In Vivo Activity and Hydrophobicity of Cytostatic Aziridinyl Quinones

Ed E. Moret,* Mark de Boer, Hans W. Hilbers, Jan P. Tollenaere, Lambert H. M. Janssen, Joost J. M. Holthuis, Reinoud J. Driebergen, Willem Verboom,† and David N. Reinhoudt†

Departments of Pharmaceutical Chemistry and Analytical Pharmacy, Faculty of Pharmacy, Universiteit Utrecht, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands, and Department of Organic Chemistry, Universiteit Twente, Twente, The Netherlands

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For a series of 3,6-disubstituted bisaziridinylbenzoquinones the in vivo and in vitro activities against murine tumors, as well as the in vivo toxicity, are analyzed. Properties describing biochemical and physicochemical reactions are also incorporated in the analyses. The important 1-octanol/water partition coefficients were determined, using a fast variation of the shake flask method. New π'-values were calculated for the substituents in this series. These quinone π'-values deviate strongly from the standard π-values, especially for hydrogen-bonding substituents. To discriminate between the toxic and therapeutic activity of the compounds, principal components and partial least squares analyses were applied. Evidence is presented for selective antitumor action of the investigated compounds. The L_{1210} clonogenic assay only seems to relate to the general cytotoxicity and has no predictive value for in vivo activity for these compounds. The activity is correlated to the hydrophobicity of the quinones. The toxicity correlates with the ease of reduction, contrary to the hypothesis of bioreductive activation as a mechanism for selectivity.

Introduction

Already in 1962 Wheeler[1] presented an impressive list of possible interactions of aziridinyl compounds with biochemical processes. A large number of drugs, currently used in chemotherapy, possess a quinone moiety. At the National Cancer Institute (NCI) of the USA, 2,5-bis(1-aziridinyl)-1,4-benzoquinone (BABQ) compounds with 3,6-aminoalkyl substituents were synthesized and tested.[2,3] On the basis of the NCI study, more derivatives were synthesized and tested in vitro and in vivo.[4] Several mechanisms for cytotoxicity have been proposed and investigated for the highly reactive BABQ compounds. DNA strand breaks and interstrand cross-linking have been reported.[5−10] Generation of reactive oxygen species and initiation of redox cycling was reported equally often.[11−16] However, some doubts about the relationship between redox cycling and anti-leukaemic activity have also been published.[17−19] Reactions with glutathione (GSH) and proteins have been observed by others.[20,21] The concept of bioreductive alkylation as a mechanism of selectivity for quinones was proposed 20 years ago.[22] High levels of DT-diaphorase in tumor cells might enhance bioreductive alkylation, since it has been shown that DT-diaphorase reduces quinones to hydroquinones.[23,24] The prospects of tumor cell hypoxia for selectivity were reviewed by Kirkpatrick.[25] Tannock et al.[26] reviewed the potential of low pH values in tumors for therapeutic exploitation. For the BABQ compounds, increased acidity causes increased protonation of the aziridinyl group and, hence, increased tendency for ring opening and alkylation. This low pH value in tumor cells has been considered significant by many authors (see Vaupel et al.[27] for a review). However, Griffiths[28] denies this phenomenon based on NMR measurements of pH values of the intracellular fluid.

The majority of quantitative structure−activity relationships (QSAR) involves hydrophobicity properties like log $P_{o/w}$ or $\pi_{o/w}$ (the corresponding substituent constant of benzene substitution[29]), where o/w is the 1-octanol/water partitioning system. Such relationships reflect either transport to the site of action or binding to a hydrophobic site in a receptor molecule. Measurement of partition coefficients can be achieved with a shake-flask procedure using 1-octanol/water[30] or with reversed-phase HPLC[31] (RP-HPLC). The stationary phase is usually an octadecyl ether of silanol.

The capacity factor in RP-HPLC was determined for BABQ compounds in order to test correlation with cytostatic activity by Driebergen et al.[4] Significant relationships were found between in vivo activity and decreasing hydrophobicity as well as hydrogen-bonding capacity. The correction for hydrogen-bonding substituents with an indicator variable, simply counting the number of possible hydrogen bonds, may point to a difference between the biological system and the system of measurement[32] as in a Collander type equation.[33] Measurement in a new partitioning system might be worthwhile and is reported here. Yoshimoto et al.[34] reported relationships between in vivo anti-leukaemic activity of alkyl-substituted BABQ compounds and decreasing hydrophobicity as well as the Swain and Lupton electronic parameters. No explanation was given for the occurrence of higher activity at decreased hydrophobicity values. The electronic parameters could represent the same substituent effects as the hydrogen bond indicator in the study by Driebergen et al.[4]

Thus far, neither selectivity for tumor cells nor the mechanism of bioreductive activation has been shown to occur in vivo for these compounds. Pattern recognition techniques, like multivariate statistics, will be used in order to investigate activity and selectivity. Similar

* Author to whom correspondence should be addressed at the Universiteit Utrecht.
† Universiteit Twente.
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techniques have been successfully used on other cytostatic activity data.[35−37] These papers discouraged the use of one single experiment for the determination of biological activity. Principal component analysis helps in reducing data from a number of experiments to the underlying patterns of information.[38−40] New properties, called principal components, are derived by linear combination of the old properties, assigning contributions to the old properties in an interative manner. The principal components are then orthogonalized so as to prevent internal correlation. The data reduction is achieved by replacing all old properties by a smaller number of principal components, thus removing superfluous intercorrelated data. The principal components are ordered in a decreasing percentage of explained variance (eigenvalue) and numbered principal component 1 (PC1), principal component 2 (PC2), and so on. The old properties appear as loadings in the newly derived components in the form of a correlation coefficient. This enables one to understand the nature of the principal components.

