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A Comparison of the Inhibition of Bovine and Murine Leukemia Dihydrofolate Reductase by 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(3-X-phenyl)-s-Triazines

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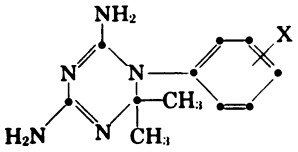
SUMMARY

A comparison has been made of the inhibition of two mammalian dihydrofolate reductases, one from bovine liver and the other from a murine tumor (L5178 YR-C_3), by 40 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(3-X-phenyl)-*s*-triazines. The K_i values obtained were used to formulate quantitative structure-activity relationships. The 3-X substituents were found to bind in a hydrophobic pocket of the enzyme. Binding was well correlated by the hydrophobic parameter π up to π_0 of 1.6–1.7. Distinct differences were found in the inhibition constants for the two different enzymes. However, only one substituent not large enough to extend beyond the hydrophobic pocket showed selectivity. Those substituents, whose π values were $\geq$1.66, showed no selectivity. These results confirm the hypothesis of Baker [*Design of Active-Site-Directed Irreversible Enzyme Inhibitors.* Wiley, New York (1967)] that one should not search for selective inhibitors by making variations on that part of a parent molecule binding in hydrophobic space.

INTRODUCTION

We recently made a comparison (1) of the inhibitory potency of Congeners I with 2-X substitution on bovine liver and murine (L5178YR-C_3) leukemia DHFR.[4]



CONGENERS I

This was an exploratory study to determine whether significant differences could be found in the interactions of Congeners I with DHFR from different sources. Because of its availability, bovine DHFR was chosen as a representative enzyme from normal mammalian tissue. Gene-amplified DHFR from tumor cells was chosen as a representative enzyme from cancerous mammalian tissue. Thirteen variations of Congeners I with substituents only in position 2 were tested on both enzymes. Three substituents (2-CF_3, 2-Cl, and 2-CH_2CN) were 4- to 6-fold more inhibitory against the bovine enzyme than against murine tumor enzyme. The reason for concentrating on the 2-substitution of Congeners I in this first study was that earlier work (2) with triazine inhibitors had indicated that the enzyme region contacted by groups in this position did not appear to be typically hydrophobic. If not hydrophobic, then one would assume that this space was composed of polar amino acid residues. It seems likely that in such a region one might be able to uncover more specific interactions with polar and hydrogen-bonding substituents which would vary with DHFR from different sources. Although the differences which we uncovered in these two mammalian enzymes are not spectacular, they do lend support to our hypothesis, as well as suggest that there may be significant differences between DHFR from normal and tumor tissue.

The converse of the above thinking is that one might expect relatively small differences in the interaction of ligand substituents with a hydrophobic pocket in isozymes or the same enzyme from different sources. In order to increase our understanding of structural parameters in enzyme inhibitors for drug development in cancer chemotherapy, we now report on the inhibition of the

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[4] The abbreviations used are: DHFR, dihydrofolate reductase; DMF, dimethylformamide; QSAR, quantitative structure-activity relationships.

