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Full Length Research Paper

Quantitative structure activity relationship studies for new antimicrobial N2- substituted phenazines

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The increased availability of iminophenazines with polar substitute in the N^2- side chain (notably B826 with a (3- diethylaminopropyl) imino substituent) in the spleen of mice following oral administration prompted an investigation of a possible correlation between the partition coefficient (log P) values and electronic properties expressed by σ* with the concentration of the iminophenazines in the spleen of mice. The correlation studies between log P values and the concentration of the iminophenazines in the spleen of mice showed a poor correlation coefficient (r = 0.515), while a much better correlation was obtained using the electronic parameter σ*, where the correlation coefficient was 0.911, indicating the significance of the electronic factor in relation to the transport of the compounds to the spleen of mice, despite the high lipophilicity of the iminophenazines.

Key words: QSAR, iminophenazines, partition coefficient, electronic parameter.

INTRODUCTION

Iminophenazines (Figure 1) exhibit antibacterial activity against species of *Mycobacterium* including *M. leprae*, the causative organism in leprosy. Clofazimine (British Pharmacopoeia, 1988) has been used for many years, either alone or in combination with a sulphone, dapsone, in the treatment of leprosy.

The development of clofazimine-resistant strains of *M. leprae* has led to the synthesis and testing of new analogous of clofazimine. (O'Sullivan et al., 1988).The N^2-substituted phenazines used in this study are shown in Table 1. These compounds are incompletely absorbed from the gastro-intestinal tract and accumulate in relatively large amounts in the spleen, liver, lungs and fat from which they are slowly released. The partitioning and distribution behaviors of solutes between the organic and aqueous phases have been widely studied in quantitative structure activity relationships (Lien et al., 1986; Dearden and Bresnen, 2005). Various solvents pairs have been studied (Beezer et al., 1980; Beezer et al., 1983; Rogers and Davis, 1980; Anderson et al., 1983; Kojima and Davis, 1984; Betageri and Rogers, 1987). The n-octanol/water system is frequently employed as a model system (Hansch and Leo, 1979; Kellogg and Abraham, 2000).

Quantitative structure relationships for many drugs using log P and other steric and electronic parameters have been extensively studied (Johnson et al., 1998; Hansch and Leo, 1995; Warhurst et al., 1998; Thakur et al., 2004).

Quantitative structure/protein binding relationships indicate that lipophilicity is the principle physicochemical determinant correlating protein binding to chemical structure (Austel and Kutter, 1983). In addition to lipophi-licity the polar character of substituents, as expressed by Hammett σ* values was also found to correlate with the observed protein binding (Martin, (1979).

In a previous study the thermodynamics of the distribution and interfacial transfer of antimicrobial iminophenazines in two phases system of n-octanol/ aqueous buffer has been reported (Fahelelbom et al., 1989). In the present work correlations study between the Partition coefficient and polar electronic constant σ* with the concentration of the iminophenazines in the spleen of mice have been investigated in order to get a suitable quantitative structure relationship for these highly lipophilic

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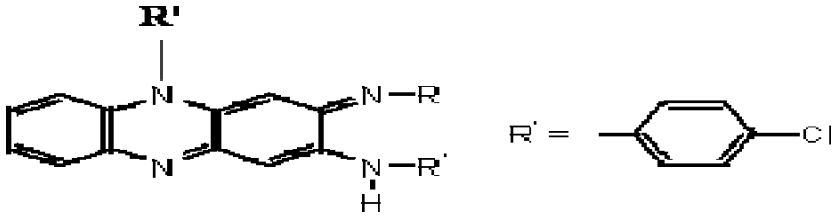

Figure 1. Chemical structure of Iminophenazines.

Table 1. The antimicrobial activity and accumulation of the iminophenazines in the spleen and fatty tissue.

Compound	R	Growth inhibition of *M. smegmate* 607(μg/ml)		Concentration (μg/ml)
		Sens. to clofazimine	Res. to clofazimine	Spleen
B663	$CH\ Me_2$	1.3	30.0	16.5
B826	$(CH_2)_3NEt_2$	0.2	1.0	257.0
B3779	$CH(Me)(CH_2)_3NEt_2$	0.2	2.0	146.0
B3770	$(CH_2)_3N(CH_2)_4CH_2$	0.2	2.0	250.0
B3785	$(CH_2)_3N(CH_2)_3\ CH_2$	0.3	0.8	103.0
B749	$CH_2CH_2N(C_2H_5)_2$	0.6	12.0	262.0
B3955	$CH_2(CH_2)_4NHCH_2\ CH_2$	0.4	6.0	110.0

B663* = Clofazimine.

compounds.

