A Comparison of the Inhibition of Growth of Methotrexate-Resistant and -Sensitive Leukemia Cells in Culture by Triazines.[1] Evidence for a New Mechanism of Cell Resistance to Methotrexate

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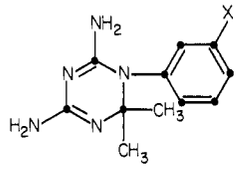
Forty-five 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(3-X-phenyl)-*s*-triazine inhibitors of dihydrofolate reductase (DHFR) and methotrexate (MTX) were tested on L5178Y/R murine tumor cell culture. The concentration of inhibitor causing a 50% decrease in growth rate was determined, and from these results a quantitative structure–activity relationship (QSAR) was developed. This QSAR is compared with QSAR for the same inhibitors acting on isolated DHFR and on L5178Y cell culture sensitive to MTX. The results show that very potent triazine inhibitors of resistant tumor cell growth can easily be made by making the triazines strongly hydrophobic. The optimum π value for inhibition of MTX-sensitive cell culture is 0.8, while π_0 for the resistant cell culture is about 6.0. The QSAR for MTX-sensitive and -resistant tumor cell culture inhibition is compared with the corresponding QSAR for *Lactobacillus casei* cells. Both the mammalian and bacterial cells appear to protect themselves from the highly hydrophilic MTX by erecting lipophilic barriers.

In this report we continue our discussion of the inhibition of dihydrofolate reductase (DHFR) and the growth of tumor cells [both resistant and sensitive to methotrexate (MTX)] by triazines I. The following quantitative



I

structure–activity relationships (QSAR) were recently formulated for the action of congeners I on purified DHFR[3] and on tumor cell culture.[4] In comparing eq 1

Inhibition of Bovine Liver DHFR

$$\log 1/K_{i\,app} = 1.08\pi' - 1.19 \log (\beta \cdot 10^{\pi'} + 1) + 7.27 \quad (1)$$

$n = 38$; $r = 0.903$; $s = 0.288$; $\pi_0 = 1.62$; $\log \beta = -0.656$

Inhibition of Murine Leukemia (L5178Y) DHFR

$$\log 1/K_{i\,app} = 1.13\pi' - 1.33 \log (\beta \cdot 10^{\pi'} + 1) + 0.42I + 6.44 \quad (2)$$

$n = 38$; $r = 0.920$; $s = 0.315$; $\pi_0 = 1.44$; $\log \beta = -0.675$

50% Inhibition of Growth of Leukemia (L5178Y) Cell Culture

$$\log 1/C = 1.32\pi - 1.70 \log (\beta \cdot 10^{\pi} + 1) + 0.44I + 8.10 \quad (3)$$

$n = 37$; $r = 0.929$; $s = 0.274$; $\pi_0 = 0.76$; $\log \beta = -0.215$

and 2, we were struck by the fact that the dependence of inhibitory power on the hydrophobicity of X of the triazines was essentially the same for bovine and murine L5178Y DHFR (note coefficients with π terms and π_0). However, an additional term ($0.42I$) was needed in eq 2 for the murine tumor DHFR. This indicator variable (I) is assigned the value of 1 for congeners where X = 3-CH_2Z-C_6H_4-3′-Y (Z = O or NH). These congeners are 2 to 3 times more inhibitory than π' alone predicts for the tumor enzyme. Compounds with very similar structure (e.g., 3-$OCH_2C_6H_4$-Y) do not show this effect. Although this is certainly not a large effect, it may offer a lead which can be developed to design drugs more selective for tumor enzyme. Since this initial study we have found that chicken liver DHFR yields the same equation as bovine DHFR.[5] Although one's first reaction might be to consider the difference between eq 1 and 2 to be species related (mouse vs. cow), the fact that both the normal bovine and chicken DHFR yield identical equations suggests the possibility that the I term arises from the difference between tumor and normal vertebrate DHFR.

