Brief Articles

QSAR Based on Biological Microcalorimetry

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In this paper we describe a QSAR based on biological microcalorimetry for a set of antimicrobial hydrazides acting against *Saccharomyces cerivisiae* and *Escherichia coli*. Results show that an extrathermodynamic relationship exists based upon partitioning (log P_{TA}) and microcalorimetrically measured biopotencies using the same cell systems. Moreover, the extrathermodynamic relationship between drug potencies for these two cell systems shows that both cellular systems appear to behave in the same way with respect to the importance of partitioning. This means that the same set of congeneric compounds experience a similar environment in the two systems. This represents a lateral validation of the method and discloses the validity of the QSAR model.

Introduction

Quantitative structure−activity relationship (QSAR) studies rely upon the correctness of *quantitative* measurement of drug potencies, generally via in vitro screening.[1] Many different kinds of biological data can be used in QSARs.[2] To derive biological data establishing group additivity contributions, we have developed a method of screening drugs using *biological microcalorimetry*[3−8] to derive quantitative biological potency values. In this method the live biological cell systems (the metabolic energies) are recorded upon interaction with bioactive molecules.

The classical way of dealing with parameters for drug interaction with cellular systems is to derive a lipophilicity measurement (partition coefficient, *P*). Thus, we have also developed a technique, using the Taylor−Aris diffusion process, to measure the partition coefficient (P_{TA}), in the same cells used for the microcalorimetric biological screening.[9]

In this paper we show a QSAR that is developed between hydrazide[10−17] potencies against *Escherichia coli* and *Saccharomyces cerivisiae* and log P_{TA}. It shows also that an extrathermodynamic relationship can be established between these two different cell systems.

Experimental Section

Hydrazides syntheses are described elsewhere.[19]

Cells Preparation and Storage.[18,19] Chemicals: Oxoid supplied all complex media; all other chemicals were of analytical grade.

***S. cerevisiae* and *E. coli*:** Aliquots of *S. cerevisiae*, NCYC 239, and *E. coli*, NCTC 10418, were grown, harvested, resuspended, and stored in liquid nitrogen as described previously.[6−8,19]

Microcalorimeter. A flow microcalorimeter (LKB type 10700-1; LKB Produkter AB, S-161 25 Bromma 1, Sweden) fitted with a flow-through calorimetric vessel (0.5-mL working volume) was used throughout this study. The thermostatic airbath was maintained at 37 °C in a room kept at 25 ± 0.2 °C. The voltage was amplified with a Keithley 150B microvoltammeter (nominal setting of 3 μv equivalent to 43.2 μv full scale deflection) The power-time curves were recorded on a potentiometric recorder, and the procedure was as described previously[4,19] and is briefly outlined below.

Calorimetric Medium. The glucose buffer solution used for the calorimetric medium was phosphate buffer, pH 7.0, containing (g dm^{-3}): D-glucose, 1.80; K_2HPO_4, 3.68; KH_2PO_4, 1.32; made to volume with distilled water.

Calorimetric Method. 45 mL of glucose buffer was added to 3 mL of DMSO in a three-necked vessel, thermostated and stirred at 37 °C in a bath external to the microcalorimeter. This volume of DMSO was necessitated by the working solubility limits of the higher homologues.

A pump rate of 0.78 mL min^{-1} was used (calculated from an independent volume of reaction medium expelled over 5 min). The ampule of cells was removed from a liquid nitrogen cryostat, thawed in a water bath at 37 °C for 3 min and shaken for 1 min. Five minutes after commencement of thawing, 0.5 mL of the cell suspension was pipetted into the reaction vessel. One minute later 1.5 mL of DMSO (carrying the appropriate amount of study compound to give the final concentration as required in the reaction vessel or control blank) was added to the mixture, with continuous stirring to promote homogeneity of the reaction mixture. Thus, the total volume of the incubation medium was 50 mL.

This medium flowed through the calorimeter and the heat change associated with the metabolism of glucose, under the particular conditions of each assay, was registered. The flow microcalorimeter was washed out after each incubation with water followed by thermostated glucose buffer prior to the establishment of each reaction loop. The calorimeter was left overnight in 10% RBS solution − a commercial surfactant.

