Quantitative Structure–Activity Relationships of the Inhibition of *Pneumocystis carinii* Dihydrofolate Reductase by 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(X-phenyl)-*s*-triazines†

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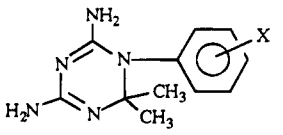
The inhibitory activities of 60 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(X-phenyl)-*s*-triazines versus purified, recombinant *Pneumocystis carinii* (*Pc*) dihydrofolate reductase (DHFR) have been determined at pH 7.0. Utilization of these K_{iapp} values has led to the formulation of appropriate quantitative structure–activity relationships (QSAR's) for both meta- and para-substituted derivatives. The QSAR's from *Pc* are compared with other triazine QSAR's derived versus chicken, murine tumor, *Escherichia coli*, and particularly human DHFR. Selectivity indices indicate that hydrophobic triazines are particularly effective versus *Pc* DHFR; they have lower K_i values for *Pc* DHFR than for human DHFR.

Pneumocystis carinii pneumonia is one of the premier causes of morbidity and mortality in patients with acquired immunodeficiency syndrome (AIDS). Early studies have indicated that trimetrexate, a nonclassical antifolate, has great potential as a treatment for *P. carinii* (*Pc*) pneumonia.[1] These results suggested that other antifolates could also be potential inhibitors of the target enzyme dihydrofolate reductase (DHFR) from *Pc*. Recently Queener has demonstrated that several analogs of pyrimethamine, methotrexate, and trimetrexate can be effective inhibitors of *Pc* DHFR.[2] DHFR is critical to cell growth because of its pivotal role in providing one-carbon cofactors for DNA synthesis. It comprises a useful target because it has been well characterized in bacterial, mammalian, and some microbial sources.[3] Moreover, it can also be selectively inhibited as trimethoprim and tetroxoprim have amply demonstrated.[4] The quantitative structure–activity relationships of the interaction of various antifolates with different DHFR's have also been well established.[5,6] In this study we examine the interactions of a set of antifolates, namely, the 4,6-diamino-2,2-dimethyl-1-(X-phenyl)-*s*-triazines **I** with DHFR from *Pc* and formulate an appropriate QSAR.



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Results

From the inhibition data in Table 1, the following mathematical models were developed for meta-substituted **I**.

$$\log 1/K_i = 6.97\ (\pm 0.37) + 0.29\ (\pm 0.15)\pi_3' \quad (1)$$

$$n = 43,\ r = 0.534,\ s = 0.870,\ F_{1,41} = 16.39$$

$$\log 1/K_i = 6.54\ (\pm 0.24) + 1.02\ (\pm 0.19)\pi_3' - 1.25\ (\pm 0.28) \log (\beta \cdot 10^{\pi 3'} + 1) \quad (2)$$

$$n = 43,\ r = 0.874,\ s = 0.513,\ F_{2,39} = 39.51,\ \text{optimum } \pi_3' = 2.54\ (\pm 0.72),\ \log \beta = -1.888$$

$$\log 1/K_i = 6.58\ (\pm 0.22) + 0.95\ (\pm 0.16)\pi_3' - 1.12\ (\pm 0.26) \log (\beta \cdot 10^{\pi 3'} + 1) - 0.71\ (\pm 0.43) I_{OR} \quad (3)$$

$$n = 43,\ r = 0.903,\ s = 0.460,\ F_{1,38} = 10.55,\ \text{optimum } \pi_3' = 2.89\ (\pm 3.27),\ \log \beta = -2.123$$

$$\log 1/K_i = 6.48\ (\pm 0.23) + 0.73\ (\pm 0.12)\pi_3' - 1.36\ (\pm 0.35) \log(\beta \cdot 10^{\pi 3'} + 1) - 0.78\ (\pm 0.42) I_{OR} + 0.28\ (\pm 0.21) MR_y \quad (4)$$

$$n = 43,\ r = 0.916,\ s = 0.435,\ F_{1,37} = 5.51,\ \text{optimum } \pi_3' = 3.99\ (\pm 0.68),\ \log \beta = -3.925$$

