Comparison of Quantitative Structure–Activity Relationships of the Inhibition of Leukemia Cells in Culture with the Inhibition of Dihydrofolate Reductase from Leukemia Cells and Other Cell Types

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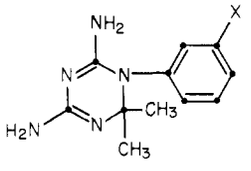
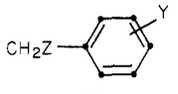
A set of 2,4-diamino-5-(3-X-phenyl)-s-triazines was used to inhibit the growth of tumor cells (L5178 leukemia) in culture. The molar concentration (C) of triazine causing 50% reduction in the rate of cell growth was used to develop a quantitative structure–activity relationship: $\log 1/C = 1.32\pi - 1.70 \log (\beta \cdot 10^{\pi} + 1) + 0.44I + 8.10$, where π is the hydrophobic constant for X, β is a disposable parameter, and I is an indicator variable for congeners containing a $-CH_2Z\text{-}C_6H_4\text{-}Y$ moiety (Z = O or NH). This equation is compared with similar equations derived for the inhibition of dihydrofolate reductase from leukemia cells and bovine liver.

Since the enzyme dihydrofolate reductase (DHFR) is crucial for the synthesis of DNA and since DHFR from different sources varies considerably in its reaction with various inhibitors, this enzyme has been the subject of many studies in the search for new drugs. The success of methotrexate (MTX) as an antitumor drug and the great success of trimethoprim as an antibacterial agent have further stimulated work in the search for selective inhibitors of DHFR. In an effort to more clearly delineate the mechanism of ligand interaction with DHFR, quantitative structure–activity relationships (QSAR) have recently been formulated[2–9] for various DHFR inhibitors. The QSAR embodied in eq 1–6 have been developed from studies of inhibitors of type I with DHFR from various sources.



I

While X represents many relatively simple substituents, there are many examples where X represents structures of type II. In II, Z = O or NH and Y = various hydro-



II

phobic and hydrophilic groups, both large and small, most of which are in position 3′ of the second phenyl ring.

There is at present an enormous effort being devoted to the study of the interaction of organic compounds with an extremely wide variety of living systems: microorganisms, plants, animals, and insects. These studies are, for the most part, being carried out with whole organisms in order to develop better drugs and agriculture chemicals and to understand the influence of environmental toxicants on living systems. In recent years there has been considerable success in rationalizing the perturbations caused in whole organisms by large sets of congeneric compounds by means of the QSAR paradigm.[10,11] However, the correlations are often not as sharp as one would like and, in some cases, reasonable correlations between structure and activity cannot be obtained. While there are of course many reasons for failure in such a complex undertaking, which ones are operative in a given situation are never clear. Does the trouble reside in the mathematical model or in the complexity of the definition of the biological response? The end result, observed in the whole organism (e.g., an ED_{50} or LD_{50}), is a composite result that may be exceedingly complex; that is, numerous enzyme systems and/or membranes may be disturbed, all of which may or may not make contributions to the observed ED_{50} or LD_{50}. The opposite extreme, from studying the effects of a set of congeners on a whole organism, is studying the action on a purified enzyme. Work with purified enzymes is much simpler and the increasing knowledge of structures obtained from X-ray studies enormously simplifies interpretation; nevertheless, many attempts to design drugs by developing inhibitors on isolated enzymes have not been successful. It is our belief that the careful step-by-step study of the interaction of well-designed sets of congeners first on purified enzymes, then on isolated cells, and finally on whole animals will increase our understanding of the horrendously complex reactions that can occur between a complex set of organic compounds and living systems.

It is important in undertaking such a program to select a biological receptor that is of central concern to medicinal chemistry and whose structure is being studied via X-ray crystallography with enzyme from a variety of sources. We have, for these reasons, elected to use DHFR for comparative QSAR.

One of the first questions with which we are concerned is, does the isolated enzyme respond to a set of inhibitors in the same way as enzyme in the whole cell? Although such studies have often been made on a few assorted inhibitors, very few studies have been published on well-developed sets of congeners with the objective of QSAR in mind.