In eq 1 and 2, π' indicates that π_Y has been set = 0 for substituents of the type 3-CH_2O-C_6H_4-3′-Y; that is, $\pi_{CH_2ZC_6H_4\text{-}3'\text{-}Y} = \pi_{CH_2ZC_6H_5}$. The initial reason for making this assumption was that variations in Y did not produce significant variations in log $1/C$, regardless of whether Y was hydrophobic, hydrophilic, or large or small. Recent studies using molecular graphics in Professor Langridge's laboratory at the University of California at San Francisco have confirmed the possibility that Y can be held by the ring to which it is attached in such a fashion that it projects away from the enzyme into aqueous space. Thus, Y cannot produce a hydrophobic effect in the isolated enzyme; however, in cell culture the drug must pass through cell membranes as well as other hydrophobic cellular material before reaching the DHFR. In this partitioning process Y plays a role, and this is seen in eqs 3–5 where π' does not yield as good a correlation as π when π refers to all of 3-X.

To gain further understanding of the potential of the triazines as antitumor drugs, we measured their 50% inhibitory concentration (C in eq 3) on tumor cell culture growth. The correlation eq 3 from this study also requires a term of $0.44I$, providing evidence that I in eq 2 is not simply an artifact. The DHFR on which eq 2 is based was isolated and provided to us by Professor Bertino of Yale University. The enzyme comes from cells that are gene amplified by MTX which are extremely resistant to MTX, while the cells used to derive eq 3 are the normal cells

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(2) Visiting Scientist from the Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China.
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which are quite sensitive to MTX. The problem of cells developing resistance to drugs is of extreme importance in cancer chemotherapy. Therefore, we decided to undertake a study of the action of triazines I on L5178Y cells resistant to MTX. It was hoped that such a study might uncover differences in DHFR in situ in the resistant cell compared to DHFR in the normal tumor cell. Such differences had already been uncovered in MTX-resistant and -sensitive *L. casei* cells.[6]

Results and Discussion

Using the data in Table I we have formulated eq 4–7 for

$$\log 1/C = 0.31\ (\pm 0.06)\ \pi + 4.90\ (\pm 0.17) \quad (4)$$

$$n = 42;\ r = 0.839;\ s = 0.415;\ F_{1,40} = 94.7$$

$$\log 1/C = 0.41\ (\pm 0.07)\ \pi - 0.16\ (\pm 0.07)\ \mathrm{MR} + 5.24\ (\pm 0.21) \quad (5)$$

$$n = 42;\ r = 0.895;\ s = 0.344;\ F_{1,39} = 19.2$$

$$\log 1/C = 0.59\ (\pm 0.10)\ \pi - 0.14\ (\pm 0.06)\ \mathrm{MR} - 0.038\ (\pm 0.017)\ \pi^2 + 5.14\ (\pm 0.18) \quad (6)$$

$$n = 42;\ r = 0.933;\ s = 0.282;\ F_{1,38} = 20.3;\ \pi_0 = 7.7\ (6\text{–}12)$$

$$\log 1/C = 0.57\ (\pm 0.09)\ \pi - 0.15\ (\pm 0.06)\ \mathrm{MR} - 0.35 \log (\beta \cdot 10^{\pi} + 1) + 5.12\ (\pm 0.18) \quad (7)$$

