Comparative Structure-Activity Relationships of Antifolate Triazines Inhibiting Murine Tumor Cells Sensitive and Resistant to Methotrexate[1]

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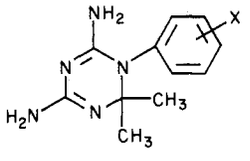
The inhibitory effect of 108 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(substituted-phenyl)-*s*-triazines on murine L5178Y tumor cells, resistant and sensitive to methotrexate (MTX), has been studied. From the pI_{50} values, quantitative structure–activity relationships have been formulated which show that the lipophilic triazines are much more inhibitory against resistant cells than methotrexate or hydrophilic triazines. The results are compared with the behavior of other antifolate drugs that have been used in chemotherapy, as well as with eight antitumor drugs that are not antifolates. The acquired resistance of these cells toward hydrophilic antifolates may be attributed to the combined effect of an impaired active-transport system, a change in the conformation of dihydrofolate reductase in the resistant cells, and an amplified production of dihydrofolate reductase in the resistant cells.

There is a trend among those searching for new drugs to begin by investigating the interaction of inhibitors with enzymes or isolated receptors. From the point of view of increasing our basic understanding of which structural parameters influence ligand binding, working with enzymes whose structures have been established by X-ray crystallography offers great advantages. With the crystallographic coordinates it is possible to build, by computer, stereo models of the active sites in order to study how ligands might or might not bind.[2-6] While such an approach is an excellent way to explore the intrinsic inhibitory power of potential drugs, one cannot predict with much confidence how such compounds will behave in animals. As a first step toward a more comprehensive understanding of how enzymes react in vivo, we have been studying antifolate inhibitors at the level of the isolated enzyme and in cell culture.[7,8] In this report we consider the inhibition of L5178Y murine tumor cells by triazines (I).



I

Equations 1–3 for the inhibition of highly purified dihydrofolate reductase (DHFR) from various vertebrate sources provide reference equations with which to compare our results.

Inhibition of DHFR from Chicken Liver by 3- and 4-Substituted Triazines[6]

$$\log 1/K_{i\,app} = 0.85\pi' - 1.04 \log (\beta \cdot 10^{\pi'} + 1) + 0.57\sigma + 6.36 \quad (1)$$

$$n = 101,\ r = 0.910,\ s = 0.294,\ \pi_0 = 2.03$$

substituents poorly fit: 3-$COOC_2H_5$ (−1.84), 3-CN (+0.71), 3-$CH_2N(CH_3)_3{}^+Cl^-$ (−2.65), 3-$CH(OH)C_6H_5$ (−1.01), 3-OCH_2-adamantyl (−0.98), 4-$COOCH_3$ (−1.30), 4-$COOC_2H_5$ (−1.97), 4-$NHCOCH_3$ (−0.98), 4-CN (−1.01), 4-OCH_2CO-morpholine (+1.47), 4-$CH_2SC_6H_5$ (0.59), 4-C≡CC_6H_5 (−2.07), 4-C≡CH (−0.56), 4-C≡$CSi(CH_3)_3$ (−1.61)

The minus sign indicates that the analogue is less active than expected, while the plus sign indicates greater than expected activity. The figures in parentheses are the differences between calculated and observed values on a logrithmic scale (i.e., −1.0 means 10 times less active than calculated).

Inhibition of DHFR from Human Lymphoblastoid Cells[9]

3-Substituted Triazines

$$\log 1/K_{i\,app} = 1.07\pi'_3 - 1.10 \log (\beta \cdot 10^{\pi'_3} + 1) + 0.50I + 0.82\sigma + 6.07 \quad (2)$$

$$n = 60,\ r = 0.890,\ s = 0.308,\ \pi_0 = 1.84$$

substituents poorly fit: 3-$COOCH_3$ (−1.67), 3-$CH_2N(CH_3)_3{}^+Cl^-$ (−1.76), 3-$CH(OH)C_6H_5$ (−0.74), 3-$O(CH_2)_2CH_3$ (−0.93), 3-OC_2H_5 (−0.66)

4-Substituted Triazines

$$\log 1/K_{i\,app} = 0.78\pi'_4 - 0.78 \log (\beta \cdot 10^{\pi'_4} + 1) + 1.26I - 0.88\nu + 5.83 \quad (3)$$

$$n = 35,\ r = 0.953,\ s = 0.361,\ \pi_0 = 3.43$$

substituents poorly fit: 4-$COOCH_3$ (−1.33), 4-CN (−1.33), 4-OCH_2CO-morpholine (+1.46), 4-$OCH_2C_6H_3$-3′,4′-Cl_2 (−0.72)

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Inhibition of DHFR from Murine L5178Y Tumor Cells[10]

$$\log 1/K_{i\,app} = 1.19\pi'_3 - 1.38 \log (\beta \cdot 10^{\pi'_3} + 1) + 0.50I + 0.90\sigma + 6.20 \quad (4)$$

$n = 38$, $r = 0.935$, $s = 0.289$, $\pi_0 = 1.56$

substituents poorly fit: 3-$COOCH_3$ (−1.81), 3-CN (+0.97), 3-$O(CH_2)_3CH_3$ (−0.63)

In these expressions, $K_{i\,app}$ is the apparent inhibition constant,[12] and π' is the hydrophobic parameter derived from the partitioning of benzene derivatives between octanol and water.[11] The "prime" denotes qualifications on π for certain types of compounds. For eq 1, π' for 3-OR and 4-OR groups is set equal to zero. Such derivatives have a more or less constant inhibitory effect on chicken DHFR independent of the length of R. For substituents of the type $-CH_2ZC_6H_4$-Y (Z = O, NH, S, or S), π_Y is set equal to zero. This also applies to substituents of the type $-ZCH_2C_6H_4$-Y (Z = O or S). The reason for this is that Y, regardless of its character, had almost no effect on $K_{i\,app}$. Graphics analysis using X-ray coordinates of 3-$CH_2OC_6H_4$-3′-$NHCOCH_3$-I bound to chicken liver DHFR shows that Y does not contact the enzyme.[6] This is, of course, strong support for the lack of influence Y has on K_i. We have found other instances where, in enzyme studies, substituents located in certain positions must be assigned π values of zero, and the results are supported by graphics analysis.[2] Thus, direct evidence is beginning to accumulate to confirm the power of QSAR in delineating hydrophobic effects.

In eq 1, 2, and 4, the term in σ holds only for 3-substituents; 4-substituents do not show a similar electronic effect. This is unusual, but the fact that it has been found with DHFR from several sources convinces one that the effect is real, although its nature is not clear.

In eq 2–4, the indicator variable I is assigned the value of 1 for substituents of the type $-ZCH_2C_6H_4$-Y and $-CH_2ZC_6H_4$-Y. This bridged phenyl is reminiscent of a similar structure in folic acid. The positive coefficient with I reveals that this moiety imparts increased inhibitory potency beyond that accounted for by its hydrophobic character alone.

In eq 3, ν is Charton's steric parameter,[13] which is similar to Taft's E_s. Its negative coefficient brings out the detrimental steric effect of 4-substituents which is not seen with 3-substituents. Although this term does not occur in eq 1, if this equation is factored in two equations, one for 3-substituents and one for 4-substituents, a minor role for ν can be found for 4-substituents.[6]

Equation 1 is unusual in that it does not contain a term in I as the other equations do. This reveals a less specific binding mode for the bridged phenyl inhibitors with the avian DHFR.

Equation 4 for the murine tumor DHFR is quite similar to eq 2 for human DHFR. However, equations 2 and 3 for 3- and 4-substituted triazines are so different for human DHFR that we cannot merge them without loss in the quality of the fit. The much larger coefficient with I for 4-substituents brings out the greater specific effect of the bridged substituents in the 4-position compared with the 3-position.

Results

Equations 5 and 6 compare the action of 3-substituted triazines on L5178Y cells sensitive and resistant to methotrexate.

50% Inhibition of Growth of L5178Y Sensitive Cells by 3-Substituted Triazines

$$\log 1/C = 1.40\ (\pm 0.23)\ \pi - 1.65\ (\pm 0.26) \log (\beta \cdot 10^{\pi} + 1) + 0.88\ (\pm 0.57)\ \sigma + 0.52\ (\pm 0.20)\ I - 0.25\ (\pm 0.24)\ \mathrm{OR} + 0.63\ (\pm 0.33)\ \mathrm{DO} + 7.94\ (\pm 0.21) \quad (5)$$

$n = 64$, $r = 0.904$, $s = 0.298$, $\pi_0 = 0.89\ (\pm 0.29)$, $\log \beta = -0.054$

poorly fit points: 3,5-$(Me)_2$ (+0.68), 3-$CH(OH)C_6H_5$ (−1.25)

50% Inhibition of Growth of L5178Y Resistant Cells by 3-Substituted Triazines

$$\log 1/C = 0.63\ (\pm 0.20)\ \pi - 0.26\ (\pm 0.25) \log (\beta \cdot 10^{\pi} + 1) - 0.17\ (\pm 0.07)\ \mathrm{MR} - 0.33\ (\pm 0.24)\ \mathrm{OR} + 5.11\ (\pm 0.19) \quad (6)$$

$n = 61$, $r = 0.878$, $s = 0.335$, $\pi_0 = 4\text{–}5$, $\log \beta = -0.748$

poorly fit points: 3-SO_2NH_2 (−0.74), 3-$CH_2SC_6H_5$ (+0.64), 3-$O(CH_2)_8CH_3$ (+0.75), 3-$CH_2OC_6H_4$-3′-C_6H_5 (−0.64), 3-$CH_2OC_6H_2$-2′,4′,5′-Cl_3 (−0.84), 3-$CH_2NHC_6H_4$-4′-SO_2NH_2 (+0.64), 3-$CH(OH)C_6H_5$ (−0.92)

In both of the above expressions, π gives better correlations than π', presumably because Y *does* make hydrophobic contact in its random movement through the living cells.

Insofar as the common variables are concerned, eq 5 can be compared with our previously derived eq 7.

