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Electrochemistry of potentially bioreductive alkylating quinones. Part 4. Qualitative and quantitative structure–activity relationships of aziridinylquinones

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Abstract. The concept of bioreductive alkylation as a mechanism of action of aziridinylquinoid anticancer agents has been investigated. The influence of quinone substituents on quinone reduction, on protonation of the aziridines prior to and following quinone reduction and on partitioning properties of the compound was examined. Parameters obtained from a combined electrochemical, chemical-stability and lipophilicity study describing these processes were determined and correlated quantitatively in a Hansch-type QSAR study with biological data obtained from three experimental tumor models. Poor quantitative correlations between cytotoxicity in a L_{1210} clonogenic assay and the parameters were obtained. Good linear relationships, however, between antitumor activity *in vivo* (*vs.* L_{1210} leukemic mice and *vs.* B_{16} melanoma-bearing mice) and the lipophilic properties of the quinone were found. These relationships, showing a negative correlation between antitumor activity and lipophilicity, can be used to predict the activity of new, unknown compounds. No trend was evident between antitumor activity and other parameters, although some indications for potential importance of electronic and steric properties of the substituents and of their ability to form hydrogen bonds were found.

Introduction

Sartorelli and co-workers have extensively studied the possible role of bioreductive alkylating quinones, from which the class of mitomycins is the most widely known, in the treatment of solid tumors[1-7]. It is believed, that the alkylating potency of these compounds is enhanced upon reduction of the quinone moiety under anaerobic conditions, making them more selectively cytotoxic to hypoxic cells present in solid tumors than to aerobic tumor types.

Although conflicting evidence on the state of reduction of the quinone moiety required to form the alkylating species has been reported, *i.e.*, both, the necessity of a *one-electron* reduction of the quinone moiety[8,9], occurring under aerobic conditions, and a *two-electron* reduction[7,10], occurring under anaerobic conditions, it has been recognized that reduction indeed is required. Reduction of the quinone moiety has, on the other hand, also been considered to be a *deactivating* reaction, assuming that the quinoid form is the more activated state[11-13,15,16].

Based on the latter hypothesis, research on the development of new analogs of the aziridinylquinone type has been focussed mainly on compounds carrying amino substituents (*e.g.*, AZQ and BZQ, Figure 1) or alkyl groups (*e.g.*, Carboquone, Figure 1), which were assumed to thwart quinone reduction[17-23]. Other reports, however, suggest or prove a bioreductive activation[14] mechanism of AZQ, despite its amino functions[9,10,24].

The same holds for current drug design in mitomycin analogs: although mitomycins are considered to be prototypes of bioreductive alkylating quinones[1,2,4-6], new analogs carrying amino functions attached to the quinone function have been designed: quinone reduction will be thwarted by the electron-donating amino function, aiming to decrease systemic toxic side effects without loss of antitumor activity[25-28]. On the other hand, mitomycin analogs have been designed carrying electron-withdrawing alkoxy substituents[29-30], thus facilitating quinone reduction.

It is evident from the examples given above that more insight into the potential mechanism(s) of action is essential for successful development of new quinone-type antitumor agents.

The use of quantitative structure–activity relationships (QSAR), using parameters describing the individual steps of the hypothetical mechanism of action, may be a helpful guide for new drug research. Several QSAR models for the correlation of biological data with physico-chemical parameters have been developed[31], from which the *Hansch* model has been used frequently in antitumor-drug design[*e.g.*, 18,23,28,32,35].

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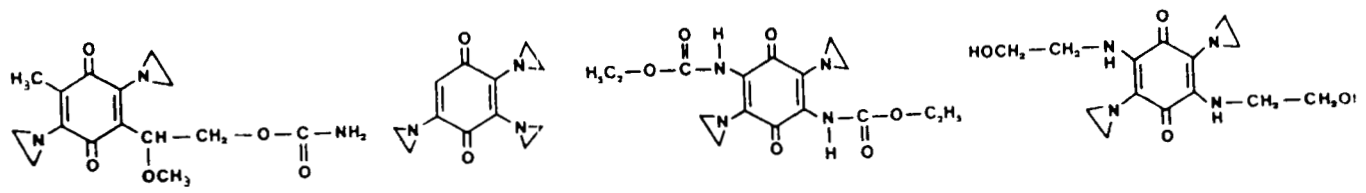
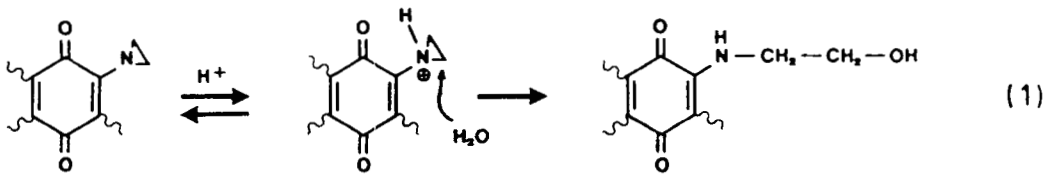
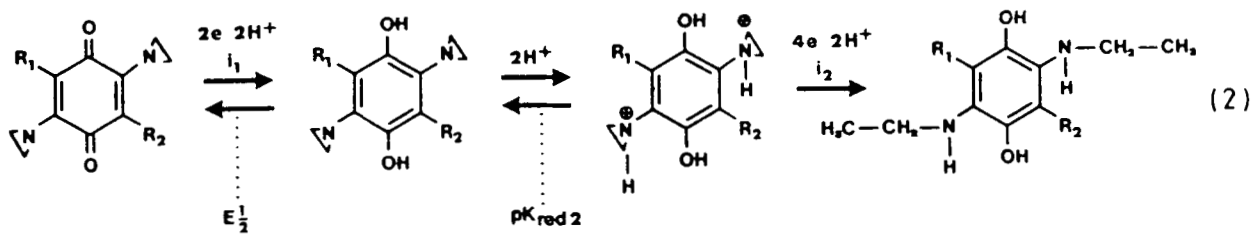


Carboquone (Compound 51) | Trenimon (Compound 58) | AZQ (Compound 41) | BZQ (Compound 40)

Figure 1. Structures of cytostatic aziridinylquinones.



Scheme 1. Protonation of aziridinyl moiety prior to quinone reduction, *followed by hydrolysis. Parameter* k_{obs} *reflects observed rate constant for hydrolytic reaction.*



Scheme 2. Reduction of quinone to corresponding hydroquinone (described by $E_{1/2}$*) and subsequent protonation of aziridinyl moieties at mercury electrode (described by* pK_{red2}*), followed by their reductive opening.*

QSAR studies of potential bioreductive alkylating quinoid antitumor agents are limited. Aerobic tumor models have been mainly used, such as L_{1210} and P_{388} leukemia. The importance of lipophilicity of antitumor agents has been emphasized by *Hansch*[32] and *Rekker*[33]: low lipophilic compounds have a low probability to reach distant tumor tissue and may be subjected to rapid excretion and/or inactivation, whereas too high lipophilicity prevents transport of the compound through aqueous media. Lipophilicity was reported to be the most important parameter in a QSAR study of 10 mitomycin-C analogs against P_{388} murine leukemia *in vivo*, whereas the contributions of σ_p and $E_{1/2}$, parameters related to the ease of quinone reduction, were not significant[28].

