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MICROSOMAL SUPEROXIDE ANION PRODUCTION AND NADPH-OXIDATION IN A SERIES OF 22 AZIRIDINYLBENZOQUINONES

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Abstract—Several 2,5-bis(1-aziridinyl)-1,4-benzoquinones (BABQs) can be activated to alkylating species by reduction of the quinone moiety. On the other hand, cytotoxicity of these compounds can be induced by redox cycling. A series of BABQs and their methylated analogues (BMABQs) with different substituents at the 3- and 6-position was synthesized in order to investigate the influence of the substituents on the reduction of the quinone moiety and on the generation of superoxide anion radicals with rat liver microsomes. Superoxide anion production (SAP) ranged from 3.7 ± 0.1 to 742 ± 74 nmoles/min/mg protein with quinone concentrations of 10 nmoles/ml. NADPH-oxidation was measured under the same conditions and it correlated well ($r = 0.88$, $P < 0.001$) with SAP. It ranged from 1.4 ± 0.2 to 494 ± 60 nmoles/min/mg protein. SAP for 22 B(M)ABQs showed a good correlation with the summated electronic substituent constant $\sigma_{para,total}$ ($r = 0.86$, $P < 0.001$). It can be concluded that superoxide anion production by 22 B(M)ABQs in rat liver microsomes can be predicted from structural features of the compounds.

The concept of bioreductive activation has been developed since 1972 to explain the antitumor activity of quinone-containing antibiotics [1]. This concept has been used to obtain drugs with selectivity for hypoxic cells [2, 3]. Hypoxic cells exist in solid tumors in regions of poor vascularization where a reductive environment might prevail. Since oxygen-deficient cells have a limited sensitivity to radiotherapy and most chemotherapy, the development of drugs that are active to hypoxic cells is very important [4]. Well known examples of bioreductively activated cytostatic drugs are the quinone compounds Adriamycin® and mitomycin C [2, 5].

At present, two classes of bioreductively activated drugs are in clinical use: the quinone containing alkylating agents in chemotherapy and the hypoxic cell sensitizers (e.g. nitroimidazoles) in radiotherapy [4]. The clinical use of these compounds often is limited by dose-dependent side effects. These effects probably are caused by reactive oxygen intermediates that are formed by redox cycling of the quinone moiety [6]. Based on AZQ‡ and related compounds evaluated by Driscoll *et al.* [7] we synthesized a series of 2,5-bis (1-aziridinyl)-1,4-benzoquinones and methylated analogues (Table 1, Fig. 1). These compounds contain two active parts: the quinone moiety and the alkylating aziridinyl groups. Reduction of the quinone moiety to the hydroquinone facilitates protonation and opening of the aziridine ring [8]. After one-electron reduction and the formation of a semiquinone reactive oxygen species can be formed by redox cycling of the quinone moiety [10]. Semiquinone formation has been shown to occur for AZQ too [11]. For several quinones it is assumed that superoxide anions formed by redox cycling play an important role in the acute cytotoxicity [12]. In the development of quinone-containing cytostatic drugs it is important to predict these acute effects by redox cycling in an early stage.

In this study we investigated the ability of a series of 2,5-bis(1-aziridinyl)-1,4-benzoquinones for redox cycling with rat liver microsomes. The electrochemical parameters and the antitumor activity of these compounds bearing different substituents at the 3- and 6-position were studied by Driebergen *et al.* [8]. The results of measurements of redox cycling are compared with several structural parameters of the substituents.

MATERIALS AND METHODS

Compounds. The compounds that are used in this study are designated by TW-numbers and presented in Table 1.

