Correlation of Physicochemical Parameters with Antisickling Activity of Substituted Benzoic Acids

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ABSTRACT

Quantitative relationships between the antisickling activity and experimentally determined physicochemical properties of substituted benzoic acids have been investigated by means of the Hansch approach.

Results showed that increased lipophilicity of the compounds enhances the sickle-cell reversal activity and that electron donating substituents seem to play a very important role in sickle-cell reversal activity.

INTRODUCTION

The aqueous extract of Fagara zanthoxyloides roots, from which p-hydroxybenzoic acid and related acids have been isolated and characterised, has been shown to possess the ability to reverse sickled human blood cells with the S-Haemoglobin (Hbs), and crenated normal human erythrocytes (1,2). A large range of compounds, some of which include urea (3), steroid hormones (4,5), procaine hydrochloride (6), amino acids (7), 3,4-dihydro-2, 2-dimethyl-2H-l-benzopyran-6-butyric acid (DBA) (8) pyridoxal, and (+)-glyceraldehyde (9) have also been reported to possess anti-sickling properties. However, no attempt has been made at any quantitative structure-activity relationship (QSAR) studies on any of these groups of compounds. It is particularly of interest to investigate the physico-chemical similarities between these varied group of compounds that adapt them for antisickling activity.

We have therefore, decided to employ the Hansch-type of QSAR model, which is based on the assumption that the physico-chemical parameters governing the transport, and drug-receptor interaction can be factored into lipophilic, electronic, and steric properties of the drug molecule. This model has been used to correlate a wide range of chemical compounds with their biological action with very good results (10, 11, 12). The model is also very flexible in that the basic equation can be expanded to accommodate additional physicochemical terms such as the Infrared (IR) and Ultraviolet (UV) data, chromatographic factor (Rm), and molecular orbital calculations, to improve the correlation, thereby shedding light on the relative importance of each factor in the particular drug action in question, and possibly the mode of action.

In this report, using p-hydroxybenzoic acid (PHBA) as the model molecule for the synthesis of various derivatives, we have assayed some mono- and di-substituted benzoic acid derivatives for in-vitro anti-sickling activity. Attempts have been made at correlating their biological activity with experimentally determined physicochemical parameters, using the Hansch approach with a view to determining the optimum structural modifications for antisickling activity.

EXPERIMENTAL

(a) Biological Evaluation:

The identity of all commercially available samples and synthesized compounds (Table I) was confirmed spectroscopically and chromatographically prior to biological testing.

Biological assay was carried out using intact red blood cell by a modified procedure of Sofowora et al. (2) and that of Mackenzie and co-workers (13). Blood samples were collected from voluntary patients homozygous with sickle hemoglobin (HbSS) and kept in citrate -dextrose medium at $5^{o}C$ for not more than 2 days. After removal of plasma, the cells were washed with Tissue Culture Medium RPM 1640 (pH 7.4) and resuspended in five times its original volume of the medium. 10 ml of the cell suspension was pipetted into each of 20 ml capped blood bottles. After the experimental conditions with respect to the optimum exposure time to N_2, optimum incubation time at 37^{o} for sickle cell inhibitory and reversal activities and optimum dose level had been determined and standardised, sickling was experimentally induced by passing N_2 through the cell suspension for 15 min. and incubated for 6 hours at 37^{o} A fixed dose (0.044 mM) of each compound (Table I) was tested for both inhibitory and sickle-cell reversal activities as described below. The percentage of cells reversed was taken as a measure of activity, and the activity of the compounds relative to PHBA was calculated. PHBA was run with every batch of compounds tested.

Inhibition of sickling: Cell suspension in medium (10 ml) was incubated with drug (0 0145mM) for 6 hours at the end of which N_2 gas was bubbled through the suspension for 15 min. Samples were withdrawn immediately for pO_2 determination and fixed in 3% glutaraldehyde. The control was without the drug. The percentage of cells sickled was determined by counting at least 400 cells from each sample by means of light microscope. Since PHBA has no inhitory activity the biological response was calculated by subtracting the percentage of cells sickled in the test from the percentage sickled in the control. This is equivalent to the percentage of the cells prevented from sickling by the test compounds.

Reversal of sickling: Cells were first sickled and 0.1 ml of solution of drug (1.45mM) was added to the test making a final concentration of 0.0145mM solution, sealed with 3 ml of liquid paraffin, and immediately sampled for pO_2 determination and fixation. The controls were similarly treated, omitting the drug The cell suspensions were incubated at 37°C for 6 hours and fixed in glutaraldehyde. The percentage of cells sickled was determined. The biological response (BR) was calculated relative to PHBA which was set at an activity level of 100%, that is log BR for PHBA is 2.0.