To gain perspective on this question we have derived eq 1 for the inhibition of DHFR from normal mammalian

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QSAR for Inhibition of Bovine Liver DHFR by Congeners I

$$\log 1/K_{i\,app} = 1.08\ (\pm 0.19)\ \pi' - 1.19\ (\pm 0.25) \log (\beta \cdot 10^{\pi'} + 1) + 7.27\ (\pm 0.13) \quad (1)$$

$$n = 38;\ r = 0.903;\ s = 0.288;\ \pi_0 = 1.62\ (1.24\text{–}2.06);\ \log \beta = -0.656$$

tissue (bovine liver) and eq 2 for the inhibition of DHFR

QSAR for Inhibition of Murine Leukemia DHFR by Congeners I

$$\log 1/K_{i\,app} = 1.13\ (\pm 0.22)\ \pi' - 1.33\ (\pm 0.30) \log (\beta \cdot 10^{\pi'} + 1) + 0.42\ (\pm 0.24)\ I + 6.44\ (\pm 0.16) \quad (2)$$

$$n = 38;\ r = 0.920;\ s = 0.315;\ \pi_0 = 1.44\ (1.08\text{–}1.80);\ \log \beta = -0.675$$

from tumor cells (L5178Y-RC_3 murine leukemia).[12] Considerable care has been taken in the evaluation of the $K_{i\,app}$ values used to obtain the above expressions so that relatively small differences in the pattern of inhibitor–enzyme interactions can be detected via QSAR.[13,14] In the above equations, π' is the hydrophobic interaction constant for 3-X except for substituents of type II; for these substituents, π of Y is set = 0 so that π for $-CH_2ZC_6H_4$-Y is the same as π for $-CH_2ZC_6H_5$, which gives a far better correlation than using π for all of X. The interpretation of this fact is that Y does not interact hydrophobically or in any other way with the enzyme. All congeners with type II substituents have approximately the same activity (i.e., the same $K_{i\,app}$ values). For these equations, n represents the number of congeners used to formulate the equation, r is the correlation coefficient, s is the standard deviation from regression, and π_0 is the optimum value of π producing the most potent inhibitor, other factors being constant. The figures in parentheses with π_0 are the 95% confidence limits. The other figures in parentheses are for the construction of the 95% confidence limits. The mathematical model for eq 1 and 2 has been developed by Kubinyi and is usually referred to as the bilinear model.[15] Inhibitory potency initially increases linearly with π with a slope of 1.08 until it approaches π_0 (1.62). A gradual change occurs and activity then decreases linearly with a slope of −0.11 (1.08–1.19). Actually, this slope is so close to 0 that we assume that substituents with $\pi > 1.62$ (π_0) do not contact the hydrophobic pocket of the enzyme.

In terms of π, the congeners I appear to interact in much the same way with DHFR (eq 2) as with bovine DHFR (eq 1). The difference in π_0 is probably not significant; note the confidence intervals. However, eq 2 does contain an additional term in the form of the indicator variable I. For those substituents containing groups of type II with the $-CH_2-$ bridge, I takes the value of 1. For all other substituents, I is assigned the value of 0. While this term is not highly significant (note confidence limits), it does suggest that there is a difference between DHFR from bovine liver and mouse tumor. What is particularly interesting is that when we find differences in inhibitory potency for the two enzymes on a molecule-by-molecule basis,[12] significant differences occur only with those substituents large enough to interact with enzymic space outside of the hydrophobic pocket defined by π_0. Most of these groups contain the $-CH_2Z-$ bridge. The intercepts of the two equations differ, showing that—other factors being equal—isolipophilic congeners are more inhibitory to bovine enzyme.

In the present study we have investigated the interaction of congeners II with leukemia cells L5178Y sensitive to MTX. The enzyme on which eq 2 is based is from the same strain of cells, except that they are resistant to MTX. The resistant cells produce much larger amounts of DHFR via gene amplification. It seems likely that the enzyme used to develop eq 2 is the same as the enzyme in the cells upon which eq 3 and 6 are based, but there is no proof of this.

The present study of drug action on cells in culture is the next step up in complexity from the enzyme level and it allows us to make comparisons with a normal mammalian enzyme (eq 1) as well as the tumor enzyme (eq 2). We feel that this step is essential before we attempt a study in whole animals.

Results and Discussion

Using the data in Table I, we have derived eq 3–6 for

$$\log 1/C = 0.03\ (\pm 0.14)\ \pi + 7.59\ (\pm 0.29) \quad (3)$$

$$n = 37;\ r = 0.065;\ s = 0.707;\ F_{1,35} = 0.14$$

$$\log 1/C = 1.27\ (\pm 0.25)\ \pi - 1.68\ (\pm 0.32) \log (\beta \cdot 10^{\pi} + 1) + 8.14\ (\pm 0.18) \quad (4)$$

$$n = 37;\ r = 0.879;\ s = 0.347;\ F_{1,33} = 58.0;\ \log \beta = -0.361;\ \pi_0 = 0.85\ (0.59\text{–}1.13)$$