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TABLE 1

Parameters used for deriving equations correlating the inhibition of DHFR by I

No.	X	Log $1/K_{i_{app}}$							$\pi'3$	σ	I
		Bovine			|Δ Log $1/K_i$|	Murine L5178YR-C_3					
		pH 6.25 Observed	pH 7.20 Observed	pH 7.20 Calculated		pH 7.20 Observed	Calculated	|Δ Log $1/K_i$|			
1	3-SO_2NH_2	4.69 (4.65–4.74)	5.43 (5.41–5.46)	5.31	0.12	4.66 (4.62–4.70)	4.44	0.22	−1.82	0.46	0
2	3-$CONH_2$	5.07 (5.01–5.12)	5.70 (5.68–5.72)	5.66	0.04	4.74 (4.70–4.78)	4.67	0.07	−1.49	0.28	0
3	H	6.33 (6.27–6.38)	6.81 (6.77–6.85)	7.17	0.36	5.78 (5.74–5.82)	6.10	0.32	0.00	0.00	0
4	3-$COCH_3$	6.19 (6.13–6.24)	6.25 (6.24–6.27)	6.65	0.40	5.42 (5.37–5.46)	5.85	0.43	−0.55	0.38	0
5	3-CH_3	7.10 (7.05–7.15)	7.74 (7.72–7.76)	7.57	0.17	6.84 (6.80–6.88)	6.51	0.33	0.57	−0.07	0
6	3-OCH_3	6.54 (6.49–6.58)	6.89 (6.87–6.91)	7.15	0.26	6.02 (6.00–6.05)	6.19	0.17	−0.02	0.12	0
7	3-OH	5.75 (5.73–5.78)	6.38 (6.37–6.39)	6.52	0.14	5.36 (5.33–5.39)	5.48	0.12	−0.67	0.12	0
8	3-$C(CH_3)_3$	6.87 (6.81–6.92)	7.10 (7.09–7.12)	7.80	0.70	6.38 (6.34–6.42)	6.74	0.36	1.98	−0.10	0
9	3-$COOC_2H_5$	5.74 (5.64–5.82)	5.73 (5.72–5.75)	7.54	1.81	5.06 (5.03–5.10)	6.87	1.81	0.51	0.37	0
10	3-F	6.82 (6.78–6.86)	7.61 (7.58–7.64)	7.28	0.33	6.70 (6.66–6.74)	6.54	0.16	0.14	0.34	0
11	3-CF_3	7.10 (7.05–7.14)	7.60 (7.58–7.63)	7.71	0.11	7.09 (7.02–7.17)	7.12	0.03	0.88	0.43	0
12	3-Cl	7.10 (7.08–7.12)	7.87 (7.85–7.90)	7.64	0.23	7.30 (7.24–7.36)	6.99	0.31	0.71	0.37	0
13	3-NO_2	6.58 (6.55–6.61)	7.35 (7.34–7.37)	6.91	0.44	6.40 (6.35–6.46)	6.45	0.05	−0.28	0.71	0
14	3-CN	7.06 (7.02–7.10)	7.54 (7.52–7.55)	6.63	0.91	6.96 (6.91–7.00)	5.99	0.97	−0.57	0.56	0
15	3-Br	7.22 (7.20–7.24)	7.83 (7.78–7.88)	7.70	0.13	7.32 (7.28–7.37)	7.08	0.24	0.86	0.39	0
16	3-I	7.13 (7.04–7.20)	7.73 (7.71–7.74)	7.77	0.04	7.22 (7.17–7.26)	7.13	0.09	1.12	0.35	0
17	3-$O(CH_2)_2OC_6H_5$	7.35 (7.25–7.43)	8.17 (8.13–8.20)	7.81	0.36	6.75 (6.71–6.80)	6.96	0.21	1.68	0.12	0
18	3-$O(CH_2)_2OC_6H_4$-3′-CF_3	7.45 (7.40–7.50)	8.19 (8.17–8.21)	7.74	0.44	7.03 (7.00–7.06)	6.85	0.18	2.56	0.12	0
19	3-$OCH_2C_6H_5$	7.14 (7.04–7.23)	7.65 (7.61–7.68)	7.81	0.16	6.77 (6.71–6.82)	6.96	0.19	1.66	0.12	0
20	3-$O(CH_2)_3CH_3$	6.99 (6.96–7.02)	7.01 (6.98–7.05)	7.81	0.80	6.33 (6.29–6.36)	6.96	0.63	1.59	0.12	0
21	3-$OCH_2C_6H_3$-3′,4′-Cl_2	7.41 (7.38–7.45)	7.88 (7.87–7.90)	7.71	0.17	6.41 (6.36–6.47)	6.79	0.38	2.91	0.12	0
22	3-$O(CH_2)_{11}CH_3$	6.66 (6.60–6.71)	7.55 (7.49–7.62)	7.35	0.20	6.06 (6.03–6.09)	6.24	0.18	5.91	0.12	0
23	3-$O(CH_2)_8CH_3$	6.55 (6.47–6.62)	7.43 (7.40–7.46)	7.55	0.11	6.70 (5.61–6.79)	6.54	0.16	4.29	0.12	0
24	3-$CH_2NHC_6H_3$-3′,5′-$(CONH_2)_2$	6.71 (6.67–6.75)	7.74 (7.67–7.81)	7.74	0.00	6.84 (6.81–6.86)	6.78	0.06	1.00	0.00	0
25	3-$O(CH_2)_4OC_6H_5$	7.28 (7.26–7.31)	7.96 (7.92–8.01)	7.96	7.73	6.80 (6.74–6.87)	6.83	0.03	2.71	0.12	0
26	3-$O(CH_2)_4OC_6H_4$-3′-CF_3	6.90 (6.81–6.98)	7.32 (7.28–7.37)	7.63	0.31	6.83 (6.81–6.86)	6.67	0.16	3.59	0.12	0
27	3-$(CH_2)_5CH_3$	6.91 (6.87–6.95)	7.35 (7.28–7.42)	7.68	0.33	7.13 (7.08–7.19)	6.56	0.57	3.21	−0.08	0
28	3-$CH_2NHC_6H_4$-4′-SO_2NH_2	7.14 (7.10–7.17)	7.90 (7.84–7.96)	7.74	0.16	7.34 (7.30–7.39)	6.78	0.56	1.00	0.00	0
29	3-$CH(OH)C_6H_5$	6.11 (6.08–6.14)	6.49 (6.47–6.50)	7.56	1.07				0.54	0.00	0
30	3-$CH_2OC_6H_5$		7.75 (7.72–7.78)	7.81	0.06	7.71 (7.69–7.74)	7.38	0.33	1.66	0.03	1

TABLE 1—*continued*

No.	X	Log $1/K_{i_{app}}$							π'_3	σ	I
		Bovine			$\lvert\Delta$ Log $1/K_i\rvert$	Murine L5178YR-C_3					
		pH 6.25 Observed	pH 7.20 Observed	pH 7.20 Calculated		pH 7.20 Observed	Calculated	$\lvert\Delta$ Log $1/K_i\rvert$			
31	3-$CH_2OC_6H_4$-3′-Cl		7.69 (7.64–7.75)	7.81	0.12	7.48 (7.45–7.52)	7.38	0.10	1.66	0.03	1
32	3-$CH_2OC_6H_4$-3′-CN		7.83 (7.79–7.88)	7.81	0.02	7.58 (7.54–7.63)	7.38	0.20	1.66	0.03	1
33	3-$CH_2OC_6H_4$-3′-OCH_3		8.05 (8.00–8.10)	7.81	0.24	7.59 (7.56–7.63)	7.38	0.21	1.66	0.03	1
34	3-$CH_2OC_6H_4$-3′-CH_2OH		7.87 (7.85–7.90)	7.81	0.06	7.37 (7.34–7.40)	7.38	0.01	1.66	0.03	1
35	3-$CH_2OC_6H_4$-3′-CH_3		8.00 (7.96–8.04)	7.81	0.19	7.17 (7.16–7.19)	7.38	0.21	1.66	0.03	1
36	3-$CH_2OC_6H_4$-3′-C_2H_5		7.85 (7.80–7.90)	7.81	0.04	7.07 (7.03–7.10)	7.38	0.31	1.66	0.03	1
37	3-$CH_2OC_6H_4$-3′-$CH(CH_3)_2$		7.83 (7.79–7.88)	7.81	0.02	7.11 (7.07–7.14)	7.38	0.27	1.66	0.03	1
38	3-$CH_2OC_6H_4$-3′-$C(CH_3)_3$		7.96 (7.93–8.00)	7.81	0.15	7.23 (7.19–7.28)	7.38	0.15	1.66	0.03	1
39	3-$CH_2OC_6H_4$-3′-$NHCOCH_3$		8.01 (7.97–8.06)	7.81	0.20	7.48 (7.42–7.54)	7.38	0.10	1.66	0.03	1
40	3-$CH_2OC_6H_4$-3′-$NHCONH_2$	7.41 (7.37–7.45)	7.82 (7.77–7.88)	7.81	0.01	7.47 (7.43–7.51)	7.38	0.10	1.66	0.03	1
41	3-$CH_2OOC_6H_4$-3′-$NHCSNH_2$		7.76 (7.71–7.80)	7.81	0.05	7.29 (7.23–7.36)	7.38	0.09	1.66	0.03	1

