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Original article

Molecular basis of the low activity of antitumor anthracenediones, mitoxantrone and ametantrone, in oxygen radical generation catalyzed by NADH dehydrogenase. Enzymatic and molecular modelling studies

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Abstract

Synthetic antitumor anthracenedione drugs, in contrast to anthracycline antibiotics, are ineffective in free radical formation in NADH dehydrogenase system. Our results have indicated that neither the reduction potential nor the side chain conformation and the energies of border orbitals (HOMO and LUMO) determine the ability of anthracenediones to stimulate reactive oxygen species formation in NADH dehydrogenase system. It was shown that the distribution of the molecular electrostatic potential (MEP), around the quinone system was crucial for this ability. We have found for non-stimulating anthracenediones that the clouds of positive MEP cover the quinone carbon atoms while for agents effective in stimulating reactive oxygen species formation the clouds of negative MEP cover continuously the aromatic core together with the quinone system.

Keywords: Anthracenedione antitumor agents; Molecular modelling; NADH dehydrogenase; Oxygen radical generation

1. Introduction

The antitumor anthracycline antibiotics doxorubicin (DOX) and daunorubicin (DR) belong to the leading clinical cytotoxic agents. They are widely used agents in the treatment of many neoplastic diseases [1,2]. However, their clinical effectiveness is limited by, among other factors, the development of dose-dependent cardiotoxicity [3]. Their synthetic analogs, anthracenediones: mitoxantrone (MX) and ametantrone (AMET), although exhibit narrower spectrum of antitumor activity, have found the application in clinical oncology, due to the lower cost and diminished cardiotoxic effects [4,5]. Numerous data indicate that cardiac effects of anthraquinone compounds are the consequence of one-electron transfer from reduced nucleotides to molecular oxygen mediated by these compounds. This process is catalyzed by cellular oxidoreductases, mainly by NADH dehydrogenase, NADPH cytochrome P450 reductase and xanthine oxidase [6–8]. These enzymes catalyze one-electron reduction of anthraquinone compounds with the formation of semiquinone anion radical. Its non-enzymatic reoxidation by molecular oxygen generates a cascade of reactive oxygen species ($O_2^{-\bullet}$, H_2O_2, $^{\bullet}OH$, $^1O_2$) highly toxic for heart cells devoid of antioxidants, due to the peroxidation of cell membranes, proteins and nucleic acids [9]. Studies aimed at the removal of the peroxidating activity of anthraquinone compounds by their rational modifications require firstly the elucidation of the role of structural factors in their enzyme-catalyzed generation of reactive oxygen species. In our previous studies it was shown that the effectiveness of anthraquinone compounds in mediating one-electron transfer is dependent essentially on their affinity to oxidoreductases catalyzing this process. We have found that the redox properties of anthraquinones play only a secondary role [10]. It was demonstrated that anthraquinones interact with NADH dehydrogenase acting as structural analogs of its physiological substrate, ubiquinone [11]. Up to now, the structural factors essential for the enzyme substrate properties of anthraquinone compounds have not been fully identified. This problem is of

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essential importance for the rational design of non-cardiotoxic antitumor compounds. In our previous works two structural factors of anthraquinone compounds have been identified which favor the stimulation of toxic oxygen species formation. These are the quinonoid moiety with both carbonyl groups unsubstituted and the presence of at least two phenolic groups [12]. In the light of these findings, the behavior of MX needed further studies for the understanding of its low activity in stimulating free radical generation catalyzed by NADH dehydrogenase in spite of the presence of unsubstituted quinone carbonyls and two phenolic groups. We postulated that the poor substrate properties of MX for NADH dehydrogenase could be due to the steric hindrance of its side chains and/or the presence of aromatic amine groups that might disturb the interaction with the enzyme [13,14].