$$n = 42;\ r = 0.932;\ s = 0.288;\ F_{2,37} = 9.35;\ \log \beta = -2.514$$

the inhibition of cell growth of L5178Y cells highly resistant to MTX. C in these expressions is the molar concentration of drug required to produce 50% reduction in cell growth in 48 h. As with the sensitive cells (eq 3), we find π to be the parameter of importance rather than π' of eq 1 and 2. Most of the correlation depends only on π. The MR term accounts for about 10% of the "explained" variance, while the two π terms account for 77% of the "explained" variance. The parabolic (eq 6) and the bilinear (eq 7) equations give about equally good results; however, note that in eq 7 the right-hand part of the curve has a positive slope (0.57 − 0.35 = +0.22). Thus, the true optimum value for π cannot be found, and neither can good confidence limits be placed on π_0. More lipophilic compounds would have to be tested to firmly establish π_0. This is not possible because poor aqueous solubility would preclude such experiments. Equation 6, plus inspection of the log $1/C$ values, indicate that π_0 is at least >6. This is quite a change from π_0 of 0.8 found with eq 3! Another large change for the QSAR of resistant cells is the coefficient with π whose value drops from 1.3 in eq 3 to about 0.6 in eq 6 or 7; that is, with the resistant cells, each unit increase in hydrophobicity of X of I results in about half the increase (in log terms) in inhibitory potency seen with sensitive cells. However, since potency can be increased up to $\pi = 6$, one can increase log $1/C$ by 1.73 units by increasing π to about 6. With the sensitive cells, the maximum increase in log $1/C$ (compared to X = H) by increasing π is only 1 unit. The value of 1.73 is close to the size of the hydrophobic pocket of DHFR calculated from eq 2 (i.e., 1.13 × 1.44 = 1.63). The most potent of the highly lipophilic triazines of Table I is 4000 times more potent than MTX against the resistant cells.

The triazines seem to make about the same overall use of the hydrophobic pocket of the DHFR in situ as in the isolated enzyme (eq 2); however, to do this, they need much larger hydrophobic groups. We noted a similar phenomenon in the case of the inhibition of bacterial DHFR by triazines I;[7] we found an initial slope with π of about 0.5 for the bacterial QSAR but a high π_0 of about 4.4, and with mammalian DHFR we found an initial slope of about 1 with a π_0 of about 1.6. Equations 6 and 7 are reminiscent of the bacterial QSAR.

The term in MR in eq 5–7 has a negative coefficient. Since MR appears to model the volume of substituents,[8] especially when it is associated with a negative coefficient, we interpret this to mean that some kind of constraint is being imposed on the hydrophobic pocket into which X partitions in the inhibition process. This could result from a different environment surrounding the DHFR in the resistant cell compared to that in the sensitive cell. The effect of MR becomes quite significant with large substituents; for example, when MR = 6 (MR is scaled by 0.1 to make it more nearly equiscalar with π), this term in eq 6 subtracts 0.84 from log $1/C$. Although there is some collinearity between π and MR ($r^2 = 0.43$) and MR and π^2 ($r^2 = 0.40$), we believe that MR is playing a minor independent role.

Another significant difference between eq 6 and 7 and eq 2 and 3 is that the latter have no terms in I. Since I accounts for a very small effect, some small change in the conformation of the DHFR in the living resistant cell could account for this small difference in the QSAR.

The perturbation patterns from the 40-some probes are startingly different for cells sensitive to and resistant to MTX. It is obvious that the more lipophilic drugs are much more effective against resistant cells. Why?

In a recent review[9] of the molecular basis of MTX resistance, Harper and Kellems point out that three mechanisms have been identified: (1) change in the DHFR molecule so that the high affinity for MTX is reduced, (2) mutational change affecting the active transport system for MTX, and (3) a great increase in the cellular level of DHFR which protects the cell from antifolate drugs. The mechanism of action of MTX has been most recently reviewed by Wang and Loo.[10]

Of the mechanisms outlined by Harper and Kellems, all three seem likely to be operative in our view. The fact that eq 6 and 7 lack a term in I which is seen in eq 3 suggests the possibility of at least a small change in enzyme conformation. Since it has been established that MTX-resistant cells produce several hundred times as much DHFR as MTX-sensitive cells, this mechanism must also play a part in the resistance to MTX. It may be that these huge amounts of DHFR aggregate into semicrystalline clusters which make penetration of the ligand more difficult. The change in slope of the left side of the bilinear curve from eq 3 to eq 6 and 7 is remarkable and it would have to be associated either with the structure of the enzyme or the environment around the DHFR. Another possibility should also be considered. Two hydrophobic processes are involved in the whole cells: the hydrophobgic interaction between the inhibitor and the DHFR and the hydrophobic interaction between the inhibitor and cell membranes or lipophilic macromolecules. It is conceivable that in eq 3 the former hydrophobic process is dominant while the