same two enzymes by a set of 3-X-I. We have established in earlier studies with mammalian enzymes that 3-X-I appears to bind in a typical hydrophobic pocket (2) up to the point where $\pi_X = 1.6$ (3). Equation 1 gives a view of our initial results of the inhibition of bovine liver enzyme at pH 6.25 (3).

$$\text{Log } 1/C = 1.05\pi'_3 - 1.21 \log(\beta \cdot 10^{\pi'_3} + 1) + 6.64 \quad (1)$$

$$n = 28;\ r = 0.955;\ s = 0.210;\ \pi_0 = 1.56;\ \log \beta = -0.736$$

The left-hand portion of the curve in the bilinear model (4) of Eq. 1 has a slope of approximately 1, revealing the similarity of 3-X partitioning into this pocket and into octanol (π is based on partitioning between octanol and water). The slope of the right-hand portion of the curve of −0.16 (1.05–1.21) is essentially flat. The conclusion drawn from this is that substituents with $\pi > \pi_0$ (1.56) project beyond hydrophobic space and do not contact the enzyme. In Eq. 1 π' represents a modified form of π for substituents of the type 3-$CH_2ZC_6H_4$-Y, where Z = O or NH. The Y portion of 3-X substituents with negative π values has π_Y set = 0 in deriving Eq. 1. The rationale for this is that Y does not appear to contact the enzyme hydrophobically or otherwise and hence has little effect on binding (inhibition) of Congeners I to enzyme. For substituents with positive π values $>\pi_0$, the term $-1.21 \log(\beta \cdot 10^{\pi'_3} + 1)$ cuts down the effect of large π values, setting the optimum at 1.56.

Equation 1 is a good correlation based on 28 data points which correlates 91% of the variance in log $1/C$ (i.e., log $1/K_{i_{app}}$) (5, 6), where C is the molar concentration of inhibitor producing 50% inhibition. Since there were only three examples of Y, all of which had negative π values, we have now synthesized and tested additional derivatives of the type 3-CH_2O-C_6H_4-Y to establish more firmly the role of Y in the inhibition of DHFR from both bovine and murine tumor enzyme.

EXPERIMENTAL PROCEDURE

Enzyme Assays

The inhibition assays and the calculation of $K_{i_{app}}$ were performed as in our previous studies (6). The confidence limits on log $1/K_{i_{app}}$ and π_0 were carried out using the jackknife procedure (5).

Substituent Constants

The values for the substituent constants in Table 1 were taken from our recent compilation (7).

Synthesis of Triazine Inhibitors

The 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(3-substituted-phenoxymethyl)-phenyl-*s*-triazine hydrochloride salts (*A*, Fig. 1) were prepared by the three-component synthetic method of Modest (8) [as in our previous study (3)] from the appropriate 3-X-phenyl-3-aminobenxyl ethers (B): dicyandiamide, acetone, and hydrochloric acid. The anilines (*B*, Fig. 1) were obtained by reduction of the condensation products (*C*, Fig. 1) of 3-nitrobenzyl chloride and the appropriately substituted phenol (*D*, Fig. 1); see Fig. 1 and Tables 2 and 3.

Melting points (Büchi capillary apparatus) and boiling points are uncorrected. Microanalyses were performed by C. F. Geiger (Ontario, Calif.) and are within ±0.4% of the theoretical values. Thin-layer chromatography (qualitative precoated silica gel or alumina glass plates with fluorescent indicator) was routinely used to check the purity of the synthetic intermediates and of the final

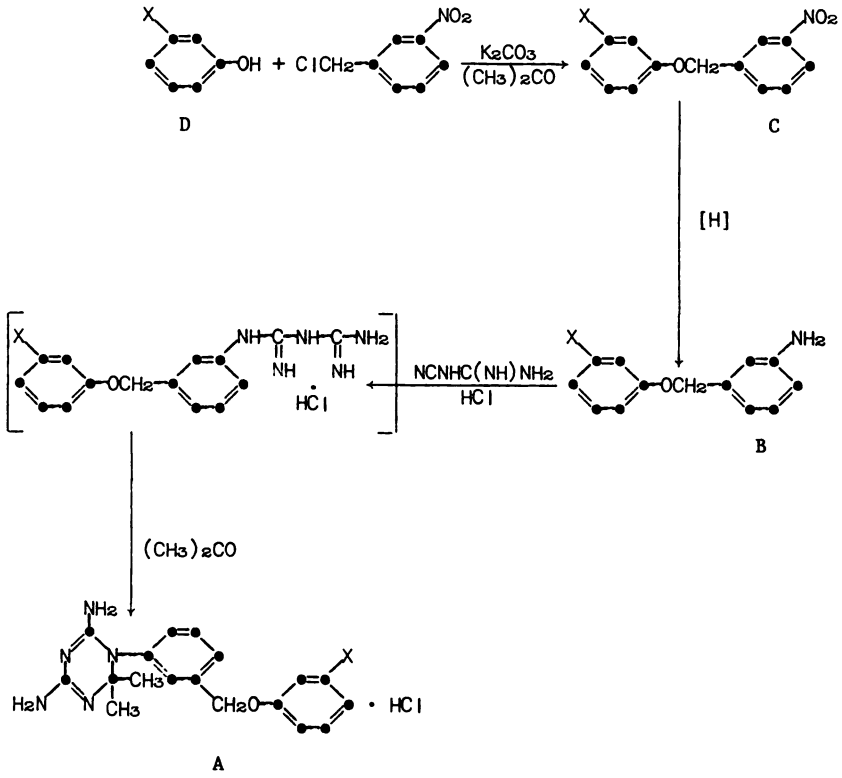


FIG. 1. *Synthetic scheme*

triazine inhibitors. UV spectra (water) of all of the triazine inhibitors were consistent with the assigned structures.