Partial least squares goes one step further by performing the same operations on the target properties. It transforms the new components back into the old properties, yielding a classical, though improved, regression equation.[41,42] The predictive ability of this equation (predictive sum of squares) can be tested by means of cross-validation, a technique excluding compounds to be predicted by the relationship based on the other compounds. The cross-validated r^2 is a measure of the predictive ability of the relationship. Positive values mean that the omitted compounds have been predicted better than the original variance would have done. A value of 0 would indicate no improvement at all, and negative values point at equations that are worse than no equation at all. Only equations with a reasonable cross-validated r^2 should be subjected to a non-cross-validated final analysis. An arbitrary, but often used, threshold is 0.4. Some striking examples of the benefit of partial least squares in contrast to multiple linear regression have been given in the literature.[43]

Results

Hydrophobicity. Tables 1 and 2 and Figure 1 show the structures of the compounds and list the capacity factor log k' (50% methanol; RP-HPLC) and the measured partition coefficient log P_m together with the error, estimated from the deviation of the duplicate or quadruplicate measurements. In the literature log $P_{o/w}$ values for 10 of the compounds are reported. The results from the fast micro-shake-flask method are evaluated by a correlation with these literature values, log P_l (eq 1). The standard errors are shown in parentheses.

$$\log P_m = -0.017(0.113) + 1.019(0.069) \log P_l \quad (1)$$

$$n = 10 \qquad r^2 = 0.993 \qquad s = 0.075 \qquad F = 1177.30$$

For aziridine the log $D_{o/w}$ values, where D is the apparent partition coefficient, were −1.06, −0.97, −0.36, and −0.31 at pH values 8, 9, 10, and 11, respectively. This agrees well with a pK_a value of 8.0 for aziridine. The average value of nine determinations at pH 10 and pH 11 was −0.35 (±0.04), the log $P_{o/w}$ value of neutral aziridine. Relationships between log $P_{o/w}$ values measured in different partitioning systems are known as Collander relations.[33] Equation 2 shows the Collander relation between the 1-octanol/water system and the RP-HPLC system with the standard error in parentheses.

$$\log P_m = 0.195 + 2.109(0.128) \log k' \quad (2)$$

$$n = 39 \qquad r^2 = 0.880 \qquad s = 0.353 \qquad F = 1271.3$$

The quality of eq 2 is some what decreased because of the different partitioning behavior of hydrogen-bonding substituents[32,44] and the relative acidity of methanol, the modifier in RP-HPLC measurement,[45] compared to octanol.[46] Figure 2 shows the Collander

Table 1. Partition Coefficients and Capacity Factors for a Series of Benzoquinones

compd	substituents	log k' [a]	log P_m	error	log P_l
1	BQ	−0.466	0.20	0.02	0.20[b]
2	2-CH_3	−0.211	0.72	0.03	0.73[c]
3	2,5-CH_3	0.139	1.28	0.03	1.24[c]
4	2,6-CH_3	0.136	1.22	0.03	
5	2,3,5,6-CH_3	0.776	2.23	0.22	2.12[c]
16	2,5-$NHCH_3$	−0.611	0.34	0.16	
17	2,5-$N(CH_3)_2$	−0.004	1.03	0.06	
25	2,5-R1	0.587	1.97	0.10	
27	2,5-AZ	−0.440	0.18	0.02	
28	2,5-AZME	0.152	1.07	0.05	
29	2,5-AZ,3,6-Br	0.474	2.03	0.12	
34	2,5-AZME,3,6-F	0.444	1.61	0.08	
35	2,5-AZ,3,6-OCH_3	−0.210	0.17	0.02	
38	2,5-AZ,3,6-$NHCH_3$	−0.370	0.13	0.04	
40	2,5-AZ,3,6-NHC_2H_4OH	−0.700			−1.48[d]
41	2,5-AZ,3,6-$NHCO_2C_2H_5$	−0.480	0.04	0.01	0.05[d]
43	2,5-AZ,3,6-$N(CH_3)C_2H_4OH$	−0.052			−0.38[d]
47	2,5-AZ,3-CH_3,6-C_2H_5	0.291	1.35	0.04	
48	2,5-AZ,3-CH_3,6-C_2H_4OH	−0.417	0.18	0.02	
49	2,5-AZ,3-CH_3,6-$C_2H_4OCONH_2$	−0.429	0.16	0.02	
51	2,5-AZ,3-CH_3,6-R3	−0.438	−0.15	0.03	
52	2,5-AZ,3-Br,6-CH_3	0.279	1.36	0.06	
56	2,5-AZ,3-Br,6-$C_2H_4OCONH_2$	−0.055	0.68	0.03	
57	2,5-AZ,3-Cl,6-CH_3	0.200	1.20	0.05	
58	2,3,5-AZ	−0.450	0.13	0.02	
59	2,3,5-AZ,6-F	−0.312	0.14	0.02	

[a] Reference 4. [b] Reference 63. [c] Reference 64. [d] Reference 3.

Table 2. Partition Coefficients and Capacity Factors for a Series of Naphthoquinones (Structures in Figure 1)

compd	substituents	log k' [a]	log P_m	error	log P_l
62	NQ	0.299	1.71	0.08	1.71[b]
64	2-CH_3	0.607	2.20	0.17	2.20[b]
66	2-Br	0.748	2.44	0.07	
67	2-Cl	0.659	2.29	0.05	2.15[b]
68	2-NH_2	0.129	1.77	0.05	1.88[b]
69	2-$NHCH_3$	0.279	1.74	0.06	
70	2-$N(CH_3)$	0.591	1.90	0.15	
72	2-NHC_2H_4OH	−0.005	1.23	0.10	
75	2-R1	0.736	2.89	0.13	
76	2-R2	0.896	2.85	0.36	
77	2-NH_2,3-Cl	0.259	2.08	0.19	2.12[b]
82	2-AZ	0.346	1.71	0.12	
83	2-AZME	0.611	2.17	0.08	

[a] Reference 4. [b] Reference 63.