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Table I. Parameters Used to Derive Equations 4-7 for the Inhibition of Growth of L5178Y/R Cells in Vitro by Congeners I

		log 1/C					
no.	X	obsd	95% CI	calcd[a]	Δ log 1/C	π	MR
1	CH(OH)Ph[b]	4.17	4.03-4.31	4.96	−0.79	0.54	3.15
2	OC_9H_{19}	6.57	6.47-6.68	6.27	0.30	4.29	4.46
3	$OC_{11}H_{23}$	6.38	6.27-6.50	6.38	0.00	5.37	5.38
4	$OC_{12}H_{25}$	6.24	6.10-6.38	6.43	−0.19	5.91	5.84
5	$OC_{13}H_{27}$	6.52	6.40-6.65	6.48	0.04	6.45	6.30
6	$OC_{14}H_{29}$	6.49	6.22-6.75	6.52	−0.03	6.99	6.77
7	H	4.84	4.72-4.96	5.12	−0.28	0.00	0.00
8	CH_3	4.97	4.92-5.03	5.36	−0.39	0.56	0.56
9	Cl	5.83	5.79-5.86	5.43	0.40	0.71	0.60
10	Br	5.90	5.83-5.96	5.78	0.42	0.86	0.89
11	I	5.91	5.86-5.97	5.55	0.36	1.12	1.34
12	F	5.24	5.16-5.32	5.19	0.05	0.14	0.09
13	C_6H_{13}	6.39	6.31-6.48	6.25	0.14	3.21	2.89
14	$CONH_2$	3.78	3.58-3.98	4.12	−0.34	−1.49	0.98
15	OH	4.22	4.16-4.28	4.70	−0.48	−0.67	0.28
16	$COCH_3$	4.56	4.49-4.62	4.64	−0.08	−0.55	1.12
17	CH_2NHPH-3',5'-$(CONH_2)_2$	3.79	3.67-3.91	3.57	0.22	−1.34	5.23
18	OCH_2PH-3',4'-Cl_2	5.80	5.75-5.85	5.96	−0.16	2.91	4.22
19	CN	4.77	4.72-4.82	4.70	0.07	−0.57	0.63
20	*t*-Bu	6.03	5.98-6.07	5.92	0.11	1.98	1.96
21	NO_2	5.03	5.00-5.07	4.85	0.18	−0.28	0.74
22	*o*-Bzl	5.19	5.13-5.24	5.57	−0.38	1.66	3.22
23	OCH_3	4.90	4.77-5.04	4.99	−0.09	−0.02	0.79
24	CF_3	5.66	5.61-5.71	5.55	0.11	0.88	0.50
25	CH_2NHPH-4'-SO_2NH_2	4.63	4.57-4.70	3.96	0.67	−0.82	4.60
26	$O(CH_2)_2OPH$	5.02	4.87-5.16	5.48	−0.46	1.68	3.90
27	$O(CH_2)_2OPH$-3'-CF_3	5.85	5.70-5.99	5.96	−0.11	2.56	3.40
28	$O(CH_2)_4OPH$	5.30	5.04-5.45	5.80	−0.50	2.71	4.82
29	$O(CH_2)_4OPH$-3'-CF_3	6.28	6.18-6.38	6.00	0.28	3.59	5.22
30	CH_2OPH-3'-Cl	5.93	5.86-6.00	5.83	0.10	2.37	3.72
31	CH_2OPH-3'-OCH_3	5.36	5.27-5.44	5.45	−0.09	1.64	3.90
32	CH_2OPH-3'-CN	5.25	5.08-5.41	5.18	0.07	1.09	3.75
33	CH_2OPH-H	5.67	5.58-5.77	5.57	0.10	1.66	3.22
34	CH_2OPH-3'-CH_3	5.59	5.48-5.79	5.77	−0.18	2.22	3.68
35	CH_2OPH-3'-CH_2CH_3	5.74	5.64-5.84	5.89	−0.15	2.68	4.15
36	CH_2OPH-3'-$CH(CH_3)_2$	5.87	5.80-5.94	5.97	−0.10	3.14	4.61
37	CH_2OPH-3'-$C(CH_3)_3$	6.08	5.93-6.22	6.03	0.05	3.64	5.08
38	CH_2OPH-3'-$NHCONH_2$	4.74	4.71-4.78	4.65	0.09	0.36	4.49
39	CH_2OPH-3'-CH_2OH	4.77	4.70-4.84	4.90	−0.13	0.63	3.84
40	$OCH_2CH_2CH_2CH_3$	5.95	5.84-6.06	5.69	0.26	1.59	2.17
41	CH_2OPH-3'-$NHCSNH_2$	4.01	3.96-4.06	4.45	−0.44	0.26	5.43
42	CH_2OPH-3'-$NHCOCH_3$	4.95	4.44-5.45	4.82	0.13	0.69	4.61
43	CH_2S-Ph	6.20	5.95-6.45	5.77	0.43	2.24	3.79
44	SO_2NH_2[b]	3.13	3.07-3.19	3.90	−0.77	−1.82	1.23
45	methotrexate[b]	2.90	2.74-3.05				
46	Baker's antifolate[b]	5.40	5.25-5.53				