50% Inhibition of Growth of L5178Y Sensitive Cells by 3-Substituted Triazines[10]

$$\log 1/C = 1.32\pi - 1.70 \log (\beta \cdot 10^{\pi} + 1) + 0.44I + 8.10 \quad (7)$$

$n = 37$, $r = 0.929$, $s = 0.274$, $\pi_0 = 0.76$

poorly fit points: 3-$CH(OH)C_6H_5$ (−1.41), 3-n-C_6H_{13} (+0.89)

A term in σ does not appear in eq 7 but becomes clear with the larger data set used to derive eq 5. In eq 5 the indicator variable OR takes the value of 1 for alkoxy groups, only a few of which were initially tested in formulating eq 7. DO is an indicator variable given the value of 1 for simple alkyl groups. It is of interest to note that these two rather similar classes of substituents behave in an aberrant fashion. The alkoxy groups are less active than expected, and the alkyl groups are, on the average, more active than expected. Note that 3-n-C_6H_{13} is not well fit by eq 7 but is well fit by eq 5 with the extra indicator variable.

Equation 7 does a reasonable job of predicting the activity of the 27 new congeners of equation 5. Only one data

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point [$CH(OH)C_6H_5$] in Table I was omitted in the derivation of eq 5. This substituent is invariably much less active than predicted by our QSAR on DHFR from a variety of different sources.

To our knowledge, no biological data on 3,5-disubstituted derivatives of I have yet been reported. Since it is apparent from graphics studies that these substituents would contact hydrophobic space in a favorable manner, two such compounds were prepared and tested: 3,5-$(CH_3)_2$ (log $1/C$ = 8.26) and 3,5-Cl_2 (log $1/C$ = 8.56) on sensitive cells. They are slightly more active than the corresponding 3-substituted analogues: 3-CH_3 (log $1/C$ = 8.01), 3-Cl (log $1/C$ = 8.46). The 3,5-$(CH_3)_2$ congener is about 5 times as active as expected. Both of these analogues approach methotrexate (log $1/C$ = 8.89) in potency, and both are considerably more active than Baker's antifol (log $1/C$ = 7.53) and triazine (I), where X = 3-Cl, 4-$OCH_2C_6H_4$-3′-$CON(CH_3)_2$.

Equation 5 for sensitive cell inhibition is radically different from eq 6 for resistant cells, especially in the depencence on π. The coefficient with the π term is about half that of eq 5, and the right-hand side of the bilinear curve has a positive slope (0.56 − 0.24 = 0.32), so that we cannot define π_0. Inspection of the results in Tables I and II reveals that π_0 would seem to be in the range of 4–5. The negative coefficient with the MR term in the QSAR for resistant cells is not detectable with sensitive cells and suggests that the binding site is more constricted in resistant cells, since MR is primarily a measure of substituent bulk. Both eq 5 and 6 have identical OR terms, showing that alkoxy groups behave in an analogous fashion with the DHFR in resistant and sensitive cells. However, alkyl groups behave "normally" in the resistant cells as evidenced by the absence of the DO term in this QSAR. Other significant differences include the lack of a σ term in the QSAR for resistant cells, as well as the disappearance of the I term, which was evident in the isolated DHFR QSAR as well as in the sensitive cell QSAR.

It is of interest to note that in the case of the sensitive cells, only two data points are badly fit, while in the case of the resistant cells, seven points, all except one of different structure from the bad points of eq 5, are poorly fit.

In sum, there are many different features in the two QSAR that have important bearing on the design of antifolates for resistant tumor cells.

Equation 8 has been previously derived for 3-substituted triazines vs. resistant cells.

50% Inhibition of Growth of L5178Y Resistant Cells by 3-Substituted Triazines[8]

$$\log 1/C = 0.59\pi - 0.35 \log (\beta \cdot 10^{\pi} + 1) - 0.15\mathrm{MR} + 5.12 \quad (8)$$

$$n = 42,\ r = 0.932,\ s = 0.288$$

poorly fit points: 3-$CH(OH)C_6H_5$ (−0.79), 3-SO_2NH_2 (−0.77), 3-$CH_2NHC_6H_4$-4′-SO_2NH_2 (+0.67)

The agreement between eq 6 and 8 is excellent, the only difference being the OR term in eq 6, for which there were not enough data points for OR groups in the set upon which eq 8 was based. Equation 8 predicts well the activity of 20 new congeners. A few years ago such predictions would have seemed spectacular, today they are rather routine in most cases.

Equation 5 for the inhibition of sensitive cells is rather similar to eq 4 for the inhibition of DHFR isolated from the L5178Y cells resistant to MTX. Although the intercepts of the two equations are different, no meaning can be attached to this, since eq 4 is based on K_i, while eq 5 is based on log $1/C$. The π_0 is slightly lower for the cell system, but the I and σ terms are identical.

This *suggests* that the DHFR from sensitive and resistant cells is much the same if not identical. The similarity between eq 4 and 5 also suggests that the conformation of the DHFR in vitro and in vivo is very similar. Otherwise one would expect significant differences in the QSAR.

We now consider eq 9 and 10 derived for 4-substituted triazines.

50% Inhibition of Growth of L5178Y Sensitive Cells by 4-Substituted Triazines

$$\log 1/C = 0.91\ (\pm 0.15)\ \pi - 1.16\ (\pm 0.21) \log (\beta \cdot 10^{\pi} + 1) - 0.35\ (\pm 0.23)\ \mathrm{OR} + 7.82\ (\pm 0.12) \quad (9)$$

$$n = 41,\ r = 0.896,\ s = 0.278,\ \pi_0 = 1.11\ (\pm 0.38),\ \log \beta = -0.542$$

poorly fit points: 4-C≡CC_6H_5 (−1.72), 4-CN (−0.53)

50% Inhibition of Growth of L5178Y Resistant Cells by 4-Substituted Triazines

$$\log 1/C = 0.61\ (\pm 0.10)\ \pi - 0.29\ (\pm 0.20) \log (\beta \cdot 10^{\pi} + 1) - 0.13\ (\pm 0.08)\ \mathrm{MR} + 4.42\ (\pm 0.18) \quad (10)$$

$$n = 42,\ r = 0.942,\ s = 0.306,\ \pi_0 \approx 5\text{–}6,\ \log \beta = -2.07$$

poorly fit points: 4-CN (−0.61), 4-$OCH_2C_6H_3$-3′,4′-Cl_2 (−0.77)

Equation 9 for 4-substituted triazines is a somewhat better correlation than eq 5 for 3-substituted triazines acting on sensitive cells (compare values of 5). It is also considerably simpler in that it does not contain terms in I, σ, or DO. Nor was it possible to establish the value of a steric parameter as for eq 3 for human[9] or chicken[6] DHFR. 4-Substituted triazines acting on *Lactobacillus casei* also require a steric term in ν for correlation.[6] The values of π_0 are similar for both 3- and 4-substituted triazines, and the coefficients with OR are the same.

Turning now to eq 10 for the action of 4-substituted triazines on the MTX-resistant cells, we find it quite different from eq 9 for the sensitive cells. π_0 cannot be sharply defined, but inspection of the data reveals that it would appear to be in the range of 5–6. There is no term for OR for eq 10 as there is for eq 5, 6, and 9, where there are identical OR coefficients. The coefficient with π is much smaller for eq 10 than for eq 9, which parallels the case for 3-substituted triazines in eq 5 and 6. The intercept of eq 10 is much smaller than for eq 9, showing that a much higher concentration of triazine is required for 50% inhibition of the resistant cells.

Note that the intercepts of eq 9 and 5 for 4- and 3-substituted triazines acting on sensitive cells are identical, as indeed one would expect. This is not true for eq 10 and 6 for triazines acting on resistant cells. The 4-substituted triazines have a lower intercept, indicating that as a group they are less effective as inhibitors. Although this difference is not parameterized by ν, it nevertheless appears to be present as with the other DHFRs in which the ν term is used to account for it.

It was deemed necessary to compare the potency of the triazines in Tables I and II with some of the more active antifolates that have been used in mammalian, bacterial, and/or protozoal chemotherapy. The results are listed in Table III. None of our triazine antifolates (Tables I and II) are quite as active as metoprine, etoprine, BW301U, or methotrexate, but quite a few of them do surpass Baker's antifols in potency. One congener, X = 3-$CH_2OC_6H_4$-3′-$NHCOCH_3$ (log $1/K_i$ = 8.66) is, for practical

Table I. Parameters Used to Derive Equations 5 and 6 for the Inhibition of Growth of L5178Y Cells by Triazines (I)