The aim of this study was to identify molecular properties of MX and AMET molecules disfavoring their interaction with NADH dehydrogenase. For this purpose, enzymatic studies, electrochemical analyses as well as molecular modelling calculations (shape of molecule, distribution of molecular electrostatic potential (MEP) and energy levels of LUMO and HOMO orbitals) have been performed. The series of selected compounds for this study contained MX, AMET, and model compounds: compound A (5,8-dihydroxy-1,4-bis[[3-dimethylamino)-propyl]amino]-9,10-anthracenedione), the amide analogs of AMET (AD2, AD7), as well as quinizarin and 1,4-diaminoquinizarin. DR was also included as a reference compound.

2. Results

The structures of examined compounds are presented in Fig. 1.

2.1. Enzymatic studies

The ability of examined compounds to stimulate free radical formation catalyzed by mitochondrial NADH dehydrogenase (EC 1.6.99.3) was measured as the rate of NADH oxidation and superoxide production detected by cytochrome *c* reduction method. The kinetic parameters (K_M, V_{max}) were determined for MX, AMET, AD2 and a reference compound DR using both methods. Because of the poor solubility of model compounds: quinizarin and 1,4-diaminoquinizarin, for these compounds dimethylsulfoxide (DMSO) (30% v/v) was used in enzymatic assays. Therefore, for these two compounds their ability to stimulate free radical formation were determined comparatively in regard to the ability of a reference compound DR in the enzymatic system in the presence of the same percentage of DMSO (30% v/v).

As it is presented in Table 1, MX and AMET in contrast to DR were ineffective in stimulating NADH oxidation and superoxide production. The ratio V_{max}/K_M reflecting catalytic efficiency and enzyme specificity was highly lower for these derivatives in comparison with DR (20- and 50-fold

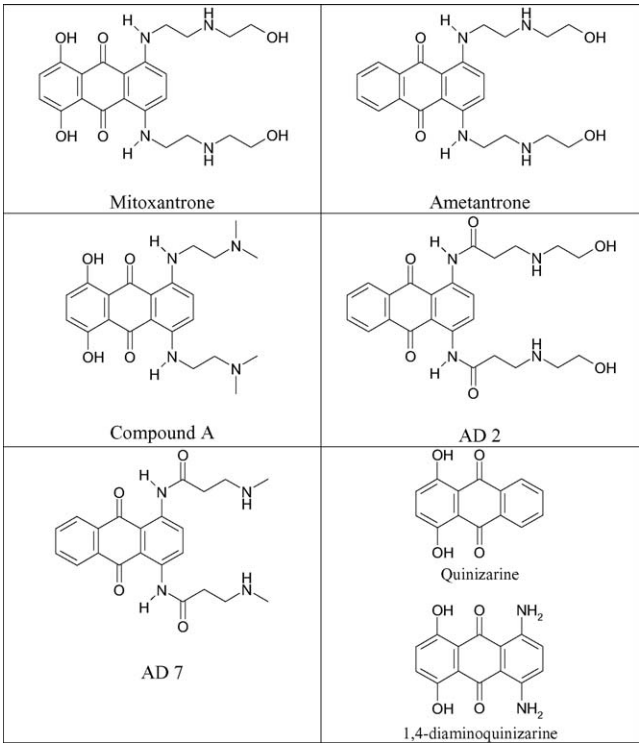


Fig. 1. The structures of examined compounds.

decreases, respectively, for MX and AMET) (Table 2). It was postulated in our previous work [13] that poor substrate properties of MX could be due to the steric hindrance of its side chains. In order to elucidate the role of inter-side chain hydrogen bond formation in the eventual steric hindrance disturbance in the interaction of MX with NADH dehydrogenase, the analog of MX namely compound A having two methyl groups per side chain has been also examined in this study. The presence of methyl substituents in side chains of this derivative disturb the formation of stable inter-chain hydrogen bonds but the activity of compound A to stimulate oxygen radical formation was almost the same as that of MX (Table 1). It suggests that the conformation of side chains of MX is not crucial for its low affinity to NADH dehydrogenase.