Partitioning Method for Obtaining log P_{TA}.[9,19] The dispersion tube was a 7.5-m length of stainless steel capillary tubing with an internal radius, specified by the manufacturer,

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Table 1. Statistical[a] Parameters for the Interaction of Hydrazides ($PhCONHNHCOC_6H_4$-X-*p*) against *E. coli* and Their Microcalorimetric Potencies

X substituent	dose (μmol·L^{-1})	% BR	log D_{50}	slope	intercept	r	s	F	r^2_{cv}
p-Br	2.619	46	2.730	0.395	−0.579	0.999	0.005	2970	0.997
	2.318	33							
	2.145	27							
	2.017	22							
	1.716	10							
p-NO_2	2.619	60	2.38	0.479	−0.640	0.999	0.008	1657	0.995
	2.318	46							
	2.145	37							
	2.017	31							
	1.716	16							
H	2.619	67	2.280	0.520	−0.687	1.000	0.005	5703	0.997
	2.318	52							
	2.145	43							
	2.017	36.5							
	1.716	20							
p-Me	2.619	54	2.540	0.462	−0.673	1.000	0.005	3781	0.998
	2.318	39							
	2.145	32							
	2.017	26							
	1.716	12							
p-OMe	2.619	51	2.600	0.419	−0.587	1.000	0.003	11760	0.999
	2.318	38							
	2.145	31							
	2.017	26							
	1.716	13							
p-*t*-Bu	2.619	34	3.214	0.276	−0.387	0.992	0.005	1680	0.992
	2.318	25							
	2.145	20							
	2.017	17							
	1.716	09							
p-C_5H_{11}	2.619	37	3.050	0.299	−0.413	1.000	0.001	50955	1
	2.318	28							
	2.145	23							
	2.017	19							
	1.716	10							
p-C_6H_5	2.619	32	3.240	0.292	−0.445	0.999	0.005	1302	0.994
	2.318	24							
	2.145	18							
	2.017	14							
	1.716	06							

[a] r = regression coefficient, s = standard error of estimation, F = F-value, r^2_{cv} = squared correlation coefficient of predictions by the "leave-many-out" procedure.

as 0.0381 cm. The tube was coiled in a 15-cm diameter helix and placed in a temperature controlled (±0.1 K) water bath. A peristaltic pump (Gilson-Minipuls 4) was used to maintain a steady flow of carrier solution in the tube. Solutes were introduced at the beginning of the tubing via a Rheodyne HPLC injector fitted with a 10-μL loop and were detected with a diode-array detector (Hewlett-Packard HP8455). Output was displayed on a computer and data manipulated with the program Origin^R (Microcal, Amherst, MA). The temperature of the water bath was kept at 298.15 ± 0.1 K.

Care was always taken to allow a proper equilibration time prior to solute injection. The carrier solution was pumped through the tubing for at least 10 min, or until a stable baseline was obtained, before the first injection was made. Measurements of retention time and peak width at half-height of the dispersion curve were made via the computer. At least two replicates were made for each solute. Standard deviations (calculated from 6 replicates of some experiments) ranged from 1.5% to 2.0%.

S. cerevisiae and *E. coli* were pipetted from an ampule (recovered from liquid nitrogen storage) into 25 mL of 1/4 Ringer's solution, the suspension swirled to homogenize and then 15 mL was removed and diluted with a further 24 mL of 1/4 Ringer's solution. This suspension was pumped through the Taylor−Aris apparatus and 10-μL samples of hydrazides were injected into the flow. The reservoir of cells was stirred using a magnetic stirrer to prevent sedimentation.

Results and Discussion

Biological microcalorimetry can, in principle, deal with small differences in potencies of a related set of compounds. The requirement is that for a set of congeneric molecules, which incorporate a small substructural modification with the same pharmacophoric group, it must be capable of disclosing any SAR. Next, one must deal with the conventional log(dose−response) curves that relate to a well-defined behavior within the set. This means that for similar compounds the mode of action must be demonstrated to be essentially the same as in this study. Table 1 shows the measured potencies and the related statistical parameters for *E. coli*.

Table 2 displays the Taylor−Aris partitioning parameters.[9] Equation 1 states that there is a linear relationship between log $1/D_{50}$ and log P_{TA} for *E. coli*. A negative slope for SARs involving drugs acting against *E. coli* has been demonstrated previously.[20] It seems that increasing hydrophobicity is detrimental to the antibacterial action of the study hydrazides. Hansch and Leo[20] have pointed out that this fact could be due to the compounds entering the cells not via passive diffusion but more likely through a polar channel, where polar porins might play an important role. Eq 1: Linear dependence of log $1/D_{50}$, for *E. coli*, versus log P_{TA}:

Table 2. Experimental Taylor−Aris Data for *E. coli*[a]