Preparation of 3-hydroxyphenyl thiourea (D, X = NHCSNH$_2$). 3-Aminophenol (0.24 mole) (26.2 g) and 0.32 mole (24.4 g) of ammonium thiocyanate were dissolved in a mixture of 20.4 ml of concentrated HCl and 40 ml of water. The solution was evaporated to dryness on a steam bath. The resulting solid was purified by dissolving it in approximately 100 ml of 25% aqueous NaOH and then reprecipitating with concentrated HCl. After recrystallization from water, there were obtained 28 g (69% yield) of yellow crystals, m.p. 172–175° (see ref. 9; m.p. 176°).

Preparation of the 3-X-phenyl-3′-nitrobenzyl ethers (C). A mixture of 30 mmoles of 3-nitrobenzyl chloride, 33 mmoles of 3-X-phenol (*D*), and 30 mmoles of anhydrous potassium carbonate in 100 ml of acetone was heated under reflux with stirring for 48 hr. The resulting potassium chloride was filtered off and the filtrate was evap-

TABLE 2

Triazine inhibitors (A)

No.	X	m.p.[a]	Yield (%)	Formula[b]
30	H	163–165°	38	$C_{18}H_{21}N_5O\cdot HCl$
31	Cl	174–174.5	61	$C_{18}H_{20}ClN_5O\cdot HCl$
32	CN	165–166	67	$C_{19}H_{20}N_6O\cdot HCl$
33	OCH_3	162–163	90	$C_{19}H_{23}N_5O_2\cdot HCl$
34	CH_2OH	108–111[c]	—[d]	$C_{19}H_{23}N_5O_2\cdot HCl$
35	CH_3	161–162[e]	72	$C_{19}H_{23}N_5O\cdot HCl$
36	C_2H_5	177–179	89	$C_{20}H_{25}N_5O\cdot HCl$
37	$CH(CH_3)_2$	179–180	70	$C_{21}H_{27}N_5O\cdot HCl$
38	$C(CH_3)_3$	164–166	86	$C_{22}H_{29}N_5O\cdot HCl$
39	$NHCOCH_3$	206–208[e]	87	$C_{20}H_{24}N_6O_2\cdot HCl$
40	$NHCONH_2$	193.5–195	—[f]	$C_{19}H_{23}N_7O_2\cdot HCl$
41	$NHCSNH_2$	222–224	—[d]	$C_{19}H_{23}N_7OS\cdot HCl$

[a] Recrystallized from CH_3CN, unless otherwise noted.
[b] Analyzed for C and H.
[c] Recrystallized from *i*-PrOH.
[d] Final purification was affected by column chromatography [silica gel; eluting with Et_2O/CH_3OH (3:1 and then 1:1)]. Yields were very low.
[e] Recrystallized from CH_3CN/EtOH.
[f] See ref. 3.

TABLE 3

Microanalysis of triazine inhibitors

No.	X	Formula	% Composition			
			Calculated		Found	
			C	H	C	H
30	H	$C_{18}H_{21}N_5O\cdot HCl$	60.07	6.16	60.40	5.86
31	Cl	$C_{18}H_{20}ClN_5O\cdot HCl$	54.83	5.37	55.02	5.55
32	CN	$C_{19}H_{20}N_6O\cdot HCl$	59.29	5.50	59.53	5.74
33	OCH_3	$C_{19}H_{23}N_5O_2\cdot HCl$	58.53	6.20	58.74	6.15
34	CH_2OH	$C_{19}H_{23}N_5O_2\cdot HCl$	58.53	6.20	58.11	6.48
35	CH_3	$C_{19}H_{23}N_5O\cdot HCl$	61.03	6.47	61.12	6.70
36	C_2H_5	$C_{20}H_{25}N_5O\cdot HCl$	61.92	6.75	61.87	6.65
37	$CH(CH_3)_2$	$C_{21}H_{27}N_5O\cdot HCl$	62.75	7.02	62.43	6.80
38	$C(CH_3)_3$	$C_{22}H_{29}N_5O\cdot HCl$	63.52	7.27	63.16	7.08
39	$NHCOCH_3$	$C_{20}H_{24}N_6O_2\cdot HCl$[a]	57.61	6.04	57.45	6.37
40	$NHCONH_2$	$C_{19}H_{23}N_7O_2\cdot HCl$	54.60	5.79	54.40	5.78
41	$NHCSNH_2$	$C_{19}H_{23}N_7OS\cdot HCl$	52.58	5.57	52.33	5.70

[a] See ref. 3.

orated to dryness. The residue was recrystallized from ethanol or distilled water (under reduced pressure).

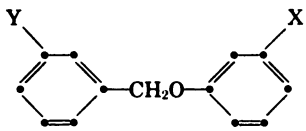
Preparation of the 3-X-phenyl-3′-aminobenzyl ethers (B, except No. 21, Table 4). A solution of 20–50 mmoles of *C* in 100–250 ml of a suitable solvent (DMF, EtOH, or *i*-PrOH) was hydrogenated (Parr hydrogenation apparatus) with 0.5–1.5 g of 5% Pd/C at room temperature. The catalyst was filtered and the solvent was evaporated. If the oily residue was treated with a small amount of ether, the free base separated as a white precipitate, which was recrystallized; if the residue was dissolved in ether and treated with HCl gas, the HCl salt of the product separated as a white precipitate which was collected, decolorized with carbon if necessary, and recrystallized.