In these equations, n represents the number of data points, r is the correlation coefficient, and s is the standard deviation from the regression, while F represents the F statistic for significance of each added variable. π_3' represents the hydrophobicity of the substituent in the meta position. I_{OR} is an indicator variable which acquires the value of 1 when X = $O(CH_2)_nCH_3$ for all n. Thus all alkoxy type derivatives are fitted with this variable. All other substituents are

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Table 1. Parameters Used To Derive Eqs 1–4 for the Inhibition of DHFR from *P. carinii* by 3-X **I**

no.	X	log $1/K_i$ obsd	log $1/K_i$ pred[a]	Δlog $1/K_i$	π_3'	I_{OR}	MR_y
1	H	6.51	6.48	0.03	0	0	0.00
2	3-SO_2NH_2	4.15	5.16	−1.00	−1.82	0	0.00
3	3-$CONH_2$	5.14	5.40	−0.26	−1.49	0	0.00
4	3-$CONH_3$	6.03	6.08	−0.05	−0.55	0	0.00
5	3-OH	5.83	6.00	−0.17	−0.67	0	0.00
6	3-CF_3	7.11	7.12	−0.01	0.88	0	0.00
7	3-Cl	7.65	7.00	0.65	0.71	0	0.00
8	3-NO_2	6.88	6.28	0.60	−0.28	0	0.00
9	3-CN	6.93	6.07	0.86	−0.57	0	0.00
10	3-CH_2CH_3	7.22	7.23	−0.01	1.03	0	0.00
11	3-$(CH_2)_8CH_3$	8.62	8.71	−0.09	4.79	0	0.00
12	3-$(CH_2)_{11}CH_3$	7.90	7.76	0.14	6.41	0	0.00
13	3-$C(CH_3)_3$[b]	6.67	7.91	−1.24	1.98	0	0.00
14	3-*d,l*-$CH(OH)C_6H_5$[b]	5.93	6.87	−0.94	0.54	0	0.00
15	3-OCH_3	6.46	5.69	0.77	−0.02	1	0.00
16	3-OCH_2CH_3	5.20	5.98	−0.78	0.38	1	0.00
17	3-$O(CH_2)_5CH_3$	7.67	7.61	0.06	2.67	1	0.00
18	3-$O(CH_2)_8CH_3$	8.17	8.11	0.06	4.29	1	0.00
19	3-$O(CH_2)_{10}CH_3$	7.73	7.62	0.11	5.37	1	0.00
20	3-$O(CH_2)_{13}CH_3$	6.48	6.62	0.14	6.99	1	0.00
21	3-$O(CH_2)_2OC_6H_4$-3′-CF_3	7.65	7.84	−0.19	1.68	0	0.50
22	3-$O(CH_2)_4OC_6H_5$	7.42	8.44	−1.02	2.71	0	0.10
23	3-$O(CH_2)_4OC_6H_5$-3′-CF_3	8.24	8.55	−0.31	2.71	0	0.50
24	3-$OCH_2C_6H_5$	7.19	7.94	−0.75	1.98	0	0.10
25	3-OCH_2-1-adamantyl	7.77	7.86	0.09	3.07	0	0.00
26	3-$CH_2OC_6H_4$-3′-Cl	7.92	7.85	0.07	1.66	0	0.60
27	3-$CH_2OC_6H_4$-3′-CN	8.08	7.86	0.22	1.66	0	0.63
28	3-$CH_2OC_6H_4$-3′-OCH_3	8.11	7.90	0.21	1.66	0	0.79
29	3-$CH_2OC_6H_4$-3′-CH_2OH	7.60	7.88	−0.28	1.66	0	0.72
30	3-$CH_2OC_6H_4$-3′-CH_3	7.71	7.84	−0.13	1.66	0	0.57
31	3-$CH_2OC_6H_4$-3′-CH_2CH_3	8.38	7.97	0.41	1.66	0	1.03
32	3-$CH_2OC_6H_4$-3′-$CH(Me)_2$	8.32	8.10	0.22	1.66	0	1.50
33	3-$CH_2OC_6H_4$-3′-C_6H_5	8.35	8.39	−0.04	1.66	0	2.54
34	3-$CH_2OC_6H_4$-3′-$NHCOCH_3$	8.49	8.10	0.39	1.66	0	1.49
35	3-$CH_2OC_6H_4$-3′-$NHCONH_2$	8.34	8.06	0.28	1.66	0	1.37
36	3-$CH_2OC_6H_4$-4′-$(CH_2)_4CH_3$	7.77	8.35	0.58	1.66	0	2.42
37	3-CH_2O-1-naphthyl	8.27	8.17	0.10	1.66	0	1.75
38	3-$CH_2SC_6H_5$	8.27	8.17	0.10	2.30	0	0.10
39	3-$CH_2SeC_6H_5$	8.42	8.22	0.20	2.37	0	0.10
40	3-$SCH_2C_6H_5$	8.55	8.17	0.38	2.30	0	0.10
41	3-$SCH_2C_6H_4$-4′-Cl	8.58	8.31	0.27	2.30	0	0.60
42	3-$CH_2OC_6H_2$-2′,4′,5′-Cl_3	8.11	8.18	−0.07	1.66	0	1.80
43	3-$COOC_2H_5$[b]	5.63	6.85	−1.22	0.51	0	0.00
44	3-$CH_2NHC_6H_3$-3′,5′-$(CONH_2)_2$	7.82	7.75	0.07	1.00	0	1.96
45	3-$CH_2NHC_6H_4$-4′-SO_2NH_2	7.34	7.55	−0.21	1.00	0	1.23
46	3-$CH_2NHC_6H_4$-4′-Cl	7.35	7.37	−0.02	1.00	0	0.60