MATERIALS AND METHODS

Chemicals

The seven iminophenazines used in this study were synthesized in the laboratories of the Medical Research Council of Ireland, Trinity College Dublin. The solvents n-octanol, methanol, glacial acetic acid and dichloromethane were all BDH- Analar grade. The Sorenson phosphate buffer was prepared using KH_2PO_4 and Na_2HPO_4 (both from BDH- Analar grade) . Tris Buffer (BDH- Analar grade) was used in pKa determinations.

Instrumentation and procedures

Determination of ionization constants: The pKa values for seven iminophenazines were determined using the spectrophotometric method described by Albert and Serjeant, 1984) . The absorbance of the iminophenazines solutions were recorded at 482 nm, using an SP8-100 spectrophotometer (Pye-Unicam). A pH-meter (Radiometer- Copenhagen) was used for the determination of the pH values at 20°C.

Determination of partition coefficient: The partition coefficients of the iminophenazines was studied using a phosphate buffer pH 5.15 and n-octanol system in a thermostatically controlled shaker water bath; a shaking time of 6 h was sufficient to produce equilibration in each case, and the absorbance of the organic layer was determined spectrophotometrically as described above. The following equation was used for the partition coefficient calculations:

$$P = P' (1-\alpha)^{-1}$$

where α is the ionization degree, and is given by:

$$\alpha = (1 + 10^{\ pH - pKa})$$

Mouse infection and treatment: This part of the work was conducted in the National Medical Research Center (Tripoli - Libya). 33 female Swiss mice (8 - 12 weeks old) weighing 18 - 20 g, were used. Before infection with *M. leprae*, the mice were housed for two weeks. Each mouse was infected in both hind footpads with a 6 × 103 CFU in a volume of 0.03 ml (Shepard, 1967). One day after infection, five mice were killed to establish baseline values of AFB and CFU counts (Degas et al., 2000), the remaining 28 were divided into two groups; a control group of 7 mice, and the other groups of 21 mice were fed orally with the phenazines derivatives.

The oral administered phenazines were prepared by dissolving 50 mg of each compound in 48 ml of mineral oil and 2 ml of water, producing a drug concentration of 1 mg/ml of the oil and water emulsion. An aliquot of 0.5 ml of the final solution (≅ 25mg/kg of body weight) was fed to the infected mice every 3 to 4 days over two weeks with an appropriate gavage needle. Control preparation of free phenazines derivatives were given also on the same schedule to each mouse every day with the appropriate gavage needle. The animals were killed 24 h later after the last treatment.
The lung, liver and spleen of the treated mice were homogenized (one part tissue and nine parts methanol/glacial acetic acid 9:1).

Determination of iminophenazines concentration: The concentration of each iminophenazines was extracted from the spleen and fat tissues of the mice using a mixture of dichloromethane and sodium hydroxide, quantified using a reversed phase C18 column and determined using the modified previous HPLC method (O'Connor et al., 1996). The HPLC analysis was carried using the Jasco – Borwin 310 model (Japan) and absorbance measurements were obtained using a multi channel UV- Visible detector at λ_{max} 384 nm.

RESULTS AND DISCUSSION

The linear regression analysis of the partition coefficients

Table 2. The ionization constant (pKa), degree of ionization (1-α) and the partition coefficients (log P) of the iminophenazines

Compound	pKa	log(1-α)	log P
B663	8.511	3.361	4.396
B826	8.813	3.663	4.496
B3779	8.662	3.512	4.533
B3770	8.800	3.650	4.782
B3785	8.592	3.446	4.380
B749	8.085	2.936	4.307
B3955	8.480	3.330	3.795

Table 3. The value of lipophilic parameter (π) obtained by comparing log P values of substituted derivative with that of parent compound (R= H)

Compound	π
B663	1.096
B826	1.196
B3779	1.233
B3770	1.482
B3785	1.080
B749	1.007
B3955	0.496

in the n-octanol/buffer system of seven iminophenazines with the concentration found in the spleen of mice following oral administration gave the following equations:

$\log (^1/_C) = 1.165 \log P + 3.015$ ---------- equation 1
t = 1.34 (P = 0.237) n = 7
r = 0.515 s = 0.398; F = 1.81; significance 76.3%

The correlation is obviously not good, and similar results were obtained using the parabolic equation:

$\log (^1/_C) = 23.630 \log P - 2.83 (\log P)^2 - 51.344$ ---------- equation 2
t = 0.52 (P = 0.237) t = 0.54 (P = 0.237)
n = 7; r = 0.562 s = 0.430