A series of 39 aziridinylquinones was evaluated in a QSAR study against L_{1210} *in vivo*[23]. Both lipophilic and electronic properties of the substituents were found to be important for the biological response, which was also concluded from a Principal Component Analysis study of the same data set[34]. Other QSAR reports on antitumor activity of simple quinoid agents deal mainly with analogs of the naphthoquinone type[35,36]. Discriminant analysis has been used to evaluate the activity of 27 naphthoquinones against three model systems: L_{1210} leukemia, solid sarcoma 180 and ascitic sarcoma 180[36]. In the first model, high lipophilicity and small substituents were found to be required for activity; in the second model, low lipophilicity and large substituents and, in the third model, low lipophilicity and small substituents. The half-wave potential of quinone reduction ($E_{1/2}$) appeared not to be important, even in the solid tumor model. In a later paper by the same authors, 12 naphthoquinones were evaluated by a *Hansch*-type analysis *vs.* ascitic sarcoma 180 of mice[35]. In this study, $E_{1/2}$ was stated to be the most important factor in determining an increase in life span to 125%. However, a doubtful parabolic relationship was reported.

Other biological effects (*e.g.*, antifungal, anti-bacterial, antimalarial or inhibitory activity of electron transport of photosynthesis) have also been quantitatively correlated with physico-chemical properties of quinones[37-40]. Generally, $E_{1/2}$ is proposed to play an important role in these biological effects.

Previous papers by us describe how the concept of bioreductive alkylation as a mechanism of action of aziridinylquinoid anticancer agents has been investigated[24,41-45]. DNA alkylation was proved to occur by the protonated reduced aziridinylquinone[24], supporting the concept of bioreductive alkylation of aziridinylquinones. Thermodynamic and quantum-chemical calculations were related to reductive activation and radical formation of quinones[45]. Electrochemical analysis of aziridinylquinones offers the possibility of determining two parameters giving information on both steps of the hypothetical mechanism of action. These parameters are $E_{1/2}$, *i.e.*, the half-wave potential of the quinone reduction wave, and pK_{red2}, reflecting the ease of protonation at the mercury electrode of two aziridines in the *hydroquinone*. Both parameters are obtained from direct-current polarography[43] and can be used in combination

with a parameter describing the ease of protonation of the *quinone* aziridines, *i.e.*, k_{obs}, describing the acid-catalyzed opening of the aziridines (Schemes 1 and 2).
Quantitative structure–electrochemistry relationships (QSER) correlate $E_{1/2}$ and pK_{red2} with physico-chemical parameters[44]. To examine substituent effects on the anti-neoplastic activity of these aziridinylquinones, a QSAR study towards their cytotoxic properties in experimental tumor models was performed, using the above-mentioned parameters describing reduction, and protonation prior to and following reduction. The present paper reports the results of this QSAR study.

Materials and methods

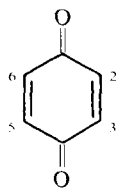
Chemicals

The structures of the compounds are given in Tables I and II. Four well-known aziridinylquinoid antitumor agents, *viz.*, BZQ, AZQ, carboquone and trenimon (Table I, compounds 40, 41, 51 and 58, respectively) were included in the series of compounds investigated. For reasons of simplicity, the numbers of the compounds are the same as in our previous paper[44], in which the sources or syntheses of the compounds have also been described. Chemicals used in the electrochemical, stability, and lipophilicity studies were of analytical grade.

Determination of parameters

The following parameters were determined: k', k_{obs} (Scheme 1), $E_{1/2}$ (Scheme 2) and pK_{red2} (Scheme 2).
As a measure of lipophilicity, the capacity factor k' of the compound obtained in a reversed-phase HPLC system was used[46,48]. Compounds were dissolved in *N*,*N*-dimethylformamide to a concentration of 1 mg/ml (about 5 mM). After determination of $t_0(LiNO_3)$, 10 µl of the solution of the compound of interest was injected into the chromatography system (Lichrosorb RP-18 (30 cm × 3.9 mm i.d., 10 µm) MeOH/0.5M NaH_2PO_4 pH 6.5 50/50 w/w, UV

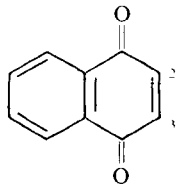
Table I Structures of 1,4-benzoquinones.



Compound No.	Substituent[a] at position:			
	2	3	5	6
27	Az	H	Az	H
28	Az-me	H	Az-me	H
29	Az	Br	Az	Br
30	Az-me	Br	Az-me	Br
31	Az	Cl	Az	Cl
32	Az-me	Cl	Az-me	Cl
33	Az	F	Az	F
34	Az-me	F	Az-me	F
35	Az	OCH_3	Az	OCH_3
36	Az-me	OCH_3	Az-me	OCH_3
37	Az	NH_2	Az	NH_2
38	Az	$NHCH_3$	Az	$NHCH_3$
39	Az-me	$NHCH_3$	Az-me	$NHCH_3$
40	Az	$NHCH_2CH_2OH$	Az	$NHCH_2CH_2OH$
41	Az	$NHCO_2CH_2CH_3$	Az	$NHCO_2CH_2CH_3$
42	Az-me	$NHCO_2CH_2CH_3$	Az-me	$NHCO_2CH_2CH_3$
43	Az	$N(CH_3)CH_2CH_2OH$	Az	$N(CH_3)CH_2CH_2OH$
44	Az	R^1	Az	R^1
45	Az-me	R^1	Az-me	R^1
46	Az	C_6H_5	Az	C_6H_5
47	Az	CH_3	Az	CH_2CH_3
48	Az	CH_3	Az	CH_2CH_2OH
49	Az	CH_3	Az	$CH_2CH_2OCONH_2$
50	Az-me	CH_3	Az-me	$CH_2CH_2OCONH_2$
51	Az	CH_3	Az	$CH(OCH_3)CH_2OCONH_2$
52	Az	Br	Az	CH_3
53	Az-me	Br	Az-me	CH_3
54	Az-me_2	Br	Az-me_2	CH_3
55	Az	Br	Az	CH_2CH_3
56	Az	Br	Az	$CH_2CH_2OCONH_2$
57	Az	Cl	Az	CH_3
58	Az	Az	Az	H
59	Az	Az	Az	F
60	Az-me	Az-me	Az-me	F
61	Az	Az	Az	Az

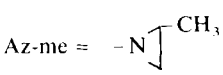
[a] For Az, etc., see bottom of Table II.

Table II Structures of 1,4-naphthoquinones.

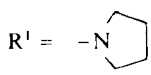


Compound No.	Substituent at position: 2	3
82	Az	H
83	Az-me	H
84	Az	Cl
85	Az	NH_2
86	Az	$NHCOCH_3$
87	Az	$NHCOCH_2Cl$
88	Az	$NHCO_2CH_2CH_3$
89	Az-me	$NHCO_2CH_2CH_3$
90	Az	$N(CH_3)CO_2CH_2CH_3$
91	Az-me	$N(CH_3)CO_2CH_2CH_3$
92	Az	$CH_2NHCO_2CH_2CH_3$

Az = 

Az-me = 

Az-me$_2$ =

R^1 = 

detection at 340 nm). The capacity factor was calculated by $k' = (t_r - t_0)/t_0$. The column was temperature-controlled at 25 °C. Compounds which could not be determined with this eluent were measured as 40/60, 30/70 and/or 20/80 mixtures, followed by linear extrapolation of $\log(k')$ to 50/50.