Preparation of 3-thioureido-phenyl-3′-aminobenzyl ether hydrochloride (B, X = NHCSNH₂; No. 21, Table 4). To a solution of 15 g of stannous chloride (dihydrate) in 25 ml of concentrated HCl were added 3 g of 3-thioureido-phenyl-3′-nitrophenyl ether (*C*, X = $NHCSNH_2$, No. 11, Table 4) in portions with stirring. After heating on a steam bath for 2 hr and then cooling, 100 ml of water were added. The separated hydrochloride was collected, dried, and recrystallized.

Preparation of the 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(3-substituted phenoxymethyl)-phenyl-s-triazine hydrochloride salts (A). A mixture of 10 mmoles of B·HCl (if free base, 10 mEq of concentrated HCl were added) and 10.7 moles of dicyandiamide in approximately 50 ml of reagent acetone was refluxed with stirring for 24 hr. (A small mount of anhydrous EtOH was added, if necessary, in order to dissolve the B·HCl.) The resulting solution (or suspension) was evaporated to dryness. If not a solid, the residue was triturated with the recrystallization solvent to solidify the crude product. The crude product, once in solid form, was dissolved in the recrystallization solvent, decolorized with carbon, and recrystallized. The crystalline triazine was repeatedly recrystallized until a negative test for aryl guanide was obtained (3, 8).

TABLE 4

Synthetic intermediates



No.	X	Y	m.p	Yield (%)	Formula[a]
1	H	NO_2	172–173/1.2°[b,c]	85	$C_{13}H_{11}NO_3$
2	Cl	NO_2	80–81[d]	77	$C_{13}H_{10}ClNO_3$
3	CN	NO_2	128.5–130.5[d]	87	$C_{14}H_{10}N_2O_3$
4	OCH_3	NO_2	65–66[d]	86	$C_{14}H_{13}NO_4$
5	CH_2OH	NO_2	222–225/0.9	90	$C_{14}H_{13}NO_4$
6	CH_3	NO_2	172–175/1.2	82	$C_{14}H_{13}NO_3$
7	C_2H_5	NO_2	150–153/0.2	70	$C_{15}H_{15}NO_3$
8	$CH(CH_3)_2$	NO_2	175–180/1.1	77	$C_{16}H_{17}NO_3$
9	$C(CH_3)_3$	NO_2	190–194/1.2	86	$C_{17}H_{19}NO_3$
10	$NHCOCH_3$	NO_2	92–95[d,e]	63	$C_{15}H_{14}N_2O_4$
11	$NHCSNH_2$	NO_2	106–107[f]	40	$C_{14}H_{13}N_3OS$
12	H	NH_2	46–47[g]	72	$C_{13}H_{13}NO$
13	Cl	NH_2	85–86[h]	38	$C_{13}H_{12}ClNO$
14	CN	NH_2	90–91.5[i]	67	$C_{14}H_{12}N_2O$
15	OCH_3	NH_2·HCl	169–170.5[d]	81	$C_{14}H_{15}NO_2$·HCl
16	CH_2OH	NH_2·HCl	131–133[i]	75	$C_{14}H_{15}NO_2$·HCl
17	CH_3	NH_2·HCl	151–153[j]	52	$C_{14}H_{15}NO$·HCl
18	C_2H_5	NH_2·HCl	133–135[i]	67	$C_{15}H_{17}NO$·HCl
19	$CH(CH_3)_2$	NH_2·HCl	153–154[i]	80	$C_{16}H_{19}NO$·HCl
20	$NHCOCH_3$	NH_2·HCl	192–195[j]	75	$C_{15}H_{16}N_2O_2$·HCl
21	$NHCSNH_2$	NH_2·HCl	275–278 (d)[h]	88	$C_{14}H_{15}N_3OS$·HCl

[a] Analyzed for C and H.
[b] B.p./*p* (°/mm Hg).
[c] See ref. 10, b.p. 182°/3 mm Hg.
[d] Recrystallized from EtOH.
[e] See ref. 11; m.p. 96–97°.
[f] Recrystallized from aqueous CH_3OH.
[g] Recrystallized from water.
[h] Recrystallized from aqueous EtOH.
[i] Recrystallized from CH_3CN.
[j] Recrystallized from CH_3CH/EtOH.

RESULTS AND DISCUSSION

Inhibition of bovine liver DHFR. We have derived Eqs. 2–4 from the data in Table 1 for the inhibition of bovine DHFR at pH 7.2.

$$\text{Log } 1/K_{i_{app}} = 0.25(\pm 0.12)\pi'_3 + 7.15(\pm 0.25) \quad (2)$$
$$n = 38;\ r = 0.568;\ s = 0.537;\ F_{1,\,36} = 17.2$$

$$\text{Log } 1/K_{i_{app}} = 1.08(\pm 0.19)\pi'_3 - 1.19(\pm 0.25)\log(\beta \cdot 10^{\pi'_3} + 1) + 7.27(\pm 0.13) \quad (3)$$
$$n = 38;\ r = 0.903;\ s = 0.288;\ F_{2,\,34} = 45.5;\ \pi_0 = 1.62\ (1.24 - 2.06);\ \log \beta = -0.656$$

$$\text{Log } 1/K_{i_{app}} = 1.10(\pm 0.19)\pi'_3 - 1.23(\pm 0.25)\log(\beta \cdot 10^{\pi'_3} + 1) + 0.61(\pm 0.62)\sigma + 7.08(\pm 0.18) \quad (4)$$
$$n = 38;\ r = 0.914;\ s = 0.277;\ F_{1,33} = 3.76;\ \pi_0 = 1.72(1.37 - 2.11);\ \log \beta = -0.789$$

Equation 4 is at best of marginal value compared with Eq. 3. The confidence intervals on the σ term are so large that one can place little weight on its value; that is, electronic effects of substituents are of little significance. There were three examples in Eq. 1 where bridges of the type $3\text{-}CH_2Z$ joined two phenyl groups of I, and all of these had Y substituents with negative π values. There are 14 examples in our present data set of congeners with this feature, and all are reasonably well fit by Eq. 3, regardless of whether Y is large or small, hydrophilic or hydrophobic. This confirms our earlier belief that Y is held in such a way by the $—CH_2Z—C_6H_5—$ moiety that it does not effectively contact the enzyme. The optimal value for π (π_0) and the coefficients with the two π terms are essentially identical with Eq. 1. The rather large difference between the intercepts of Eqs. 1 and 3 is due to difference in the pH of the medium. On the average, the triazine inhibitors are almost 5 times more potent at the higher pH; however, the structure-activity relationship is much the same.

