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A COMPARATIVE ANALYSIS OF THE SYNERGISTIC INTERACTION BETWEEN N[1]-PHENYLSULFANILAMIDES AND BENZYLPYRIMIDINES USING QSAR TECHNIQUES

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Growth inhibition of *E. coli* cell culture has been determined for a series of 4-substituted-N[1]-phenylsulfonamides tested in the presence and absence of synergistic concentrations of trimethoprim. Quantitative structure-activity relationships, established by regression analysis, exhibit an identical dependence of bacterial growth inhibition on sulfonamide pK_a irrespective of the presence or absence of trimethoprim. Examination of a small series of benzylpyrimidines in the presence or absence of 4-dimethylamino-N[1]-phenylsulfanilamide gave similar results. Since the presence of a synergistic agent affords no change in structure-activity relationships, it is concluded that no direct interaction between sulfonamides and benzylpyrimidines occurs and that the synergism observed is solely the result of the kinetic consequences of sequential blockade of the folate biosynthetic pathway.

KEY WORDS: N[1]-phenylsulphonamides, trimethoprim, benzylpyrimidines, folate biosynthetic pathway, synergism.

INTRODUCTION

The concept of utilizing an antifolate in combination with a sulfonamide was first suggested by Greenberg in 1949[1] but received little attention until Hurley[2] reported the potentiation of pyrimethamine by sulfadiazine in human malaria. In 1968, extensive investigations on the synergistic interaction of trimethoprim and sulfamethoxazole[3,4] stimulated an intense effort on the part of numerous laboratories which resulted in the marketing of this combination, generically referred to as co-trimoxazole. In spite of the large amount of research that has been conducted, the *in vivo* effectiveness as well as the exact mechanism of such combinations remains open to question.

Detailed kinetic studies by Seydel[5–7] and others[8] have provided support for the true synergistic nature of co-trimoxazole in bacterial cell culture test systems. Treatment of *E. coli* cell culture with a sulfonamide and trimethoprim produced a greater than additive growth inhibition. The normally delayed onset of sulfonamide-induced growth inhibition was eliminated. Growth inhibition by sulfonamides was considerably enhanced even in the presence of trimethoprim concentrations which were far below those which would produce inhibition by trimethoprim alone. Studies on

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mechanism of action have shown that the enzyme, 7,8-dihydropteroate synthetase, is the primary site of sulfonamide inhibition while the enzyme, dihydrofolate reductase, is accepted as the intracellular target for benzylpyrimidines such as trimethoprim. Thus, administration of a combination of these agents would be expected to result in a sequential blockade of the bacterial folate biosynthetic pathway. While each agent when used alone was bacteriostatic, the combination proved to be "bactericidal" under certain condition.[5] Based on such observations, it has been suggested that trimethoprim may also bind to synthetase, the site of sulfonamide action[9] or, conversely, that sulfonamide may bind at the primary site of trimethoprim action.[10] One means of examining such possibilities is to investigate the structure-activity relationships for a series of sulfonamides tested alone as well as in the presence of trimethoprim. If there is in fact any significant interaction at common target sites by these two types of inhibitor, a change in structure-activity relationships should result. To examine this possibility, a series of fourteen 4-substituted-N^1-phenylsulfonamides was selected for study. The sulfonamide bacterial growth inhibition kinetics were examined in *E. coli* cell culture, in the absence and in the presence of synergistic concentrations of trimethoprim. In addition, four trimethoprim derivatives were studied in the absence and in the presence of synergistic concentrations of 4-acetyl-N^1-phenylsulfanilamide. The results of these studies are the subject of this report.

METHODS

Materials

E. coli mutaflor maintained on agar slants was used as the test organism. The culture broth was a dextrose-casamino acid (vitamin-free) which has been described by Anton.[11] The medium was sterilized by filtration through cellulose ester membranes (0.22 μ) or by Seitz-Filter according to standard procedures.

Growth. A broth culture was inoculated from an agar and allowed to grow for 12–16 h. All cultures were grown at 37°C. A dilution of this culture, the preliminary culture, whose growth was measured turbidimetrically at 500 nm was allowed to grow into the logarithmic phase.