Apparatus and procedures for the determination of k_{obs} values will be reported in a separate paper[49].

Apparatus and procedures for the determination of $E_{1/2}$ and pK_{red2} values, obtained from direct current polarography, are the same as in our previous reports[42-44]. Descriptions and values of physico-chemical parameters of all substituents involved in this study were reported in our previous paper[44], as were the procedures for the determination of not-tabulated parameters.

Determination of antitumor activity

Since reduction of the quinone function, generation of free-radical species, and formation of the alkylating species strongly depend on the level of oxygenation of the tumor cells (*vide supra*), antitumor activity of bioreductive alkylating quinones should preferably be determined in both hypoxic- and aerobic-tumor models. Antitumor activity of aziridinylquinones under *aerobic* conditions may then be an indication for alkylation by the quinone or the semi-quinone free radical and/or the involvement of activated oxygen species in the mechanism of action. Activity under *anaerobic* conditions may be a result of alkylation by the hydroquinone.

In the present study, biological data were obtained from three tumor models. An attempt was made to select these models such that more insight into the difference between cytostatic activity towards anaerobic and aerobic tumor cells could be obtained. Aerobic cytotoxicity *in vitro* was studied in a leukemia L_{1210} clonogenic assay. Unfortunately, attempts to measure cytotoxicity in this assay under anaerobic conditions were not successful. Two tumor models *in vivo* were used. As a measure for cytostatic activity towards aerobic tumor cells L_{1210} leukemic mice were used, which have the added advantage that this model is a general screening system for new potentially cytostatic compounds. Antitumor activity in B_{16} melanoma-bearing mice was used as a model for antitumor action towards anaerobic tumor cells.

In-vitro *tumor model*

The L_{1210} clonogenic assay is used as a general pre-screen to identify compounds with potential antitumor activity. The end-point is the ID_{75}, *i.e.*, the concentration of the compound which causes 75% inhibition of the number of colonies relative to colonies arising from untreated cells. This value is calculated from the dose-response cure (*i.e.*, concentration *vs.* number of colonies), provided that the compound of interest shows concentration-dependent activity. An example is given in Figure 2a.

Aziridinylquinones exhibit a concentration- and time-dependent behaviour in the clonogenic assay[50]. The activity observed gives information on the cytotoxicity of the compound under aerobic conditions, although several processes may influence its ID_{75} value, such as the time of exposure and the solubility and chemical decomposition of the compound in the medium during the period of incubation. The ID_{75} value of AZQ, an experimental tumor agent, was used as reference point. Compounds with an ID_{75} lower than or equal to that of AZQ are considered to be active in the assay.

Compounds were dissolved in a suitable solvent (alcohol,

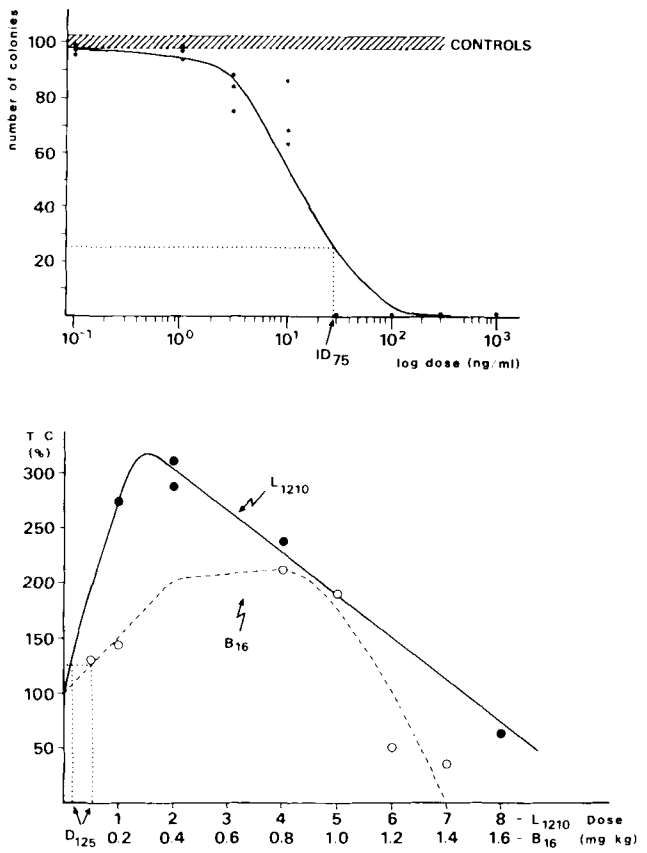


Figure 2. Dose-response curves of Carboquone (compound 51) in (top) a L_{1210} clonogenic assay and (bottom) L_{1210} leukemic and B_{16} melanoma-bearing mice.

dimethyl sulfoxide or *N*,*N*-dimethylformamide) and diluted in Hepes-buffered Hanks'-balanced salt solution to a suitable concentration series. Subsequently, 0.1 ml of each dilution was added to 0.1 ml of an L_{1210} suspension culture of 103 cells/ml and 1.0 ml agar medium in triplicate. The cells were incubated at 37°C in an atmosphere of 10% CO_2 in humidified air for eight days. After eight days, the colonies were counted. At 100% plating efficiency, 100 colonies should arise in the dishes of 100 control cells which were not treated with the drug. Cells which were inhibited in their growth by the drug did not form colonies. The response level of controls was determined as the mean number of 9 dishes. From the 3 dishes of each concentration of the compound, the mean number of colonies was calculated; variations were usually less than 15–20%. Calculation of the ID_{75} proceeded via a computerized procedure. Variations in $\log(1/ID_{75})$ values of the same compound, which was verified for 6 different compounds (41, 47, 49, 55, 59 and 60) are, in general, less than 10%. Indications for the precision in these values can be seen in Figure 3e.

In-vivo *tumor models*

Generally, only compounds which show activity in the clonogenic assay were tested in *in-vivo*-tumor models. In these experiments, the survival time of treated tumor-bearing mice was determined and expressed as median life span of treated (T) over untreated (controls, C) mice ($T/C \times 100\%$). Differences in the metabolism of tumor and normal cells may be responsible for more selective cytotoxicity of a compound against tumor cells in a particular dose range. Consequently, dose-response curves of *in-vivo* experiments may contain a part where the compound is active towards tumor cells without being toxic to normal cells. At increasing dosages, toxicity to all cells may occur and T/C values decrease. This results in a biphasic dose-response curve, containing occasionally a third, horizontal part (Figure 2b). Some compounds may have a very narrow, steep dose-response curve, demonstrating toxic properties. Several end-points can be chosen from the dose-response curve, *e.g.*, the minimum effective dose (*MED*), the maximum effective dose (or optimum dose, *OD*) or the ratio between these two doses (*OD*/*MED*), as a therapeutic index (*TI*). We usually considered that a minimum T/C value of 125% is required for a compound to be significantly active in the test system[51]. Compounds with lower T/C values were regarded as inactive.