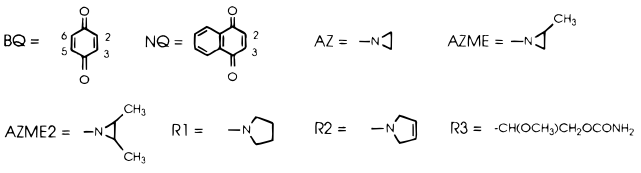

Figure 1. Structures of the basic compounds and substituents.

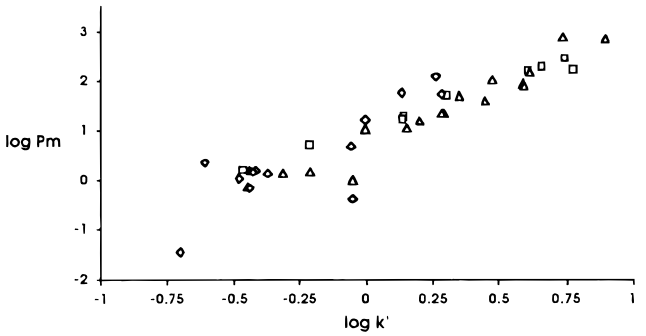

Figure 2. Collander plot of measured log $P_{o/w}$ and log k': (△) hydrogen bond accepting substituents; (□) nonhydrogen bonding substituents; (◆) amphiprotic substituents.

plot. The log $P_{o/w}$ for 1-aziridinylbenzene was measured as well (0.96). This enables us to calculate a $\pi_{o/w}$ value of −1.12 for the aziridinyl substituent. Compared to the log $P_{o/w}$ of aziridine, benzene seems to make the aziridine group more hydrophilic. Compounds **82** (naphtoquinone) and **62** (2-aziridinylnaphtoquinone) have identical log $P_{o/w}$ values, immediately showing the failure of using aromatic $\pi_{o/w}$ values for quinoid compounds. It could be concluded that quinones make aziridinyl substituents more hydrophobic. Equation 3 shows the relationship between the measured partition coefficients log P_m and the calculated log P_c, based on the use of the tabulated aromatic $\pi_{o/w}$ values of the quinone compounds **2**, **3**, **4**, **5**, **16**, **17**, **27**, **29**, **38**, **52**, **57**, **58**, and **77**. The standard error is shown in parentheses.

$$\log P_m = 1.134 + 0.351(0.085) \log P_c \qquad (3)$$

$$n = 13 \qquad r^2 = 0.608 \qquad s = 0.507 \qquad F = 17.06$$

Since these compounds only possess substituents that occur in the monosubstituted naphthoquinones, an honest comparison can be made with the use of special π' values, defined as the hydrophobic substituent constant in 1,4-quinones. Equation 4 shows the improved relationship between the log P_m values and log P'_c, calculated using these new π' values. The standard error is shown in parentheses. The substituted naphthoquinones and disubstituted benzoquinones were taken as the basis for newly derived π' values, and these were used for calculating log $P_{o/w}$ values for all compounds under investigation for which no log $P_{o/w}$ was measured.

$$\log P_m = 0.060 + 0.975(\pm 0.088) \log P'_c \qquad (4)$$

$$n = 13 \qquad r^2 = 0.918 \qquad s = 0.233 \qquad F = 122.61$$

Biological Activity. In Table 3 all properties used in the analysis are listed. They can be separated into target and explanatory properties. The biological activity of the compounds, as measured in vitro and in vivo, is always used as the target property. The biochemical experiments to determine the mechanism of action (mec), as well as the physicochemical properties (phy), form the explanatory properties. Not shown are the calculated log $P_{o/w}$ values (see Tables 1 and 2) and the therapeutic index TI, defined as 2BDB − LLD. the difference between in vivo activity and in vivo toxicity. The tabulated properties and abbreviations are as follows:

vitro	LID	log $1/ID_{75}$ for the in vitro clonogenic L_{1210} assay with ID_{75} in μmol/L[4]
vivo	LDL	log $1/D_{125}$ for dose (mmol/kg) to give 125% T/C ratio (L_{1210}) in vivo[4]
vivo	BDB	log $1/D_{125}$ for dose (mmol/kg) to give 125% T/C ratio (B_{16}) in vivo[4]
vivo	LLD	log $1/LD_{50}$ for LD_{50} (mmol/kg)[4]
mec	LFU	log 1/MIC for fungi in mmol/L
mec	LOS	log 1/concn in mmol/min·L of superoxide anion (100 μmol/L)[14] production
mec	LGS	log k_{GSH} for the rate k of glutathione (GSH) consumption in s^{-1} [59]
mec	LNK	DNA cross-link ratio at pH = 5.0 and 50 μmol/L compound[5]
phy	E12	$E_{1/2}$ for the half-wave potential at pH = 7.0 in V[57]
phy	PKR	pK_{red} for the electrochemically observed pK_a of aziridines[57]
phy	LKO	log k_{obs} for the observed rate k in s^{-1} of hydrolysis at pH = 4.0

The results of the most extensive principal component analysis, using all 13 measured properties and all compounds, with known activity in the L_{1210} clonogenic assay, are given in Figure 3. Four principal components out of a possible 13 are significant, contributing more than 7.9% (100% divided by 13), although only the first two components have a major contribution to the overall variance (PC1 30%, PC2 19%, PC3 11%, PC4 8%). The loadings plot on the left shows the correlation coefficients of all old properties with the major principal components PC1 and PC2. PC1 correlates with in vivo activity (LDL, BDB) as well as DNA cross-linking (LNK). This might suggest that principal component 1 reflects "in vivo antitumor activity". The in vitro results (LID) and the half-wave potential (E12) correlate with the biochemical properties of cell toxicity, like glutathione depletion and fungicidal activity (LGS and LFU). This suggests that principal component 2 reflects "general cytotoxicity". Not shown is the correlation between the third principal component with the rate of aziridine hydrolysis (LKO). The scores plot of the compounds on the right shows the value of the principal components of these compounds, which would now translate to in vivo antitumor activity and general cytotoxicity. Since principal component 1 (PC1) is negatively correlated with in vivo antitumor activity and principal component 2 (PC2) is positively correlated with general cytotoxicity, the more active and less toxic compounds can be found in the lower left section.