In order to elucidate the role of aromatic amine group in the anthracenedione structure in the interaction with NADH dehydrogenase, model compounds—quinizarin as well as its 1,4-diamino derivative have been studied. It was found that quinizarin presented relatively high activity in stimulating NADH oxidation and superoxide production while 1,4-diaminoquinizarin presented markedly decreased activity (about threefold) in stimulating these processes in comparison with the activity of the reference compound DR determined in the same conditions (in the presence of DMSO (30% v/v) (Table 1). Above results could suggest that the introduction of aromatic amino groups in the anthraquinone structure disturbs the interaction with NADH dehydrogenase. In fact, the amide analogs of AMET, AD2 and AD7, having the same side chains as AMET but attached to the ring moiety not through the aromatic amine function but through the amide

Table 1
NADH oxidation and superoxide ($O_2^{-\bullet}$) production by examined compounds in NADH dehydrogenase system

Compound	NADH dehydrogenase activity (U ml^{-1})	NADH oxidation[a] (μM min^{-1})	NADH dehydrogenase activity (U ml^{-1})	$O_2^{-\bullet}$ production[b] (μM min^{-1})
DR	0.2	29.4 ± 0.9	0.1	21.6 ± 2.6
MX	0.2	1.4 ± 0.5	0.1	2.6 ± 0.8
AMET	0.2	0.52 ± 0.02	0.1	0.50 ± 0.11
Compound A	0.2	1.2 ± 0.5	0.1	2.4 ± 0.8
AD2	0.2	11.2 ± 2.3	0.1	8.1 ± 0.7
AD7	0.2	14.5 ± 1.5	0.1	9.7 ± 0.9
DR (*)	1.0	21.4 ± 2.3	0.5	25.4 ± 3.0
Quinizarin (*)	1.0	18.9 ± 0.2	0.5	14.3 ± 2.6
1,4-Diaminoquinizarin (*)	1.0	8.3 ± 1.0	0.5	8.7 ± 0.9

The reaction mixture (1 ml) contained: 100 μM examined compound (a, b), 100 μM NADH (a, b), 50 μM cytochrome *c* (b), indicated activity of NADH dehydrogenase, 0.05 M Tris–HCl (pH 7.3, temperature 25 °C).
(*) 300 μl of DMSO was added.

Table 2
Kinetic parameters for a reference compound: DR, anthracenediones: MX, AMET and an amide analog of AMET (AD2) in NADH dehydrogenase system

Compound	NADH oxidation			$O_2^{-\bullet}$ production		
	NADH dehydrogenase activity 0.2 U ml^{-1}			NADH dehydrogenase activity 0.1 U ml^{-1}		
	K_M (μM)	V_{max} (μM min^{-1})	V_{max}/K_M (min^{-1})	K_M (μM)	V_{max} (μM min^{-1})	V_{max}/K_M (min^{-1})
DR	36.0 ± 9.7	39.1 ± 3.2	1.09	31.2 ± 4.6	30.1 ± 7.6	0.96
MX	57.1 ± 5.8	2.9 ± 0.3	0.05	53.3 ± 2.2	2.5 ± 0.2	0.05
AMET	68.4 ± 7.0	1.5 ± 0.2	0.02	62.4 ± 3.0	1.2 ± 0.2	0.02
AD2	n.d.	n.d.	n.d.	16.3 ± 2.8	10.8 ± 2.5	0.66

The reaction mixture (1 ml) contained: 0–100 μM examined compound (a, b), 100 μM NADH (a, b), 50 μM cytochrome *c* (b), indicated activity of NADH dehydrogenase, 0.05 M Tris–HCl (pH 7.3, temperature 25 °C).
n.d., not determined.

group were almost as effective in stimulating NADH oxidation and superoxide production as DR (Table 1). The ratio V_{max}/K_M found for AD2 was comparable to the value characteristic for DR (Table 2). It suggests that amide analogs, in contrast to anthracenediones having aromatic amine groups, are good substrates for NADH dehydrogenase.