(b) Physicochemical properties:

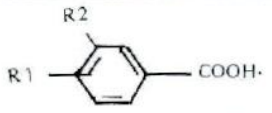
(i) Rm values determination: The chromatographic technique for the determination of the Rm values as an expression of the lipophilic character of molecules has previously been described (14). The Rm values of compounds listed in Table I were determined by a reversed phase thin-layer chromatographic technique, using a modified procedure of Boyce and Milborrow (15). The polar mobile phase was represented by acetone: water (70:30) system, while the stationary non-polar phase consisted of a silica gel G (Merck) layer impregnated with 5% v/v liquid paraffin solution in n-hexane. The solutions of the test compounds (0.5 mg /ml in EtOH) were applied at 2 cm intervals along the edge of the plates at randomized allocations and the developed plates were dried and the spots visualised by placing them in iodine tank. The experimental Rm values were calculated by means of equation (2).

$$Rm = (\log 1/Rf - 1) \text{------} (2)$$

(ii) Partition coefficient determination: The partition coefficients of compounds in Table 1 were determined in n-octanol-phosphate buffer (pH 7.4) system, using a modified procedure of Hansch and co-workers (16) and Rogers (17). To purified n-octanol (2ml) saturated with phosphate buffer and containing 2 mg of dissolved compound was added 10 ml of buffer saturated with n-octanol. The two phases were mixed vogorously by shaking for 20 min. and thereafter shaken at room temperature by a mechanical shaker for 12 hours. The phases were separated by centrifugation at-25° for 2 hours at 2,000 r.p.m. and the concentration of drug in the aqueous layer was determined by direct UV spectroscopic method, using appropriate calibration curves for each compound. The partition coefficient (P), and hence log P, was calculated from the relationship.

TABLE I

Physicochemical parameters and biological activity of substituted benzoic acids



Compound R1	R2	log P	Rm	$\Delta\lambda$ max	σ	log BRR	Log BR 1
CH	H	1.543 +0.015	−0.9649	19.5	−0.37	2.000	–
OCH3	H	1.920 +0.020	−0.5638	22.5	−0 27	2.690 +0.033	–
NH2	H	0.630 +0.110	−0.630	40.5	−0.66	1.115 +0.582	–
OH	OH	1.150 +0.49	−0.7368	25.5	−0.25	1.230 +0.219	0.360 +0.350
OH	OCH3	1.690 +0.011	−0.6529	26.5	−0.25	1.600 +0.063	0.544 +0.0135
OCH3	OCH3	1.970 +0.014	−0.5636	27.5	−0.15	–	–
OH	OEt	2.280 +0.023	−0.6057	26.5	−0.27	1.320 +0.049	0.638 +0.106
OH	NO2	1.526 +0.055	−0.8192	11.5	0.34	1.090 +0.890	0.406 +0.212
Me	Me	2.813 +0.015	−0.5062	11.5	−0.25	2.280 +0.058	1.670 +0.039

BRR = Biol. Response (Reversal Activity)
BRI = Biol. Response (Inhibitory Activity)

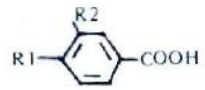
$$P = \frac{Co}{(1 - \alpha)Cw}$$

where Co is the concentration in octanol, Cw the concentration in the aqueous layer and α - the degree of ionisation.

(iii) UV spectroscopic data: The UV spectra of the compounds (Table I) were run in phosphate buffer (pH 7.4), using benzoic acid as the parent compound. The change in λmax ($\Delta\lambda$ max) was measured with respect to the B-band (18, 19).

TABLE II

Paramerters for Regression Analysis

Compound 							
R1	R2	log P	$\overline{\Pi}$*	σ*	Rm	$\Delta\lambda$max	log BR
(a) OH	H	1.54	−0.67	−0.37	−0.9649	19.5	2.00
(b) CH3	H	1.92	0.02	−0.27	−0.5638	22.5	2.69
(c) OH	OH	1.20	−1.25	−0.25	−0.7368	22.5	1.23
(d) OH	OCH3	1.69*	−0.69	−0.25	−0.6529	26.5	1.60
(e) CH3	CH3	2.81	−0.99	−0.25	−0.5062	11.5	2.28

*Obtained from compilation by Hansch and co-workers (32).

RESULTS AND DISCUSSION

The chromatographic Rm value, which is related to the logarithm of the partition coefficient of the molecule between the polar and non-polar phases of a reversed TLC system, has been used to estimate the lipophilic character of compounds (20). Furthermore, a linear relationship between the response to a drug action and the relative lipophilic character of the drug has also been established (21). The Rm value is free-energy related, and the change in the Rm value due to a substituent (ΔRm) is an extra-thermodynamic constant identical with the Hansch hydrophobicity constant $\overline{\Pi}$ (15). Since the value of $\overline{\Pi}$, and hence Rm value, for a particular substituent depends on the type of electronic interaction with the nucleus (22). it is expected that the Δ Rm values will also bear some relationship to the Hammett substituent constant The usefulness of the Rm values in studying the relationship between chemical structure and biological activity has been shown with the bis (dichloroacertamides) and vitamin K analogs (23), penicillins and cephalosporins (24) testosterone esters (25), sulphonamides (26) and steroids (27). The correlation of Rm values with biological response is discussed later.