Some of the partial least squares results are shown in Figure 4. The cross-validated r^2 for the L_{1210} clonogenic assay (LID), the LD_{50} (LLD), and the in vivo L_{1210} activity (LDL) analyses were too low to continue. For in vivo B_{16} melanoma activity (BDB) and the therapeutic index (TI), the cross-validated r^2 values were 0.469 and 0.602, respectively. Figure 4 shows the predicted versus the actual values for the target property BDB and the therapeutic index TI. The BDB and TI partial least squares regression equations can be used to determine in vivo antitumor activity and selectivity.

The statistics are summarized in Table 4. Both partial least squares regression equations were calculated with the optimum number of two components. Multiple linear regression equations are appended for comparison. They were obtained after stepwise regression with almost all properties used in the partial least squares equations. The properties DNA cross-linking (LNK) and glutathione depletion (LGS) could not be

Table 3. Biological Activity (vitro and vivo), Biochemical Mechanisms (mec), and Physicochemical Properties (phy)[a]

		vitro	vivo			mec				phy		
compd	substituents	LID	LDL	BDB	LLD	LFU	LOS	LGS	LNK	E12	PKR	LKO
20	2,5-$NHCO_2C_2H_5$	0.09				0.85						
22	2,3-CH_3,5-R1	−1.70				−0.30				−0.303		
23	2,3-CH_3,5-R2	−0.71				−0.17				−0.311		
24	2-OCH_3,3-CH_3,5-R1	−1.66				−0.26				−0.315		
25	2,5-R1	−1.61				−0.38				−0.439		
26	2,5-R2	−1.62				−0.52						
27	2,5-AZ	1.68	2.77	3.16	1.80	2.58	2.09	−3.24	0.29	−0.105	7.5	−2.57
28	2,5-AZME	1.03	1.64	2.12	1.16	0.44	1.75			−0.115	8.1	−1.55
29	2,5-AZ,3,6-Br	0.35				1.54	2.87					−4.70
30	2,5-AZME,3,6-Br	0.77				1.28	2.80					−3.45
31	2,5-AZ,3,6-Cl	−0.31				1.63	2.74			−0.133		−4.70
32	2,5-AZME,3,6-Cl	−0.69	1.16			1.16	2.65			−0.125		−4.15
33	2,5-AZ,3,6-F	−0.29				1.75	1.72	−2.21		−0.087	7.2	−4.03
34	2,5-AZME,3,6-F	1.49			1.50	1.50	1.77			−0.093	7.9	−0.03
35	2,5-AZ,3,6-OCH_3	0.15	2.38	3.53						−0.179	8.0	−3.26
36	2,5-AZME,3,6-CH_3	−0.54	1.47	2.27	1.40					−0.187	8.6	−2.61
37	2,5-AZ,3,6-NH_2	1.16				−1.66						
38	2,5-AZ,3,6-$NHCH_3$	0.32	2.49	3.14						−0.415	8.5	
39	2,5-AZME,3,6-$NHCH_3$	−0.37	2.19	2.33						−0.400	9.3	
40	2,5-AZ,3,6-NHC_2H_4OH	0.42	3.39	3.94						−0.385	9.5	−2.80
41	2,5-AZ,3,6-$NHCO_2C_2H_5$	−0.57	2.26	2.32	1.54	−0.29	1.56	−4.64	0.14	−0.149	8.3	−4.25
42	2,5-AZME,3,6-$NHCO_2C_2H_5$	−1.00	1.59	1.94	0.69	−1.41	1.26		0.02	−0.145	8.5	−3.57
43	2,5-AZ,3,6-$N(CH_3)C_2H_4OH$	0.09				−1.47				−0.225	9.7	
44	2,5-AZ,3,6-R1	−0.39	2.07	2.40						−0.315	10.6	
45	2,5-AZME,3,6-R1	−1.12								−0.303	11.1	
47	2,5-AZ,3-CH_3,6-C_2H_5	−0.31		3.25	1.46	0.77	0.87	−5.00	0.23	−0.227	8.7	−3.59
48	2,5-AZ,3-CH_3,6-C_2H_4OH	1.51	3.37	3.60	1.39	0.44	0.95			−0.209	8.6	−4.00
49	2,5-AZ,3-CH_3,6-$C_2H_4OCONH_2$	1.82	2.86	3.49	1.99	−0.14	1.02	−4.92	0.27	−0.213	8.9	−4.05
50	2,5-AZME,3-CH_3,6-$C_2H_4OCONH_2$	−0.15	1.89	2.31			0.56		0.13	−0.235	9.7	−3.67
51	2,5-AZ,3-CH_3,6-R3	1.05	3.21	3.32	1.73	−1.19	0.57	−5.15	0.26	−0.182	8.6	−3.65
52	2,5-AZ,3-Br,6-CH_3	2.76		2.09	1.44	1.45	2.45			−0.185	8.7	−4.45
53	2,5-AZME,3-Br,6-CH_3	2.66	1.79	1.71	1.59	1.01	2.50		0.03	−0.194	9.2	−3.85
54	2,5-AZME2,3-Br,6-CH_3	−0.16	0.69		0.63	−0.47						−1.15
55	2,5-AZ,3-Br,6-C_2H_5	2.82	1.41	2.60	1.39	1.57	2.42	−3.00	0.15	−0.210	8.8	−4.30
56	2,5-Az,3-Br,6-$C_2H_4OCONH_2$	2.45	2.00	2.68	1.05	0.25	2.48	−3.60		−0.201	8.9	−4.49
57	2,5-AZ,3-Cl,6-CH_3	2.41	1.99	2.68			2.42			−0.197	8.5	−4.32
58	2,3,5-AZ	2.72	3.52	3.80	2.66	1.06	1.32	−3.14		−0.171	8.0	−2.85
59	2,3,5-AZ,6-F	2.32	1.95		1.62	1.79	1.72	−2.24	0.17	−0.171	7.8	−3.75
60	2,3,5-AZME,6-F	2.01			1.45	1.18				−0.195	8.2	
61	2,3,5,6-AZ	0.90				0.95				−0.245	7.7	−3.70