[a] Calculated using eq 7. [b] These points not used in the derivation of eq 4-7.

latter process is dominant in eq 6 and 7.

It is seen from Table I that log 1/C for MTX causing 50% inhibition of resistant cells is 2.90 (i.e., about 10^{-3} M MTX is required). Log 1/C of MTX for sensitive cells is 8.89; hence, a 1,000,000-fold higher concentration of MTX is needed to inhibit the resistant cells. It seems unlikely that this could be accounted for by a small change in the conformation of the DHFR or by an increase of a few hundred-fold in the concentration of DHFR. It therefore seems likely to us that a change in the active transport system must be highly important. In support of this, it can be seen in Table I that the most lipophilic drugs are quite active against the resistant cells. The highest log 1/C of about 6.5 is only 2.4 log units below that found for MTX in the sensitive cells. This difference could easily be accounted for by the few hundred-fold increase in the DHFR in resistant cells.

The difference we find between the parameters of eq 3 and eq 6 and 7 suggest a fourth mechanism that cells may use to protect themselves from hydrophilic toxic compounds. This appears to be the construction of a barrier to hydrophilic molecules that is readily overcome by hydrophobic congeners.

It is of interest to compare equations for the inhibition by triazines of mammalian DHFR and cell culture with corresponding bacterial equations (eq 8–10).[6] *I* in these

Inhibition of *L. casei* DHFR

$$\log 1/K_{i\,app} = 0.46\pi' - 0.57 \log (\beta \cdot 10^{\pi'} + 1) + 1.38I + 3.16 \quad (8)$$

$$n = 44;\ r = 0.947;\ s = 0.333;\ \pi_0 = 4.39$$

Inhibition of *L. casei* Cell Culture Sensitive to MTX

$$\log 1/C = 0.86\pi' - 1.11 \log (\beta \cdot 10^{\pi'} + 1) - 0.94MR_Y + 0.79I + 4.31 \quad (9)$$

$$n = 32;\ r = 0.948;\ s = 0.328;\ \pi_0 = 2.88$$

Inhibition of *L. casei* Cell Culture Resistant to MTX

$$\log 1/C = 0.43\pi + 1.06I - 0.49MR_Y + 3.38 \quad (10)$$

$$n = 37;\ r = 0.964;\ s = 0.268$$

equations has the same meaning as in eq 2 and 3, although the effect of the $-CH_2OC_6H_4-$ moiety is much more pro-

nounced with bacterial cells than with mammalian cells. As with the inhibition of mammalian DHFR, π' gives a better correlation than π in eq 8. A totally different effect of Y in $CH_2OC_6H_4$-Y is found with the bacterial cells; Y in these cells appears to encounter a barrier not seen with isolated enzyme (eq 8) which reduces the effectiveness of substituents containing this unit. This appears to be a "curtain" open in isolated DHFR but closed in DHFR in situ. The curtain appears to be movable, since large groups like *tert*-butyl are active, even though their activity is greatly depressed. This depression is proportional to MR and occurs regardless of whether the substituent is polar (e.g., $NHCONH_2$) or nonpolar [e.g., $C(CH_3)_3$].