		L5178Y/S			L5178Y/R								
		log 1/C		Δ log	log 1/C		Δ log						
no.	X	obsd	calcd	1/C	obsd	calcd	1/C	σ	I	OR	π	DO	MR
1	H	7.39	7.49	−0.10	4.84	5.07	−0.23	0.0	0	0	0.0	0	0.10
2	3-SO_2NH_2 [d]	5.61	5.79	−0.18	3.13	3.76	−0.63	0.46	0	0	−1.82	0	1.23
3	3-$CONH_2$	6.10	6.08	0.02	3.78	4.01	−0.23	0.28	0	0	−1.49	0	0.98
4	3-$COCH_3$	6.96	7.34	−0.38	4.56	4.57	−0.01	0.38	0	0	−0.55	0	1.12
5	3-$COOCH_2CH_3$ [c,d]	8.13	8.02	0.11	0.00	0.00	0.0	0.37	0	0	0.51	0	1.75
6	3-OH	6.74	6.99	−0.25	4.22	4.63	−0.41	0.12	0	0	−0.67	0	0.29
7	3-CF_3	8.01	8.09	−0.08	5.66	5.48	0.18	0.43	0	0	0.88	0	0.50
8	3-F	7.94	7.87	0.07	5.24	5.15	0.09	0.34	0	0	0.14	0	0.09
9	3-Cl	8.46	8.04	0.42	5.83	5.38	0.45	0.37	0	0	0.71	0	0.60
10	3-Br	8.42	8.06	0.36	5.90	5.41	0.49	0.39	0	0	0.86	0	0.89
11	3-I	8.40	8.00	0.30	5.91	5.45	0.46	0.35	0	0	1.12	0	1.39
12	3-NO_2	7.66	7.91	−0.25	5.03	4.80	0.23	0.71	0	0	−0.28	0	0.74
13	3-CN	7.55	7.49	0.06	4.77	4.64	0.13	0.56	0	0	−0.57	0	0.63
14	3-CH_3	8.01	8.27	−0.26	4.97	5.31	−0.34	−0.07	0	0	0.56	1	0.57
15	3-CH_2CH_3	8.48	8.28	0.20	5.31	5.46	−0.15	−0.07	0	0	1.03	1	1.03
16	3-$(CH_2)_5CH_3$	7.97	7.79	0.18	6.39	6.08	0.31	−0.08	0	0	3.21	1	2.43
17	3-$(CH_2)_8CH_3$	7.67	7.39	0.28	6.15	6.37	−0.22	−0.08	0	0	4.83	1	4.29
18	3-$(CH_2)_{11}CH_3$	7.07	6.99	0.08	6.10	6.73	−0.63	−0.08	0	0	6.45	1	5.68
19	3-$C(CH_3)_3$	7.59	8.08	−0.49	6.03	5.70	0.33	−0.10	0	0	1.98	1	1.96
20	3-DL-$CH(OH)C_6H_5$ [c,d]	6.41	7.66	−1.25	4.00	4.87	−0.87	−0.04	0	0	0.54	0	3.15
21	3-OCH_3	7.93	7.33	0.60	4.90	4.62	0.28	0.12	0	1	−0.02	0	0.79
22	3-OCH_2CH_3	7.66	7.49	0.17	4.50	4.77	−0.27	0.10	0	1	0.38	0	1.25
23	3-$O(CH_2)_2CH_3$	7.24	7.54	−0.30	4.48	5.03	−0.55	0.10	0	1	1.05	0	1.71
24	3-$O(CH_2)_3CH_3$	7.14	7.46	−0.32	4.63	5.17	−0.54	0.10	0	1	1.55	0	2.17
25	3-$O(CH_2)_4CH_3$	6.85	7.34	−0.49	4.95	5.30	−0.35	0.10	0	1	2.08	0	2.63
26	3-$O(CH_2)_5CH_3$	6.83	7.21	−0.38	5.32	5.43	−0.11	0.10	0	1	2.62	0	3.07
27	3-$O(CH_2)_8CH_3$	6.93	6.80	0.13	6.50	5.81	0.69	0.10	0	1	4.29	0	4.46
28	3-$O(CH_2)_{10}CH_3$	6.67	6.53	0.14	6.38	6.06	0.32	0.10	0	1	5.37	0	5.38
29	3-$O(CH_2)_{11}CH_3$	6.41	6.39	0.02	6.24	6.18	0.06	0.10	0	1	5.91	0	5.84
30	3-$O(CH_2)_{12}CH_3$	6.22	6.26	−0.04	6.52	6.30	0.22	0.10	0	1	6.45	0	6.30
31	3-$O(CH_2)_{13}CH_3$	6.17	6.12	0.05	6.49	6.42	0.07	0.10	0	1	6.99	0	6.77
32	3-$O(CH_2)_2OC_6H_5$	7.34	7.68	−0.34	5.02	5.27	−0.25	0.10	0	0	1.68	0	3.90
33	3-$O(CH_2)_2OC_6H_4$-3′-CF_3	7.37	7.48	−0.11	5.85	5.53	0.32	0.10	0	0	2.56	0	4.30
34	3-$O(CH_2)_4OC_6H_5$	6.97	7.44	−0.47	5.30	5.51	−0.21	0.10	0	0	2.71	0	4.82
35	3-$O(CH_2)_4OC_6H_4$-3′-CF_3	7.14	7.22	−0.08	6.28	5.76	0.52	0.10	0	0	3.59	0	5.22
36	3-$OCH_2C_6H_5$	7.78	7.69	−0.09	5.19	5.38	−0.19	0.10	0	0	1.66	0	3.17
37	3-$OCH_2C_6H_3$-3′,4′-Cl_2	7.15	7.39	−0.24	5.80	5.68	0.12	0.10	0	0	2.91	0	4.17
38	3-$OCH_2C_6H_4$-4′-$CONH_2$	7.21	7.67	−0.46	4.34	4.52	−0.18	0.10	0	0	0.17	0	4.05
39	3-OCH_2-1-adamantyl	7.38	6.97	0.41	5.70	5.52	0.18	0.10	0	1	3.61	0	4.70
40	3-CH_2O-c-C_6H_{11}	7.62	7.70	−0.08	5.38	5.26	0.12	0.06	0	0	1.43	0	3.31
41	3-$CH_2NHC_6H_3$-3′,5′-$(CONH_2)_2$	7.01	6.61	0.40	3.79	3.40	0.39	0.06	1	0	−1.34	0	5.23
42	3-$CH_2NHC_6H_4$-4′-SO_2NH_2 [d]	7.42	7.27	0.15	4.63	3.83	0.80	0.06	1	0	−0.82	0	4.60
43	3-$CH_2OC_6H_5$	8.20	8.17	0.03	5.67	5.38	0.29	0.06	1	0	1.66	0	3.17
44	3-$CH_2OC_6H_4$-3′-Cl	8.10	8.01	0.09	5.93	5.57	0.36	0.06	1	0	2.37	0	3.67
45	3-$CH_2OC_6H_4$-3′-CN	8.09	8.27	−0.18	5.25	5.06	0.19	0.06	1	0	1.09	0	3.70
46	3-$CH_2OC_6H_4$-3′-OCH_3	8.02	8.17	−0.15	5.36	5.26	0.10	0.06	1	0	1.64	0	3.86
47	3-$CH_2OC_6H_4$-3′-CH_2OH	8.32	8.27	0.05	4.77	4.81	−0.04	0.06	1	0	0.63	0	3.79
48	3-$CH_2OC_6H_4$-3′-CH_3	8.16	8.04	0.12	5.59	5.51	0.08	0.06	1	0	2.22	0	3.64
49	3-$CH_2OC_6H_4$-3′-CH_2CH_3	8.11	7.93	0.18	5.74	5.61	0.13	0.06	1	0	2.68	0	4.10
50	3-$CH_2OC_6H_4$-3′-$CH(CH_3)_2$	8.13	7.80	0.33	5.87	5.72	0.15	0.06	1	0	3.19	0	4.57
51	3-$CH_2OC_6H_4$-3′-$C(CH_3)_3$	7.30	7.69	−0.39	6.08	5.81	0.27	0.06	1	0	3.64	0	5.03
52	3-$CH_2OC_6H_4$-3′-C_6H_5	7.33	7.68	−0.35	5.33	5.73	−0.40	0.06	1	0	3.69	0	5.61
53	3-$CH_2OC_6H_4$-3′-$NHCOCH_3$	8.66	8.28	0.38	4.95	4.72	0.23	0.06	1	0	0.69	0	4.56
54	3-$CH_2OC_6H_4$-3′-$NHCSNH_2$	8.08	8.19	−0.11	4.01	4.37	−0.36	0.06	1	0	0.26	0	5.29
55	3-$CH_2OC_6H_4$-3′-$NHCONH_2$	8.18	8.22	−0.04	4.74	4.56	0.18	0.06	1	0	0.36	0	4.44
56	3-$CH_2OC_6H_4$-4′-$(CH_2)_4CH_3$	7.52	7.52	0.00	5.56	5.99	−0.43	0.06	1	0	4.33	0	5.50
57	3-CH_2O-2-naphthyl	7.63	7.86	−0.23	5.32	5.62	−0.30	0.06	1	0	2.98	0	4.72
58	3-CH_2O-1-naphthyl	7.53	7.86	−0.33	5.39	5.62	−0.23	0.06	1	0	2.98	0	4.72
59	3-$CH_2SC_6H_5$	8.31	8.02	0.29	6.05	5.52	0.53	0.06	1	0	2.30	0	3.79
60	3-$CH_2SC_6H_4$-3′-CH_3	7.86	7.89	−0.03	5.26	5.65	−0.39	0.06	1	0	2.86	0	4.26
61	3-$CH_2SeC_6H_5$	7.98	8.01	−0.03	5.22	5.52	−0.30	0.06	1	0	2.37	0	3.96
62	3-$SCH_2C_6H_5$	7.68	7.48	0.20	5.05	5.52	−0.47	0.03	0	0	2.30	0	3.79
63	3-$SCH_2C_6H_4$-4′-Cl	7.76	7.31	0.45	5.22	5.70	−0.48	0.03	0	0	3.01	0	4.29
64	3-$CH_2OC_6H_2$-2′,4′,5′-Cl_3 [d]	7.47	7.65	−0.18	5.16	5.93	−0.77	0.06	1	0	3.79	0	4.67
65	3,5-Cl_2	8.56	8.30	0.26	5.51	5.61	−0.10	0.74	0	0	1.42	0	1.20
66	3,5-$(CH_3)_2$	8.26	7.57	0.69	5.36	5.49	−0.13	−0.14	0	0	1.12	0	1.14

[a] Calculated by eq 5. [b] Calculated by eq 6. [c] Not used in the derivation of eq 5. [d] Not used in the derivation of eq 6.

purposes, equipotent to methotrexate in the sensitive cell line. However, when it comes to the resistant cells, many of our compounds are much more active than any of those in Table III. We may conjecture that the differences in the QSAR between sensitive and resistant cells might be attributed to the erection of a nonspecific lipophilic barrier by the resistant cells in order to exclude hydrophilic drugs or antimetabolites. If this were true, one might expect to

Table II. Parameters used to Derive Equations 9 and 10 for the Inhibition of Growth in L5178Y Cells by 4-Substituted Triazines (I)