[a] Calculated using eq 4. [b] Not used in the derivation of the equations.

assigned a value of zero. This serves to pinpoint irregularities in behavior at a localized position, in this case the oxygen of the ether. MR_y (MR values scaled by 0.1 to ensure parity with π) represents the molar refractivity of the substituent on the second phenyl ring. MR as defined by the Lorenz–Lorentz equation is primarily a measure of bulk with a minor polarizability component. All these equations are significant at the 95% confidence level. The 95% confidence interval for each variable is denoted in parentheses. K_i is the Michaelis inhibition constant, while β is a disposable parameter which is obtained by an interactive procedure.[7] The correlation matrix (r) for the variables in equs 1–4 revealed the following: π_3' vs $I_{OR} = 0.37$, π_3' vs $MR_Y = 0.05$, and I_{OR} vs $MR_Y = 0.33$.

An examination of the equations indicates that the hydrophobic parameter π_3' accounts for nearly 76% of the variance in the data while the minor variables I_{OR} and MR_y represent approximately 6% and 2% of the variance, respectively. The overriding importance of hydrophobicity in the inhibition of *Pc* DHFR is established by the strong presence of π_3'. The hydrophobicity variable π_3' indicates that for substituents of the type $CH_2ZC_6H_4$-Y or $ZCH_2C_6H_5$-Y where Z = oxygen, sulfur, selenium, or NH, the value of Y is set at 0, i.e., Y does not make hydrophobic contact with the enzyme. This anomalous behavior has previously been seen in the case of *Leishmania major* DHFR, chicken liver DHFR, and many other types.[5,8] This critical finding was substantiated in the case of chicken DHFR via X-ray crystallography of a ternary complex of DHFR, NADPH, and triazine **I** where X = 3-$CH_2OC_6H_4$-3′-$NHCOCH_3$.[9] The acetylamino substituent did not make hydrophobic contact with the enzyme at all but hovered in polar space. The coefficient with π_3' in eq 4 suggests that partial desolvation of the substituent occurs at the binding site. The coefficient with π_3' is very similar to that obtained from *L. major* DHFR.[8] In fact, the overall QSAR models are remarkably similar. It does indicate that the rise in inhibition parallels the increase in hydrophobicity up to a certain hydrophobic point (4.00), and then it drops off with a slope of −0.63 (−1.36 + 0.73). The relatively large optimum value in π_3' indicates that the hydrophobic surface in *Pc* is rather extensive. Examination of the model obtained with *L. major* DHFR indicates a strong similarity between the

two enzymes. See eq 5.