BABQ (TW 13), BMABQ (TW 29), 3,6-dichloro-BABQ (TW 14), 3,6-dichloro-BMABQ (TW 18), 3,6-dibromo-BABQ (TW 16) and 3,6-dibromo-BMABQ (TW 17) were prepared as described previously [13]. 3,6-Difluoro-BABQ (TW 19) 3,6-difluoro-BMABQ (TW 20) and 3-(1-aziridinyl)-6-fluoro-BABQ (TW 26) were prepared as described by Chou *et al.* [14]. 3-Methyl-6-bromo-BABQ (TW 22), 3-ethyl-6-bromo-BABQ (TW 25) and 3-[(2-carbamoyloxy)-1-ethyl]-6-bromo-BABQ (TW 50), 3-[2-(carbamoyloxy)-1-ethyl]-6-methyl-BMABQ (TW 87) were synthesized as described by Nakao *et*

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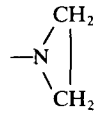
‡ Abbreviations used: AZQ, diaziquone; BABQ, 2,5-bis(1-aziridinyl)-1,4-benzoquinone; BMABQ, 2,5-bis(2-methyl-1-aziridinyl)-1,4-benzoquinone; CQ, carboquone; SOD, superoxide dismutase; SAP, superoxide anion production; SCC, succinoylated cytochrome *c*; and Tre, Trenimon.

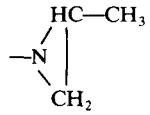
Table 1. Aziridinylbenzoquinones with their substituents and structural features [9]

TW nr.	Substituents R1	R2	Log k' (HPLC capacity factor)	$\sigma_{para,total}$	$E_{1/2}$ (mV)
13	H	H	0.44	−0.50	−105
29-Me	H	H	0.15	−0.54	−115
14	Cl	Cl	0.35	−0.04	−113
18-Me	Cl	Cl	0.88	−0.08	−125
16	Br	Br	0.48	−0.04	—
17-Me	Br	Br	1.00	−0.08	—
19	F	F	−0.15	−0.38	−87
20-Me	F	F	0.44	−0.42	−93
22	Br	CH_3	0.28	−0.44	−185
53-Me	Br	CH_3	0.76	−0.48	−194
25	Br	C_2H_5	0.51	−0.42	−210
50	Br	$C_2H_4OCONH_2$	0.19	−0.36	−201
81	Cl	CH_3	0.20	−0.44	−197
Tre	Az	H	−0.45	−0.75	−171
26	Az	F	−0.31	−0.69	−171
AZQ	$NHCO_2C_2H_5$ (2×)		−0.48	−0.80	−149
73-Me	$NHCO_2C_2H_5$ (2×)		0.04	−0.84	−145
32	CH_3	C_2H_5	0.29	−0.82	−227
40	CH_3	C_2H_4OH	−0.42	−0.83	−209
39	CH_3	$C_2H_4OCONH_2$	−0.43	−0.76	−213
87-Me	CH_3	$C_2H_4OCONH_2$	0.19	−0.80	−235
CQ	CH_3	$CHOCH_3CH_2OCONH_2$	0.44	−0.74	−182

— = not measured.
Log k' = HPLC capacity factor $(r_f-r_o)/r_o$ for 50% methanol/0.5 M Na-phosphate pH 6.5.
$\sigma_{para,total}$ = summated electronic substituent (Hammett) constant.
$E_{1/2}$ = electrochemically determined half wave potential.

Az = aziridinyl group Me = methylaziridinyl group





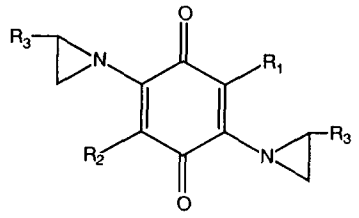


Fig. 1. Chemical structure of aziridinylbenzoquinones. R_1 and R_2 are different substituents and R_3 is a hydrogen or a methyl group.

al. [15]. 3-Methyl-6-bromo-BMABQ (TW 53) and 3-methyl-6-chloro-BABQ (TW 81) were prepared by treatment of the corresponding 2-halo-5-methyl-1,4-benzoquinone with 1-(2-methylaziridine) analogously as described by Nakao *et al.* [16]. 3-Ethyl-6-methyl-BABQ (TW 32), 3-(2-hydroxyethyl)-6-methyl-BABQ (TW 40) and its corresponding carbamate ester (TW 39) were prepared as described by Nakao *et al.* [16]. 3,6-Bis(ethoxycarbonylamino)-BMABQ (TW 73) was synthesized according to Khan and Driscoll [17]. AZQ [2,5-bis(1-aziridinyl)-3,6 - bis(ethoxycarbonylamino) - 1,4-benzoquinone] was a gift from the Drug Synthesis and Chemistry Branch (National Cancer Institute, Maryland) and CQ [2,5-bis-(1-aziridinyl)-3-{2-(carbamoyloxy)-1-(methoxyethyl)}-6-methyl-1,4-benzoquinone] was a gift from the Chemical Research Laboratories (Sankyo Co., Ltd, Tokyo, Japan). 3-(1-Aziridinyl)-BABQ (Trenimon) was a gift from Bayer AG (Pharma Research Center, Wuppertal, F.R.G.).