The following compounds were not included in the derivation of Eqs. 2–4 in Table 1: $3\text{-}COOC_2H_5$, 3-CN, $3\text{-}CH(OH)C_6H_5$. Two of these are much less active than expected, but the CN derivative is always unusually active. The reasons behind the high activity of the CN group are not known. Our equations have ruled out explanations in terms of π or σ and, since this is such a small group, steric effects seem unlikely. Some kind of special polarizability seems to be the most likely cause of the unusual activity.

TABLE 5

Microanalysis of synthetic intermediates of Table 4

No.	X	Y	Formula	Composition			
				Calculated		Found	
				C	H	C	H
1	H	NO_2	$C_{13}H_{11}NO_3$	68.11	4.84	68.36	5.01
2	Cl	NO_2	$C_{13}H_{10}ClNO_3$	59.21	3.82	59.26	3.81
3	CN	NO_2	$C_{14}H_{10}N_2O_3$	66.13	3.96	66.48	3.99
4	OCH_3	NO_2	$C_{14}H_{13}NO_4$	64.86	5.05	64.63	5.17
5	CH_2OH	NO_2	$C_{14}H_{13}NO_4$	64.86	5.05	64.59	4.94
6	CH_3	NO_2	$C_{14}H_{13}NO_3$	69.12	5.39	68.85	5.50
7	C_2H_5	NO_2	$C_{15}H_{15}NO_3$	70.02	5.88	70.12	6.20
8	$CH(CH_3)_2$	NO_2	$C_{16}H_{17}NO_3$	70.83	6.32	71.02	6.37
9	$C(CH_3)_3$	NO_2	$C_{17}H_{19}NO_3$	71.56	6.71	71.71	7.07
10	$NHCOCH_3$	NO_2	$C_{15}H_{14}N_2O_4$	62.93	4.93	63.25	4.97
11	$NHCSNH_2$	NO_2	$C_{14}H_{13}N_3OS$	55.43	4.32	55.75	4.49
12	H	NH_2	$Cl_3H_{13}NO$	78.36	6.58	78.42	6.66
13	Cl	NH_2	$C_{13}H_{12}ClNOO$	66.81	5.18	66.48	5.52
14	CN	NH_2	$C_{14}H_{12}N_2O$	74.98	5.40	75.16	5.49
15	OCH_3	$NH_2 \cdot HCl$	$C_{14}H_{15}NO_2 \cdot HCl$	63.27	6.07	62.89	6.36
16	CH_2OH	$NH_2 \cdot HCl$	$C_{14}H_{15}NO_2 \cdot HCl$	63.27	6.07	63.62	6.17
17	CH_3	$NH_2 \cdot HCl$	$C_{14}H_{15}NO \cdot HCl$	67.33	6.46	66.91	6.64
18	C_2H_5	$NH_2 \cdot HCl$	$C_{15}H_{17}NO \cdot HCl$	68.30	6.82	68.45	6.99
19	$CH(CH_3)_2$	$NH_2 \cdot HCl$	$C_{16}H_{19}NO \cdot HCl$	69.33	7.27	69.64	7.29
20	$C(CH_3)_3$	$NH_2 \cdot HCl$	$C_{17}H_{21}NO \cdot HCl$	69.97	7.60	70.02	7.92
21	$NHCOCH_3$	$NH_2 \cdot HCl$	$C_{15}H_{16}N_2O_2 \cdot HCl$	61.53	5.85	61.69	5.47
22	$NHCSNH_2$	$NH_2 \cdot HCl$	$C_{14}H_{15}N_3OS \cdot HCl$	54.27	5.21	54.40	5.68

In order to bring out more clearly the effect of pH on the structure-activity relationship, we have correlated log $1/K_i$ values for the action of inhibitors at two pH values, 6.25 and 7.20, for 30 examples where $K_{i_{app}}$ was measured at both pH levels. The result is shown in Eq. 5:

$$\text{Log } 1/K_{i(7.2)} = 0.98(\pm 0.11)\log 1/K_{i(6.25)} + 0.73(\pm 0.73) \quad (5)$$
$$n = 26;\ r = 0.968;\ s = 0.180$$

Four congeners were poorly correlated by Eq. 5 and were not used in its derivation. These poorly fit substituents with their deviations from Eq. 5 are as follows: 3-$COOC_2H_5$, −0.63; 3-$O(CH_2)_3CH_3$, −0.58; 3-$COCH_3$, −0.55; and 3-$CH_2NHC_6H_3$-3′,5′-$(CONH_2)_2$, 0.43. Equation 5 is similar to an equation correlating the same pH effect for 2-X-I (1). The slope of the correlation is essentially 1 in each example. The over-all structure-activity relationship is very much the same at the two different pH levels, even though the triazines are more active at the higher pH. The cause of the activity at the higher pH is unknown. Since the triazines have pK_a values of about 11 (8), they would be completely monoprotonated at either pH 6.25 or 7.20; hence the difference in activity must be associated with the effect of pH on the enzyme.

In order to be sure that the effect of pH is not due simply to a change in the partition coefficient with pH, we measured log *P* for two triazines at the two pH values:

X-triazine-I	log *P*, pH 6	log *P*, pH 7
4-C_6H_5	−1.04 ± 0.04	−1.08 ± 0.04
3-$CH_2OC_6H_4$-3′-$NHCOCH_3$	−1.52 ± 0.03	−1.61 ± 0.04

Since log *P* of protonated solutes varies with solute concentration, log *P* values were determined at a number of concentrations and extrapolated to infinite dilution for good comparative values.