$$\log 1/K_i = 5.05\ (\pm 0.16) + 0.65\ (\pm 0.08)\pi_3' - 1.22\ (\pm 0.29)\log(\beta \cdot 10^{\pi 3'} + 1) - 1.12\ (\pm 0.29)I_{OR} + 0.58\ (\pm 0.16)MR_y \quad (5)$$

$$n = 41,\ r = 0.965,\ s = 0.298,\ \text{optimum}\ \pi_3' = 4.54,\ \log\beta = -4.491,\ F_{1,35} = 45.2$$

Both equations signal the importance of critical variables—π_3', I_{OR}, and MR_y. After being corrected for their unique hydrophobicities, the alkoxy derivatives are still approximately 13 and 6 times less inhibitory than other substituents versus *L. major* DHFR and *Pc* DHFR, respectively. Perhaps the oxygen with its two p orbitals destroys the coplanarity of the substituent with the ring.

The positive coefficient with MR_y suggests that the bulkier substituents slightly enhance inhibitory potency. These parameters do not appear in the QSAR's for other DHFR's, suggesting that unlike in chicken liver DHFR or L1210 DHFR, Y substituents do make contact, albeit polar in nature, with *Pc* and *L. major* DHFR's. The bulk term, however, only accounts for 2% of the variance in the data, and the *F* test indicates that it is significant at the 97.5 level.

Three data points were not included in the analysis—X = $3C(CH_3)_3$, $3\text{-}COOC_2H_5$, and 3-*d,l*-$CH(OH)C_6H_5$. With the *tert*-butyl derivative, the observed value is 17-fold less than predicted and its log $1/K_i$ value (6.67) is about three standard deviations outside the correlation value. Normally the *tert*-butyl derivative is slightly askew in its interactions with both mammalian and bacterial DHFR's but not to the extent seen in both *L. major* and *Pc*. This suggests that with both these enzymes the binding site, although hydrophobic in nature, is restrictive in its spatial attributes. This suggests that some bulky amino acid residue such as Trp, Ile, or Phe may be constricting the entrance to the hydrophobic binding area. The two other derivatives, $3\text{-}COOC_2H_5$ and 3-*d,l*-$CH(OH)C_6H_5$, are also off their mark by at least two standard deviations. All three of these substituents have branching at the α-carbon attached to the primary phenyl ring. This is a critical position for orienting the substituents within the hydrophobic milieu, and any untoward steric effect immediately compromises the effectiveness of the substituent.

The data were also examined for electronic effects, since σ effects have been observed in a significant number of 3X-triazine QSAR's, e.g., chicken liver DHFR, human liver DHFR, L1210 leukemia DHFR, etc. However, no electronic effect was discernible. Again it parallels what has been observed with *L. major* DHFR. A comparison of the inhibitory potencies of the triazines versus *L. major* DHFR and *Pc* DHFR resulted in the following equation.

$$\log 1/K_i\ (Pc) = 0.91\ (\pm 0.12)\log 1/K_i\ (L.\ major) + 1.94\ (\pm 0.72) \quad (6)$$

$$n = 41,\ r = 0.930,\ s = 0.387,\ F_{1,39} = 250.66$$

From eq 6 it is apparent that the triazines have approximately 90-fold (antilog of 1.94) greater affinity for the *Pc* DHFR than for the *L. major* DHFR.

Table 2. Parameters Used To Derive Eqs 7–9 for the Inhibition of DHFR from *P. carinii* by 4-X **I**

		log $1/K_i$				
no.	X	obsd	pred[a]	Δlog $1/K_i$	π_4'	I_{OR}
1n	H	6.51	6.12	0.39	0	0
2n	$4\text{-}CONH_2$	4.70	4.81	−0.11	−1.49	0
3n	4-OH	5.60	5.53	0.07	−0.67	0
4n	$4\text{-}CF_3$	6.88	6.88	0	0.88	0
5n	4-Cl	6.96	6.73	0.23	0.71	0
6n	$4\text{-}CH_3$	6.78	6.61	0.17	0.56	0
7n	$4\text{-}O(CH_2)_3CH_3$	6.20	6.22	−0.02	1.55	1
8n	$4\text{-}O(CH_2)_4CH_3$	6.59	6.65	−0.06	2.13	1
9n	$4\text{-}O(CH_2)_5CH_3$	7.00	6.92	0.08	2.67	1
10n	$4\text{-}(CH_2)_5CH_3$	8.10	8.24	−0.14	3.22	0
11n	$4\text{-}(CH_2)_6CH_3$	8.20	8.18	0.02	3.77	0
12n	$4\text{-}(CH_2)_7CH_3$	8.20	8.05	0.15	4.32	0
13n	$4\text{-}(CH_2)_9CH_3$	7.80	7.90	−0.10	4.87	0
14n	$4\text{-}SCH_3$	6.60	6.65	−0.05	0.61	0
15n	4-SH	5.80	6.46	−0.66	0.39	0

[a] Calculated using eq 9.