[a] The abbreviations used are explained in the text.

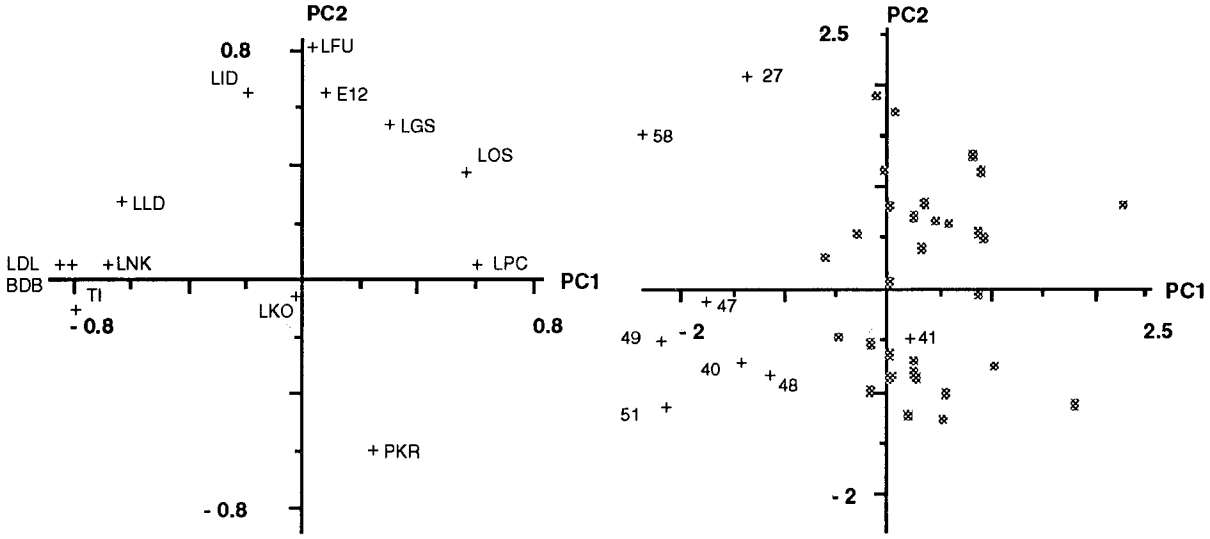

Figure 3. Loadings and scores of all compounds in PC1 and PC2. Only those compounds are numbered that are relevant for the discussion.

included in the stepwise multiple linear regression, because for none of the compounds are all data available.

Conclusions

Hydrophobicity. Equation 1 shows very good agreement between the log $P_{o/w}$ values measured with the micro-shake-flask method, described here, and the literature values for 10 compounds. The speed, accuracy, and small amount of compound necessary make this method an interesting modification of the time-consuming shake-flask method. The intercept and coefficient of the Collander eq 2 tells us that 1-octanol

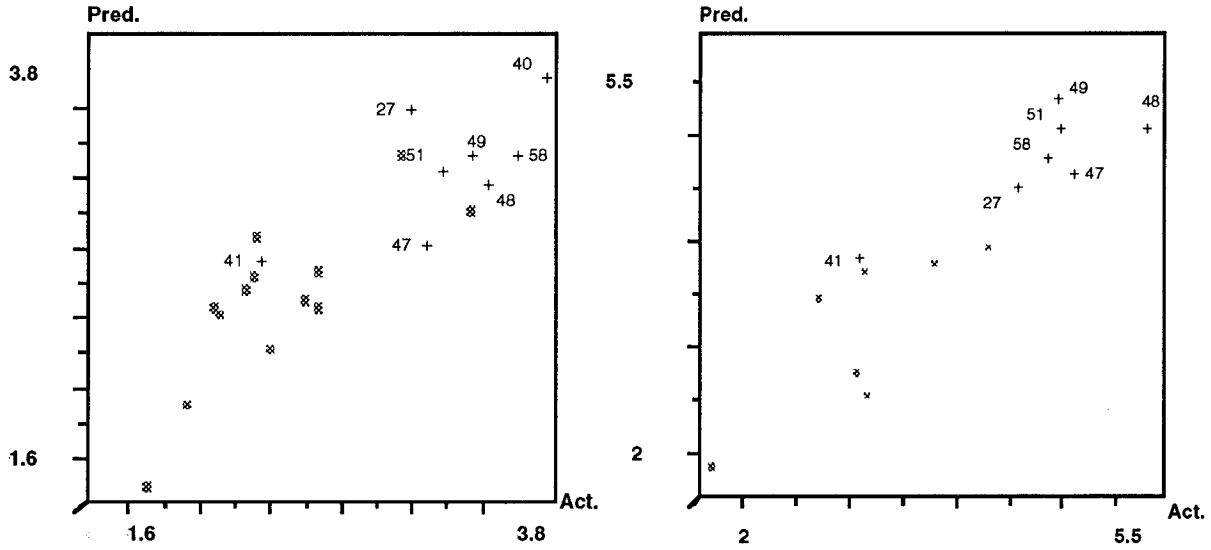

Figure 4. Predicted versus actual values for partial least squares analysis of BDB (left) and TI (right). Only the most noteworthy compounds are numbered.