These results indicate that lipophilicity of the new potent analogous is not by itself a significant physicochemical parameter in determining the transport of these compounds to the spleen.The high log P values of clofazimine and derivatives with polar N^2- substituents using n-octanol/buffer system and the lipophilic parameter (π) (Tables 2 and 3) may reflect an extensive binding to lipoproteins, as the mechanism of absorption of the phenazines has not been established. Electrophortic studies of the blood serum of orally treated mice with clofazimine have shown the binding of clofazimine to lipoproteins of the $\alpha-$ and $\beta-$ globulin fraction. These lipoprotein-clofazimine complexes are taken up by the macrophages and concentrated in the reticulo-endothlial system. Following fusion with liposomes, it appears that the lipoprotein carrier is enzymatically split off from the complex, with the resultant as the crystallization of clofazimine (Barry et al., 1960; Conalty et al., 1971). It has also been advanced that clofazimine inhibits the multiplication of organisms by binding to the guanine bases of DNA (Morrison and Marley, 1976).

However, correlations of protein binding with the polar properties of the substituents of certain drug molecules have been reported, and this showed that the extent of plasma protein binding is significantly dependent on the frontier electron density on the hetero-atom at the terminal portion of the alkyl side chains. The greater the frontier electron density, the greater was the degree of the drug binding to plasma protein (Austel and Kutter, 1983). Several studies have been conducted on the anti-microbial and the structure activity relationships of clofa-zimine analogues (Kumar, 2008; Huygens et al., 2005; Oliva et al., 2004; Scollard et al., 2006; Carta et al., 2008).

The relatively high concentration of phenazines with polar substituents at the N^2- position recovered from the spleen of mice may be due to the contribution of the polar groups to protein binding. This finding prompted the calculations of the polar substituent constants for the alkyl amino and cycloalkyl amino groups at the N^2- posi-tion.

It was recommended that the use of $\sigma*$ might give a better discrimination between the various substituents than $\sigma 1$. The $\sigma*$ was proposed by Taft, (1956) for evaluating the polar effects of substitutes R in the ester RCO2 R'.

$\sigma* = [\log (K/K_0) B - \log (K/K_0) A] 2.48$ --------- equation 3

where $\sigma*$ is the polar substitute constants for R, the rate constants (K) refer to the reactions of RCO2 R' and K_0 to those of CH3-CO2 R' standard. B and A refer to basic and acidic hydrolyses carried out with same R' solvent and temperature. The factor 2.48 puts the $\sigma*$ values into about the numerical range as Hammett's σ which has been used extensively in QSAR.

The linear regression analysis using σ values for the polar N^2- substituents of six compounds and the concentration extracted from the spleen of mice following oral administration gave the following equations:

$\text{Log} (^1/_C) = - 3.740 \sigma* - 2.094$ ---------- equation 4
t = 3.88 (P = 0.018); n = 6
r = 0.889 s = 0.238; significance 98.2%

A combination of log P (n-octanol/buffer) and $\sigma*$ values gave the following regression equation:

$\text{Log} (^1/_C) = 0.706 \log P - 3.217 \sigma* + 1.032$ ------equation 5
t = -0.84 (P = 0.465); t = -2.72 (P = 0.073)
significance = 53.3%; significance = 92.7%
n = 6; r = 0.911 s = 0.248; F = 7.286 (sig. = 92.7%) A

Table 4. The σ* of the polar N^2- substituted iminophenazines.

Compound	σ*
B663	-0.190
B826	0.040
B3779	0.055
B3770	0.042
B3785	0.045
B749	0.125

linear regression analysis using the lipophilic parameter for the six phenazines gave a poorer correlation (r = 0.640). It is clear from the results that the electronic properties of the N^2-substituents, as expressed by their σ* values are of great significance in relation to the transport of these compounds to the spleen of the mice than the lipophilicity of the molecules. The negative value of the coefficient of the σ* term (Table 4) indicates the enhancement of the electron- donating substituents. These recent results indicate that the absorption process is affected by not only lipophilicity, and the absorption may be dependent on protein binding of these lipophilic molecules.

Conclusion

The recent results suggest that despite the high log P values, transport to the spleen where the compounds accumulate is not, apparently, a simple absorption process but is more likely to be dependent to a significant extent on protein binding, as these iminophenazines with polar substituents have a pKa values within a narrow range (8.66 - 8.80). This suggests their capabilities, therefore, of binding to proteins by electrostatic interacttions.

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