Compounds were dissolved in Hanks-Hepes, containing max. 20% EtOH or 30% dimethyl sulfoxide, if necessary. Insoluble compounds were suspended in 2% carboxymethylcellulose. Dosages were initially based upon LD_{50} values which had first to be determined. Later, relationships between lipophilic properties and antitumor activity were used to calculate the dosage at which antitumor activity was expected to occur (see Discussion). Injections of 0.01 ml per gram mouse body weight were given intraperitoneally (i.p.). Five leukemic mice were injected i.p. on day 1 with each dose of the compound; ten leukemic mice were used as untreated controls. After determination of the survival time, the median life-span of treated over control mice was determined and expressed at $T/C \times 100\%$. For B_{16} melanoma treatment, mice were injected i.p. on day 1, 2, 3, ... 9 with the drug.

D_{125} and *OD* values were determined from the dose-response curves as described by Dunn[52]. Only one experiment was performed for each compound.

Data analysis

Linear, non-linear and step-wise regression analyses were performed using the HP 98820 A Statistical Library program on a HP 310 microcomputer, equipped with an HP 7475 A3 plotter (all Hewlett-Packard). For equiscalarity reasons, in regression analysis, *MR* values of the benzoquinone series, having 4 substituents attached to the quinone moiety, were divided by 20, those of the naphthoquinone series, bearing 2 substituents, by 10.

Results

Data obtained from electrochemical, chemical-stability and lipophilicity studies of the compounds are summarized in Table III. Results from biological-activity studies are described in Tables IV and V.

QSAR against data obtained from L_{1210} clonogenic assay

1,4-Benzoquinones. From 32 aziridinylbenzoquinones examined, 27 revealed a higher cytotoxic activity than AZQ, 13 than Carboquone and 2 than Trenimon. A qualitative trend between cytotoxicity in this assay of a limited number of compounds and aziridine protonation of the hydroquinone has been reported previously[41]. However, poor quantitative correlations between $\log(1/ID_{75})$ and $\log(k')$, $\log(k_{obs})$, $E_{1/2}$, $\sigma_{p,tot}$, pK_{red2} or $MR/20_{tot}$, separately or in combination, were obtained. Plots of $\log(1/ID_{75})$ of the benzoquinone series *vs.* some of the parameters mentioned above are presented in Figure 3.

1,4-Naphthoquinones. Much lower cytotoxic activity is observed in the naphthoquinones series (Table IV). Although no well-known reference compound of the mono(1-aziridinyl)naphthoquinone type is available for

Table III Data of aziridinylquinones.

Compound No.	log(*k*)[a]	log(k_{obs})[b]	$E_{1/2}$[c]	pK_{red2}[d]	$\sigma_{p,tot}$[e]	F_{tot}[e]	R_{tot}[e]	MR_{tot}[f]	$HB_{1,tot}$
27	−0.44*	−2.57	−0.105	7.5	−0.50	−0.10	−0.40	1.46	2
28	0.15	−1.55	−0.115	8.1	−0.54	−0.12	−0.42	1.93	2
29	0.48	−4.70	–	–	−0.04	0.74	−0.76	2.24	2
30	1.00	−3.45	–	–	−0.08	0.72	−0.78	2.71	2
31	0.35	−4.70	−0.113	–	−0.04	0.72	−0.70	1.96	2
32	0.88	−4.15	−0.125	–	−0.08	0.70	−0.72	2.43	2
33	−0.15	−4.03	−0.087	7.2	−0.38	0.76	−1.08	1.45	2
34	0.44	−3.25	−0.093	7.9	−0.42	0.74	−1.10	1.92	2
35	−0.21*	−3.26	−0.179	8.0	−0.74	−0.30	−0.44	2.15	4
36	0.29	−2.61	−0.187	8.6	−0.78	−0.32	−0.46	2.62	4
37	−0.83*	–	–	–	−1.82	−0.04	−1.78	1.90	8
38	−0.37*	–	−0.415	8.5	−2.18	−0.48	−1.70	2.39	6
39	0.15	–	−0.400	9.3	−2.22	−0.50	−1.72	2.86	6
40	−0.70*	−2.80	−0.385	9.5	−2.24	−0.42	−1.82	3.05	6
41	−0.48*	−4.25	−0.149	8.3	−0.80	0.18	−0.96	3.48	8
42	0.04	−3.57	−0.145	8.5	−0.84	0.16	−0.98	3.95	8
43	−0.05	–	−0.225	9.7	−0.88	−0.16	−0.72	3.53	4
44	0.50	–	−0.315	10.6	−2.06	0.42	−1.64	3.65	4
45	1.04	–	−0.303	11.1	−2.10	−0.44	−1.66	4.12	4
46	1.05	–	–	–	−0.52	0.08	−0.58	3.89	0
47	0.29	−3.59	−0.227	8.7	−0.82	−0.17	−0.65	2.16	2
48	−0.42*	−4.00	−0.209	8.6	−0.83	−0.19	−0.64	2.24	2
49	−0.43*	−4.05	−0.213	8.9	−0.76	−0.18	−0.60	2.71	2
50	0.19	−3.67	−0.235	9.7	−0.80	−0.18	−0.62	3.18	2
51	0.44*	−3.65	−0.182	8.6	−0.74	−0.16	−0.38	3.05	3
52	0.28	−4.45	−0.185	8.7	−0.44	0.30	−0.70	2.09	2
53	0.76	−3.85	−0.194	9.2	−0.48	0.28	−0.72	2.56	2
54	1.12	−1.15	–	–	–	–	–	–	2
55	0.51	−4.30	−0.210	8.8	−0.42	0.29	−0.67	2.32	2
56	−0.06	−4.49	−0.201	8.9	−0.36	0.32	−0.64	2.87	2
57	0.20	−4.32	−0.197	8.5	−0.44	0.27	−0.68	1.95	2
58	−0.45*	−2.85	−0.171	8.0	−0.75	−0.15	−0.60	2.08	3
59	−0.31*	−3.75	−0.171	7.8	−0.69	0.28	−0.94	2.08	3
60	0.51	–	−0.195	8.2	−0.75	0.25	−0.97	2.97	3
61	−0.46*	−3.70	−0.245	7.7	−1.00	−0.20	−0.80	2.72	4
82	0.35	−2.85	−0.259	–	−0.25	−0.04	−0.21	1.35	1
83	0.61	−1.87	−0.252	–	−0.27	−0.03	−0.24	1.82	1
84	0.77	−4.77	−0.287	–	−0.02	−0.37	−0.36	1.96	1
85	0.25	−3.25	−0.414	–	−0.91	−0.02	−0.89	1.90	4
86	−0.21*	−4.65	−0.285	–	−0.25	0.24	−0.47	2.85	4
87	−0.04	−4.80	−0.269	–	−0.28	0.19	−0.46	3.33	4
88	0.21	−4.35	−0.285	–	−0.40	0.10	−0.49	3.47	4
89	0.42	−3.75	−0.261	–	−0.42	0.11	−0.52	3.93	4
90	0.59	−4.70	−0.288	–	−0.40	0.10	−0.49	3.93	4
91	0.84	−4.03	−0.283	–	−0.42	0.11	−0.52	4.40	4
92	0.54	−4.65	−0.317	–	−0.36	−0.05	−0.31	3.93	3

[a] Determined in [or extrapolated (*) to] MeOH/0.5M-NaH_2PO_4 50/50 w/w. [b] At pH 4.0, in s^{-1} (Scheme 1). [c] Obtained from direct-current polarography at pH 7.0, in V (Scheme 2). [d] Obtained from direct-current polarography (Scheme 2). [e] From Ref. 44. [f] Read *MR*/10 for compounds 82–92, *MR*/20 for all other compounds.

comparison of activities, four quinones (84, 86, 87 and 91) reveal relatively high cytotoxicity. In analogy to the benzoquinone series, poor quantitative relationships were found.