The data in Table 2 was utilized to generate eqs 7–9 for the inhibition of *Pc* DHFR by 4-X-triazines.

$$\log 1/K_i = 6.05\ (\pm 0.36) + 0.48\ (\pm 0.15)\pi_4' \quad (7)$$

$$n = 15,\ r = 0.887,\ s = 0.481,\ F_{1,13} = 47.81$$

$$\log 1/K_i = 6.14\ (\pm 0.33) + 0.50\ (\pm 0.14)\pi_4' - 0.59\ (\pm 0.61)I_{OR} \quad (8)$$

$$n = 15,\ r = 0.919,\ s = 0.428,\ F_{1,12} = 4.41$$

$$\log 1/K_i = 6.12\ (\pm 0.22) + 0.88\ (\pm 0.21)\pi_4' - 1.17\ (\pm 0.59)\log(\beta \cdot 10^{\pi 4'} + 1) - 1.22\ (\pm 0.51)I_{OR} \quad (9)$$

$$n = 15,\ r = 0.973,\ s = 0.274,\ F_{2,10} = 9.67,\ \text{optimum}\ \pi_4' = 3.22\ (\pm 1.51),\ \log\beta = -2.741$$

The correlation matrix (*r*) values for the variables in eqs 7–9 are as follows: π_4' vs $I_{OR} = 0.15$.

Equation 9 again establishes the bilinear dependence of inhibitory potency on the hydrophobicity of the substituents. The deleterious effect of the alkoxy linkage is also clearly evident. The small number of data points, particularly in the hydrophobic sphere, precludes the inclusion of any other parameters such as the MR term. Careful examination of the data does suggest, however, that bulky substituents on the phenyl ring enhance binding to the receptor. The high optimum hydrophobicity argues for the existence of an extensive hydrophobic cleft with marginal bulk tolerance. These results with the para-substituted triazines confirm what has been observed with the meta-substituted triazines. They also suggest that both types of substituted triazines can access the same binding site on *Pc* DHFR.

Discussion

A comparison of the various QSAR's developed by meta-substituted triazines versus DHFR's from different sources is made in Table 3. The statistical parameters in all eight systems are comparable. Four main facets of the QSAR's will be discussed. These include the coefficient with the hydrophobic term, the ϱ value (or coefficient with the σ term), the numerical value of

Table 3. Comparison of Various QSAR's Generated in the Interactions of 3-X Triazines and Various DHFR's

no.	type of DHFR	n	r	s	a π_3'	bσ	intercept	other	optimum π_3'	ref
1	chicken	59	0.906	0.267	1.01	0.86	6.33	−1.89	5	
2	human	60	0.890	0.308	1.07	0.82	6.07	0.50I	2.10	20
3	rat	18	0.977	0.171	1.12	0.46	6.23	−1.72	5	
4	L1210	58	0.900	0.264	0.98	0.79	6.12	$0.44I_{CN}$	1.76	6
5	*E. coli*	31	0.930	0.280	1.16	1.36	5.08	0.41*I*	~3.00	17
6	*L. casei*	44	0.953	0.319	0.53	0.70	2.93	1.49*I*	4.31	18
7	*L. major*	41	0.965	0.298	0.65		−5.05	$-1.12I_{OR} + 0.58MR_y$	4.54	10
8	*P. carinii*	43	0.916	0.435	0.73		−6.48	$-0.78I_{OR} + 0.28MR_y$	4.00	–

the intercept, and the optimum hydrophobic requirement for maximal inhibition of binding.