		L5178Y/S			L5178Y/R					
		log 1/C		Δ log	log 1/C		Δ log			
no.	X	obsd	calcd[a]	1/C	obsd	calcd[b]	1/C	OR	π	MR
1	H	7.39	7.69	−0.30	4.84	4.41	0.43	0	0.0	0.10
2	4-SO_2NH_2	5.97	6.16	−0.19	3.11	3.14	−0.03	0	−1.82	1.23
3	4-SO_2CH_3	6.28	6.33	−0.05	3.12	3.25	−0.13	0	−1.63	1.35
4	4-$CONH_2$	6.03	6.46	−0.43	3.15	3.38	−0.23	0	−1.49	0.98
5	4-$COCH_3$	7.40	7.28	0.12	3.95	3.94	0.01	0	−0.55	1.12
6	4-OH	7.48	7.18	0.30	4.13	3.97	0.16	0	−0.67	0.29
7	4-NH_2	7.14	6.69	0.45	3.88	3.60	0.28	0	−1.23	0.54
8	4-$NHCOCH_3$	6.79	6.92	−0.13	3.17	3.63	−0.46	0	−0.97	1.49
9	4-CF_3	7.91	8.04	−0.13	4.74	4.89	−0.15	0	0.88	0.50
10	4-F	8.15	7.78	0.37	4.87	4.49	0.38	0	0.14	0.09
11	4-Cl	8.27	8.01	0.26	4.84	4.77	0.07	0	0.71	0.60
12	4-Br	8.21	8.04	0.17	4.80	4.83	−0.03	0	0.86	0.89
13	4-I	7.96	8.06	−0.10	4.90	4.92	−0.02	0	1.12	1.39
14	4-CN	6.73	7.26	−0.53	3.39	4.00	−0.61	0	−0.57	0.57
15	4-OCH_2CO-morpholine	7.04	6.55	0.49	3.31	3.06	0.25	0	−1.39	4.00
16	4-$O(CH_2)_2$-OC_6H_4-4′-NH_2	7.61	7.93	−0.32	3.65	4.14	−0.49	0	0.45	4.34
17	4-CH_3	8.13	7.97	0.16	4.68	4.69	−0.01	0	0.56	0.57
18	4-$(CH_2)_3CH_3$	8.37	7.92	0.45	5.34	5.38	−0.04	0	2.13	1.96
19	4-$(CH_2)_8CH_3$	7.65	7.27	0.38	6.29	6.04	0.25	0	4.83	4.29
20	4-$C(CH_3)_3$	7.62	7.95	−0.33	5.75	5.31	0.44	0	1.98	1.96
21	4-CCC_6H_5[c]	6.08	7.80	1.72	5.75	5.43	0.32	0	2.65	3.32
22	4-C≡CH	7.53	7.91	−0.38	4.27	4.54	−0.27	0	0.40	0.95
23	4-C≡C$Si(CH_3)_3$	7.74	7.93	−0.19	4.92	5.17	−0.25	0	2.06	3.35
24	4-OCH_3	7.43	7.33	0.11	4.49	4.31	0.18	1	−0.02	0.79
25	4-OCH_2CH_3	7.59	7.55	0.04	4.56	4.49	0.07	1	0.38	1.25
26	4-$OCH_2CH{=}CH_2$	7.97	7.68	0.29	4.56	4.72	−0.16	1	0.81	1.50
27	4-$O(CH_2)_2CH_3$	7.73	7.70	0.03	4.58	4.84	−0.26	1	1.05	1.71
28	4-$O(CH_2)_3CH_3$	7.38	7.67	−0.29	4.86	5.06	−0.20	1	1.55	2.17
29	4-$O(CH_2)_5CH_3$	7.63	7.45	0.18	5.75	5.44	0.31	1	2.62	3.07
30	4-$O(CH_2)_7CH_3$	7.10	7.21	−0.11	5.78	5.68	0.10	1	3.63	4.00
31	4-$O(CH_2)_{10}CH_3$	6.77	6.78	−0.01	5.97	6.07	−0.10	1	5.37	5.38
32	4-$O(CH_2)_{11}CH_3$	6.42	6.65	−0.23	6.13	6.19	−0.06	1	5.91	5.84
33	4-$OCH_2C_6H_5$	7.95	8.01	−0.06	4.95	4.99	−0.04	0	1.66	3.17
34	4-$OCH_2C_6H_3$-3′,4′-Cl_2	7.54	7.74	−0.20	4.68	5.41	−0.73	0	2.91	4.17
35	4-$OCH_2C_6H_4$-4′-SO_2NH_2	7.63	7.58	0.05	4.23	3.77	0.46	0	−0.16	4.30
36	4-$OCH_2C_6H_4$-4′-$CONH_2$	7.78	7.80	−0.02	4.34	4.00	0.34	0	0.17	4.05
37	4-$OCH_2C_6H_4$-4′-CH_2OH	7.89	7.99	−0.10	4.53	4.32	0.21	0	0.63	3.79
38	4-$CH_2SC_6H_5$	8.06	7.88	0.18	5.35	5.22	0.13	0	2.30	3.79
39	4-$CH_2SC_6H_4$-2′-CH_3	7.85	7.75	0.10	5.69	5.38	0.31	0	2.86	4.26
40	4-$CH_2SC_6H_4$-3′-CH_3	8.02	7.75	0.27	5.54	5.38	0.16	0	2.86	4.26
41	4-$SCH_2C_6H_5$	7.96	7.88	0.08	5.07	5.22	−0.15	0	2.30	3.79
42	4-$SCH_2C_6H_4$-4′-Cl	7.34	7.72	−0.38	5.01	5.43	−0.42	0	3.01	4.29

[a] Calculated by eq 9. [b] Calculated by eq 10. [c] Not used in the derivation of Eq 9.

Table III. Comparison of the 50% Inhibition of Growth of Sensitive and Resistant Tumors Cells by Certain Antifolates

compd	log 1/C (L5178Y/S)	log 1/C (L5178Y/R)	Δ log 1/C	log P
metoprine	8.68 ± 0.05	5.58 ± 0.10	3.10	2.82
etoprine	8.55 ± 0.04	5.53 ± 0.07	3.02	3.19
BW 301U	8.75 ± 0.05	5.27 ± 0.08	3.48	2.13[b]
Baker's antifol I	7.53 ± 0.14	5.39 ± 0.14	2.14	−2.46
Baker's antifol II	6.74 ± 0.25	4.99 ± 0.14	1.75	2.42[c]
methotrexate	8.73 ± 0.11	2.93 ± 0.06	5.80	−2.59[a]
trimethoprim	5.19 ± 0.08	3.73 ± 0.06	1.46	0.82[b]
tetroxoprim	4.20 ± 0.11	3.16 ± 0.14	1.04	0.60[b]

[a] Determined at pH 2. [b] Measured in 0.1 N NaOH. [c] At pH 7.4.

see some difference in the activity of other antitumor drugs against the two types of cells. The results from such experiments are shown in Table IV.

With the possible exception of cisplatin, in no case is there a significant difference between the activity of these drugs on sensitive and resistant cells. Fischer has earlier observed that the sensitivity of resistant cells to certain antimetabolites (cytosine arabinoside, 6-azauridine, and 5-fluorodeoxyuridine) did not differ from that of the sensitive cells.[19b]

Table IV. 50% Inhibition of L5178Y Cell Growth by Various Antitumor Agents

compd	log P	log 1/C (L5178Y/S)	log 1/C (L5178Y/R)
hydroxyurea	−1.80	4.33 ± 0.08	4.36 ± 0.05
puromycin	0.03	6.46 ± 0.17	6.41 ± 0.03
daunomycin	0.66	7.17 ± 0.12	7.10 ± 0.18
adriamycin	0.10	7.18 ± 0.16	7.23 ± 0.21
actinomycin D	3.21	7.75 ± 0.04	7.76 ± 0.09
mitomycin C	−0.47	7.11 ± 0.05	7.13 ± 0.06
cytosine arabinoside	−2.13	7.18 ± 0.07	7.20 ± 0.15
cisplatin	−1.45	6.70 ± 0.05	7.10 ± 0.09

Except for hydroxyurea, all of the other compounds are highly active against the MTX-resistant cells, and thus it would seem that any of these agents would be suitable to use in combination chemotherapy with MTX to prevent the rise of MTX-resistant cells, in cases where resistance has been developed specifically for antifolates.

Discussion

One of the focal points of interest is the similarity between eq 4 for purified DHFR from L5178Y resistant cells

and that of eq 5 for the testing of the 3-substituted triazines against L5178Y cell culture sensitive to methotrexate. Unfortunately, we have not been able to get further quantities of enzyme for testing more inhibitors, which would enable us to evaluate whether or not the OR and DO terms would appear in eq 4, if a reasonable number of the alkoxy and alkyl congeners were included. However, the major features of the two active sites as reflected in eq 4 and 5 must be rather similar. It is highly unlikely that the coefficients with σ and I would by chance be identical. Also, there are interesting specific differences between the enzymic and in vivo equations.

The most useful clues for the design of selective inhibitors for a given type of DHFR are the poorly fit points. These vary with the DHFR, depending on its source. These points should also be of service to those doing X-ray crystallography in directing attention to critical differences in the various DHFR.

With purified DHFR, chicken,[6] L5178Y tumor,[14] bovine,[14] rat liver,[15] and *L. casei*[16] (but not human), the 3-CN substituent is 7 to 10 times more active than our QSAR predicts. With human DHFR, it is only 2.5 times as active as expected. Even from a study of the graphics model based on the X-ray crystallographic coordinates of the congener bound to chicken DHFR,[17a] there is no apparent reason for this anomaly. In light of these results, it is surprising to find that the 3-CN congener well fit by both eq 5 and 6. However, in the case of both *L. casei* DHFR and cell culture (both sensitive and resistant), the 3-cyanotriazine is more active than expected. These ambiguous results with the 3-CN derivative show that it is not always possible to extrapolate the biological activity of an inhibitor from the cell-free system to the drug–enzyme interaction in situ. This point has also been stressed by Sirotnak et al.[17b]

The 4-CN group behaves in a manner opposite to that of the 3-CN group, generally being less active than envisioned. With human, chicken, and *L. casei* DHFR, it is 20, 10, and 6 times less potent, respectively, than expected.

The 4-$OCH_2CON(CH_2CH_2)_2O$ congener is more active than calculated with human DHFR (30 times), with *L. casei* (6 times), and with chicken (40 times). With L5178Y sensitive cells, the aberrant behavior is minimal (about 3 times as active as calculated). With the resistant cells, it is well fit; its deviation being less than the standard deviation of eq 10.

Another derivative that is generally overpredicted for purified DHFR is the 4-$C{\equiv}C$-C_6H_5 analogue. It is 100 times less potent against chicken DHFR and 30 times less potent against *L. casei* but only 3 times less potent against human DHFR than calculated. With the sensitive L5178Y cells it is 50 times less active than expected, but with the resistant cells its activity is reasonably well predicted, being off only slightly more than the standard deviation of eq 10. A graphics model of this congener bound to chicken DHFR, in the same fashion as determined by X-ray crystallography for several other triazines, shows that it makes a very bad contact with the enzyme, which can only be avoided by pulling the 2,4-diaminotriazine group away from its usual binding position[6] or by a conformational change in the enzyme. A similar rigid substituent, 4-$C{\equiv}CSi(CH_3)_3$ is badly fit by the chicken and *L. casei* DHFR QSAR, but is well fit by both eq 9 and 10 for the in vivo studies.