All 22 TW-compounds were freshly dissolved (5 mM) in dimethylsulfoxide (Merck, scintillation grade). Cytochrome *c* (horse heart, type III) was from Boehringer–Mannheim; NADPH (type X) and SOD (EC 1.15.1.1) were purchased from Sigma Chemical Co., trinitrobenzenesulphonic acid was from Serva and succinic anhydride was from Janssen Chimica.

Methods. Liver microsomes were prepared from male Wistar rats (Hsd/Cpb:WU, Harlan-CPB, Zeist, the Netherlands) in 100 mM Na-phosphate containing 0.1 mM EDTA pH 7.4. Protein was determined according to Lowry *et al.* [18]. Partial succinoylation of cytochrome *c* was performed as described by Kuthan *et al.* [19]. The initial velocity of the SOD-inhibitable reduction of SCC (50 μM) was used for quantitative detection of superoxide

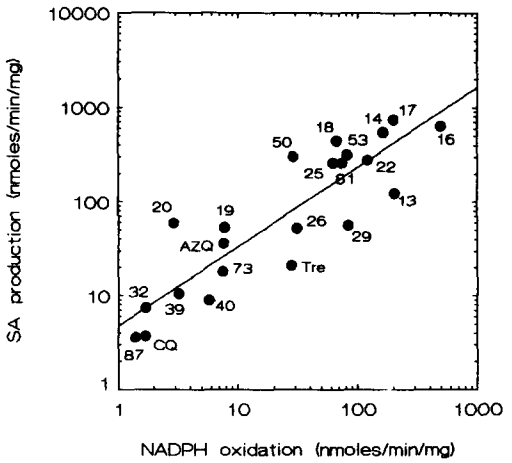


Fig. 2. Microsomal superoxide anion (SA) production (nmoles succinoylated cytochrome *c* reduction/min/mg protein) vs NADPH oxidation (nmoles/min/g protein) for 22 B(M)ABQs (10 μM) under carbogen gassing at 37°. Each point represents the mean of 3 separate measurements for one compound. The numbers refer to the compounds mentioned in Table 1. SAP ranges from 3.7 to 742 nmoles/min/mg and NADPH oxidation ranges from 1.4 to 494 nmoles/min/mg.

anion radicals. The reduction of SCC at 37° under 95% O_2/5% CO_2 was monitored at 550 nm and $\varepsilon_{550} = 19.5\ mM^{-1}$ was used for calculations. NADPH-oxidation was performed under the same conditions and monitored at 340 nm. The reactions were carried out in Milli-Q (Millipore) purified water containing 100 mM Na-phosphate + 0.1 mM EDTA pH 7.4, 10 μM B(M)ABQ in dimethylsulfoxide (1% v/v), SOD (10 IU/ml, if relevant), 0.04 mg/ml microsomal protein and were started with NADPH (400 μM). Basal rates of microsomal NADPH-oxidation and SCC reduction were subtracted from quinone-mediated measurements. Statistical analysis was performed using a multivariate least-squares program from SYSTAT [20]. Physicochemical properties (lipophilicity determined as the HPLC capacity ratio (k') and the electrochemically determined half wave reduction potential $E_{1/2}$) were taken from Driebergen [9] (see Table 1). Substituent constants for electronic (σ_{para}) and steric (MR) effects were from Hansch *et al.* [21] or were calculated by Driebergen [9].