2.2. Electrochemical studies

Because it was often postulated by other authors that electrochemical properties of anthraquinone compounds were essential for their ability to stimulate oxygen radical formation in enzymatic systems, the reduction potentials were measured for some of examined compounds (Table 3). This parameter was determined by cyclic voltammetry method. As can be seen, anthraquinone compounds containing an aromatic amine function in their structures (MX, AMET, 1,4-diaminoquinizarin) had more negative reduction potential than quinizarin and an amide analog of AMET—AD2. It suggests that these derivatives are less susceptible to undergo an electrochemical reduction. However, in our opinion these relatively small differences in reduction potential could not explain the essential differences in stimulating NADH oxidation and superoxide production observed for these compounds (for example, AD2 had about 20-fold higher activity in stimulating oxygen radical formation in comparison to AMET (Table 1)).

Table 3
The reduction potentials of examined compounds

Compound	Reduction potential (mV)
DR	–630
MX	–780
AMET	–780
Quinizarin	–630
1,4-Diaminoquinizarin	–700
AD2	–670

Reduction potential measurements were carried out in 5 ml of electrolyte contained 0.1 M KCl/0.05 M Tris–HCl buffer, pH 7.3 and DMSO in a ratio 40:60 (v/v). Compound concentration was equal to 1.2×10^{-4} M. The values of reduction potentials are reported against saturated calomel reference electrode (±20 mV).

2.3. Shape of the molecule

It was shown above that amide analogs of AMET, AD2 and AD7, in contrary to *N*-alkyl derivatives (MX and AMET), are relatively good substrates for NADH dehydrogenase. Our preliminary supposition was that this difference results from different shapes of the molecules belonging to the two families. We have tried to verify this assumption by molecular dynamics simulations. It might expect that side chains of amides of 1,4-diaminoanthraquinone, due to the presence of a rigid amide part, adopt different conformation(s). Molecular dynamics simulations performed for compound AD2 indicated that during sufficiently long simulation side chains might exist in three different conformations (Fig. 2). A "closed" conformation, very similar to "closed" conformations of MX and AMET, was predominant. In each type of

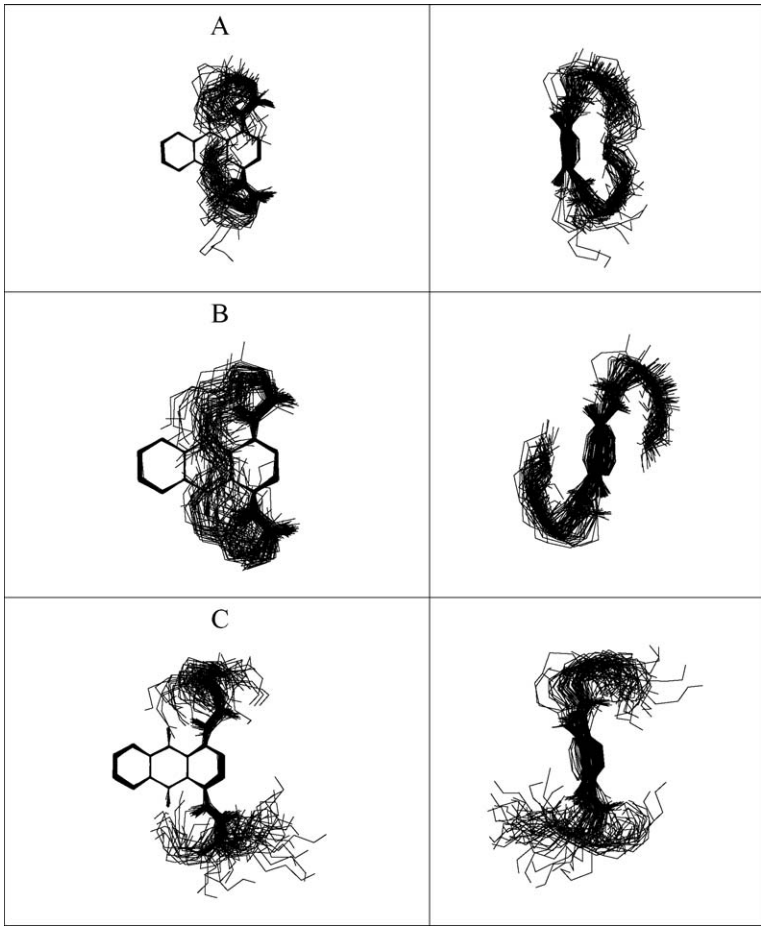


Fig. 2. Orthogonal view of composites of 40 snapshot geometries of three different conformations of AD2 obtained during 300 ps simulation: (A) *closed*, (B) *anti* and (C) *open*. Before superimpositions molecules have been shifted and rotated to obtain least square fitting of quinone system to its position in the first snapshot.

conformations the side chains formed hydrogen bonds with the quinone system. In addition, in conformations "closed" and "anti" side chains were located under or over the ring system. The molecule with such a shape could not be a good analog of the flat natural enzyme substrate: ubiquinone.