Table 4. Partial Least Squares Equations for Activity and Selectivity[a]

Partial Least Squares Regression Equations

BDB = 3.60 − 1.47E12 − 0.41LPC − 0.10PKR − 0.05LKO + 0.11LFU − 0.20LOS + 3.80LNK + 0.13LGS

$n = 21$ $r^2 = 0.75$ $F_{2,18} = 26.24$ $P(r^2 = 0)_{2,18} = 0.000$ $s = 0.35$

TI = 4.45 − 11.15E12 − 0.58LPC − 0.20PKR − 0.17LKO + 0.13LFU − 0.58LOS + 6.02LNK + 0.27LGS

$n = 14$ $r^2 = 0.84$ $F_{2,11} = 27.97$ $P(r^2 = 0)_{2,11} = 0.000$ $s = 0.51$

Relative Contributions to the Equations

	BDB equation	TI equation
E12	0.10	0.17
LPC	0.26	0.17
PKR	0.05	0.04
LKO	0.03	0.06
LFU	0.09	0.06
LOS	0.11	0.16
LNK	0.28	0.26
LGS	0.09	0.10

Multiple Linear Regression Equations

BDB = 9.28 − 22.24(2.45)E12 − 0.54(0.09)LPC − 1.05(0.26)PKR + 0.31(0.09)LKO

$n = 13$ $r^2 = 0.96$ $F_{4,8} = 44.22$ $s = 0.18$

TI = 16.44 − 32.35(7.41)E12 − 0.75(0.27)LPC − 2.07(0.72)PKR

$n = 13$ $r^2 = 0.83$ $F_{4,9} = 14.61$ $s = 0.55$

[a] The probability P of $r^2 = 0$ is the probability of obtaining a chance correlation. Multiple linear regression equations are appended for comparison.

is twice as sensitive to hydrophobic changes as the stationary phase in RP-HPLC.[31] 1-Octanol is more susceptible to hydrogen-bonding substituents than is the stationary phase in the chromatographic experiment.

It has been stated before that additivity of $\pi_{o/w}$ values is low for quinones.[3] The difficulty in calculating log $P_{o/w}$ based on aromatic $\pi_{o/w}$ values is best illustrated with eq 3. The log $P_{o/w}$ of benzoquinone (0.20) is much lower than log $P_{o/w}$ of benzene (2.10), due to the carbonyl groups, which (as potential hydrogen bond acceptors) are hydrophilic. Apart from the intrinsic hydrophobic contribution of the substituents, substitution of a quinone will also have an effect on the interaction of these carbonyl groups with the solvents. The character of this influence can be steric or electronic or can involve intramolecular hydrogen bonding. This nonadditivity of $\pi_{o/w}$ values and the necessity for quinoid π' values is best demonstrated by the AZQ (compound **41**) results. For estimating the hydrophobicity of the ethoxycarbonylamino groups, we must use eq 2, since only the capacity factor of 2-NHCOOC$_2$H$_5$-NQ is known (log k' = 1.500). Equation 2 predicts 3.36 for log $P_{o/w}$, suggesting a π' value of 1.65 for the (ethoxycarbonyl)amino group. Hydrophobic substituents should enhance penetration of the blood−brain barrier and should increase activity against CNS tumors.[2,3] However, with a vicinal aziridinyl group, the hydrophobicity of the (ethoxycarbonyl)amino substituent vanishes. Benzoquinone (compound **1**) has a log $P_{o/w}$ of 0.20. The disubstituted aziridinyl compound **27** has a log $P_{o/w}$ of 0.18; although the $\pi_{o/w}$ value of the aziridinyl substituent is −1.12, the π' value is 0. The log $P_{o/w}$ value of AZQ (compound **41**), 0.05, is therefore not only unexpected, but might also be a disadvantage for the postulated CNS antitumor activity. The fact that the most hydrophilic compound BZQ (compound **40**) is currently in clinical trials for CNS antitumor activity[47] is also somewhat surprising. Another striking example of the necessity of using π' instead of $\pi_{o/w}$ is the π' value of NH_2, derived from compound **68**, 2-NH_2NQ. This π' value of 0.06 deviates strongly from the tabulated $\pi_{o/w}$ value −1.23.[48] Tautomerization could be the cause of this phenomenon, as well as an intramolecular hydrogen bond between the amino substituent and the oxygen atom.[49]

Figure 2 shows the Collander plot of the quinones. The highest statistical quality is obtained when the compounds are subdivided into three groups according to their hydrogen-bonding behavior (equations not shown here, see De Boer et al.[50]). The "non hydrogen bonding" group contains compounds with only alkyl and halogen substituents. The "hydrogen bond acceptor" group contains compounds with substituents like aziridine and the "amphiprotic" group contains compounds with substituents that can both donate and accept hydrogen bonds. An explanation for the different behavior of the three groups has been given[46] by pointing at the difference in acidity of the two "non-water phases" (the stationary phase in HPLC versus 1-octanol). Hydrogen bond accepting compounds are known to show higher affinity for acidic solvents[51] than do amphiprotic compounds. Water, being more acidic than octanol, favors the solvation of hydrogen bond acceptors over amphiprotic compounds.

Rich et al.[52] compared the partition coefficients of some substituted quinones and their corresponding hydroquinones. In the 1-octanol/water partitioning system, quinones and hydroquinones have almost identical partition coefficients. In the cyclohexane/water partitioning system, however, the hydroquinones are less hydrophobic, and partitioning shows a different dependence on substituent effects compared to quinones (see also Huang[53]).