	X = H	X = Me	X = Me_3C	X = C_5H_{11}	X = C_6H_5	X = NO_2	X = MeO	X = Br
D_w	9.940	12.240	17.500	21.150	16.980	10.879	9.860	13.470
D_{obs}	9.200	11.020	15.100	17.300	14.600	10.150	8.980	11.900
f	0.074	0.099	0.137	0.182	0.140	0.067	0.089	0.116
P	0.080	0.111	0.159	0.222	0.163	0.072	0.979	0.132
log P_{TA}	−1.094	−0.956	−0.799	−0.652	−0.787	−1.140	−1.001	−0.879

[a] Adapted from ref 9: D_w = normal diffusion coefficient in water, D_{obs} = observed diffusion coefficient for the organic solute, f = fraction of organic molecules present in the phase.

$$\log 1/D_{50}(E.c.) = -1.986(\pm 0.55) \log P_{TA}(E.c.) + 1.419(\pm 0.45)$$

$$n = 8;\ r^2 = 0.765;\ s = 0.198;\ F = 19.50;\ r^2_{cv} = 0.496$$

Equation 1 is a linear activity−lipophilicity relationship of the type $\log 1/D_{50} = a \log P_{TA} + b$, where $\log 1/D_{50}$ is the potency taken from Table 1 and $\log P_{TA}$ is the dependence on log P, from the Taylor−Aris method. The logarithm of the experimentally determined partition coefficient for transfer of the drugs from a cell culture medium is obtained directly for the same cellular system used for screening and not from models.[9] The statistical parameters are: n = number of compounds tested, r = correlation coefficient, s = standard deviation, F = Fischer test parameter, r^2_{cv} = cross-validation term for the "leave-many-out" procedure. They were calculated via the TSAR software package.[21]

From the regression parameters displayed in Table 1, $\log 1/D_{50}$ can be calculated when the response is 50%, i.e., when cell metabolism is diminished by 50% (cell killing). The potencies so calculated allow a normalized and much better scaling contribution term to elicit biological responses for a set of related compounds. In terms of quantitative measurements, the contribution to biological activity of the various constituent groups of the active drug molecules can be accurately calculated.

The negative slope in eq 1 is almost 2; i.e., it seems that transport from the outer to the inner cell system is driven not through a passive diffusion process but by an active transport process via a system with hydrophilic properties that takes the drug across the lipid membrane. On the other hand a receptor could be located near the cell surface so that a drug might gain access to the cytoplasm via a polar channel (as above). In addition, the results shown here may also indicate that the negative slope of eq 1 can either imply that the drug molecules are completely hydrated or are located deep inside a hydrophilic cavity or groove of a biomacromolecule.

Partitioning processes are usually studied through solvent models, e.g., octanol/water, or through more structured systems such as micelles[22−24] and liposomes.[25−28] The π_{oct} lipophilic constant for the octanol/water system can be used to determine the importance of substructural contributions to the biopotencies of drugs. Thus, we have explored the behavior of the antimicrobial hydrazides through this physicochemical descriptor, and eq 2 shows a clear relationship with π_{oct}. There is a difference between the slope shown in eq 1 and that for eq 2, i.e., in the coefficient of log P_{TA} and in π_{oct}, but nevertheless, both of them are negative. The difference in the slopes does indicate the differing character of the nonpolar phase in each case: it seems that the lipid phase in the cellular system is more hydrophilic than that in the octanol/water system. Eq 2: Linear dependence of $\log 1/D_{50}$, for *E. coli*, versus π:

$$\log 1/D_{50}(E.c.) = -0.308(\pm 0.07)\pi + 3.533(\pm 0.02)$$

$$n = 8;\ r^2 = 0.810;\ s = 0.177;\ F = 25.66;\ r^2_{cv} = 0.592$$

One of the common problems in dealing with physicochemical descriptors is related to the existence of a matrix correlation between them. The π_{oct} lipophilic constant is often found to be collinear with molar refractivity, MR ($r^2 = 0.876$). Equation 3 shows that a linear relationship exists between $\log 1/D_{50}$ and MR. However, π_{oct} and MR cannot be separated, and certainly they describe the same trend. Nevertheless, the slope is still negative, and its meaning is that the less bulky the substituent, the more potent the drug. Thus, eq 3 incorporates a further improvement to the model by including the substituent's size; i.e., substituents must not only have hydrophilic character but must also be small. Eq 3: Linear dependence of $\log 1/D_{50}$, for *E. coli*, versus MR:

$$\log 1/D_{50}(E.c.) = -0.389(\pm 0.06)(0.1)MR + 3.721(\pm 0.04)$$

$$n = 8;\ r^2 = 0.901;\ s = 0.128;\ F = 54.56;\ r^2_{cv} = 0.832$$

It is clearly seen that eq 3 gives slightly better results through MR than π or log P_{TA}. However, one should also recognize that log P_{TA} is well-correlated to MR, $r^2 = 0.762$.