Comparing eq 8 and 9, we note that π_0 drops significantly in cell culture compared to isolated enzyme. The same effect can be found by comparing eq 2 and 3. The higher coefficient with π' in eq 9 compared to eq 8 suggests that increased hydrophobicity aids transport across lipophilic barriers in normal cells. Again, as in the mammalian equations, we note a large drop in the coefficient with π in going from sensitive to resistant cells (eq 9 to eq 10).

Most significant is the much higher π_0 of eq 10 compared to eq 8 and 6. For both types of cells it would appear from inspection of the data in Table I that π_0 will be near 6. It is fascinating that both types of cells seem to use a similar mechanism for protecting themselves from the hydrophilic triazines or MTX.

It is possible that changes in the membrane structure or other structural changes in the cell account for this great difference in the way lipophilic drugs penetrate the two types of cells. Burns et al.[11] recently showed that the lipid composition of L1210 murine leukemia cells was dependent upon the fat fed to the host animal. They also showed that MTX penetrated cells with different lipid composition at different rates. Hence, it seems reasonable that some change in membrane structure may account for our results.

Still another problem to consider in the action of antifolates on tumor cells is the observation by Greco and Hakala[12] that although 2,4-diaminopyrimidine antifolates resemble MTX in their action on various tumor cells, they do seem to differ in action, which suggests the possibility of a second relatively sensitive folate-independent site of action.

There is of course great concern about the feasibility of making drugs that can effectively inhibit DHFR in the resistant cells. Evidence is accumulating to show that attaching MTX to proteins or peptides yields compounds that are taken up by resistant cells and then apparently hydrolyzed to release MTX inside the cell.[13-15] An alternative to this approach is to use drugs that are more hydrophobic than MTX. In addition to the large range of quantitative results of Table I, there is now other evidence that more lipophilic inhibitors of DHFR are effective against tumor cells.[16,17] However, one cannot blindly increase lipophilicity to obtain more potent drugs. One must establish log P_0 (the optimum lipophilicity for a given type of congener in a given system[18]) for DHFR inhibitors acting in whole animals. Once log P_0 has been determined, there is no value in making inhibitors much more lipophilic than this limit. While maintaining potency and getting drugs to the active site (in the present case, DHFR) are of high importance, we believe that large advances in cancer chemotherapy cannot be attained without designing drugs that show selectivity for tumor cells. It is for this reason that the I term in eq 2 and 3 is of such special interest. It is our hope that structural variations can be made to enhance this property of the triazines. Why the I term does not occur in eq 6 or 7 for the resistant cells is fascinating. This missing term suggests a different enzyme conformation in the resistant cell.

It should be noted that two data points (1 and 44) have not been included in the derivation of eq 4–7. The 3-CH(OH)C_6H_5 congener is invariably much less active than expected, both in cell culture and with isolated DHFR. All DHFR show this same response to this substituent; hence, it is an interesting marker derivative which indicates that the triazines are indeed slowing cell growth by inhibition of DHFR. It is known that inhibitors of DHFR may also inhibit thymidylate synthetase.[19] It is possible that congeners I may be inhibiting other enzymes rather than DHFR to cause inhibition of cell growth. Although methotrexate does inhibit thymidylate synthetase, Chello et al.[20] have shown that Baker's antifol [I, X = 3-Cl-4-$OCH_2C_6H_4$-3'-$CON(CH_3)_2$] at 10^{-3} M had no significant effect on thymidylate synthetase or thymidine kinase. Other workers[21,22] support our view that resistance to MTX is related to changes in membrane properties.