Total count method. Samples of the experimental cultures were diluted with a particle free saline (0.85%) – formaldehyde (1%) solution so that a count of 10,000 – 30,000 organisms was obtained. Diluted samples were counted with a Coulter counter Model B equipped with a 30-μ orifice. Counts per 50 μl are usually obtained with this instrument. Instrument settings which gave satisfactory results were: 1/aperture current, 1; 1/amplification, 1; matching switch 40 K; gain 10; lower threshold, 7; and upper threshold, maximum.

Addition of antibacterials. When used separately the sulfonamide or benzylpyrimidine were added to the experimental cultures shortly after inoculation (15–30 min). When used in combination the sulfonamide was added first and after 1.5–1.75 h of growth the benzylpyrimidine was added. In this way the onset of inhibition caused by both antibacterials would occur at approximately the same time and multiphasic growth curves were avoided.

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Growth Inhibition

Each of the fourteen sulfonamide derivatives was evaluated to determine an I_{50} value in the absence of trimethoprim and, in a parallel experiment, to determine an I_{50} value in the presence of a synergistic concentration trimethoprim (0.15 μM). In addition, a small set of four trimethoprim analogs was evaluated in the absence and presence of a synergistic concentration (2 μM) of 4-acetyl-N^1-phenylsulfanilamide. Figures 1a and b provide an illustration of the results of a typical experiment. In these plots, the ordinate is *E. coli* cell count on a logarithmic scale and the abscissa is time in hours. Figure 1a shows the influence of bacteriostatic concentrations of 4-dimethylamino-N^1-phenylsulfanilamide on bacterial growth rate while Figure 1b demonstrates the synergism observed upon addition of a 0.15 μM concentration of trimethoprim. A curve-fitting procedure on a Wang 2200 microcomputer was used to compute I_{50} values. Inhibition experiments were repeated until the computed 95% confidence interval was less than $\pm$15%.

Correlation Analysis

Correlation analyses of the biological data and the physicochemical parameters associated with the set of sulfonamides were conducted using a multiparameter

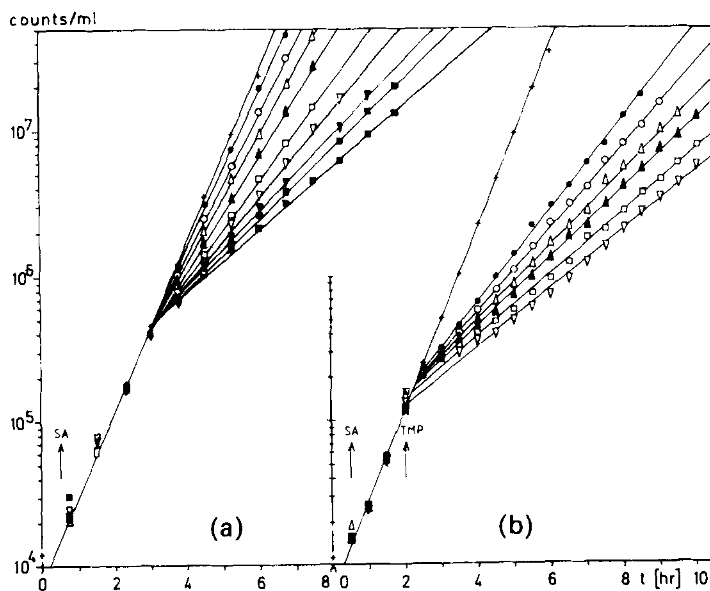

FIGURE 1 *E. coli* Growth Inhibition Kinetics. (a) In the presence of increasing concentrations of 4-dimethylamino-N^1-phenylsulfanilamide (SA). ↑ = point of drug addition, x = control; ● = 5 μmol/l SA, ○ = 10 μmol/l SA; △ = 16 μmol/l SA, ▲ = 24 μmol/l SA; □ = 36 μmol/l SA, ▽ = 50 μmol/l SA; ▼ = 64 μmol/l SA, ■ = 80 μmol/l SA; ⊠ = 100 μmol/l SA. (b) In the presence of increasing concentrations of 4-dimethyl-N^1-phenylsulfanilamide (SA) and a constant concentration of trimethoprim (TMP). ↑ = point of drug addition, x = control; ● = 0.15 μmol/l TMP; ○ = TMP + 0.25 μmol/l SA; △ = TMP + 0.50 μmol/l SA; △ = TMP + 1.00 μmol/l SA; □ = TMP + 1.75 μmol/l SA; ▽ = TMP + 2.50 μmol/l SA.