A bulky substituent, 3-OCH_2-adamantyl, is poorly fit by the chicken and *L. casei* QSAR and marginally so by the human QSAR. This bulky substituent is also well fit by both eq 5 and 6; thus, although the similarity of the equations augurs well for a similarity in receptor geometry, the activity of certain substituents accentuates some highly significant difference.

The only substituent that we have found to be invariably poorly fit with all DHFRs, as well as with sensitive and resistant cells, is 3-$CH(OH)C_6H_5$. The consistent behavior of this substituent makes it a useful marker group. It will be interesting to see if there are any cases where it will be well behaved in cell-culture systems.

One generalization that seems in order is that resistant cells seem more tolerant of bulky or rigid groups than isolated DHFR or sensitive cells. This is best illustrated by the following substituents: 4-$C{\equiv}CSi(CH_3)_3$, 4-$C{\equiv}CC_6H_5$, 3-CH_2O-adamantyl.

We believe that the general similarity of eq 5 and 9 with their counter eq 1–4 indicates that cells are indeed being inhibited in their growth primarily by inhibition of DHFR and the subsequent depletion of reduced folate pools by the triazines.

It is through the study of variations in exceptional substituents that one hopes to be able to develop drug selectivity. The good agreement between eq 4 and 5 offers us some reassurance that data obtained on an isolated enzyme can be extrapolated to the whole cell in general while exercising a certain degree of caution.

However, a comparison of the results from sensitive and resistant cells not only reveals gross differences in the QSAR but also great discrepancies among the behavior of individual congeners. Comparing eq 5 and 6, we find many more badly fit points with the equation for resistant cells. The negative coefficient with the MR term, which is not found for isolated DHFR or sensitive cells, suggests to us that the active site in the resistant cells is under greater constraint and is less flexible. This could explain some of the deviant points of the 3-substituted triazines.

Harper and Kellems have recently discussed the molecular basis of methotrexate resistance.[18] They consider three possible mechanisms: "1. alterations in the DHFR molecule such that the normal high affinity for methotrexate is reduced or lost (these changes presumably result from mutations in the structural gene), 2. mutational alterations affecting some component of the active transport system involved in the uptake of methotrexate into cells, resulting in a low intracellular concentration of the drug, and 3. an increase in the level of DHFR such that there is an excess of enzyme relative to the concentration of drug in the cells (thus some of the enzyme is free to carry out the conversion of dihydrofolate to tetrahydrofolate)."

Harper and Kellems note that several other instances have been found where development of resistance by cells to certain chemicals has resulted in a concomitant incrrease in enzyme activity. Therefore, such differences, as are found between eq 5 and 6 as well as 9 and 10, may not be so unusual. In fact, we have found very similar differences between *L. casei* cells resistant and sensitive to MTX.[7] In the case of the MTX-resistant *L. casei* cells at

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(17) (a) X-ray Coordinates of this complex were kindly supplied to us by D. A. Matthews. (b) Sirotnak, F. M.; Chello, P. L.; Moccio, D. M.; Piper, J. R.; Montgomery, J. A.; Parham, J. C. *Biochem. Pharmacol.* **1980**, *29*, 3293.
(18) Harper, M. E.; Kellems, R. E. *Cancer Bull.* **1981**, *33*, 43.

100 000 000-fold greater concentration is required for 50% inhibition of growth.

It has been shown that L5178Y cells resistant to MTX contains hundreds of times the normal amount of DHFR. This actually amounts to 10% of the total cellular protein being in the form of the enzyme.[19a] Harper and Kellems report an instance where 20% of all cell protein is DHFR.[18]

In stable, mutant, MTX-resistant cell lines, the overproduction of DHFR has been well documented.[19a] DHFR gene amplification has also been recently demonstrated in a leukemia patient after treatment with MTX.[48] The increased synthesis of the enzyme, which is due to the selective amplification of the DHFR genes in resistant cells, protects against the cytotoxic effects of MTX and other hydrophilic antifolates.

Although DHFR production in resistant cells may be several hundred times as great as in sensitive cells, it is hard to envision that this factor alone could account for cells being 10^6 to 10^8 times more resistant to MTX.

Wang and Loo[20] consider the evidence for loss of MTX activity through metabolism but find little evidence that this could be a route for high loss of activity in cell culture.

Hakala has pointed out[21] that although in vitro MTX is a "stoichiometric" inhibitor of DHFR, this inhibition is pH dependent, so that it is concluded that stoichiometric inhibition intracellularly would require drug concentrations $>10^{-5}$ M. However, it is difficult to see how this could account for the insensitivity of resistant L5178Y cells to MTX.

It is well known that some resistant cells transport MTX poorly. Kamen et al.[22] have demonstrated that L1210 resistant cells incorporate only 5% as much MTX as sensitive cells, even though they contain 100 times as much DHFR. Huennekens et al.[23,24] have reviewed the problem of active transport of MTX in mammalian and *L. casei* cells and conclude that transport is mediated by a single protein with an active site sufficiently flexible to accommodate both large and small molecules where the only common feature is a negative charge.[24]

Sirotnak et al.,[25] in reviewing the potential for exploitation of transport systems in anticancer drug design, have shown that different antifolates have different rates of influx and efflux from tumor cells.[25,26,49]

In a study of normal and resistant Reuber rat hepatoma cells, Galivan[27] has shown that the resistant cells that did not allow MTX to gain access to the intracellular compartment had an altered transport system. Furthermore, the great sensitivity of the resistant cells to heat and trypsinization suggested an alteration in the membranes of these cells, which appeared to allow the entrance of MTX only by diffusion.

McCormick et al.[28] have recently characterized a MTX transport defect in a resistant L1210 lymphoma cell line. Their results suggested that a large amount of MTX was not being transported into the intracellular compartment but may have instead bound to functional receptors for MTX on the exterior surface of the plasma membrane in the absence of a functioning MTX–THF carrier system. One wonders if these binding sites might not constitute normal carrier protein displaced so as to be incapable of displaying its normal function.

Table V. Differences in Potency between Four of the Most Hydrophilic and Four of the Most Hydrophobic Triazines for Resistant and Sensitive Cells

substituent	π	log 1/C (sensitive cells)	log 1/C (resistant cells)	Δ log 1/C
4-SO_2NH_2	−1.82	5.97	3.11	−2.86
4-SO_2CH_3	−1.63	6.28	3.12	−3.16
3-SO_2NH_2	−1.82	5.61	3.13	−2.48
3-$CONH_2$	−1.49	6.10	3.78	−2.32
				av −2.71
3-$OC_{14}H_{29}$	6.99	6.17	6.49	+0.32
3-$OC_{13}H_{27}$	6.45	6.22	6.52	+0.30
4-$OC_{12}H_{25}$	5.91	6.42	5.97	−0.45
4-$OC_{11}H_{23}$	5.37	6.77	6.13	−0.64
				av −0.12

Evidence for an impaired transport system in L5178Y resistant cells was uncovered many years ago by Harrap et al.[29] In a continuation of these studies, Hill et al.[30] have concluded that the MTX–tetrahydrofolate transport carriers have undergone a marked reduction in affinity for their substrates. These findings led them to suggest that the bulk of the transport of MTX, AMT, and 5-CH_3-H_4-folate occurs by a mechanism other than the MTX–THF cofactor carrier. Recently, Hill et al.[31] in a further comparison of resistant and sensitive L5178Y cells showed that at low MTX concentrations (10^{-6} M) sensitive cells rapidly take up MTX but resistant cells do not. At high MTX concentrations (10^{-4} M) both sensitive and resistant cells take up the drug in almost identical fashion! There was little competition between MTX and 5-CH_3-H_4folate or folinic acid, suggesting that MTX was not being taken up by the normal folate transport system. The resistant cells were found to be markedly less affected by MTX cytotoxicity, despite the fact that potentially cytocidal intracellular concentrations of drug were achieved. They conclude that in addition to impaired transport and extra DHFR, the DHFR may be present in an altered form.

Our results point to the same conclusions reached by Hill et al.[31] It seems to us that the lack of an effective transport system may be the single most important factor in accounting for the 10^6-fold difference between sensitive and resistant L5178Y cells and the 10^8-fold difference between sensitive and resistant *L. casei* cells. No doubt overproduction of DHFR plays a part, but in addition, we also feel that the DHFR has an altered conformation in resistant cells.

Our earlier conclusion from a comparison of eq 5 and 6 suggested that a lipophilic barrier had been erected in the resistant cells, preventing the entrance of highly polar MTX, the polar triazines,[8] and the polar benzylpyrimidines.[32] The lipophilic triazines, which are much

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less active than MTX against isolated DHFR, are 3000–4000 times more potent than MTX against resistant cells.

If the active-transport system for MTX in resistant cells were in some way or another completely impaired, then this highly polar, negatively charged molecule would have to gain entry by passive diffusion though a lipid membrane. Thus, in effect the resistant cells would have erected a hydrophobic barrier against hydrophilic compounds.

Hakala[33] points out that ionogenic groups on mammalian cell surfaces give the cells a net negative charge, revealed by an isoelectric point between 3.2 and 3.7. It was noted that a 30.5-kcal activation energy is required for MTX influx. Thus, it is not surprising that the highly polar, negatively charged MTX does not readily enter cells by passive diffusion.

While this could account for the huge difference in MTX, one wonders how the triazines are affected. Table V shows the difference in activity between four of the most hydrophilic and four of the most hydrophobic triazines on the two types of cells. On the average, the four hydrophilic drugs are 500 times less effective against the resistant cells, while the hydrophobic drugs, on the average, are only slightly less active. In fact, the two most hydrophobic congeners are actually more active against resistant cells than sensitive cells.

Of course a factor of 500 for the triazines is a far cry from a factor of 1 000 000 for MTX. We believe that a large part of this difference is due to the difference in the resistance met by the negatively charged MTX and positively charged triazines at the negatively charged cell surface.

In addition to a crippled transport system and overproduction of DHFR to protect them from polar antifolates, resistant cells seem to have DHFR with a different conformation. Since eq 4 for the action of triazines on DHFR isolated from resistant L4178Y cells is so similar to eq 5 for the action of triazines on sensitive cells, we conclude that the basic structure of the DHFR in each case is similar. However, as discussed above, the markedly different way certain inhibitors, especially those with bulky substituents, react with sensitive and resistant cells suggest a different conformation of DHFR in resistant cells.