In QSAR studies the determination of biological descriptors, i.e., quantitative drug potencies, can be crucial since they can be time-consuming to measure and not truly *quantitative*. Thus, this is a "bottleneck" which can, of course, obstruct model development. This seems not to be true for biological microcalorimetrically measured biopotencies where the experimental bioassay time required is only ca. 30 min! Moreover, the potentiality for automation, and hence its application to high-throughput screening (HTS), can also be envisaged. Nonetheless, it has to be pointed out that in order to carry out an experiment in only 30 min routine procedures must be available − particularly, for instance, to prepare the cell system. Of course, this is now not a problem since the development of frozen storage cells is well-established.[29] However, it should be noted that what is needed in biological microcalorimetry is to expand the range of new cell targets that would be used for *mass* screening.

The interactions of the study hydrazide antimicrobials can be ranked in potency according to substitution at

the 4-position of the aryl ring. They reveal relationships between potencies and the Gibbs function of binding (or partitioning) in terms of "extrathermodynamic relationships" that can then be established through eqs 1−3, above. The graded responses shown in Table 1 indicate the sensitivity concentration of the time-related response available via biological microcalorimetry for such small subunit moiety alterations. The success of the QSAR analyses shown in eqs 1−3 constitutes evidence for the additivity of group contributions to biological activity values.

Extrathermodynamic relationships can be found among biological activities.[2,30] Thus, we have analyzed the possibility of obtaining a relationship for the antimicrobial hydrazides between the same series of compounds and two different cell systems. Equation 4 shows that the log $1/D_{50}$(*S.c.*) values[8] are linearly correlated with those for log $1/D_{50}$(*E.c.*). Eq 4: Extrathermodynamic relationship between *E. coli* and *S. cerevisiae*:

$$\log 1/D_{50}(E.c.) = 1.368(\pm 0.13) \log 1/D_{50}(S.c.) - 1.605(\pm 0.48)$$

$$n = 8;\ r^2 = 0.978;\ s = 0.06;\ F = 269.69;\ r^2_{cv} = 0.938$$

Equation 4 indicates the existence of an extrathermodynamic relationship for antimicrobial activity between the same series of compounds but an interaction with different cellular systems. The fact that the slope is close to unity is probably a consequence of similar modes of action, including transport processes and binding interactions with receptors.

We have tested the hypothesis of hydrazides behaving through a similar transport process, and eq 5 suggests it is upheld. Eq 5: Extrathermodynamic relationship between log P_{TA}(*E.c.*) and log P_{TA}(*S.c.*):

$$\log P_{TA}(E.c.) = 0.833(\pm 0.05) \log P_{TA}(S.c.) - 0.323(\pm 0.05)$$

$$n = 8;\ r^2 = 0.955;\ s = 0.038;\ F = 128.40;\ r^2_{cv} = 0.931$$

Lipophilic properties do not differ significantly in the cell systems. The environment in which transport takes place is similar. Thus, the binding modes of these hydrazides should experience a similar physicochemical environment in the two different cell systems.

Conclusions

Statistically significant classical QSAR models were developed. log P_{TA} and biological microcalorimetry can be used to derive QSARs. This method seems to be a good alternative to the octanol/water system largely because the cell suspension more closely represents the natural system. Microcalorimetry is a promising tool for such QSAR studies.

Biological microcalorimetry is efficient, fast, and reproducible to better than 3%. Inconsistencies in biological data can be envisaged promptly from derived power-time curves. It can be used instead of other techniques such as agar diffusion or tube assays (serial dilution). In vitro screening can be performed in complex and defined medium using frozen cells. Calorimetric output can reveal the biocidal and biostatic activity of compounds directly; this is very important in order to control drug doses.

Overall, biological microcalorimetry can be applied to screening and for sensitive differentiation between related compounds, thereby generating a good quantitative biological descriptor. log *P* can be used directly from the same biological systems through the Taylor−Aris diffusion technique, and microcalorimetry can also give an insight into the mode of action of antimicrobial drugs.

The approach outlined in this paper offers not only procedures for detecting similar modes of drug action but also suggestions on how to proceed toward further clarification of the mode of drug action. Since the same series of compounds can be tested against two different cell systems, determination of partitioning can be achieved using the biological cells themselves, not models!

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