The coefficient of the π term for the chicken, human, rat, murine leukemia, and *Escherichia coli*[10] enzymes approaches unity which argues strongly for total desolvation of the substituents within the confines of the binding site. However, with *Lactobacillus casei*,[11] *L. major*,[8] and *Pc* DHFR's this is not the case; the coefficient implies that partial desolvation or surface binding is operative.[12]

Electronic effects are not visible with *L. major* DHFR and *Pc* DHFR. The positive ϱ value suggests that electron-withdrawing substituents on the phenyl ring enhance binding between inhibitor and enzyme; *E. coli* DHFR (1.36) is unusually high compared to either chicken (0.86) or human (0.82) DHFR. It may indicate a type of dipolar interaction between the negatively charged bacterial enzyme and the electron-deficient nucleus of the triazine. On the other hand, with *Pc* DHFR this electronic effect is not apparent. The intercepts indicate that the triazines **I** are generally more potent inhibitors of *Pc* DHFR than of human or *E. coli* DHFR. Hydrophobic space is also more extensive in the case of *Pc* DHFR ($\pi_0 = 4.00$) in comparison to the human enzyme ($\pi_0 = 2.10$). These differences in the interactions of **I** with *Pc* DHFR and human DHFR indicate that a favorable selectivity index may be attainable.

Pc DHFR (206 residues) compares favorably with human DHFR (186 residues). An alignment of the conserved residues indicates that 61 residues are identical. Both enzymes also reveal a glutamate residue in the active site. However, since the X-ray crystallography coordinates of *Pc* DHFR have not been reported, it is not possible to do any indepth comparisons.

The following QSAR eqs 10 and 11 have been established for the inhibition of DHFR from human lymphoblastoid cells (WIL2).[13] Inhibition of human DHFR by 3-X **I**:

$$\log 1/K_i = 6.07\ (\pm 0.21) + 1.07\ (\pm 0.23)\pi_3' - 1.10\ (\pm 0.26)\log(\beta \cdot 10^{\pi 3'} + 1) + 0.50\ (\pm 0.19)I + 0.82\ (\pm 0.66)\sigma \quad (10)$$

$$n = 60,\ r = 0.890,\ s = 0.308,\ F_{1,54} = 6.04,\ \text{optimum } \pi_3' = 2.10\ (\pm 0.87),\ \log \beta = -0.577$$

Inhibition of human DHFR by 4-X **I**:

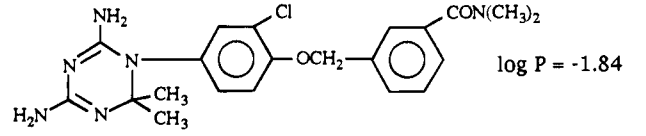
$$\log 1/K_i = 5.83\ (\pm 0.34) + 0.78\ (\pm 0.20)\pi_4' - 0.78\ (\pm 0.29)\log(\beta \cdot 10^{\pi 4'} + 1) + 1.26\ (\pm 0.32)I - 0.88\ (\pm 0.45)\nu \quad (11)$$

$$n = 35,\ r = 0.953,\ s = 0.361,\ F_{1,29} = 14.7,\ \text{optimum } \pi_4' = 3.43\ (\pm\alpha),\ \log \beta = -0.926$$

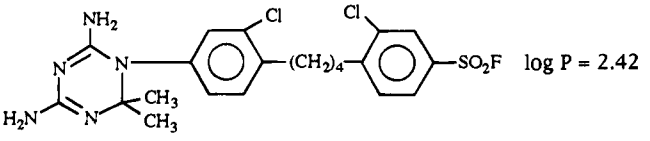
An important variable that crops up in these QSAR's is the indicator variable *I*. *I* is assigned a value of 1 for substituents containing a -OCH_2-, -SCH_2-, -CH_2S-, or -CH_2NH- moiety between the parent phenyl group and an adjoining phenyl group. This "bridge" is reminiscent of the -CH_2NH- bridge in folic acid, and it confers added potency to the triazines that incorporate this functionality. A steric parameter is also evident with the 4-X triazines; Charton's parameter, ϑ, based on the van der Waals' radii of the substituents, is used to illustrate this effect.[14] These two QSAR's are useful in predicting the activities of potential inhibitors.