Much of the experience with QSAR in the last 20 years advocates that the dominant feature affecting *passive* drug movement in biological material is the hydrophobic character, as represented by the octanol/water partition coefficient (log P or π).[34-37] In instances where the differences in molecular size are very great, molecular weight is also a factor.[38] Specific steric factors appear to play little, if any, role. Therefore, we attribute the differences in poorly fit congeners for eq 5 and 6 as well as 9 and 10 to be most likely due to a conformational difference in the DHFR in sensitive and resistant cells. It is hard to envision how a congener such as $C{\equiv}C\text{-}C_6H_5$, which is grossly misfit in the sensitive cells equation, would fit the equation for resistant cells, except by means of some change in the DHFR.

An especially important difference in both eq 6 and 10 is the necessity for identical MR terms with negative coefficients. We see no way of interpreting this other than a generally constrained binding site in the resistant cells. No role for MR in eq 5 and 9 could be found.

Table VI

substituent	yield, %	mp, °C (solvent)	formula
4-OCH_2CH_3	90	237-239 (EtOH)	$C_8H_{11}NO{\cdot}HCl$
4-$O(CH_2)_3CH_3$	67	113.5-115 (EtOAc)	$C_{10}H_{15}NO{\cdot}HCl$
4-$O(CH_2)_7CH_3$	40	102-103 (EtOH)	$C_{14}H_{23}NO{\cdot}HCl$

Table VII

substituent	yield, %	mp, °C (solvent)	formula
3-$CH_2OC_6H_2$-2',4',5'-Cl_3	70	140-141 (EtOH-benzene)	$C_{13}H_{10}Cl_3NO$
4-$OCH_2CH{=}CH_2$	33	211-214 (EtOH)	$C_9H_{11}NO{\cdot}HCl$

Whether or not the change in the DHFR in phenotypic resistance is more than just conformational is difficult to say. Considerable evidence exists which indicates that DHFR from sensitive and resistant cells is the same.[39,40,50] On the other hand, evidence has been uncovered for other structurally altered forms of DHFR.[41-46]

While we cannot rule out the presence of a second form of DHFR in the resistant cells, the similarity between eq 4 and 5 points to the majority of the DHFR in sensitive and resistant cells to being very similar, if not identical.

The results in this report, as well as those in an earlier one,[7] illustrate the power of the QSAR paradigm in making inferences about the differences as well as the similarities between isolated purified enzyme and enzyme in the living cell. It is this kind of understanding that is so necessary for some of the modern approaches to drug design in which the starting point is purified enzyme or isolated receptor. We believe that studies of the well-characterized enzymes will eventually pave the way for better understanding of bioreceptors in general.

Sirotnak and his colleagues[25,26,47] have emphasized that in the development of better antitumor drugs, more attention should be paid to the SAR of active transport. They have stated that only therapeutically responsive tumors have been shown to possess an efficient mechanism for transporting and concentrating antifolates intracellularly.[47]

While our results indicate that in the design of antifolates more potent against MTX-resistant tumors, highly lipophilic congeners are potent on isolated DHFR and

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Table VIII

substituent	yield, %	mp, °C (solvent)	formula
$3,5\text{-}(CH_3)_2$	61	205.5-207 (EtOH)	$C_{13}H_{19}N_5 \cdot HCl$
$3\text{-}CH_2OC_6H_2\text{-}2',4',5'\text{-}Cl_3$	53	203-204.5 (EtOH)	$C_{16}H_{18}Cl_3N_5O \cdot HCl$
$4\text{-}OCH_2CH_3$	77	221-222 (EtOH)	$C_{13}H_{19}N_5O \cdot HCl$
$4\text{-}OCH_2CH{=}CH_2$	80	212-213.5 (EtOH)	$C_{14}H_{19}N_5O \cdot HCl$
$4\text{-}O(CH_2)_3CH_3$	30	211-212 (EtOH)	$C_{15}H_{23}N_5O \cdot HCl$
$4\text{-}O(CH_2)_7CH_3$	38	201-202 (EtOH-benzene)	$C_{19}H_{31}N_5O \cdot HCl$

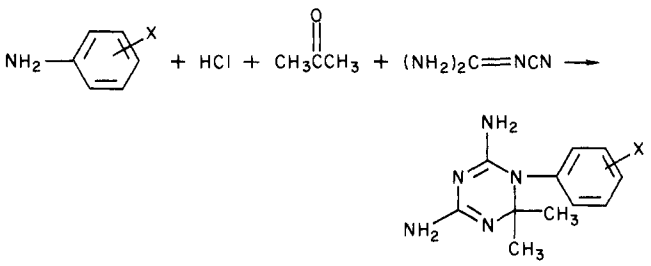
especially in tumor cells, this does not mean that one can automatically exploit this potential in the whole animal. It is all too well known that each series of drugs possess an ideal log *P* for optimum movement in animals. To date, the value of this parameter has not been established for the antifolates.

The log *P* for the parent I (X = H) in water at pH 6 or buffer is estimated to be −3.0. Thus, it might be reasonable to add a substituent X of π about 5. This would give an overall log *P* of 2.0, which has often been found to be near the optimum for movement of *neutral* drugs in animals. However, the triazines do not constitute a neutral class of drugs. At physiological pH they are completely protonated. This creates an additional problem, since the partition of a charged species depends heavily on the type and concentration of the counterion associated with it. Since we do not know what the counterion in vivo would be, we cannot at this time make a good estimate of what log P_0 would be for an animal system. This important parameter will have to be experimentally determined.

Experimental Section

Biology. The original L5178Y/S and L5178Y/R cells were kindly provided by Dr. J. Bertino, Department of Pharmacology, Yale University School of Medicine, New Haven, CT. Cytotoxicity assays in 48-h cultures were performed as previously described.[8]

Chemistry. The syntheses of most of the triazines used in this study have been reported elsewhere, with the exception of the following compounds, which were synthesized by the three-component condensation method of Modest et al.[6,51] The necessary aromatic amines were generally prepared from the corresponding nitro compounds, the syntheses of which, are reported below.



Nitro Compounds. Method A. 3-Nitrobenzyl 2,4,5-Trichlorophenyl Ether. A suspension of 2,4,5-trichlorophenol (40 g, 0.2 mol), 3-nitrobenzyl chloride (35 g, 0.2 mol), and potassium hydroxide (11.2 g, 0.2 mol) in a mixture of 200 mL of absolute ethanol and diglyme was refluxed for 20 h. The flocculent precipitate was collected, washed with water, and crystallized from methanol to yield 49 g (63%) of the desired product, mp 176–177 °C. Anal. ($C_{13}H_8NO_3Cl_3$) C, H.

Method B. A suspension of either *p*-hydroxyacetanilide or *p*-nitrophenol (0.1 mol), the appropriate alkyl bromide (0.1 mol) and potassium carbonate (0.1 mol) in acetone (200 mL) was heated at reflux for 24 h. The suspension was filtered, the solvent was evaporated, and the oily residue was dissolved in 100 mL of ether. The ethereal layer was washed successively with 10% potassium hydroxide (100 mL) and water (2 × 100 mL) and dried ($MgSO_4$). After removal of the drying agent and evaporation of the ether, the crude product was generally used without further purification. Crude yields were as follows: $4\text{-}OCH_2CH_3$, 43%; $4\text{-}OCH_2CH{=}CH_2$, 70%; $4\text{-}O(CH_2)_3CH_3$, 40%; $4\text{-}O(CH_2)_7CH_3$, 80%.

Method C. A suspension of *p*-hydroxyacetanilide (0.1 mol), octyl bromide (0.1 mol), and potassium carbonate (0.1 mol) in acetone (100 mL) was refluxed with stirring for 48 h. The acetone was evaporated, and the residue was poured into 2% sodium hydroxide (50 mL) and stirred for 1 h. The product was filtered and dried: yield 80%; mp 97–99 °C.

A mixture of *p*-(octyloxy)acetanilide (0.05 mol), ethanol (50 mL), and concentrated hydrochloric acid (50 mL) was refluxed for 2 h. The resulting crystalline solid of *p*-(octyloxy)aniline hydrochloride was collected, washed with ether, and dried: yield 5.2 g (40%); mp 102–103 °C. Anal. ($C_{14}H_{23}NO \cdot HCl$) C, H.

Aromatic Anilines. Method A. The nitro compound was dissolved in 100 mL of absolute ethanol and hydrogenated on a Parr low-pressure apparatus with a 1:10 ratio of catalyst (PtO_2) to substrate. After subsequent removal of the catalyst and solvent, the residue was dissolved in ether, and HCl gas was passed through the solution. The precipitate was collected and crystallized. All compounds analyzed correctly for carbon and hydrogen (Table VI).

Method B. A suspension of the appropriate nitro compound (5 g), iron powder (25 g), and acetic acid (1 mL) in water (200 mL) was mechanically stirred at 80–90 °C for 28 h. The resulting slurry was neutralized with 10% Na_2CO_3 (50 mL) and filtered, and the filtrate was extracted with hot benzene (3 × 100 mL). The combined benzene fractions were washed with water (2 × 100 mL) and dried over $MgSO_4$. After removal of the solvent, the residue was either recrystallized from an appropriate solvent or converted into the hydrochloride salt (Table VII).

Triazines. A mixture of the substituted aniline hydrochloride (1.0 equiv) and dicyandiamide (1.05 equiv) was heated at reflux in acetone for 24 h. The solvent was removed, and the residue was crystallized from an appropriate solvent. In the case of an aniline free base, 1.0 equiv of concentrated HCl was added to the reaction mixture. All the compounds analyzed correctly for C and H (Table VIII).

QSAR. The values for the substituent constants in Tables I and II were taken from our recent compilation.[11] The stepwise development of the final equations obtained in the QSAR is outlined below.