We have used π values from the benzene system in our correlations. To be sure that these are reasonably close to π from the triazines, we have measured log *P* for four triazines in 0.1 N NaOH (neutral form of triazine). From these results we can compare π values from the two systems as follows:

3-X of I	π from triazine	π from benzene
H	0.00	0.00
NO_2	−0.34	−0.28
CN	−0.64	−0.57
$COCH_3$	−0.62	−0.55
OC_4H_9	1.63	1.55

There is a small, consistent difference between the two systems, but it is insignificant when compared with the standard deviation of our correlation equations.

Inhibition of murine tumor DHFR. Equations 6–8 correlate the inhibition of DHFR from murine tumor [from the mouse L5178YR-C_3 clone, a gene-amplified lymphoblastoid tumor cell line (12)]. Two data points in Table 1 were omitted in the derivation of Eqs. 6–9: 3-$COOC_2H_5$ and 3-CN.

$$\text{Log } 1/K_{i_{app}} = 0.23(\pm 0.15)\pi'_3 + 6.43(\pm 0.31) \quad (6)$$
$$n = 38;\ r = 0.454;\ s = 0.687;\ F_{1,\ 36} = 9.35$$

$$\text{Log } 1/K_{i_{app}} = 1.11(\pm 0.19)\pi'_3 - 1.41(\pm 0.28)\log(\beta \cdot 10^{\pi'_3} + 1) + 6.39(\pm 0.16) \quad (7)$$
$$n = 38;\ r = 0.895;\ s = 0.353;\ F_{1,\ 34} = 50.9;\ \pi_0 = 1.57\ (1.26 - 1.88);\ \log \beta = -0.992$$

$$\text{Log } 1/K_{i_{app}} = 1.13(\pm 0.22)\pi'_3 - 1.33(\pm 0.30)\log(\beta \cdot 10^{\pi'_3} + 1) + 0.42(\pm 0.24)I + 6.44(\pm 0.16) \quad (8)$$

$$n = 38;\ r = 0.920;\ s = 0.315;\ F_{1,\,33} = 9.92;\ \pi_0 = 1.44\ (1.08 - 1.80);\ \log \beta = -0.675$$

$$\text{Log } 1/K_{i_{app}} = 1.19(\pm 0.21)\pi'_3 - 1.38(\pm 0.28)\log(\beta \cdot 10^{\pi'_3} + 1) + 0.50(\pm 0.24)I + 0.90(\pm 0.68)\sigma + 6.20(\pm 0.22) \quad (9)$$

$$n = 38;\ r = 0.935;\ s = 0.289;\ F_{1,\,32} = 7.14;\ \pi_0 = 1.56\ (1.19 - 1.93);\ \log \beta = -0.750$$

As usual, the 3-CN group is more active than expected, and the 3-$COOC_2H_5$ group is much less active than expected. In Eqs. 8 and 9, the indicator variable I is assigned the value of 1 for congeners containing the —CH_2O— bridge between the two phenyl groups; so doing yields a small but significant improvement over Eq. 7. Equation 9 indicates a marginal role for electron-withdrawing substituents. If Congeners 20 and 29–31 are dropped and the data refit, the term in I is not found to be significant, although the term in σ is still significant with a coefficient of 0.72 (± 0.69), close to that in Eq. 9. Thus there is uncertainty as to whether or not the —CH_2Z— bridge plays a small role in the QSAR for the murine tumor enzyme. It does not appear of importance for bovine liver DHFR.

In order to seek out congeners having selectivity for one of the two enzymes, we compared activities via Eqs. 10 and 11:

$$\text{Log } 1/K_{i\ (\text{murine})} = 1.04(\pm 0.16)\log 1/K_{i\ (\text{bovine})} - 1.05(\pm 1.2) \quad (10)$$

$$n = 40;\ r = 0.908;\ s = 0.335$$

$$\text{Log } 1/K_{i\ (\text{murine})} = 1.11(\pm 0.09)\log 1/K_{i\ (\text{bovine})} - 1.53(\pm 0.65) \quad (11)$$

$$n = 31;\ r = 0.979;\ s = 0.174$$

All of the data are correlated in Eq. 10. In forming Eq. 11, nine data points which were misfit by more than 1.5 SD by Eq. 10 were omitted. The comparison is given in Table 6, where calculated values are obtained by Eq. 11.

The results for the first 16 congeners in Table 6 show that, when 3-X is a relatively small substituent, one finds close correlation between the effect of these inhibitors on the two enzymes. All nine poorly fit congeners of Table 6, denoted by footnote *b*, are among the large substituents. Of course, it is these substituents which would interact with parts of the enzymes most remote from the active site. Baker (13) and others have long proposed that it is in these more remote regions of an enzyme that one should seek differences between isozymes or enzyme from different sources. Our results confirm the value of this philosophy in drug design.

From our earlier work (1) with 2-X-I, we believe that 2-X is not reaching a hydrophobic pocket. Indeed, rather small substituents (CF_3, SCH_3, Cl, CH_2CN) showed significant differences in activity with the bovine and murine tumor enzymes. However, CF_3 and Cl are closely correlated for 3-X substituents in the two systems. Even larger substituents, such as $C(CH_3)_3$, $COOC_2H_5$, and $O(CH_2)C_6H_5$, are well correlated. In fact, it is only in those substituents which would extend beyond the hydrophobic pocket of 1.6 defined by π_0 that one finds significant differences in the interactions with the two enzymes. Again, these results confirm our expectation that, as long as substituents are binding with corresponding hydrophobic pockets of different forms of the same enzyme, one would not expect large differences in the interactions. We define a hydrophobic pocket by the observation that the binding of congeneric ligands is correlated by π or log P; that is, ligand partitioning onto the enzyme is correlated with its partitioning between octanol and water.