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regression analysis program designed for structure-activity work. Physicochemical parameters examined were the Hansch octanol/water π constant, the Hammett σ constant, the pK_a value, the n.m.r. shift for the amino hydrogens and the n.m.r. shift for the 2/6 hydrogens of the sulfonamide ring. N.m.r. shifts were determined in deuterated dimethylsulfoxide and converted to shift differences with respect to the unsubstituted N^1-phenylsulfonamide. Thus, the signs of the shift differences correspond directly to Hammett σ constants where negative signs indicate electron donation and positive signs indicate electron withdrawal by substituent.

RESULTS AND DISCUSSION

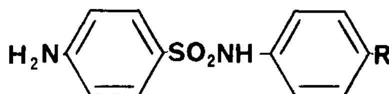
The *E. coli* growth inhibition data and the physicochemical parameters associated with the 14 sulfonamide derivatives are summarized in Table I. Table II is the correlation matrix for all parameters investigated. Correlation of the data resulting from evaluation of the sulfonamides in the absence of trimethoprim afforded equations (1)–(3).

$$\text{Log } 1/I_{50} = -0.51\ (0.10)\, pK_a + 9.22\ (0.84) \tag{1}$$

$$n = 14 \quad s = 0.166 \quad r = 0.957 \quad F = 128$$

TABLE I

Bacterial Growth Inhibition and Physicochemical Parameters for 4-R-N′-Phenylsulfonamides



Compounds		Growth Inhibition: Alone		Growth Inhibition: with Trimethoprim		Parameters		
No.	R	log $1/I_{50}$[a] (obs)	log $1/I_{50}$[b] (calc)	log $1/I_{50}$[c] (obs)	log $1/I_{50}$[d] (calc)	pK_a^e	$\Delta^{2/6}$ppm[f]	π^g
1	NO_2	5.53	5.66	6.72	6.85	6.97	0.123	−0.28
2	NH_2	4.34	4.20	5.62	5.46	9.83	−0.137	−1.23
3	H	4.60	4.57	6.06	5.81	9.10	0.000	0.00
4	$N(CH_3)_2$	4.27	4.26	5.43	5.51	9.72	−0.100	0.18
5	OC_2H_5	4.30	4.32	5.39	5.57	9.60	−0.088	0.38
6	$COCH_3$	5.71	5.38	6.95	6.58	7.52	0.084	−0.55
7	Cl	4.74	4.85	6.25	6.07	8.56	−0.004	0.71
8	CN	5.66	5.46	6.77	6.66	7.36	0.087	−0.57
9	F	4.33	4.60	5.44	5.84	9.05	−0.039	0.14
10	CF_3	4.96	5.14	6.21	6.36	7.98	0.074	0.88
11	OCH_3	4.45	4.45	5.85	5.70	9.34	−0.082	−0.02
12	C_2H_5	4.57	4.44	5.66	5.69	9.35	−0.014	1.02
13	SO_2CH_3	5.53	5.57	6.61	6.76	7.14	0.105	−1.63
14	$CONHNH_2$	5.08	5.17	6.27	6.38	7.94	0.050	−1.92

[a] I_{50} is moles per liter affording 50% growth inhibition of *E. coli*.
[b] Calculated by equation (1).
[c] I_{50} is moles per liter affording 50% growth inhibition of *E. coli* in the presence of trimethoprim.
[d] Calculated by equation (4).
[e] Values taken from ref 12 or determined in this work.
[f] This work.
[g] Taken from[13].

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TABLE II
Correlation Matrix (r^2) Between Parameters used in the Development of Equations (1)–(6)

	log $1/I_{50}$ (S)[a]	log $1/I_{50}$[b]	$\Delta^{2/6}$ppm	pK_a	π
log $1/I_{50}$ (S)	1.0				
log $1/I_{50}$	0.96	1.0			
$\Delta^{2/6}$ppm	0.78	0.84	1.0		
pK_a	0.85	0.91	0.93	1.0	
π	0.14	0.17	0.05	0.14	1.0

[a] In the presence of trimethoprim
[b] Sulfonamides alone

$$\text{Log } 1/I_{50} = 5.89\ (1.63)\ \Delta^{2/6}\text{ppm} + 4.84\ (0.13) \quad (2)$$

$$n = 14 \quad s = 0.228 \quad r = 0.890 \quad F = 62$$

$$\text{Log } 1/I_{50} = -0.50\ (0.\ 11)\ pK_a - 0.04\ (0.12)\ \pi + 9.10\ (0.95) \quad (3)$$

$$n = 14 \quad s = 0.165 \quad r = 0.958 \quad F = 61$$

In these equations, I_{50} is the molar concentration of sulfonamide affording 50% growth inhibition, $\Delta^{2/6}$ ppm is the n.m.r. shift difference for the 2/6 hydrogens, and π is the octanol-water substituent constant.