QSAR for 3-Substituted Triazines

50% Inhibition of Growth of L5178Y/S Cells

Points Not Included: $3\text{-}COOC_2H_5$, 3-*dl*-$CH(OH)C_6H_5$

$$\log 1/C = -0.07\ (\pm 0.08)\ \pi + 7.70\ (\pm 0.23) \qquad (11)$$

$$n = 64,\ r = 0.217,\ s = 0.647,\ F_{1,62} = 3.1$$

$$\log 1/C = 1.23\ (\pm 0.28)\ \pi - 1.53\ (\pm 0.32) \log(\beta \cdot 10^{\pi} + 1) + 8.10\ (\pm 0.17) \qquad (12)$$

$$n = 64,\ r = 0.785,\ s = 0.417,\ \pi_0 = 0.85\ (\pm 0.33),\ \log \beta = -0.229,\ F_{2,60} = 44.6$$

$$\log 1/C = 1.40\ (\pm 0.28)\ \pi - 1.63\ (\pm 0.31) \log(\beta \cdot 10^{\pi} + 1) - 0.59\ (\pm 0.25)\ \mathrm{OR} + 8.34\ (\pm 0.17) \qquad (13)$$

$$n = 64,\ r = 0.851,\ s = 0.357,\ \pi_0 = 0.81\ (\pm 0.35),\ \log \beta = -0.031,\ F_{1,59} = 22.8$$

$$\log 1/C = 1.43\ (\pm 0.27)\ \pi - 1.66\ (\pm 0.30) \log(\beta \cdot 10^{\pi} + 1) - 0.46\ (\pm 0.25)\ \mathrm{OR} + 0.29\ (\pm 0.19)\ I + 8.31\ (\pm 0.19) \qquad (14)$$

$$n = 64,\ r = 0.873,\ s = 0.335,\ \pi_0 = 0.76\ (\pm 0.31),\ \log \beta = -0.027,\ F_{1,58} = 9.2$$

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$$\log 1/C = 1.40\ (\pm0.26)\ \pi - 1.66\ (\pm0.29) \log(\beta \cdot 10^{\pi} + 1) - 0.34\ (\pm0.25)\ \text{OR} + 0.38\ (\pm0.20)\ I + 0.39\ (\pm0.31)\ \text{DO} + 8.25\ (\pm0.18) \quad (15)$$

$n = 64$, $r = 0.886$, $s = 0.320$, $\pi_0 = 0.72\ (\pm0.30)$, $\log \beta = -0.023$, $F_{1,57} = 6.3$

$$\log 1/C = 1.40\ (\pm0.23)\ \pi - 1.65\ (\pm0.26) \log(\beta \cdot 10^{\pi} + 1) - 0.25\ (\pm0.24)\ \text{OR} + 0.52\ (\pm0.20)\ I + 0.63\ (\pm0.33)\ \text{DO} + 0.88\ (\pm0.57)\ \sigma + 7.94\ (\pm0.21) \quad (5)$$

$n = 64$, $r = 0.904$, $s = 0.298$, $\pi_0 = 0.80\ (\pm0.29)$, $\log \beta = -0.054$, $F_{1,56} = 9.7$

Equation 16 has been derived using all data points.

$$\log 1/C = 1.34\ (\pm0.25)\ \pi - 1.58\ (\pm0.28) \log(\beta \cdot 10^{\pi} + 1) - 0.19\ (\pm0.27)\ \text{OR} + 0.60\ (\pm0.22)\ I + 0.76\ (\pm0.36)\ \text{DO} + 1.18\ (\pm0.60)\ \sigma + 7.75\ (\pm0.21) \quad (16)$$

$n = 66$, $r = 0.882$, $s = 0.332$, $\pi_0 = 0.85\ (\pm0.33)$, $\log \beta = -0.108$

Squared Correlation Matrix

	π	OR	I	σ	DO
π	1.00	0.12	0	0.14	0.03
OR	0.12	1.00	0.12	0	0.02
I	0.0	0.12	1.00	0.07	0.05
σ	0.14	0.00	0.07	1.00	0.14
DO	0.03	0.02	0.05	0.14	1.00

50% Inhibition of Growth of L5178Y/R Cells

Points Not Included: 3-$COOC_2H_5$ (not tested), 3-SO_2NH_2, 3-*dl*-$CH(OH)C_6H_5$, 3-CH_2NH-C_6H_4-4′-SO_2NH_2, 3-$CH_2OC_6H_2$-2′,4′,5′-Cl_3

$$\log 1/C = 0.28\ (\pm0.06)\ \pi + 4.81\ (\pm0.16) \quad (17)$$

$n = 61$, $r = 0.787$, $s = 0.417$, $F_{1,59} = 96.0$

$$\log 1/C = 0.39\ (\pm0.07)\ \pi - 0.16\ (\pm0.07)\ \text{MR} + 5.10\ (\pm0.20) \quad (18)$$

$n = 61$, $r = 0.843$, $s = 0.367$, $F_{1,58} = 18.1$

$$\log 1/C = 0.43\ (\pm0.07)\ \pi - 0.18\ (\pm0.07)\ \text{MR} - 0.37\ (\pm0.24)\ \text{OR} + 5.16\ (\pm0.19) \quad (19)$$

$n = 61$, $r = 0.867$, $s = 0.342$, $F_{1,57} = 9.7$

$$\log 1/C = 0.63\ (\pm0.20)\ \pi - 0.26\ (\pm0.25) \log(\beta \cdot 10^{\pi} + 1) - 0.33\ (\pm0.24)\ \text{OR} - 0.17\ (\pm0.07)\ \text{MR} + 5.11\ (\pm0.19) \quad (6)$$

$n = 61$, $r = 0.878$, $s = 0.335$, $\pi_0 = 5$–6, $\log \beta = -0.718$, $F_{2,55} = 2.2$

The corresponding bilinear equation with all points included was not obtainable.

Squared Correlation Matrix

	π	OR	MR
π	1.0	0.12	0.52
OR	0.12	1.0	0.02
MR	0.52	0.02	1.0

QSAR for 4-Substituted Triazines

50% Inhibition of Growth of L5178Y/S Cells

Points Not Included: 4-C≡CC_6H_5

$$\log 1/C = 0.07\ (\pm0.10)\ \pi + 7.42\ (\pm0.22) \quad (20)$$

$n = 41$, $r = 0.219$, $s = 0.589$, $F_{1,39} = 1.96$

$$\log 1/C = 0.82\ (\pm0.16)\ \pi - 1.12\ (\pm0.22) \log(\beta \cdot 10^{\pi} + 1) + 7.70\ (\pm0.13) \quad (21)$$

$n = 41$, $r = 0.867$, $s = 0.309$, $\pi_0 = 1.15\ (\pm0.46)$, $\log \beta = -0.711$, $F_{2,37} = 52.3$

$$\log 1/C = 0.91\ (\pm0.15)\ \pi - 1.16\ (\pm0.21) \log(\beta \cdot 10^{\pi} + 1) - 0.35\ (\pm0.23)\ \text{OR} + 7.82\ (\pm0.12) \quad (9)$$

$n = 41$, $r = 0.896$, $s = 0.278$, $\pi_0 = 1.11\ (\pm0.38)$, $\log \beta = -0.542$, $F_{1,36} = 4.8$

Equation 22 has been derived using all data points.

$$\log 1/C = 0.96\ (\pm0.23)\ \pi - 1.21\ (\pm0.30) \log(\beta \cdot 10^{\pi} + 1) - 0.27\ (\pm0.31)\ \text{OR} + 7.89\ (\pm0.19) \quad (22)$$

$n = 42$, $r = 0.811$, $s = 0.387$, $\pi_0 = 0.96\ (\pm0.47)$, $\log \beta = -0.372$

Squared Correlation Matrix

	π	OR
π	1.00	0.13
OR	0.13	1.00

50% Inhibition of Growth of L5178Y/R Cells

No Data Points Dropped

$$\log 1/C = 0.42\ (\pm0.06)\ \pi + 4.21\ (\pm0.14) \quad (23)$$

$n = 42$, $r = 0.904$, $s = 0.374$, $F_{1,40} = 177.9$

$$\log 1/C = 0.51\ (\pm0.08)\ \pi - 0.15\ (\pm0.09)\ \text{MR} + 4.65\ (\pm0.19) \quad (24)$$

$n = 42$, $r = 0.928$, $s = 0.330$, $F_{1,39} = 12.4$

$$\log 1/C = 0.61\ (\pm0.10)\ \pi - 0.29\ (\pm0.20) \log(\beta \cdot 10^{\pi} + 1) - 0.13\ (\pm0.08)\ \text{MR} + 4.42\ (\pm0.18) \quad (10)$$

$n = 42$, $r = 0.942$, $s = 0.306$, $\pi_0 = 5$–6, $\log \beta = -2.07$, $F_{2,37} = 4.2$

Squared Correlation Matrix

	π	MR
π	1.00	0.47
MR	0.47	1.00

Acknowledgment. We thank Cynthia D. Strong for the synthesis of triazines I, where X = 4-OCH_2CH_3 and 4-$O(CH_2)_3CH_3$. We also thank Dr. Kenneth Chan, University of Southern California School of Pharmacy for the adriamycin and Dr. D. S. Duch of Buroughs Wellcome for a sample of BW 301U. We are indebted to P. Y. C. Jow for determining several of the partition coefficients of compounds in Table II.