The other poorly fit congener is 44. The reason for this is not clear, since this congener is normally well fit using purified enzyme data or data from the sensitive cells. Including all data points for the triazines of Table I yields eq 11 and 12. The parameters of eq 11 and 12 do not

$$\log 1/C = 0.64\ (\pm 0.11)\ \pi - 0.15\ (\pm 0.07)\ \mathrm{MR} - 0.043\ (\pm 0.018)\ \pi^2 + 5.09\ (\pm 0.20) \quad (11)$$

$$n = 44;\ r = 0.927;\ s = 0.326;\ \pi_0 = 7.39$$

$$\log 1/C = 0.65\ (\pm 0.11)\ \pi - 0.15\ (\pm 0.07)\ \mathrm{MR} - 0.41\ (\pm 0.18)\ \log(\beta \cdot 10^{\pi} + 1) + 5.06\ (\pm 0.21) \quad (12)$$

$$n = 44;\ r = 0.925;\ s = 0.335;\ \log \beta = -2.12$$

differ significantly from eq 6 and 7, although the fit of the data is somewhat poorer.

In summary then, our work appears to have developed evidence for a fourth mechanism in addition to the three now recognized whereby cells can develop resistance to MTX. The fact that both mammalian and bacterial cells show similar changes in QSAR for MTX-resistant and -nonresistant cell types suggests a common approach that cells in general may have for protecting themselves from toxic hydrophilic agents.

Our work, plus that cited in this report, shows that despite about 30 years of studying antifols' action on cells, we are still rather ignorant about the various ways cells can react to such toxic agents.

QSAR is a useful means for uncovering parameters which cells can vary to develop resistance to drugs. The QSAR paradigm is often discussed as being a tool for optimizing biological activity for a given set of congeners; in

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fact, as Unger[23] has pointed out, it is QSAR-gained insight into drug action and design that is of the greatest importance.

Experimental Section

Chemistry. The syntheses of the triazines used in this study have been previously reported[3] except for congener **43**.

3-Nitrobenzyl Phenyl Sulfide. A suspension of thiophenol (4.4 g, 40 mmol), 3-nitrobenzyl chloride (6.8 g, 40 mmol), and potassium carbonate (5.6 g, 40 mmol) in acetone was refluxed for 48 h. The potassium chloride was filtered off, and the filtrate was concentrated under reduced pressure. Distillation in vacuo yielded 8.0 g (85%) of a yellow viscous oil, bp 150–160 °C (2.8 mm). Anal. ($C_{13}H_{11}O_2NS$) C, H.

3-[(Phenylthio)methyl]aniline Hydrochloride. 3-Nitrobenzyl phenyl sulfide (7.5 g, 30 mmol), acetic acid (0.2 mL), and iron powder (40 g, 720 mmol) were stirred in 150 mL of water at 85–95 °C for 10 h. The slurry was alkanized with sodium carbonate, filtered, and washed with hot benzene. The filtrate was collected and extracted with benzene, and the combined extracts were concentrated on a rotary evaporator. The resulting syrupy liquid was dissolved in ether, and HCl gas was passed through the solution until a yellow flocculent solid was formed. The hydrochloride was collected and recrystallized from acetonitrile to yield 6.6 g (87%) of a white solid, mp 152–153.5 °C. Anal. ($C_{13}H_{14}ClNS$) C, H.

Congener 43. 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-[3-[(phenylthio)methyl]phenyl]-*s*-triazine Hydrochloride. A suspension of 3-[(phenylthio)methyl]aniline hydrochloride (2.5 g, 10 mmol) and cyanoguanidine (0.85 g, 10 mmol) in reagent grade acetone was refluxed for 20 h. The pale yellow solid that precipitated was collected by filtration and recrystallized from acetonitrile–ethanol to yield 3.6 g (95%) of a white solid, mp 165–167 °C. Anal. ($C_{18}H_{22}N_5SCl$) C, H.

Methotrexate was supplied by the Division of Cancer Treatment of the National Cancer Institute.

Biology. The original L5178Y/R cells were kindly provided by Dr. J. Bertino, Department of Pharmacology, Yale University School of Medicine, New Haven, CT. For routine passage and during dose–response experiments, L5178Y/R murine leukemia cells were maintained in asynchronous logarithmic growth at 37 °C in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum and 1% (v/v) penicillin–streptomycin. The population doubling time was 15–18 h.[21] Twice a week, cells in the mid to late logarithmic stage of growth were diluted (v/v) 1:10- to 1:20-fold with fresh medium and serum in order to keep a portion of the cell stocks in the logarithmic stage of growth at all times. The stock solutions of the triazines were made with unsupplemented medium.