The numbers in parentheses are the 95% confidence intervals associated with the respective coefficients, n is the number of molecules included in the regression, s is the standard deviation, r is the correlation coefficient, and F is the value used to test signifiance. Equations (1) and (2) clearly show that most of the variance in biological activity can be attributed to changes in electronic influence of the sulfonamide substituents. Qualitatively similar relationships were obtained with Hammett σ or with Δ^{NH_2}ppm as electronic parameters. The experimentally determined parameters; pK_a, Δ^{NH_2}ppm, or $\Delta^{2/6}$ppm, were in all cases superior to Hammett σ. Of the various equations between electronic parameters and Log $1/I_{50}$, equation (1) with pK_a proved to be the best and is very similar to that developed previously for a larger series of N^1-phenylsulfonamides with minimum inhibitory concentration as a measure of antibacterial activity.[12] Consequently, the enhancement of growth inhibition by electron withdrawing substituents which lower the pKa was not unexpected. Addition of a hydrophobic term, π, to equation 1 offers no improvement in correlation as can be seen in equation (3). It should be noted here that the negative sign associated with pK_a in equation (1) and the positive sign associated with $\Delta^{2/6}$ppm in equation (2) are reflections of the same electronic influence on activity .

Next, the growth inhibition data for the sulfonamides tested in the presence of a constant synergistic concentration of trimethoprim were subjected to regression analysis to give equations (4)–(6).

$$\text{Log } 1/I_{50} = -0.48\ (0.13)\ pK_a + 10.22\ (1.10) \quad (4)$$

$$n = 14 \quad s = 0.216 \quad r = 0.992 \quad F = 68$$

$$\text{Log } 1/I_{50} = 5.60\ (1.86)\ \Delta^{2/6}\text{ppm} + 6.06\ (0.15) \quad (5)$$

$$n = 14 \quad s = 0.261 \quad r = 0.884 \quad F = 43$$

$$\text{Log } 1/I_{50} = -0.48\ (0.15)\ pK_a - 0.02\ (0.17)\ \pi + 10.17\ (1.26) \quad (6)$$

$$n = 14 \quad s = 0.225 \quad r = 0.923 \quad F = 31$$

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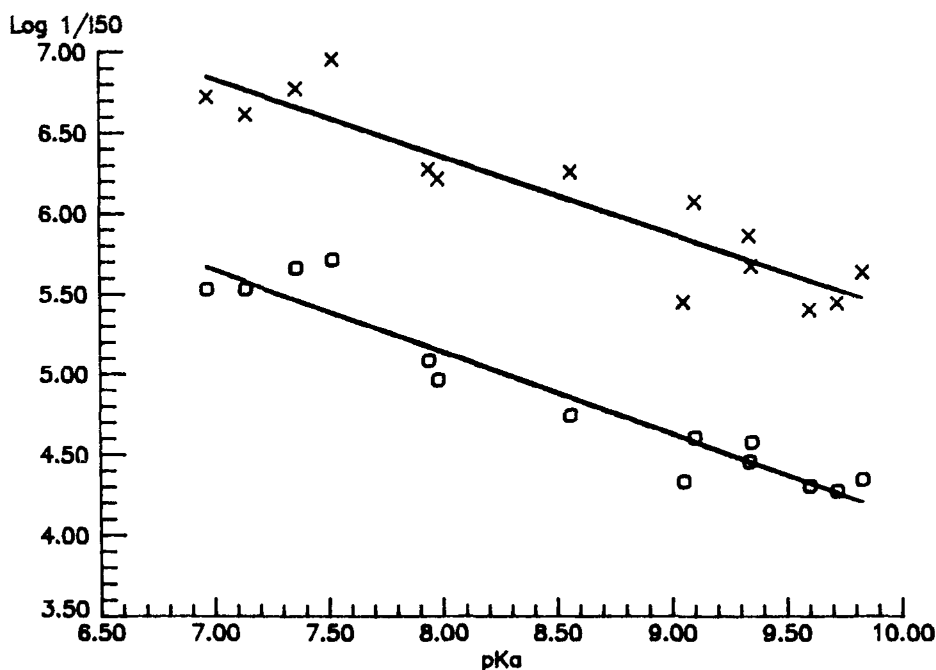