Registry No. I (X = H), 4022-58-6; I (X = 3-SO_2NH_2), 70579-32-7; I (X = 3-$CONH_2$), 70579-33-8; I (X = 3-$COCH_3$), 70579-34-9; I (X = 3-$COOCH_2CH_3$), 70650-62-3; I (X = 3-OH), 70579-35-0; I (X = 3-CF_3), 1492-81-5; I (X = 3-F), 3850-94-0; I (X = 3-Cl), 13351-02-5; I (X = 3-Br), 24849-96-5; I (X = 3-I), 51012-14-7; I (X = 3-NO_2), 17711-74-9; I (X = 3-CN), 70743-55-4; I (X = 3-CH_3), 4038-60-2; I (X = 3-CH_2CH_3), 87739-76-2; I (X = 3-$(CH_2)_5CH_3$), 70650-60-1; I (X = 3-$(CH_2)_8CH_3$), 87739-77-3; I (X = 3-$(CH_2)_{11}CH_3$), 87739-78-4; I [X = 3-$C(CH_3)_3$], 70579-36-1; I [X = 3-DL-$CH(OH)C_6H_5$], 75153-70-7; I (X = 3-OCH_3), 17711-73-8; I (X = 3-OCH_2CH_3), 46985-98-2; I [X = 3-$O(CH_2)_2CH_3$], 14052-49-4; I [X = 3-$O(CH_2)_3CH_3$], 70606-63-2; I [X = 3-$O(CH_2)_4CH_3$], 87739-98-8; I [X = 3-$O(CH_2)_5CH_3$], 74798-21-3; I [X = 3-$O(CH_2)_8CH_3$], 70579-30-5; I [X = 3-$O(CH_2)_{10}CH_3$], 70579-55-4; I [X = 3-$O(CH_2)_{11}CH_3$], 70579-29-2; I [X = 3-$O(CH_2)_{12}CH_3$], 79508-88-6; I [X = 3-$O(CH_2)_{13}CH_3$], 70579-52-1; I [X = 3-$O(CH_2)_2OC_6H_5$], 19161-84-3; I [X = 3-$O(CH_2)_2OC_6H_4$-3-CF_3], 70579-37-2; I [X = 3-$O(CH_2)_4OC_6H_5$], 70579-31-6; I [X = 3-$O(CH_2)_4OC_6H_4$-3′-CF_3], 70579-41-8; I [X = 3-$OCH_2C_6H_5$], 70579-38-3; I (X = 3-$OCH_2C_6H_3$-3′,4′-Cl_2), 70579-39-4; I (X = 3-$OCH_2C_6H_4$)-4′-$CONH_2$), 87739-82-0; I (X = 3-OCH_2-1-adamantyl), 87871-38-3; I (X = 3-CH_2O-c-C_6H_{11}), 87871-39-4; I [X = 3-$CH_2NHC_6H_3$-3′,5′-$(CONH_2)_2$], 70579-40-7; I (X = 3-$CH_2NHC_6H_4$-4′-SO_2NH_2), 70579-42-9; I (X = 3-$CH_2OC_6H_5$), 79508-78-4; I (X = 3-$CH_2OC_6H_4$-3′-Cl), 79508-79-5; I (X = 3-$CH_2OC_6H_4$-3′-CN), 79519-97-4; I (X = 3-$CH_2OC_6H_4$-3′-OCH_3), 79508-80-8; I (X = 3-$CH_2OC_6H_4$-3′-CH_2OH), 79508-81-9; I (X = 3-$CH_2OC_6H_4$-3′-CH_3), 79508-82-0; I (X = 3-$CH_2OC_6H_4$-3′-

CH_2CH_3), 79508-83-1; I [X = 3-$CH_2OC_6H_4$-3′-$CH(CH_3)_2$], 79508-84-2; I [X = 3-$CH_2OC_6H_4$-3′-$C(CH_3)_3$], 79508-85-3; I (X = 3-$CH_2OC_6H_4$-3′-C_6H_5), 87739-80-8; I (X = 3-$CH_2OC_6H_4$-3′-$NHCOCH_3$), 79508-86-4; I (X = 3-$CH_2OC_6H_4$-3′-$NHCSNH_2$), 79508-87-5; I (X = 3-$CH_2OC_6H_4$-3′-$NHCONH_2$), 70579-43-0; I [X = 3-$CH_2OC_6H_4$-4′-$(CH_2)_4CH_3$], 87739-81-9; I (X = 3-CH_2O-2-naphthyl), 87871-40-7; I (X = 3-CH_2O-1-naphthyl), 87871-41-8; I (X = 3-$CH_2SC_6H_5$), 80239-83-4; I (X = 3-$CH_2SC_6H_4$-3′-CH_3), 87739-85-3; I (X = 3-$CH_2SeC_6H_5$), 87739-79-5; I (X = 3-$CH_2C_6H_5$), 87739-83-1; I (X = 3-$SCH_2C_6H_4$-4′-Cl), 87739-84-2; I (X = 3-$CH_2OC_6H_2$-2′,4′,5′-Cl_3), 88253-89-8; I (X = 3,5-Cl_2), 2727-10-8; I [X = 3,5-$(CH_3)_2$], 88253-90-1; I (X = 4-SO_2NH_2), 90-08-4; I (X = 4-SO_2CH_3), 74798-28-0; I (X = 4-$CONH_2$), 87871-34-9; I (X = 4-$COCH_3$), 85304-88-7; I (X = 4-OH), 74798-26-8; I (X = NH_2), 87871-35-0; I (X = 4-$NHCOCH_3$), 74798-27-9; I (X = 4-CF_3), 47071-11-4; I (X = 4-F), 1542-59-2; I (X = 4-Cl), 516-21-2; I (X = Br), 3567-84-8; I (X = 4-I), 46781-41-3; I (X = 4-CN), 17711-68-1; I (X = 4-OCH_2CO-morpholine), 50574-87-3; I [X = 4-$O(CH_2)_2$-OC_6H_4-4′-NH_2], 87871-36-1; I (X = 4-CH_3), 15233-37-1; I [X = 4-$(CH_2)_3CH_3$], 4653-73-0; I [X = 4-$(CH_2)_8CH_3$], 87739-87-5; I [X = 4-$C(CH_3)_3$], 4653-75-2; I (X = 4-C≡CC_6H_5), 87871-37-2; I (X = 4-C≡CH), 87740-00-9; I [X = 4-C≡$CSi(CH_3)_3$], 87740-01-0; I (X = 4-OCH_3), 21316-30-3; I (X = 4-OCH_2CH_3), 46985-99-3; I (X = 4-OCH_2CH=CH_2), 88253-91-2; I [X = 4-$O(CH_2)_3CH_3$], 87739-96-6; I [X = 4-$O(CH_2)_5CH_3$], 4653-82-1; I (X = 4-$O(CH_2)_5CH_3$], 4653-85-4; I [X = 4-$O(CH_2)_7CH_3$], 4653-87-6; I [X = 4-$O(CH_2)_{10}CH_3$], 87739-97-7; I [X = 4-$O(CH_2)_{11}CH_3$], 79515-25-6; I (X = 4-$OCH_2C_6H_5$), 17944-10-4; I (X = 4-$OCH_2C_6H_3$-3′,4′-Cl_2), 85304-89-8; I (X = $OCH_2C_6H_4$-4′-SO_2NH_2), 87739-88-6; I (X = $OCH_2C_6H_4$-4′-$CONH_2$), 87739-89-7; I (X = $OCH_2C_6H_4$-4′-CH_2OH), 87739-90-0; I (X = $CH_2SC_6H_5$), 87739-93-3; I (X = $CH_2SC_6H_4$-2′-CH_3), 87739-94-4; I (X = $CH_2SC_6H_4$-3′-CH_3), 87739-95-5; I (X = $SCH_2C_6H_5$), 87739-91-1; I (X = $SCH_2C_6H_4$-4′-Cl), 87739-92-2; methotrexate, 59-05-2; 2,4,5-trichlorophenol, 95-95-4; 3-nitrobenzyl chloride, 619-23-8; 3-nitrobenzyl 2,4,5-trichlorophenyl ether, 88253-92-3; *p*-nitrophenol, 100-02-7; ethyl bromide, 74-96-4; allyl bromide, 106-95-6; butyl bromide, 109-65-9; octyl bromide, 111-83-1; ethyl *p*-nitrophenyl ether, 100-29-8; allyl *p*-nitrophenyl ether, 1568-66-7; butyl *p*-nitrophenyl ether, 7244-78-2; *p*-nitrophenyl octyl ether, 49562-76-7; 4-ethoxyaniline, 6375-69-4; 4-butoxyaniline hydrochloride, 6927-73-7; 4-(octyloxy)aniline hydrochloride, 30402-00-7; 3-aminobenzyl 2,4,5-trichlorophenyl ether, 88253-93-4; 4-(allyloxy)aniline hydrochloride, 88271-75-4; dihydrofolate reductase, 9002-03-3; cyanoguanidine, 461-58-5.

Studies of the Mode of Action of Antitumor Triazenes and Triazines. 6.† 1-Aryl-3-(hydroxymethyl)-3-methyltriazenes: Synthesis, Chemistry, and Antitumor Properties

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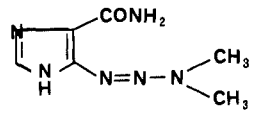
1-Aryl-3-(hydroxymethyl)-3-alkyltriazenes [ArN=NN(CH_3)CH_2OH] have been synthesized by diazonium coupling to the carbinolamine ($RNHCH_2OH$), generated in situ from the alkylamine and formaldehyde mixtures. The (hydroxymethyl)triazene structure has been confirmed by IR, NMR, and mass spectral analysis and also by the preparation of a crystalline benzoate derivative. The mass spectra of the (hydroxymethyl)triazenes suggest that they fragment by loss of formaldehyde to give the methyltriazene, which is also the product of hydrolysis in solution. The degradation of the (hydroxymethyl)triazenes in solution has been followed by UV spectroscopy and by HPLC analysis, and the half-lives were determined under a variety of conditions. The half-lives of the corresponding methyl- and (hydroxymethyl)triazenes are very similar. Both methyl- and (hydroxymethyl)triazenes decompose on silica plates during TLC analysis to give products consistent with known diazo-migration reactions. The (hydroxymethyl)triazenes have pronounced antitumor activity against the TLX5 tumor in vivo; in vivo–in vitro bioassay experiments suggest that the (hydroxymethyl)triazenes exert their in vivo antitumor activity via the degradation product, the alkyltriazene.

5-(3,3-Dimethyltriazen-1-yl)imidazole-4-carboxamide (1, DTIC, Dacarbazine, NSC 45388) has been used in the



1

treatment of malignant melanoma, soft tissue sarcoma, and Hodgkin's disease.[1] The more accessible 1-aryl-3,3-dimethyltriazenes (ArN=$NNMe_2$) are also cytotoxic agents with a broad spectrum of activity against animal tumors.[2] It has been claimed that metabolic conversion of the dimethyltriazenes to methyltriazenes (ArN=NNHMe) is essential for antitumor activity.[3] However, recent studies[4] have suggested that dimethyltriazenes are metabolized to a mixture of selective and nonselective metabolites and that methyltriazenes are nonselective cytotoxic agents. An alternative candidate for the selectively cytotoxic species is the (hydroxymethyl)triazene [ArN=NN(CH_3)CH_2OH], since oxidative metabolic demethylation of dimethylamino compounds generally is considered to proceed via 1-hydroxymethyl intermediates.

Although the 1-(hydroxymethyl)triazenes have been regarded as only transient species, Kolar has adduced evidence that suggests that (hydroxymethyl)triazenes are relatively long-lived moieties in vivo. The hydroxymethyl metabolite of DTIC has been tentatively identified in the

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