$$\log 1/C = 1.32\ (\pm 0.22)\ \pi - 1.70\ (\pm 0.27) \log (\beta \cdot 10^{\pi} + 1) + 0.44\ (\pm 0.19)\ I + 8.10\ (\pm 0.18) \quad (5)$$

$$n = 37;\ r = 0.929;\ s = 0.274;\ F_{1,32} = 20.9;\ \log \beta = -0.215;\ \pi_0 = 0.76\ (0.52\text{–}1.00)$$

$$\log 1/C = 1.34\ (\pm 0.20)\ \pi - 1.69\ (\pm 0.25) \log (\beta \cdot 10^{\pi} + 1) + 0.58\ (\pm 0.21)\ I + 0.75\ (\pm 0.59)\ \sigma + 7.87\ (\pm 0.22) \quad (6)$$

$$n = 37;\ r = 0.942;\ s = 0.254;\ F_{1,31} = 6.68;\ \log \beta = -0.249;\ \pi_0 = 0.82\ (0.59\text{–}1.05)$$

the inhibition of L5178Y cells by congeners I. Equation 6a is the correlation for all congeners in Table I. Of the

$$\log 1/C = 1.25\ (\pm 0.28)\ \pi - 1.56\ (\pm 0.35) \log (\beta \cdot 10^{\pi} + 1) + 0.62\ (\pm 0.29)\ I + 0.87\ (\pm 0.80)\ \sigma + 7.72\ (\pm 0.29) \quad (6a)$$

$$n = 40;\ r = 0.873;\ s = 0.368;\ \log \beta = -0.283;\ \pi_0 = 0.89\ (0.56\text{–}1.22)$$

above, eq 3 is not significant (note F value and r); however, all of the other equations are significant as judged by the F statistic ($F_{1,30(\alpha=0.05)} = 7.17$; $F_{1,30(\alpha=0.01)} = 7.56$; $F_{1,30(\alpha=0.001)} = 13.3$). Note that in eq 3–6 π and not π' has been employed. Using π' in place of π in eq 6 results in a much poorer correlation ($r = 0.878$, $s = 0.369$). This is to be expected because the penetration of drugs in whole cells through hydrophobic membranes and other compartments is highly dependent on log P or π. Moreover, the culture medium contains fetal calf serum which contains hydrophobic material that restricts movement of the more hydrophobic compounds. Although there is now so much work in hand which shows that sets of congeners inevitably have an optimum π_0 or log P value for movement through biological material, the value of 0.8 is surprising.

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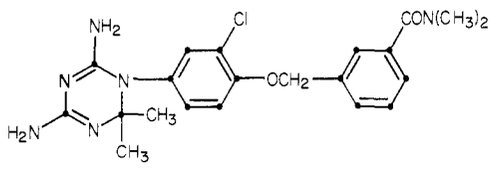
Table I. Parameters Used to Derive Equations 3-6 for the Inhibition of Murine Leukemia L5178Y Cell Culture by Congeners I