Cell cultures were seeded at 4.0–6.0 × 10^4 cells/mL in duplicate for each drug concentration in a plastic microtiter plate (0.2 mL/well). The triazines that were added to the cell cultures in 1:10 dilution to achieve the desired drug concentration were tested at a minimum of eight different concentrations. After 48 h of continuous drug exposure in a humidified incubator supplied with 95% air and 5% carbon dioxide, the cells were harvested and counted using a Coulter Counter, Model B (Coulter Electronics, Hialeah, FL). A control untreated set of cultures and four duplicate sets of MTX-treated cells were included for each separate dose–response experiment. Duplicate counts were taken on each well and were usually in agreement with each other (±10%).

From the data obtained, a dose–response curve was drawn and the ID_{50} was calculated as in our previous studies.[4] The ID_{50} is defined as the concentration of inhibitor that halves the growth rate, i.e., doubles the generation time. The confidence limits on log $1/ID_{50}$ and π_0 were calculated by utilizing the jackknife procedure.[22]

Substituent Constants. The values for the substituent constants in Table I were taken from our recent compilation.[7]

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Synthesis of Pseudo Cofactor Analogues as Potential Inhibitors of the Folate Enzymes

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Reaction of 5,6,7,8-tetrahydrofolic acid (THF, **7**) with phosgene, thiophosgene, and cyanogen bromide gave the bridged derivatives, 5,10-(CO)-THF (**8**), 5,10-(CS)-THF (**9**), and 5,10-(C=NH)-THF (**11**), respectively. Catalytic hydrogenation of 10-(chloroacetyl)folic acid (**2**) gave 5,10-(CH_2CO)-THF (**12**). A similar reaction with 10-(3-chloropropionyl)folic acid (**3**) gave 10-($ClCH_2CH_2CO$)-THF (**14**) rather than 5,10-(CH_2CH_2CO)-THF (**13**). In the catalytic hydrogenation of 10-ethoxalylfolic acid (**5**), the initial product 10-(EtO_2CCO)-THF (**22**) rearranged readily to give 5-(EtO_2CCO)-THF (**21**). Acylation of THF with chloroacetyl chloride gave a N^5,N^{10}-diacylated product (**18** or **19**), which could not be converted to 5,10-($COCH_2$)-THF (**17**). Reductive alkylation of THF with glyoxylic acid and 5-hydroxypentanal, respectively, gave 5-(HO_2CCH_2)-THF (**24**) and 5-[$HO(CH_2)_5$]-THF (**25**). Reductive dialkylation of THF with formaldehyde gave 5,10-$(CH_3)_2$-THF (**27**), whereas glyoxal gave 5,10-(CH_2CH_2)-THF (**10**). Also, both folic acid and 5-(CHO)-THF were reductively alkylated with formaldehyde to give 10-methylfolic acid (**6**) and 5-(CHO)-10-(CH_3)-THF (**28**), respectively. These compounds were tested as inhibitors of the enzymes involved in folate metabolism and for activity against lymphocytic leukemia P388 in mice.

The six biologically active cofactor forms of 5,6,7,8-tetrahydrofolic acid (THF) (**7**) are substrates for at least 15 enzymes. Key enzymes are GAR and AICAR transformylase (EC 2.1.2.2 and EC 2.1.2.3), serine transhydroxymethylase (EC 2.1.2.1), and thymidylate synthetase (EC 2.1.1.b). The transformylases provide carbon-8 and carbon-2 of inosinic acid, and the methylase and synthetase enzymes catalyze transformations that provide the 5-methyl group of thymidylic acid. Inhibitors of these enzymes will result in the blockage of purine and pyrimidine synthesis, followed by the arrest of DNA synthesis.[1]

In the search for inhibitors of the folate enzymes, the preparation of 5- and 10-substituted, 5,10-disubstituted,

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