Table 4 examines a representative set of triazines and their biological activities versus human and *Pc* DHFR's. These differences in binding behavior suggest that some of the triazines, particularly those with enhanced hydrophobicities, have the ability to discriminate between human and *Pc* DHFR's. Particularly noteworthy are those **I** with substituents such as $(CH_2)_7CH_3$, $(CH_2)_8CH_3$, and CH_2O-1-adamantyl. The *Pc* activities of trimethoprim and epiroprim were obtained by Margosiak et al.[15] and are included for comparative purposes.

Baker's antifols **I** and **II** are triazines that have been used in chemotherapy, and thus their chemical safety profiles have been well developed and studied.[16] They



Baker's Antifol I



Baker's Antifol II

both have the physicochemical attributes to act as selective inhibitors of *Pc* DHFR. The sensitivity index of Baker's antifol **I** surpasses that of trimethoprim and epiroprim. However, this may be due to differences in assay procedures. Nevertheless, this suggests that there exist significant differences in the active site of the two enzymes which should be probed further with a more extensive set of inhibitors. Nuances in behavior are clearly discernible and should be manipulated to yield more effective and selective inhibitors.

Queener et al. have tested a series of meta- and para-substituted **I** versus *Pc* DHFR and rat liver DHFR.[17]

Table 4 Comparison of Various QSAR's Generated in the Interactions of 3-X Triazines and Various DHFR's

no.	compound[a]	log $1/K_i$[b] human DHFR	log $1/K_i$[b] *P. carinii* DHFR	index sensitivity, $K_i(h)/K_i(Pc)$
1	3-H, T	5.78	6.51	0.73
2	3-Cl, T	7.03	7.65	0.62
3	3-$(CH_2)_8CH_3$, T	6.66	8.62	1.96
4	4-$(CH_2)_7CH_3$, T	(6.01)[c]	8.20	2.19
5	3-$SCH_2C_6H_5$, T	7.37	8.55	1.18
6	3-CH_2O-1-naphthyl, T	6.89	8.27	1.38
7	3-CH_2O-adamantyl, T	6.11	7.77	1.66
8	3-$(CH_2)_{11}CH_3$, T	6.52	7.90	1.38
9	trimethoprim[d]	6.71	6.82	0.11
10	epiroprim[d]	7.23	7.76	0.53
11	Baker's antifol I, T	7.11	(8.73)[e]	(1.62)
12	Baker's antifol II, T	7.65	(8.99)[e]	(1.34)

[a] T= 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(X-phenyl)-*s*-triazines. [b] Reference 20. [c] Calculated using eq 11. [d] Reference 22. [e] Calculated using eq **4**.

However, the analogs tested did not span adequate hydrophobic substituent space, and a cursory analysis of seven mono- and meta-substituted analogs yielded a tenuous bilinear relationship between potency and hydrophobicity. Despite the paucity of data points, it was clearly apparent that the inhibitory activity increased with hydrophobicity up to the π_o value (approximately 2) and then decreased beyond π_o. Their selectivity ratios using rat liver DHFR in lieu of human DHFR were clearly not favorable to the triazines. However, selectivity ratios utilizing human DHFR should be more accurate and desirable. The study of Queener et al. also revealed that trimethoprim proved to be a less potent but more selective inhibitor of *Pc* DHFR.

The results with the triazines **I** versus human and *Pc* DHFR's indicate that this type of comprehensive analysis would be extremely useful in identifying potent and perhaps selective antifolates as therapeutic agents for *P. carinii* pneumonia. However, further studies should be done in culture to determine the other pharmacokinetic parameters necessary for efficacy.

Experimental Section

Materials. *Pc* DHFR was obtained according to the procedure of Sirawaraporn et al.[18] and stored at −20 °C in 50 mM Tes, 5 mM DTT, 1 mM EDTA, 20% glycerol, and 1 mg/mL BSA at pH 7.0. NADPH (Sigma, type I) was dissolved in water at 2 mM and stored at −70 °C. DHF (dihydrofolate) was prepared by partial reduction of folic acid according to the procedure of Friedkin.[19] The DHF concentration was checked by UV absorption and confirmed by enzymatic conversion to THF (tetrahydrofolate).