QSAR against data obtained from L_{1210} leukemic mice

1,4-Benzoquinones. Dose-response curves of 30 compounds are available (Tables IV and V). It is apparent from these data that only seven analogs (31, 33, 34, 45, 52, 60, 94) are inactive. Three compounds (32, 54, 55) reveal low activity (*T/C* 125%), whereas the remaining 20 derivatives are more active, with *T/C* values of 256 (compound 56), 313 (compound 51, Carboquone) and 400 (compound 40, BZQ).

The following equations could be obtained (D_{125} in mmol/kg, data of compound 39 not included, see below):

$$\log(1/D_{125}) = -1.308(\pm 0.189)\cdot\log(k') + 2.217(\pm 0.095) \quad (3)$$

$n = 20$ $r^2 = 0.728$ $sd = 0.425$ $F = 48.08$ $F_{1,18,0.999} = 15.38$

$$\log(1/D_{125}) = -1.415(\pm 0.191)\cdot\log(k') - 0.085(\pm 0.051)\cdot HB_{1,tot} + 2.487(\pm 0.190) \quad (4)$$

$n = 20$ $r^2 = 0.766$ $sd = 0.406$ $F = 27.85$ $F_{2,17,0.999} = 10.66$

n is number of compounds
r^2 is square of correlation coefficient
sd is standard deviation
F is distribution of variance ratio
$F_{x,y,z}$ is F value for a regression analysis using x parameters, y data points/observations with a confidence of z

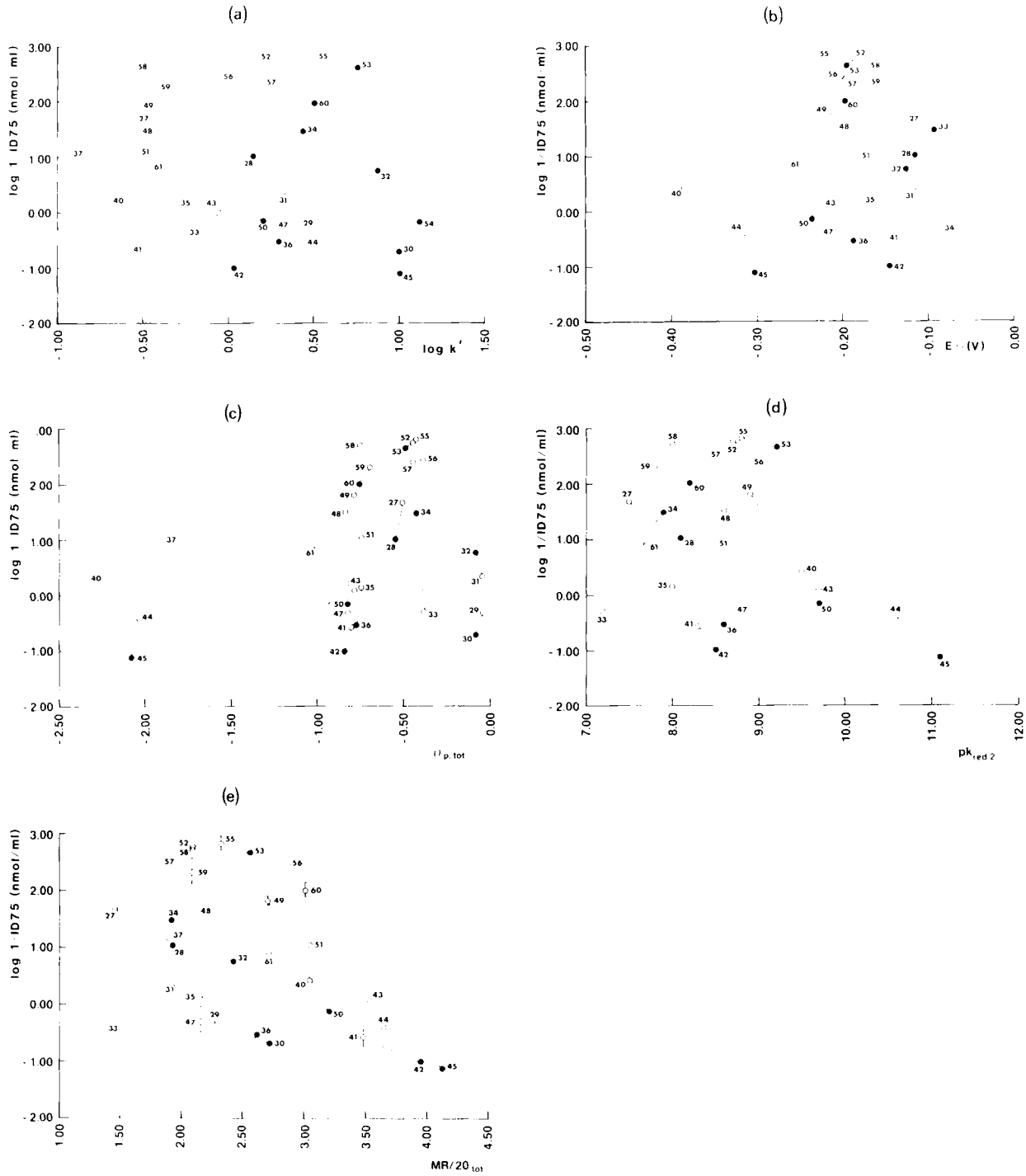


Figure 3. Plot of $\log(1/ID_{75})$ vs. *(a)* $\log(k')$, *(b)* $E_{1/2}$, *(c)* $\sigma_{p,\mathrm{tot}}$, *(d)* pK_{red2} *and (e)* $MR/20_{tot}$ *of aziridinylbenzoquinones* o.....● *represents influence of methyl substitution of the aziridines:* o *aziridine,* ● *methylaziridine, numbers correspond to compound code in Table I.*

The only parameters relevant for activity in this tumor model appeared to be $\log(k')$ and HB_1. Correlations of $\log(1/OD)$ yielded virtually similar relationships with, however, slightly less statistical significance. Other parameters, alone or in combination, yielded poor correlations ($r^2 < 0.5$, data not presented). A plot of $\log(1/D_{125})$ *vs.* $\log(k')$ and a graphical description of Eqn. 3 are given in Figure 4a.

Relationships such as Eqns. 3 and 4 were used as an indication for the dose range to be used in the *in-vivo* testing procedure. Initially, the calculated D_{125} values, using relationships similar to Eqns. 3 and 4, corresponded fairly with those obtained from the dose-response curves. However, when Eqns. 3 and 4 were used to calculate D_{125} values of compound 39, relatively large deviations from the calculated values were obtained (Table V).

1,4-Naphthoquinones. From the 11 naphthoquinones available, 7 were tested in the L_{1210} tumor model. Unfortunately, the decrease in toxicity, as reflected by an increase of the LD_{50}, is also accompanied by a decrease in toxicity towards the leukemic cells, since no significant antitumor activity was observed with these compounds (Table IV).

Table IV Biological data of aziridinylquinones.