Biological Activity. Multivariate statistical techniques were used to study the biological activities of BABQs. The large number of missing data in Table 3 did not allow reliable multiple linear regression calculations. After transforming the data to principal components with principal component analysis, possible conclusions are based on all available data, despite the amount of missing data. In previous principal component analyses,[54] DNA cross-linking was shown to be highly related to DNA alkylation.[55] Despite the limited number of data on DNA cross-linking, the relationship with other DNA alkylation experiments gives more credibility to the use of cross-linking data in this paper. The technique of cross-validation, used in conjunction with partial least squares, gives even better estimates of the significance of the QSARs.

The principal component analysis results in Figure 3 may allow for some important conclusions to be drawn. The two main components of information discriminate in vitro assays from in vivo assays. The in vitro L_{1210} clonogenic assay is orthogonal to the in vivo activity against L_{1210} tumors. It is, however, collinear to the fungitoxicity experiment. This suggests that in the clonogenic assay acute cell toxicity is measured, regardless of the type of cell. The use of this clonogenic assay as a screening for antitumor activity should be questioned. Figure 3 shows that one might compare principal component 1 (PC1) with in vivo activity and principal component 2 (PC2) with general cytotoxicity. The lower left section of Figure 3 would then be the section of the desired selective toxicity: high in vivo antitumor activity and low general cytotoxicity. In Figure 3 it can be seen that the therapeutic index (TI) is in this section. The LD_{50} experiment is in the section of in vivo activity, but also general cytotoxicity. The properties glutathione depletion (LGS), superoxide anion production (LOS), and fungitoxicity (LFU) correlate mainly with general cytotoxicity, as could be expected. These tests seem to reflect better acute cell toxicity, as does the clonogenic assay, in contrast to in vivo activity. The DNA cross-linking (LNK), however, is clustered with the measured in vivo activities. This suggests that cross-linking of DNA strands is an important mechanism for in vivo antitumor activity.

An immediate question may arise: what physicochemical properties describe activity best and what properties describe toxicity best? Hydrophobicity (LPC) correlates with in vivo activity with a negative slope, as was shown earlier.[4,34] It would be hard to imagine that activity would continuously increase with decreasing hydrophobicity. Most likely, more negative hydrophobicity values would show a parabolic relationship, as it was reported for acridines.[37] The general belief that more hydrophobic compounds would have better anticancer activity[56] does not hold for quinones and acridines. A possible explanation for the observed inverse relation with hydrophobicity is the difference in polarity between the highly charged DNA backbone and the more hydrophobic cellular proteins; attack of the latter would be considered nonselective for tumor cells. The fast division of tumor cells is selectively inhibited by DNA cross-linking. This resembles the selectivity of methotrexate for tumor cell DHFR, which is an opportunistic and nondiscriminative selectivity, more than the selectivity based on the more elegant concept of bioreductive activation. The half-wave potential correlates best with general cytotoxicity and is orthogonal to in vivo activity. The occurrence of redox cycling may be the cause of this relationship. This is substantiated by the data for superoxide anion production (LOS). It might mean that the concept of bioreductive activation is valid for toxicity, instead of in vivo antitumor activity. This important finding is of course restricted to the compounds under consideration.

High pK_a values seem to have an adverse effect on toxicity. Again, this property is orthogonal to in vivo activity, confirming the findings of Griffiths[28] that tumor cells are not as acidic as was the general belief for quite some time. However, it is also possible that the adverse effects of basicity (PKR) only reflect the fact that compounds with methylaziridinyl substituents have higher pK_a values but lower activity, due to steric hindrance. Chemical instability of the aziridine rings (LKO) shows discrimination between LD_{50} and in vivo activity in the third principal component. According to Figure 3 high in vivo activity is expected for the compounds **27** (DZQ), **58** (Trenimon), **51** (Carboquone), **40** (BZQ), **47**, **48**, and **49**. Although compound **41** (AZQ) is currently in clinical trials, it is not one of the most active compounds, presumably because of its higher partition coefficient and easy reduction. The recipe for selective antitumor BABQ compounds would be as follows: hydrophilic, hard to reduce, easy to protonate, and composed of two chemically unstable aziridine rings. The latter two properties rely both on electronic effects as well as on steric factors.[57] Carboquone (compound **51**) seems to fit these criteria. BZQ (compound **40**) is more active in the vivo tests; however, its low score in the principal component analysis may be caused by the fact that no LD_{50} data were available for this compound.

Partial least squares and particularly cross-validation can tell us more about the significance of a relationship

and its predictive power. Good equations were obtained for the in vivo activity against B_{16} melanoma and the therapeutic index (TI). The major contributing properties in the "activity" or BDB equation are hydrophobicity and cross-linking. In the "selectivity" or TI equation, cross-linking and hydrophobicity have a positive contribution and "ease of reduction" and generation of superoxide anions have a negative contribution. Comparing both equations, the increased importance of reduction and superoxide anion production in the selectivity equation is apparent. Within this selection of compounds ($n = 21$, $n = 14$), the equations would also favor an increase of the less contributing properties glutathione depletion (LGS) and superoxide anion production (LOS) which were related to toxicity in the principal component analysis. Unfortunately, for the most active compound in the B_{16} melanoma test, BZQ (compound **40**), no LD_{50} was measured, so no therapeutic index could be calculated. Compound **48** may be a promising candidate for CNS tumor chemotherapy, being more hydrophobic than BZQ, a compound that has been tested in clinical trials against CNS tumors.[47]

The multiple linear regression equations immediately show the inherent problems of missing data. Not all independent properties could be used in the multiple linear regression, because no compound has known data for all properties. Although the multiple linear regression equations are statistically quite good, they do not reflect the SAR of all compounds and cannot point at important mechanisms like cross-linking and depletion of glutathione.

