was initiated by mixing a solution of FAH_2 with a solution of enzyme which had been preincubated with NADPH and inhibitor (if any). With this assay procedure, initial reaction velocities were found to increase as the reaction proceeds, leveling off at a constant velocity within about 5–30 s. V_i values were, as above, taken once equilibrium appeared to be established. Each compound (for both enzymes at pH 7.20) exhibited equal activities when assayed by these two procedures, thereby confirming that sufficient time was being allowed for equilibrium to be established.

As in our previous studies,[2,6-8] we have assumed that, under our assay conditions (i.e., saturating NADPH concentration), the triazine inhibitors of type I are acting as competitive inhibitors for FAH_2; hence, the log $(1/C)$ values were calcuated as log $[1/K_{i(app)}]$ values as in our previous study,[2] utilizing the jackknife procedure.[18] These $K_{i(app)}$ values are, for this study, equal to I_{50}, the concentration of inhibitor necessary for 50% inhibition of the enzyme, since the enzyme concentration was negligible compared to $K_{i(app)}$ (as determined by methotrexate titration of the enzymes; see ref 2).

No attempt was made in this study to further analyze the kinetics of the slow DHFR/NADPH/FAH_2/inhibitor equilibrium or how the structural features of the 2-substituents of I influence this equilibrium (as has been done with other inhibitors of DHFR[15]). As mentioned above, for the three disubstituted compounds (15–17, Table III), the rate of attainment of equilibrium was too slow (compared to the time scale of the assay) to permit reliable estimation of their log $[1/K_{i(app)}]$ values at this point. In this study we did observe, however, that the rate of approach to equilibrium does appear qualitatively to be inversely related to the bulk and/or number of X substitutents of I (e.g., especially the disubstituted compounds just mentioned).

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Synthesis and Antiallergy Activity of 4-Oxopyrano[3,2-*b*]indoles

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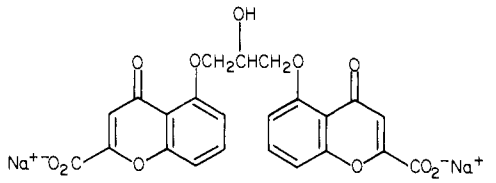
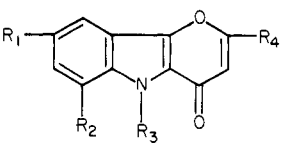
A series of 4-oxopyrano[3,2-*b*]indole carboxylic acids, tetrazoles, and carboxamidotetrazoles has been prepared and tested for antiallergy potential in the rat passive cutaneous anaphylaxis (PCA) assay. Many of the compounds showed activity comparable or superior to that of cromolyn sodium or doxantrazole. Several compounds were orally active.

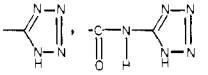
Allergic reactions initiated by antigen–antibody interactions are thought to result from the release of mediators [histamine, slow-reacting substance of anaphylaxis (SRS-A), and others] of immediate hypersensitivity. The antiallergy agent cromolyn sodium (1) appears to act by inhibition of mediator release.[2]

Numerous research groups have reported antiallergy properties for additional compounds in a variety of chemical classes.[3] In some instances, a common structural feature is the presence of an acidic function located on, or in conjugation with, an aromatic or heteroaromatic ring.



1



2, R_4 = CO_2H, 

A novel chemical series of this type is represented by acidic 4-oxopyrano[3,2-*b*]indoles, **2**.

We now report preliminary results concerning the preparation and antiallergic activity of a series of compounds of general structure **2**. Many of these compounds show high potency in the standard rat passive cutaneous

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