These results of molecular modelling strongly suggest that the shape of the molecule is not a crucial factor determining substrate properties of anthraquinones in relation to NADH dehydrogenase. Therefore, we focused our attention on electronic properties of the anthraquinone system.

2.4. Energy of LUMO and HOMO

It is interesting to note that all compounds studied, which were less effective in stimulating oxygen radical formation, possess amino groups in positions 1 and 4 of the anthraquinone system (Fig. 1, Tables 1 and 2). Thus, we tried to examine electronic properties of the anthraquinone core characteristic for such chemotype. In order to broaden quantum mechanics calculations and focus attention on the ring system, the calculations have been done for model compounds in which the side chains were limited to two carbon atoms only. Thus, 1,4-diethylaminoquinizarine has been taken into consideration instead of MX and compound A, 1,4-diethylaminoanthraquinone instead of AMET, and 1,4-diacetamidoanthraquinone instead of AD2 and AD7.

Our investigations have been started by studying the energy of border orbitals. The obtained HOMO and LUMO energies are shown in Table 4. The HOMO energies for compounds effective in stimulating formation of oxygen reactive species

Table 4
The energy of HOMO and LUMO obtained for examined compounds by semi-empirical methods AM1

Compound	HOMO energy (eV)	LUMO energy (eV)	Activity in stimulating $O_2^{-\bullet}$ formation
1,4-Diethylaminoquinizarine	–8.23	–1.25	–
1,4-Diethylaminoanthraquinone	–8.06	–1.11	–
1,4-Diacetamidoanthraquinone	–8.83	–1.63	+
1,4-Diaminoquinizarine	–7.91	–1.31	–
Quinizarine	–9.07	–1.62	+

were slightly more negative than for compounds for which such effect was not observed. Similar relations were observed for results characterizing LUMO energies of examined derivatives. The observed differences seem to be significant, however, too small for explaining great differences in the ability to stimulate oxygen reactive species formation observed between the two groups of examined compounds (Tables 1 and 2).

2.5. MEP distribution

A distribution of MEP around the quinone system is the other electronic property that might distinguish between the two groups of anthraquinone derivatives. Fig. 3 presents the comparison of MEP distribution in the plane containing quinone carbon atoms and perpendicular to the ring system for quinizarine and 1,4-diaminoquinizarine. It is interesting to note that for 1,4-diaminoquinizarine, which was less active in stimulating oxygen radical formation, a cloud of positive MEP covered the quinone system. In the case of quinizarine, having relatively high activity in stimulating oxygen radical production, clouds of negative MEP surrounded the carbon atoms of the quinone system.

More detailed comparison of MEP distributions around the two compounds studied revealed that these significant differences were also observed at about 2 Å under and over the plane of the ring system (Fig. 4). The clouds of negative MEP covered not only the quinone but also practically the whole ring system of quinizarine. In the case of 1,4-diaminoquinizarine the clouds of negative MEP were not continuous and positive clouds covered the quinone carbon atoms.

The same difference was observed in MEP distribution for 1,4-diethylaminoanthraquinone and 1,4-diacetamidoanthraquinone (Fig. 5). It was also found that the characteristic of MEP distribution around the quinone system of 1,4-diethylaminoquinizarine was the same as for 1,4-diethylaminoanthraquinone (data not shown).