RESULTS

Without added microsomes no direct reaction was observed between the B(M)ABQs and NADPH or between the B(M)ABQs and SCC. In the presence of NADPH, B(M)ABQs and microsomes the reduction of SCC was inhibited more than 95% by SOD (10 IU/ml). Superoxide anion production (SAP) was determined for 22 B(M)ABQs at quinone concentrations of 10 μM. Under nitrogen gassing the reduction of SCC was below 1.0 nmole/min/mg for all compounds. Under carbogen gassing SAP ranged from 3.7 ± 0.1 to 724 ± 74 nmoles/min/mg protein (Fig. 2). For 4 B(M)ABQs bearing two bromo- or chloro-substituents (TW 14,18,16,17) the maximal SAP was already reached at 10 μM. BABQs with one or two fluoro-substituents (TW 19,20,26) showed a much lower SAP at this concentration. For BABQs with one bromo- or chloro-substituent (TW 22,25,50,53,81) a higher SAP was found at 10 μM than with the unsubstituted BABQ (TW 13). The lowest SAP was found for compounds bearing one methyl and one ethyl derivative function (TW 32,39,40, CQ). When the SAP of 7 BMABQs was compared with that of the corresponding non-methylated BABQs there were 4 that showed no significant difference (Table 2). In contrast TW 29, TW 73 and TW 87 showed a significantly lower SAP at 10 μM when compared with the corresponding non-methylated compounds TW 13, AZQ and TW 39.

Table 2. Microsomal superoxide anion production (SAP) and NADPH-oxidation of 22 aziridinylbenzoquinones under carbogen gassing at 37°. Mean ± SE

TW n.	SAP (nmoles/min/mg)	NADPH-oxidation (nmoles/min/mg)
13	123 ± 19	204 ± 24
29-Me	56 ± 9	83 ± 8
14	545 ± 81	164 ± 20
18-Me	445 ± 67	67 ± 9
16	742 ± 74	494 ± 60
17-Me	635 ± 86	200 ± 25
19	53 ± 7	7.7 ± 1.7
20-Me	59 ± 8	2.9 ± 1.3
22	279 ± 39	121 ± 23
53-Me	317 ± 31	81 ± 8
25	260 ± 30	62 ± 8
50	304 ± 26	29 ± 6
81	260 ± 29	74 ± 7
Tre	21 ± 3	28 ± 4
26	52 ± 3	31 ± 5
AZQ	36 ± 4	7.6 ± 1.2
73-Me	18 ± 2	7.5 ± 1.0
32	7.4 ± 1	1.7 ± 0.2
40	8.9 ± 1	5.7 ± 0.6
39	10.4 ± 2	3.2 ± 0.3
87-Me	3.6 ± 0.2	1.4 ± 0.2
CQ	3.7 ± 0.1	1.7 ± 0.2

See Table 1 for substituents.

Microsomal NADPH-oxidation was measured for the 22 compounds; at 10 μM it ranged from 1.4 ± 0.2 to 494 ± 60 nmoles/min/mg (Fig. 2, Table 2). At quinone concentrations of 10 μM microsomal NADPH-oxidation correlated well with superoxide anion production ($r = 0.88$, $P < 0.001$) for the 22 quinones. For most compounds SAP was between 1.0 and 5.0 times NADPH-oxidation (nmoles/min/mg).

In view of the good agreement of quinone-mediated microsomal SAP with NADPH-oxidation we only used SAP for correlations with several experimentally determined physicochemical parameters (log k', $E_{1/2}$) and with electronic substituent constants ($\sigma_{para,total}$) describing both the inductive field and the resonance component of the substituents [9]. SAP for 22 B(M)ABQs showed a statistically significant correlation with log k' ($r = 0.67$,

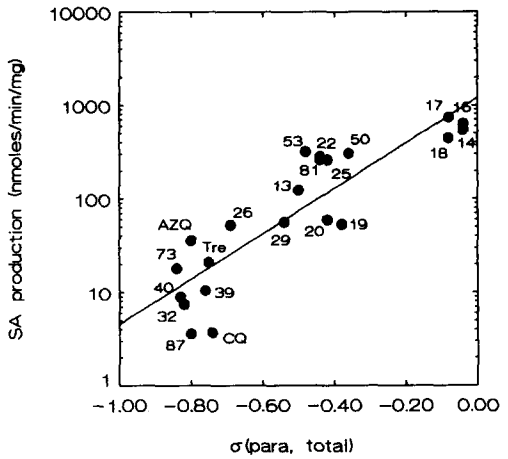


Fig. 3. Microsomal superoxide anion (SA) production (nmoles succinoylated cytochrome *c* reduction/min/mg protein) vs the electronic substituent constant $\sigma_{para,total}$ for 22 B(M)ABQs (10 μM) under carbogen gassing at 37°. Each point represents the mean of 3 separate measurements for one compound. The numbers refer to the compounds mentioned in Table 1. SAP ranges from 3.7 to 742 nmoles/min/mg.