FIGURE 2 *E. coli* Growth Inhibition versus pK_a of 4-R-N[1]-Phenylsulfonamides in the Presence (x) and Absence (□) of Trimethoprim.

As in the relationships correlating sulfonamide bacterial growth inhibition in the absence of trimethoprim, pK_a in equation (4) or $\Delta^{2/6}$ppm in equation (5) account for most of the variance in the biological data. Here as well, equation (4) was not significantly improved by the addition of a hydrophobic parameter as shown by equation (6). Comparison of equations (1)–(3) for single agent treatment with equations (4)–(6) for synergistic action clearly establishes that the same structural features are related to *E. coli* growth inhibition by N[1]-phenylsulfonamides. Furthermore, the degree of influence by these structural features is virtually identical as evidenced by the coefficients for pK_a, -0.51 versus -0.48 in equations (1) and (4) respectively. The only differences between the two sets of equations are in the intercepts which merely reflect the increased potency of the sulfonamides in the presence of trimethoprim. Figure 2, which is a plot of the data used to derive equations (1) and (4), provides a graphic illustration of these results. While it is evident that the presence of trimethoprim does not produce changes in sulfonamide structure-activity relationships as reflected by I_{50} values, there is the slight possibility that the slopes of the dose response relationships may have changed. As a simple check on this possibility, equations (1) and (4) were recomputed using I_{25} values in place of I_{50} values. Once again, the relationships for the sulfonamides in the absence and presence of trimethoprim were identical with the exception of the intercepts.

While it seems clear that trimethoprim does not directly influence the interaction of sulfonamides with dihydropteroate synthetase in *E. coli*, it is feasible that the sulfonamides may affect the interaction of trimethoprim with dihydrofolate reduc-

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tase. Consequently, a small set of four trimethoprim analogs was studied alone and in the presence of a constant concentration of 4-acetyl-N^1-phenylsulfanilamide. The purpose here was not to develop structure-activity relationships for the benzylpyrimidines but rather to provide an assessment of the effect of the presence of a synergistic concentration of sulfonamide. Table III summarizes the data obtained. Equation (7) illustrates that the two sets of I_{50} values are completely parallel with a slope of 1, thus affording additional support for the concept that no direct interaction between sulfonamides and benzylpyrimidines occurs.

$$\text{Log } 1/I_{50} (+\text{sulfonamide}) = 0.96\ (0.26)\ \text{Log } 1/I_{50} + 1.30\ (0.76) \quad (7)$$

$$n = 4 \quad s = 0.08 \quad r = 0.99 \quad F = 177$$

The identical nature of the synergistic and nonsynergistic structure-activity equations for the sulfonamides and the highly significant one to one relationship observed for the four trimethoprim congeners provide no evidence that modes of binding are changed and therefore no indication of direct interaction between sulfonamides and

TABLE III

Observed *E. coli* Growth Inhibition for Trimethoprim Derivatives

NH_2, CH_2-R, N, H_2N, N

R	log $1/I_{50}$[a]	log $1/I_{50}$[b] (with sulfonamide)
OCH_3, SCH_3, OCH_3	7.10	8.36
OCH_3, OCH_3	5.85	7.25
Cl, $NHCH_2CH_3$, Cl	5.57	6.82
OCH_3, $OCH_2CH_2OCH_3$, OCH_3	6.09	7.35

[a] I_{50} in moles/liter
[b] I_{50} in moles/liter in presence of 4-acetyl-N^1-phenylsulfanilamide

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benzylpyrimidines. It must be concluded that sulfonamides and benzylpyrimidines exert their respective inhibitory actions independently. Although the result of combined treatment is clearly synergistic, this effect must arise from the enzyme kinetic consequences of a reduction in the concentration of these agents required to inhibit the target enzymes. The observed synergism must then occur solely as a result of sequential blockade of the folate biosynthetic pathway.

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