Multiple Enzyme Assay Analysis. Solutions of inhibitors were prepared by dissolving 1−5 mg of triazine with the appropriate amount of DMSO to give a 50 mM solution which was diluted with assay buffer to give a final concentration of not greater than 2% DMSO. This concentration was found to inhibit the velocity by not more than 10%. Inhibitor solutions were found to be stable in DMSO at −70 °C for months.

IC_{50} determinations were performed simultaneously on eight compounds per 96-well microtiter plate utilizing a Molecular Devices thermax plate reader. Data was collected and analyzed using Deltasoft software from Biometallics, Inc. A 6.7 μL sample of the DMSO inhibitor solution was placed in the first well of each of eight rows of a Falcon 3072 96-well microtiter place. The inhibitor in each row was diluted with 162 μL of assay buffer (50 mM Tes, 75 mM BME, 1 mM EDTA, pH 7.0) while 125 μL was added to subsequent rows. The plate was mixed by placing briefly on a orbital plate shaker. Fourfold serial dilutions were then accomplished by hand using an eight-channel pipetman or a Perkin-Elmer Pro/Pette on a single plate or multiple plates diluted using a Zymark robot. Transfer of 42 μL of solution to the next row (nine total rows) with thorough mixing gave uniform serial dilutions with concentrations of inhibitor which ranged, after final addition of substrates and enzyme, from 1 mM to 15 nM. Row 10 was used as a control, while rows 11 and 12 were blanks with no enzyme in order to subtract out the background decomposition of NADPH. A 100 μL portion of substrate solution (assay buffer plus 2.5 mg/mL BSA) was added such that the final concentration, in a total volume of 250 μL, was 25 μM for DHF and 100 μM for NADPH. Enzyme (25 μL, 4 nM final concentration) was used to initiate the reaction by addition to all wells but blanks. After thorough mixing, data was collected with the plate reader for 10 min by following the decrease in absorbance at 340 nM.[18] The IC_{50} was determined for each compound (row) using the Deltasoft program which performs a sigmoidal curve fit to a plot of activity verses log inhibitor concentration. Calculation of K_i values was performed using the equation $K_i = K_m(IC_{50}/[S])$ where $[S] > K_m = 1.36 \pm 0.15$ μM, and competitive kinetics are assumed as has been shown for *s*-triazines with other DHFR's.[8,18,20]

Table 5. 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(4-X-phenyl)-*s*-triazines

no.	X	mp, °C	yield,[a] %	formula[b]
10n	4-$(CH_2)_5CH_3$	209−211	58	$C_{17}H_{27}N_5$·HCl
11n	4-$(CH_2)_6CH_3$	195−199	73	$C_{18}H_{29}N_5$·HCl
12n	4-$(CH_2)_7CH_3$	200−202	68	$C_{19}H_{31}N_5$·HCl·H_2O
13n	4-$(CH_2)_9CH_3$	209−211	79	$C_{21}H_{35}N_5$·HCl
14n	4-SCH_3	206−212	93	$C_{13}H_{19}N_5S$·HCl·H_2O
15n	4-SH	209−211	64	$C_{12}H_{17}N_5S$·HCl

[a] Crude yield before recrystallization. [b] Calculated from combustion analysis.

Synthesis. The syntheses of most of the triazines used in this study have been previously reported.[9] However, six new compounds (entries **10n**−**15n**) were synthesized in the usual manner.[21] Their properties are outlined in Table 5. Melting points are uncorrected. Combustion analyses were performed by the UC Berkeley Analytical Facilities. Substituted anilines were commercially available from Aldrich Chemical Co. NMR spectral analysis was consistent with the assigned structure for newly synthesized analogs **10n**−**15n**.

General Method of Synthesis of 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(X-phenyl)-*s*-triazines. The general procedure used here for synthesis of the six new 4-substituted analogs was similar to that previously reported.[9,21] To a 1.5 M acetone solution of 1 equiv of a substituted aniline was added 1.1 equiv of dicyanamide. One equivalent of concentrated HCl was added, and the solution was refluxed overnight. Upon cooling, the triazine crystallized out of solution, was isolated by filtration, and then recrystallized from hot water. The triazine was then dried under vacuum at 55 °C.

QSAR Analysis. The physicochemical constants π and σ were taken from the compilations of Hansch and Leo.[22,23] The regression analysis was undertaken by using the program C-QSAR (BioByte, Inc.).[24]

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