With the exception of compound 27 of Table 6, all of the large substituents which show activity greater than expected (Eq. 11) against the tumor enzyme contain the

TABLE 6

Comparison of log 1/K_i for triazines I with bovine liver and murine tumor (L5178YR-C_3) DHFR

No.	3-X	Log 1/K_i murine DHFR		
		Observed	Calculated[a]	Δ
1	$SONH_2$	4.66	4.52	0.14
2	$CONH_2$	4.74	4.81	−0.07
3	H	5.78	6.04	−0.26
4	$COCH_3$	5.42	5.42	0.00
5	CH_3	6.84	7.06	−0.22
6	OCH_3	6.02	6.13	−0.11
7	OH	5.36	5.56	−0.20
8	$C(CH_3)_3$	6.38	6.36	0.02
9	$COOC_2H_5$	5.06	4.85	0.21
10	F	6.70	6.92	−0.22
11	CF_3	7.09	6.91	0.18
12	Cl	7.30	7.21	0.09
13	NO_2	6.40	6.63	−0.23
14	CN	6.96	6.84	0.12
15	Br	7.32	7.16	0.16
16	I	7.22	7.05	0.17
17	$O(CH_2)_2OC_6H_5$	6.75[b]	7.54	−0.79
18	$O(CH_2)_2OC_6H_4$-3′-CF_3	7.03[b]	7.56	−0.52
19	$O(CH_2)C_6H_5$	6.77	6.94	−0.19
20	$O(CH_2)_3CH_3$	6.33	6.26	0.07
21	$OCH_2C_6H_3$-3′,4′-Cl_2	6.41[b]	7.21	−0.80
22	$O(CH_2)_{11}CH_3$	6.06[b]	6.85	−0.79
23	$(CH_2)_6CH_3$	6.70	6.72	−0.02
24	$CH_2NHC_6H_3$-3′,5′-$(CONH_2)_2$	6.84	7.06	−0.22
25	$O(CH_2)_4OC_6H_5$	6.80[b]	7.31	−0.51
26	$O(CH_2)_4OC_6H_4$-3′-CF_3	6.83	6.60	0.23
27	$(CH_2)_5CH_3$	7.13[b]	6.63	0.50
28	$CH_2NHC_6H_4$-4′-SO_2NH_2	7.34	7.24	0.10
29	$CH_2OC_6H_5$	7.71	7.07	0.64
30	$CH_2OC_6H_4$-3′-Cl	7.48[b]	7.01	0.47
31	$CH_2OC_6H_4$-3′-CN	7.58[b]	7.16	0.42
32	$CH_2OC_6H_4$-3′-OCH_3	7.59	7.41	0.18
33	$CH_2OC_6H_4$-3′-CH_2OH	7.37	7.21	0.16
34	$CH_2OC_6H_4$-3′-CH_3	7.17	7.35	−0.18
35	$CH_2OC_6H_4$-3′-C_2H_5	7.07	7.18	−0.11
36	$CH_2OC_6H_4$-3′-$CH(CH_3)_2$	7.11	7.16	−0.05
37	$CH_2OC_6H_4$-3′-$C(CH_3)_3$	7.23	7.31	−0.08
38	$CH_2OC_6H_4$-3′-$NHCOCH_3$	7.48	7.36	0.12
39	$CH_2OC_6H_4$-3′-$NHCONH_2$	7.47[b]	7.15	0.32
40	$CH_2OC_6H_4$-3′-$NHCSNH_2$	7.29	7.08	0.21

[a] Calculated using Eq. 11.

[b] These congeners not used in the derivation of Eq. 11.

—CH_2O— bridge. This point was brought out by the I term in Eqs. 8 and 9; that is, this bridge does seem to provide some specificity, but it is not consistent and depends on the constitution of the rest of the substituent.

The differences between the responses of the two enzymes are not large, but they are significantly beyond experimental error (note the 95% confidence limits on log $1/K_i$). We have estimated (1) that the experimental error in log $1/K_i$ would not be greater than 0.1, and in general we find it to be much less. Since K_i comes from an equilibrium process, the free energy of interaction of an inhibitor with an enzyme = $\Delta G_o = -RT \ln K_i = -1.36 \log K_i$. Then the difference in free energy of interaction of an inhibitor with the two enzymes = $\Delta\Delta G_o = \Delta G_o$ (first enzyme) − ΔG_o (second enzyme) = -1.36[log K_i (first enzyme) − log K_i (second enzyme)]. If the difference in log $1/K_i$ values is 0.7 or if the difference between observed and expected log $1/K_i$ is 0.7, then we are dealing with a $\Delta\Delta G_o$ value of approximately 1 kcal. This is a small energy difference which could be due to a small difference in the conformation of the enzymes or the possibility of an inhibitor's making a good hydrogen bond interaction with one enzyme and a poor interaction with the other. That such small energy differences make large differences in K_i is a boon to drug development, since it means that small differences in enzymes could result in large differences in K_i. In fact, one might not need any difference in the basic structure of the isolated enzymes. Simply having the enzyme in different environments in two different cells might produce conformational changes in an enzyme that would enable it to react quite differently with an inhibitor in the normal cell as compared with the modified cell.

We believe that one of the best opportunities for developing better antitumor drugs is to uncover differences in DHFR from normal cells and tumor cells. As pointed out above, differences would not have to be large in order to yield K_i values that differ greatly (10- to 100-fold) from normal DHFR. The results in this report confirm that there are significant differences between two mammalian enzymes, one from a normal cell and the other from a tumor cell. Although the differences may be due to the different species from which the DHFRs come, it is possible that the differences are the result of tumor versus normal DHFR. We hope that through further QSAR studies larger differences can be uncovered and that these differences can be correlated with the physical and structural features of the inhibitors so that we can learn to maximize specificity in a rational, systematic manner.

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