Compound No.[l]	L_{1210}[a] ID_{75}[d]	LD_{50}[e]	L_{1210}[b]				B_{16}[c]			
			D_{125}[f]	OD[g]	T/C_m[h]	TI[i]	D_{125}[f]	OD[g]	T/C_m[h]	TI[i]
27	0.02	16	1.7	5.3	178	3.1	0.7	1.6	159	2.3
28	0.09	92	22.9	45.9	150	2.0	7.6	18.3	165	2.4
29	2.06	–[j]	–	–	–	–	–	–	–	–
30	4.93	–	–	–	–	–	–	–	–	–
31	0.45	–	*[k]	*	113	*	–	–	–	–
32	0.17	–	89.3	89.3	125	1.0	–	–	–	–
33	1.93	–	*	*	100	*	–	–	–	–
34	0.03	–	–	–	–	–	–	–	–	–
35	0.71	–	4.2	8.0	175	1.9	0.3	0.8	169	2.8
36	4.69	–	33.8	71.9	150	2.1	–	–	–	–
37	0.07	–	–	–	–	–	–	–	–	–
40	0.53	–	0.4	5.8	400	14.1	0.1	1.3	238	11.8
41	4.06	29	5.5	16.4	189	3.0	4.8	5.5	135	1.1
42	9.97	> 204	25.5	102.0	188	4.0	–	–	–	–
43	0.81	–	–	–	–	–	–	–	–	–
44	2.46	–	8.5	30.5	188	3.6	–	–	–	–
45	13.29	–	*	*	100	*	–	–	–	–
47	2.02	< 34	–	–	–	–	0.6	1.3	159	2.3
48	0.03	< 40	0.4	1.6	200	3.8	0.3	0.8	190	3.2
49	0.02	11	1.4	5.4	211	3.9	0.3	1.8	200	5.4
50	1.42	–	12.5	25.1	200	2.0	–	–	–	–
51	0.09	19	0.6	6.3	313	10.0	0.5	2.5	212	5.2
52	0.002	35	*	*	100	*	8.1	10.6	139	1.3
53	0.002	> 80	16.1	40.2	163	2.5	19.3	27.3	129	1.4
54	1.44	> 236	208.0	236.0	125	1.1	–	–	–	–
55	0.002	47	39.1	40.4	125	1.0	–	–	–	–
56	0.004	90	10.1	70.2	256	7.0	2.1	22.5	182	10.7
57	< 0.004	–	10.2	20.9	200	2.1	–	–	–	–
58	0.002	2	0.3	1.3	211	4.3	0.2	0.9	224	5.4
59	0.005	24	11.1	16.1	156	1.5	*	*	105	*
60	0.01	28	*	*	100	*	–	–	–	–
61	0.13	–	–	–	–	–	–	–	–	–
82	11.86	553	*	*	100	*	*	*	100	*
83	8.94	> 188	*	*	100	*	–	–	–	–
84	0.06	26	*	*	100	*	*	*	100	*
85	9.27	–	–	–	–	–	–	–	–	–
86	1.39	> 156	*	*	100	*	–	–	–	–
87	2.06	–	–	–	–	–	–	–	–	–
88	10.08	455	*	*	100	*	*	*	100	*
89	15.50	> 267	*	*	100	*	–	–	–	–
91	1.92	> 80	*	*	100	*	–	–	–	–
92	16.24	–	–	–	–	–	–	–	–	–

[a] *In vitro* clonogenic assay. [b] *In vivo*, single i.p. (intraperitoneal) injection on day 1. [c] *In vivo*, i.p. injection on day 1, 2, 3 ... 9. [d] Concentration (in nmol/ml) required to inhibit growth of 75% of control cells. [e] LD_{50} (μmol/kg). [f] Dose required to give T/C of 125% (μmol/kg). [g] Dose giving optimum (maximum) T/C (μmol/kg). [h] Maximum T/C. [i] OD/D_{125}. [j] –: data not yet available. [k] *: $T/C_m < 125\%$. [l] No data available for compounds 38, 39, 46 and 90.

Table V Effect of size of quinone substituents on biological activity in L_{1210} and B_{16} in-vivo tumor models of aziridinylquinones with equal lipophilic properties (data from Tables III and IV).

Compound No.	MR_{tot}	L_{1210}	B_{16}
		D_{125}	D_{125}
58	2.08	0.30	0.16
48	2.24	0.42	0.25
51	3.05	0.62	0.48
49	2.71	1.37	0.33
27	1.46	1.68	0.68
41	3.48	5.48	4.77

QSAR against data obtained from B_{16} melanoma-bearing mice

1,4-Benzoquinones. Dose-response curves of 23 compounds were obtained (Table IV). Compounds 32, 34, 38, 59 and 60 did not show significant activity in this system, despite the high activities of most of these compounds in the clonogenic assay. Consequently 16 compounds having significant antitumor activity against B_{16} melanoma remain for statistical evaluation of the biological data. It can be concluded from Tables IV and V, that high antitumor activity is observed with this type of compounds.

Step-wise regression analysis of the data revealed a potentially important role for lipophilicity [$\log(k')$, Figure 4b] and electronic effects ($\sigma_{p,tot}$). The most relevant equations

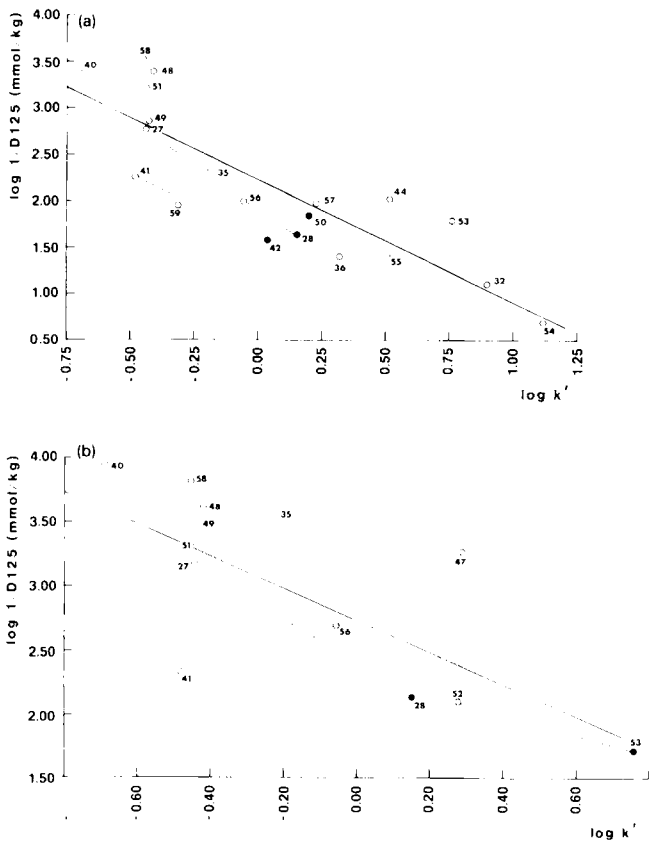


Figure 4. Plot of $\log(1/D_{125})$, *obtained from (top)* L_{1210} *and (bottom)* B_{16} in vivo *experiments, vs.* $\log(k')$ *obtained from reversed-phase HPLC of (top) 20, (bottom) 13 aziridinylbenzoquinones; numbers correspond to compound code in Table I.* ○ ..... ● *represents influence of methyl substitution of aziridines:* ○ *aziridine,* ● *methylaziridine. Regression lines correspond to (a) Eqn. 3 and (b) to Eqn. 5.*

obtained are (D_{125} in mmol/kg, data of compounds 39, 42 and 44 not included):

$$\log(1/D_{125}) = -1.293(\pm 0.350)\cdot\log(k') + 2.788(\pm 0.153) \quad (5)$$