		log 1/C						
no.	X	obsd	95% CI	calcd[a]	Δ log 1/C	π	σ	I
1[b]	$CH(OH)C_6H_5$	6.41	6.29-6.53	7.82	−1.41	0.54	0.03	0
2[b]	C_6H_{13}	7.97	7.89-8.06	7.08	0.89	3.21	−0.08	0
3[b]	$COOC_2H_5$	8.13	7.02-9.23	8.07	0.06	0.51	0.37	0
4	SO_2NH_2	5.61	5.50-5.73	5.78	−0.17	−1.82	0.46	0
5	$CONH_2$	6.10	6.03-6.17	6.08	0.02	−1.49	0.28	0
6	$OC_{12}H_{25}$	6.41	6.24-6.57	6.26	0.15	5.91	0.12	0
7	OH	6.74	6.60-6.87	6.98	−0.24	−0.67	0.12	0
8	OC_9H_{19}	6.93	6.81-7.04	6.85	0.08	4.29	0.12	0
9	$COCH_3$	6.96	6.84-7.08	7.31	−0.35	−0.55	0.38	0
10	$O(CH_2)_4OPh$	6.97	6.88-7.06	7.41	−0.44	2.71	0.12	0
11	CH_2NHPh-3′,5′-$(CONH_2)_2$	7.01	6.93-7.07	6.64	0.37	−1.34	0.00	1
12	$O(CH_2)_4OPh$-3′-CF_3	7.14	7.06-7.23	7.10	0.04	3.59	0.12	0
13	OCH_2Ph-3′,4′-Cl_2	7.15	6.88-7.41	7.34	−0.19	2.91	0.12	0
14	$O(CH_2)_2OPh$	7.34	7.14-7.54	7.75	−0.41	1.68	0.12	0
15	$O(CH_2)_2OPh$-3′-CF_3	7.37	7.33-7.42	7.46	−0.09	2.56	0.12	0
16	H	7.39	7.32-7.45	7.54	−0.15	0.00	0.00	0
17	CH_2NHPh-4′-SO_2NH_2	7.42	7.22-7.63	7.29	0.13	−0.82	0.00	1
18	CN	7.55	7.42-7.68	7.43	0.12	−0.57	0.56	0
19	*tert*-butyl	7.59	7.52-7.66	7.49	0.09	1.98	−0.10	0
20	NO_2	7.66	7.55-7.78	7.84	−0.18	−0.28	0.71	0
21	$OCH_2C_6H_5$	7.78	7.62-7.93	7.76	0.02	1.66	0.12	0
22	OCH_3	7.93	7.83-8.02	7.62	0.31	−0.02	0.12	0
23	F	7.94	7.77-8.11	7.89	0.05	0.14	0.34	0
24	CH_3	8.01	7.91-8.11	7.75	0.26	0.56	−0.07	0
25	CF_3	8.19	8.07-8.31	8.15	0.04	0.88	0.43	0
26	I	8.40	8.23-8.56	8.06	0.34	1.12	0.35	0
27	Br	8.42	8.31-8.53	8.08	0.34	0.86	0.34	0
28	Cl	8.46	8.35-8.57	8.10	0.36	0.71	0.37	0
29	CH_2OPh-3′-$C(CH_3)_3$	7.30	7.11-7.49	7.59	−0.29	3.64	0.03	1
30	CH_2OPh-3′-OCH_3	8.02	7.76-8.28	8.28	−0.26	1.64	0.03	1
31	CH_2OPh-3′-CN	8.09	7.91-8.26	8.40	−0.31	1.09	0.03	1
32	CH_2OPh-3′-Cl	8.10	7.91-8.29	8.04	0.06	2.37	0.03	1
33	CH_2OPh-3′-C_2H_5	8.11	7.92-8.28	7.93	0.18	2.68	0.03	1
34	CH_2OPh-3′-$CH(CH_3)_2$	8.13	7.92-8.34	7.77	0.36	3.14	0.03	1
35	CH_2OPh-3′-$NHCSNH_2$	8.08	7.89-8.26	8.30	−0.22	0.26	0.03	1
36	CH_2OPh-3′-CH_3	8.16	8.05-8.27	8.09	0.07	2.22	0.03	1
37	CH_2OPh-3′-$NHCONH_2$	8.18	7.95-8.40	8.34	−0.16	0.36	0.03	1
38	CH_2OPh-H	8.20	8.06-8.34	8.27	−0.07	1.66	0.03	1
39	CH_2OPh-3′-CH_2OH	8.32	8.11-8.52	8.41	−0.09	0.63	0.03	1
40	CH_2OPh-3′-$NHCOCH_3$	8.66	8.57-8.74	8.42	0.24	0.69	0.03	1
41	Baker's antifolate	7.53	7.39-7.66					
42	methotrexate	8.89	8.82-8.96					

[a] Calculated using eq 6. [b] These points not used in the derivation of eq 3-6.

Log P for the parent congener (I, X = H) was calculated by subtracting π for phenyl of 1.96 from log P of −1.03 for congener I (X = C_6H_5)[17] (−1.03 − 1.96 = −3.0) to get log P of −3.0. Hence, from π_0 of 0.82 we get log P of −2.2. It is clear from this that one needs quite hydrophilic triazines for optimum activity against tumor cells in culture. The above log P values are for the protonated form of the triazine which is the form present under physiological conditions.

It is of interest to compare log P of −2.2 with log P for Baker's antifol (III), a triazine now undergoing clinical



III

trials. In terms of eq 6, the log P of −2.46[17] for Baker's antifol is almost ideal; however, whether log P_0 for the triazines in whole animals is −2.2 remains to be seen. The evidence in hand suggests that one needs quite hydrophilic triazines to inhibit leukemia DHFR in vivo; however, what is surprising is the degree of hydrophilicity. It has been pointed out[18] that most antileukemia drugs have low log P values, but there is no solid evidence of a log P_0 so low as −2.

Since the triazines are completely protonated at physiological pH and the log P values cited above are for this protonated form, the meaning of log P_0 is more complex than for neutral drugs. The log P value for an ion pair is highly dependent on the ionic strength of the medium and the nature of the counterion. The octanol/water log P values were determined at low ionic strength, and the actual value was calculated by extrapolation to a concentration of infinite dilution for the triazine.[19] Since we do not know what the counterion would be in a living system, we cannot be sure just how low the effective in vivo log P is; nevertheless, the octanol/water scale will be of great value in the design of triazines for antitumor studies. What is now needed are data from animal studies to bracket log P_0 in mice.