Although the concept of bioreductive activation may only be connected to toxicity, the results presented in this paper clearly show a distinction between the desired in vivo antitumor activity and the general cytotoxicity of some of these BABQ compounds.

Experimental Section

The synthesis of the compounds has been described elsewhere.[57]

Measurement of Hydrophobicity. The usual shake-flask procedure for the determination of partition coefficients has been adapted for this series, to allow handling of small volumes and to decrease the shaking time. Small volumes of the two immiscible phases were thoroughly shaken. A sample of the water phase was injected on a RP-HPLC column. Comparison of the peak areas in the chromatograms of the shaken water phase and the nonshaken water phase yields the partition coefficient.

The aqueous solutions were prepared by dissolving 15 mg of the compound in 10.0 mL of *N*,*N*-dmethylformamide (Merck p.a.) and diluting this solution 40.0 times with a phosphate buffer (0.05 M; pH = 6.50). Of the homogeneous, buffered solution (500 μL) was added to an equal volume of previously water-saturated 1-octanol at room temperature in a capped plastic test tube which was vigorously shaken three times for 120 s. Afterward, the tube was centrifuged at 14000*g* for 15 min. The water phase was carefully extracted.

The high-performance liquid chromatography (Waters Associates WISP 710 B) apparatus was equipped with a Waters Associates model 510 pump, a Lichrosorb RP-18 (10 μm, 30 cm) column, and a Waters Associates model 440 UV detector. Eluents were prepared from methanol (Merck p.a.) and phosphate buffer (0.05 M; pH = 6.50). Concentrations of modifier (methanol) were chosen as 30, 50, and 70 wt %, depending on the previously determined capacity factors[4] of the compounds. Capacity factors are calculated as $k' = (t_R - t_0)/t_0$, where t_R and t_0 are retention times of respectively the compound and the eluent. The flow was kept constant at 1.0 mL/min at a pressure of 2000 psi. UV detection occurred at 254 nm. Volumes of 10 μL (for the standard, non-shaken solution) and 50 μL (for the sample) were injected automatically. The whole procedure was exectued in duplicate and if necessary in quadruplicate. The partition coefficients were calculated from peak areas as follows:

$$P_m = (PA_{reference} - PA_{sample})/PA_{sample} \quad (5)$$

$PA_{reference}$ and PA_{sample} are the peak areas of the nonshaken and the shaken solutions, respectively, corrected for different injection volumes.

The partition coefficient of 1-aziridinylbenzene was measured in the same way. The apparent coefficient of aziridine was measured in a normal shake-flask procedure.[48] The apparent partition coefficient D was determined at pH values 8, 9, 10, and 11. The average value of nine determinations at pH 10 and pH 11 was considered to be the log $P_{o/w}$ of neutral aziridine. Detection was performed according to the assay described by Pomonis et al.[58] To discriminate measured log $P_{o/w}$ values from values taken from the literature, the terms log P_m and log P_l are used, respectively, in this paper. To discriminate log $P_{o/w}$ values, calculated with $\pi_{o/w}$ values (based on benzene-substitution) from log $P_{o/w}$ values, calculated with $\pi'_{o/w}$ values (based on quinone substitution), the terms log P_c and log P'_c will be used, respectively.

Measurement of the hydrophobicity by means of RP-HPLC capacity factors k' is described by Driebergen et al.[4]

Measurement of Other Properties. Measurement of the in vitro and in vivo activities is described by Driebergen et al.[4] As a measure for tumor selective action, a therapeutic index TI is calculated as the sum of the in vivo activity against B_{16} melanoma and the difference between this activity and the LD_{50} toxicity, so TI = 2BDB − LLD. Measurement of the biochemical properties was described elsewhere.[5,14,59] The minimal inhibition concentration (MIC) against fungi was measured with the fungitoxicity TLC bioassay.[60] The half-wave potential $E_{1/2}$ and basicity pK_a were described by Driebergen et al.[57]

The observed rate of hydrolysis was measured, using cyclic voltammetry (CV). Then, 0.1 M sodium acetate buffers of pH 5.0−4.5−4.0−3.5 (μ 0.1 M) and 0.02 M NaH_2PO_4 buffers of pH 3.5 and 3.0, adjusted to μ 0.1 M with 2 M $NaNO_3$, were used, and the degradation was followed at 20 °C. The degradation was started by adding 50 μL of the stock solution of the compound to 5.0 mL of the deaerated buffered solution. After an additional deaeration of 30 s, CV curves were recorded at appropriate intervals of time, and the decrease of the cathodic peak of the parent compound in time was determined. The degradation of BABQ derivatives follows pseudo-first-order kinetics with respect to its concentration and the observed pseudo-first-order rate constant k_{obs} for the overall degradation can be calculated by linear regression analysis of a plot of the logarithm of the concentration of the compound versus time.

Statistical Analysis. A Hewlett-Packard 310 microcomputer and the HP proprietary Basic Statistics and Data Manipulation (1984) program were used to perform multiple linear and nonlinear regression calculations, as well as the NCSS[61] program on IBM PS2/80. The principal component analysis and partial least squares analyses were performed with the QSAR module of Sybyl 6.0[62] on a Silicon Graphics Crimson Elan workstation. During all multivariate correlations, randomly generated numbers were taken into account but omitted in the final results. Missing values were replaced by the mean value of that property. All data were autoscaled to unit variance and zero mean. In the partial least squares analysis the number of cross-validations was taken equal to the number of compounds that had known target properties. A maximum of 10 orthogonal latent components was extracted in the cross-validated analyses. The biological activities were transformed to reflect as closely as possible the logarithm of an equilibrium or rate constant. A distinction can be made between predetermined dose and predetermined effect experiments.

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