$n = 13$ $r^2 = 0.554$ $sd = 0.511$ $F = 13.65$ $F_{1,11,0.995} = 12.23$

$$\log(1/D_{125}) = -1.577(\pm 0.379)\cdot\log(k') - 0.129(\pm 0.084)\cdot HB_{1,\mathrm{tot}} + 3.139(\pm 0.271) \quad (6)$$

$n = 13$ $r^2 = 0.638$ $sd = 0.482$ $F = 8.83$ $F_{2,10,0.990} = 7.56$

$$\log(1/D_{125}) = -1.318(\pm 0.342)\cdot\log(k') - 0.199(\pm 0.076)\cdot HB_{1,\mathrm{tot}} - 0.714(\pm 0.322)\cdot\sigma_{p,\mathrm{tot}} + 2.846(\pm 0.265) \quad (7)$$

$n = 13$ $r^2 = 0.767$ $sd = 0.408$ $F = 9.85$ $F_{3,9,0.995} = 8.72$

Using $\log(1/OD)$, similar relationships were obtained. A graphical description of Eqn. 5 is presented in Figure 4b. Compound 47 reveals remarkably high activity in this system, which is not expected from its lipophilic properties. AZQ (41) reveals too low activity, despite its proper lipophilic properties. Since data of 13 compounds are not sufficient to allow the use of three parameters in regression analysis[53], Eqn. 7 should be considered with care: the improved correlation may be misleading. An indication for the validity of these relationships was found by calculating D_{125} values for compounds 39, 42 and 44. It appeared that the improved statistical features of the regression equation upon addition of $\sigma_{p,\mathrm{tot}}$ (Eqn. 7) are not accompanied by more accurate D_{125} values when calculated using this equation.

1,4-Naphthoquinones. The 3 compounds of the naphthoquinone type tested in the B_{16} model so far did not show a significant increase in life-span.

Discussion

Substituent effects on cytotoxicity in L_{1210} *clonogenic assay*

Unfortunately, our attempts failed to develop a clonogenic assay with which cytotoxicity under *anaerobic* conditions could have been determined. Under aerobic conditions, cytotoxicity of the aziridinylquinones may originate from several mechanisms, as outlined previously. The absence of cytotoxic activity of quinones lacking the aziridinyl moieties ($ID_{75} > 10$ μg/ml, data not presented) indicates that the aziridinyl rings are required for cytotoxicity, most likely because of their alkylating properties.

Lipophilicity seems to be the most important factor for antitumor activity *in vivo*, as will be discussed later in this section. In the clonogenic assay, however, lipophilic properties of the quinones appear to be less important (Figure 3a), probably due to the fact that a long period of interaction for drug and cells is available. In the upper part of this plot, where compounds with high activity are positioned, a range in $\log(k')$ from -0.75 to $+0.85$ occurs for these compounds. This indicates that, as well as lipophilicity, other processes are also involved, *e.g.*, generation of toxic oxygen or halide radicals and/or alkylation processes.

It can be deduced from Figures 3b and 3c that electronic effects may be of importance for cytotoxicity in this assay. Nearly all analogs with high cytotoxic activity [$\log(1/ID_{75}) > 1.0$, $ID_{75} < 0.1$ nmol/ml] have an $E_{1/2}$ value more positive than -0.25 V (at pH 7.0), or a $\sigma_{p,\mathrm{tot}}$ value smaller than -1.0; compounds 37 and 40 (BZQ) also reveal relatively high activity, despite the presence of strongly electron-donating substituents and negative $E_{1/2}$ values (*e.g.*, -0.385 V for compound 40).

In the plot of $\log(1/ID_{75})$ *vs.* pK_{red2} (Figure 3d), analogs having high activity show a pK_{red2} range from 7.5 to 9.2. However, in this range, compounds with low activity are also observed. Compounds with pK_{red2} values higher than 9.2 also seem to have low activity in this test system. In the latter case, large substituents are attached at the quinone moiety, which seems to decrease activity (Figure 3e). Substitution of the aziridine with a methyl group results in an increase of lipophilicity, pK_{red2}, $\log(k_{obs})$ and $MR/20_{tot}$ (Figure 3, Table III) and, generally, in loss of activity, which is in accordance with the reported decrease in DNA alkylation upon aziridine methylation[24].

Only qualitative interpretation of the data obtained from the clonogenic assay seems possible. The highest cytotoxic action was observed for compounds with three aziridinyl rings (58, 59 and 60) or two aziridine substituents and one halogen and one alkyl substituent (52, 53, 55, 56 and 57). Chemical decomposition during the period of incubation of compounds 29, 30, 31, 32 and 54, observed during electrochemical-analysis and chemical-stability studies, may have influenced their activities in the assay. Steric hindrance of the compound to reach a nucleophilic alkylation center by relatively large substituents at positions 3 and 6 of the quinone moiety may explain the low activity of compounds 41, 42, 43, 44 and 45. Too low pK_{red2} values of compounds 31 (estimated) and 33 may be responsible for their low cytotoxic actions; methyl substitution of the aziridines improv-

ing activity (compounds 32 and 34), although generally this decreases activity.

The naphthoquinones examined in this study contain, in general, only one alkylation center and may thus exert their cytotoxic action via mono-alkylation and/or via a different, *e.g.*, radical-involving, mechanism. Relatively low activities were observed compared with the bis(1-aziridinyl)benzoquinones. The activity observed seems not to correlate with any of the parameters investigated in this study. Since $E_{1/2}$ values of the naphthoquinones reveal potential reduction of the quinone moiety under physiological conditions, thus enabling the formation of activated oxygen species, the present results may point to a minor role for such species in the mechanism of action of these quinones in this system. The toxic effect may be a result of mono-functional alkylation by the semi-quinone free radical or the hydroquinone.

Substituent effects on cytotoxicity in L_{1210} leukemic mice

The reference aziridinylquinoid cytostatics AZQ, Carboquone and Trenimon reveal almost identical log(k') values, whereas BZQ is more hydrophilic. BZQ reveals very high anti-leukemic activity *in vivo* (T/C 400%). AZQ and its methyl-substituted analog 42 also cause a significant increase of life-span, T/C 188%, which was not predicted by the clonogenic assay. Carboquone (T/C 213%) and compound 56 (T/C 256%) also show high activity in this tumor model. Compounds 52, 55 and 60 do not exhibit antitumor activity in L_{1210} leukemic mice, despite their high cytotoxicity in the clonogenic assay (Table IV). Compounds 27, 28, 53 and 59 also reveal a lower activity than expected from the assay.