The octanol/water partition coefficient substituent constant, $\overline{\Pi}$, suggested by Hansch (28) has been found to be rather spectacularly successful in correlation of a wide spectrum of biological activities. The partition coefficient of each of the acids studied was then determined in octanol/phosphate buffer system and the log P values calculated.

The U.V. absorption on the other hand depends on the electronic disposition of a molecule; therefore, a study of the displacements of the U.V. absorption maximum (λ max) as a result of introducing additional substitutents to a pharmacologically active molecule, could give an insight into how the modified structure might behave with respect to the parent compound. Since the interaction of a drug with the receptor site depends on its steric and electronic disposition, the change in the λmax for a substituted benzoic acid can be assumed to be a measure of the electron attracting or contributing (λ max) power of the substituent, and analogous to σ values, can be related to biological activity.

Table I gives a summary of the physicochemical and biological data. Only five of the compounds tested showed significant sickling reversal activity, while only one compound, 3,4-dimethylbenzoic acid, showed significant sickling inhibitory activity.

Using the former five compounds, the quantitative relationship between the sickling reversal activity and physicochemical properties was investigated, employing the method of linear regression analysis (16, 29). Table II shows the parameters used for the regression analysis, giving rise to the following equations:

	n	s	r			
log BR = 2.291 log P^2 − 5.87 log P + 1.18 σ	5	0.570	0.451	-	-	(3)
log BR = 0.597 log P + 0.865	5	0.570	0.635	-	-	(4)
log BR = 0.813 log P - 1.454 σ + 0.37	5	0.570	0.564	-	-	(5)
log BR = 0.490 TT + 2.120	5	0.570	0.736	-	-	(6)
log BR = 1.248 Rm + 2.815	5	0.570	0.393	-	-	(7)
log BR = -1.783 σ + 1.464	5	0.570	-0.163	-	-	(8)
log BR = -0.040 $\Delta\lambda$ + 2.780	5	0.570	-0.570	-	-	(9)
log P = 0.201 Rm + 1.053	5	0.179	0.679	-	-	(10)

Comparision of equations (3) and (5) shows an improvement in the correlation of the data (r = 0.564) when the term of log P^2 is omitted. This term is introduced into the equation to describe the parabolic dependence of biological activity to the partition coefficient of the drug molecule, which is more relevant in an in-vivo study. The fact that the term is not significant in this correlation is to be expected since the compounds tested were completely soluble in the medium at the dose level (0.0044 mM) employed, and being an in-vitro experiment, the drugs were introduced directly to the immediate vicinity of cells where theywere expected to elicit their actions. The poor correlation, however, implies that the sickled-cell reversal activity cannot be clearly separated into hydrophobic and electronic factors in the group of compounds investigated in the light of the present information.

The partition coefficient (log P) and the hydrophobicity constant (Π) correlated relatively well with the biological response (r = 0.635, and 0.736 respectively). However, correlation with the chromatographic factor (Rm) was relatively unsatisfactory (r = 0.393) to be used directly for correlation in place of octanol/water partition coefficient, or the hydrophobicity constant. On the other hand, the electronic factor denoted by the Hammett sigma constant, σ, or the UV data ($\Delta\lambda$ max), do not appear to play any significant role in eliciting the biological response for the set of compounds employed in this study as shown by the poor correlation coefficients (r = -0.163 and -0.397 respectively). This result is contrary to expectation since electronic factors usually play essential roles in any drug-receptor interaction, especially as it is believed that specific receptor sites are involved It is however recognised that the σ values of the compounds used in the analysis are not sufficiently varied, and this might have contributed to this parameter not making any contribution to the biological response of the compounds. The correlation between log P and Rm values (eq. 10) shows that they are linearly related.

Although the correlation of the sickle-cell reversal activity of the substituted benzoic acid derivatives with the hydrophobic and electronic parameters did not yield statistically significant results, the slopes of the regression equation 4-10 are significant from the point of view of designing more active derivatives of this series. When parameters representing the lipophilic character of the molecules (log P, Rm, and Π) are employed, the slopes of the curves are positive. This indicates that the biological response (sickle-cell reversal activity) in these compounds could be enhanced by increasing the lipophilicity of the substituted derivatives. Similarly, the negative slope of the electronic parameters is an indication that substituents having negative sigma values would enhance the drug activity; and these are usually electron donating substituents. However, this does not clarify the substituent position which would be crucial if specific receptor sites are involved in sickle-cell reversal activity.

The results of the study suggest that increased lipophilicity (or hydrophobicity) of the molecule enhances sickle-cell reversal activity, which at the molecular level is in agreement with the postulate that hydrophobic binding was important in the reversible gelling of Hbs (30). In addition, electron donating substituents seem to play very important role in the sickle-cell reversal activity. It is hoped that the results of this on-going investigation will throw more light on the specificity, or otherwise, of the sickle-cell reversal activity, or perhaps it is a kind of non-specific binding in which any innocuous agent that can be bound to the region involved in sickling should alter the binding site significantly enough to prevent or reverse sickling. (31).

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