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The negative portion of the bilinear part of eq 6 has a slope of −0.35 (1.34–1.69). Thus, activity does not simply level off at π_0 (0.8) as it does with isolated enzyme; instead, when π of X exceeds the optimum value, inhibitory potency falls.

There are two reasons one might advance for the lower π_0 in cells compared to isolated enzyme. One may simply be the random walk problem as considered above. The other could be that the enzyme in situ may have a different conformation which restricts the effective size of the hydrophobic pocket into which 3-X fits. As of the present time, there is insufficient evidence to make any judgment on this score. However, study of the two systems with more probes may provide insight on this problem.

A most interesting aspect of eq 6 is the positive coefficient with the indicator variable I. This brings out the fact that compounds with the $-CH_2Z-$ bridge are about four times more potent (other factors being equal) than those not having this feature. This term appears more important in eq 6 than in eq 3, although the coefficients are about the same size. Such a term was not significant in eq 1, which suggests that there may be some possibility for developing selectivity around this feature. However, studies should be made using human DHFR before we can attach any significance to this factor.

Adding a term in σ to eq 5 does result in a small but significant (in terms of the F statistic) improvement. One must remember that this may be just a correction on π. The π values used are those from the benzene system.[20] It is known that π varies somewhat from system to system and that these variations are correlated with σ. The π and σ constants of Table I are not highly collinear ($r = 0.346$).

It is unexpected to find that the intercept of eq 6 is larger than that of eq 2. Since the initial dependence on π for both equations is very similar, the higher intercept of eq 6 shows that only 0.1 the amount of isolipophilic inhibitor (other factors being constant) is needed to produce 50% inhibition of the cell culture as is needed to produce 50% inhibition of the purified enzyme. Considering that a fair amount of inhibitor must be bound to serum protein in the media as well as to other sites of loss in lipophilic sites in the cells, it would seem that considerably less than 50% of the DHFR in the living cells is inhibited, to cause an overall reduction of 50% in the growth rate.

The three data points omitted in Table I were not used in the formulation of eq 3–6. The $CH(OH)C_6H_5$ congener is always less active than expected, regardless of the type of DHFR on which it is tested. We believe that this is due to some kind of steric problem. We did not include the $COOC_2H_5$ derivative, even though it is well predicted, because of erratic test results (note confidence limits on log $1/C$). This congener is usually less active than expected on isolated enzyme. The C_6H_{13} congener is poorly predicted, although it is well predicted on isolated enzyme.

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It was surprising to find that many of the compounds of Table I are more potent against the leukemia cells than Baker's antifol. Several of the inhibitors approach MTX in potency. Hence, we feel that this series is an excellent one for further study. Of course the main problem in cancer chemotherapy is not potency but rather selectivity. We believe that by more careful study of isolated enzymes and cell cultures we should be able to more clearly define the structural features which make for selectivity. If we can establish selectivity on these less expensive and simpler to understand systems, we hope that we can then design more selective drugs for the whole animal studies.

Experimental Section

Chemicals. The syntheses of the triazines used in this study have been previously reported.[12] MTX was supplied by the Division of Cancer Treatment of the National Cancer Institute (Bethesda, MD). Fetal calf serum and powdered RPMI-1640 medium were purchased from Grand Island Biological Co., Grand Island, NY.

Cell Culture. The original L5178Y 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 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 12–13 h. The stock solutions of the triazines were made with unsupplemented medium and passed through a millipore filter under sterile conditions. However, compounds **2**, **6**, **8**, **10**, **12**, **13**, **15**, **29**, and **32–34** were not filtered (Gelman Acrodisc 4192), as appreciable amounts of these triazines seemed to adhere to the filter assembly. The ability to be filtered correlated quite well with their π values; i.e., compounds with a value less than 2.5 were easily filtered.

Cell cultures were seeded at $4.0–6.0 \times 10^4$ cells/mL in duplicate for each drug concentration in a plastic microtiter plate (0.2 mL/well).[21] 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%).

A dose–response curve was drawn from the data obtained, and the ID_{50} was calculated as in our previous studies.[13] The ID_{50} is defined as the concentration of inhibitor that causes a 50% inhibition of the growth of leukemia L5178Y cells in culture. The confidence limits on log $1/ID_{50}$ and π_0 were calculated by utilizing the jackknife procedure.[14]

Substituent Constants. The values for the substituent constants in Table I were taken from our recent compilation.[17]

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(21) Details of this technique will be published elsewhere.