$P = 0.001$) but the correlation with the electrochemically determined half wave potential $E_{1/2}$ was not statistically significant ($r = 0.31$, $P > 0.05$). However, the correlation of SAP with $E_{1/2}$ was considerably improved when compounds with bromo- or chloro-substituents were omitted ($N = 13$, $r = 0.72$, $P < 0.001$). SAP for 22 B(M)ABQs showed a good correlation with the electronic substituent constant σ_{para} summated for all substituents: $r = 0.88$, $P < 0.001$ (Fig. 3). Inclusion of lipophilicity (log k') or steric (MR) parameters in a multiple linear regression analysis did not improve this correlation.

DISCUSSION

Reduction of the quinone moiety is known to initiate the bioactivation of BABQs like AZQ and CQ to alkylating agents [11]. Therefore, a series of BABQs and corresponding BMABQs was synthesized to investigate the influence of electron-withdrawing and electron-donating substituents on the reduction of the quinone moiety and on the biological activation of these compounds. Their capacity for redox cycling was measured in a microsomal system by the oxidation of NADPH and by the production of superoxide anions (SAP) as measured by the reduction of SCC. The selective inhibition of SCC-reduction in the presence of SOD or a nitrogen atmosphere demonstrate that in the presence of 95% O_2 superoxide anions or other reactive oxygen species are formed. The good correlation of quinone-mediated SCC-reduction with microsomal NADPH-oxidation—both exceeding the quinone concentration—implies that under the chosen conditions (95% O_2) both can be used as parameters for redox cycling of B(M)ABQs. The one-electron reduction by NADPH-cytochrome P-450 reductase may play an important role in the reductive activation of B(M)ABQs. This is suggested by the findings of Powis and Appel [22]. With a series of 1,4-benzoquinones they found a linear relationship beween quinone-dependent superoxide formation by NADPH-cytochrome P-450 reductase and by the hepatic microsomal fraction in the presence of NADPH.

We used experimentally determined physicochemical parameters (log k' and $E_{1/2}$) and electronic substituent constants (Hammett constant σ_{para}) [9] to correlate the capacity of B(M)ABQs for redox cycling (SAP, NADPH-oxidation) with structural parameters. The correlation of SAP and $E_{1/2}$ was poor, probably because $E_{1/2}$ represents a combined 2-electron transfer and protonation by which the hydroquinone is formed. SAP and $E_{1/2}$ were better correlated for compounds without bromo- or chloro-substituents. This can be explained by assuming that these substituents can hinder the protonation or the second electron-transfer [9]. Correlation of SAP with log k' was significant, but it should be noted that for the 22 compounds used log k' and $\sigma_{para,total}$ are intercorrelated ($r = 0.61$, $P < 0.003$). However, when the influence of methyl substitution on the aziridinyl functions is considered, no (positive) effect of 2 additional methyl groups on SAP was found despite a considerable positive (+0.55) effect on the log k'-value. This is in agreement with the observation of Driebergen *et al.* [8] that methylation of the aziridines has a minor effect on electrochemical reduction of the quinone ring as measured by the half wave potential.

Concluding, it can be said that the ability of B(M)ABQs for redox cycling, as measured by superoxide anion production and NADPH-oxidation in a microsomal system, can be reasonably predicted from structural features using the electronic substituent constant only. Therefore, introduction of electron-donating substituents in B(M)ABQs diminishes their redox cycling in a microsomal system. Thus, if the production of reactive oxygen radicals is a good predictive parameter for acute cytotoxicity of bioreductively activated quinones the introduction of such substituents could be an effective way of diminishing acute toxic effects.

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