It is clear from Figure 4a and regression equations 3 and 4 that lipophilicity plays the most important role in this tumor model. The slope of the plot points to an increase in antitumor activity on increasing lipophilicity of the compound, which is in accordance with other reports[23,32,33]. In fact, it is the only parameter, separately or in combination with the hydrogen-bond-forming ability describing parameter $HB_{1,\mathrm{tot}}$, having some predictive value. The observation that addition of the latter parameter improves the relationship found may be regarded as a correction of log(k'), reflecting the partitioning properties in a biological system more properly. However, D_{125} values calculated using Eqn. 4 deviate from those observed or calculated using Eqn. 3. Addition of $HB_{1,\mathrm{tot}}$ does not, therefore, result in more accurate values of D_{125}, despite the improved statistical significance of Eqn. 4.

Evaluation of the data of compounds 58, 48, 51, 49, 27 and 41, which are analogs with virtually equal log(k') values, reveals no trend for $E_{1/2}$, k_{obs} or pK_{red2} and antitumor potency. It can be concluded that these parameters are insignificant for predicting activity of these compounds in this tumor model. For $E_{1/2}$, this is not unreasonable considering that virtually all aziridinylquinones are relatively easy to reduce.

The observed differences in antitumor activity of compounds 41, 27, 49, 51, 48 and 58 may be also affected by variations in the steric properties of the substituents. A trend towards an increase in activity is observed upon decrease in the size of the substituents (Table V). Compound 27 may have decomposed during the test period. A quantitative description of substituent effects on the therapeutic index, *TI*, defined as OD/D_{125}, could not be obtained. From Table IV, it can be concluded that an increase in the therapeutic index is generally accompanied by an increase in the maximum T/C value. In some cases, D_{125} values point to less activity (*e.g.*, compounds 41, 42), whereas the therapeutic index remains the same. Consequently, it is advisable to consider all possible end-points (D_{125}, *OD*, *TI*, T/C_{m}) to classify a compound as active or inactive.

All naphthoquinones studied are inactive, probably as a result of their increased lipophilic properties and their lack of ability to form cross-linkages.

Interestingly, the methyl analogs of AZQ and 52, *viz.*, compounds 42 and 53, show equal (42) or higher (53) activity (TI) than their non-substituted analogs. This is in contrast to the *in-vitro* data and data reported by *Lusthof* et al. on DNA alkylation[24] and on the biological activity of AZQ and some of its analogs in L_{1210} leukemic mice by *Khar* and *Driscoll*[20] which reveal a decrease of activity upon substitution of the aziridinyl ring. In addition, compound 56 was reported to be less active than, *e.g.*, compound 49[22], whereas this compound reveals a higher T/C value and a better therapeutic index in our test system.

Substituent effects on cytotoxicity in B_{16} melanoma bearing mice

Lipophilicity, which can be described by log(k') and $HB_{1,\mathrm{tot}}$ (Eqns. 5 and 6), also plays a dominant role in the observed antitumor activities in the B_{16} model, in analogy to antitumor activity in the L_{1210} system (Figure 4b). Compound 47 reveals a remarkable antitumor activity, which cannot be explained by log(k'). Again, compounds 58, 48, 51, 49, 27 and 41, which are analogs with virtually equal log(k') values, reveal large variations in activity. Although no trend is evident for $E_{1/2}$ or pK_{red2} and antitumor potency, electronic properties may play a role. Addition of these parameters or of the electronic parameter $\sigma_{p,\mathrm{tot}}$ slightly improves the relationship found (only Eqn. 7 given). The negative sign of the coefficient of $\sigma_{p,\mathrm{tot}}$ points to the necessity for electron-donating substituents to enhance activity. In contrast to the data obtained from L_{1210} experiments, electronic factors may also, therefore, play a role in the B_{16} system. It is, however, not absolutely clear from the data currently available whether quinone reduction or subsequent protonation of the aziridines is involved.

The differences in antitumor activity of the 6 compounds with equal lipophilicity may again be a result of steric hindrance (Table V). Aziridinylquinones of the 1,4-naphthoquinone type are inactive. The observed antitumor activity of most analogs of the 1,4-benzoquinone type tested up to now, however, is promising. The lower activity of AZQ (41) compared with activity in L_{1210} is in accordance with data reported by others[20]. No biological data from the series of Carboquone analogs (47–57) in B_{16} melanoma-bearing mice have been published for comparison with our data.

Prospects for future drug design

From the results of this study, we conclude that a clear correlation exists between antitumor activity *in vivo* and lipophilicity (Eqns. 3–6). During this study, similar relationships have been successfully applied to predict D_{125} values of new compounds, which has facilitated the *in-vivo* testing procedure significantly.

The use of QSAR studies of bioreductive alkylating quinones of a parameter which gives information on the alkylating step of the mechanism of action has not been reported previously. Unfortunately, no clear trend is so far evident between aziridine protonation and antitumor activity. However, an electronic effect, which may be described by $E_{1/2}$ or $\sigma_{p,\mathrm{tot}}$, may be of importance in the B_{16} system. The signs of the coefficients suggest that hydrophilic compounds with small electron-donating substituents will

show highest activity. The negative sign of the coefficient of $\sigma_{p,\,tot}$ (Eqn. 7) indicates that less easily reducible quinones are the most active. This may be an advantage, since reduction of the quinone moiety by normal cells will also occur less easily, thus probably leading to a decreased systemic toxicity. There is some room for electron-donating substituents to become attached to the quinone moiety without making quinone reduction impossible. An additional advantage of this will be that protonation of aziridines after quinone reduction is favored upon an increase of $\sigma_{p,\,tot}$ to more negative values. It is questionable, however, whether electron-donating functions stronger than the 1-pyrrolidinyl substituents (compounds 44 and 45) and methylamino groups (38 and 39) may be found. These compounds have been synthesized based upon the assumption that high pK_{red2} values might be obtained, accompanied by a negative value of $E_{1/2}$. Unfortunately, lipophilicity of these compounds is too high for good antitumor activity.

The presence of small, hydrophilic electron-donating amino functions is expected to result in enhanced antitumor activity. The high LD_{50}, *TI* and T/C_m values of compound 56 in the *in-vivo* tests and the high ID_{75} values of compounds 52, 53, 55 and 57 in the clonogenic assay has also stimulated the synthesis of new analogs of this type. Evaluation of a larger series of compounds of equal hydrophilicity may yield more information on the potential importance of steric and electronic properties in relation to antitumor potency.

The concept of bioreductive alkylation[14,54] as a mechanism of action of aziridinylquinones is supported by these results. The absence of activity of compounds lacking the aziridinyl rings, the good activity of many of these compounds towards aerobic cancer cells, the high stability towards nucleophilic attack of the quinone at physiological pH and the ease with which these quinones can be reduced at physiological pH are indications for the involvement of an alkylation mechanism by free radical species in antitumor action. The high activity observed in the B_{16} model may point to alkylation by the hydroquinone. Most of the aziridinylquinones examined in this study are relatively easy to reduce, especially when compared with other quinoid cytostatic agents (*e.g.*, mitomycin C, $E_{1/2}$ −0.350 V at pH 7.0[55]. Even compound 40 (BZQ), having a $E_{1/2}$ of −0.385 V, shows significant activity in the tumor models used. It can be concluded from these $E_{1/2}$ values and from the results of this QSAR study that reduction of the quinone moiety may not be the limiting step for antitumor activity. Subsequent protonation of the aziridines may be either non-limiting or is poorly described by pK_{red2}. More research has to be performed to explain all activities observed in the present study.

Acknowledgement

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