3. Discussion and conclusions

In our previous studies we have postulated that the substrate properties of anthraquinone compounds towards oxidoreductase enzymes catalyzing the one-electron transfer but not their redox properties are of primary importance for their activity in stimulating toxic oxygen species formation [10,12]. We have identified also some structural factors favoring the interaction of anthraquinone compounds and their congeners with NADH dehydrogenase. These are the quinonoid moiety with both carbonyl groups unmodified and the presence of at least two phenolic groups [12]. On the basis of these results it would be expected that MX could be a good substrate for NADH dehydrogenase. However, this compound exhibits a low activity in free radical generation in NADH dehydrogenase system in spite of the presence of unsubstituted quinone carbonyls and two phenolic groups [7,12,14]. Looking for other structural factors influencing the substrate properties of this compound towards the enzyme, we assumed that the shape of anthracenedione molecule, particularly due to the side chains conformation, might be of importance. To verify this assumption molecular modelling studies were performed on side chain conformations of anthracenedione derivatives with different ability to stimulate the formation of oxygen radicals. It was shown that in lipophilic environment expected in the NADH dehydrogenase active center, the side chains of MX and AMET exist in specific, “closed” conformation [13]. In this conformation the shape of the molecule is totally different than the shape of ubiquinone, the natural substrate of this enzyme. Moreover, the side chain polar groups form the network of hydrogen bonds, which at least

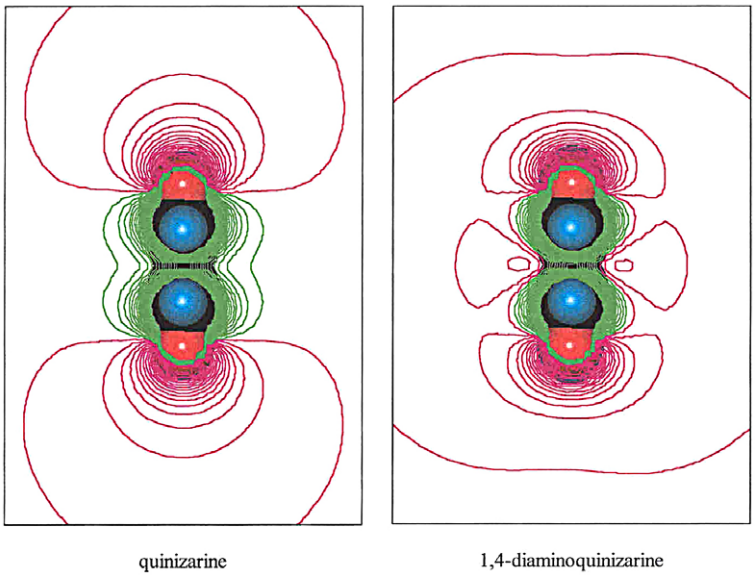
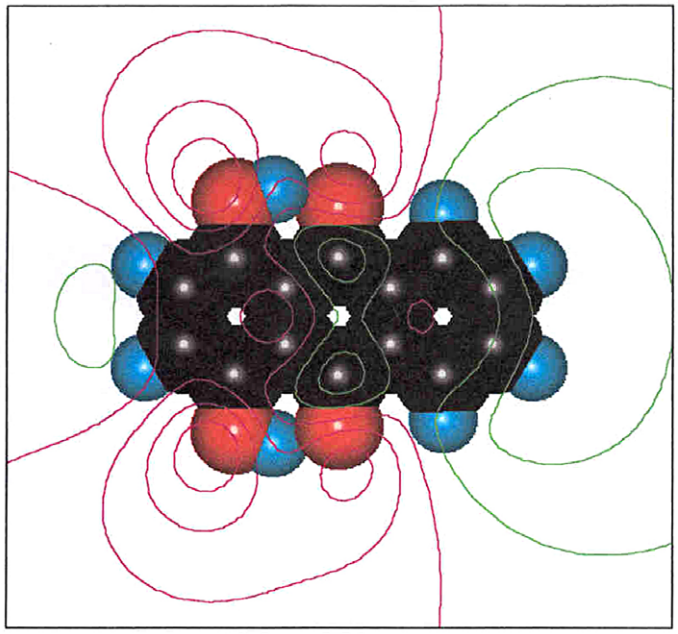
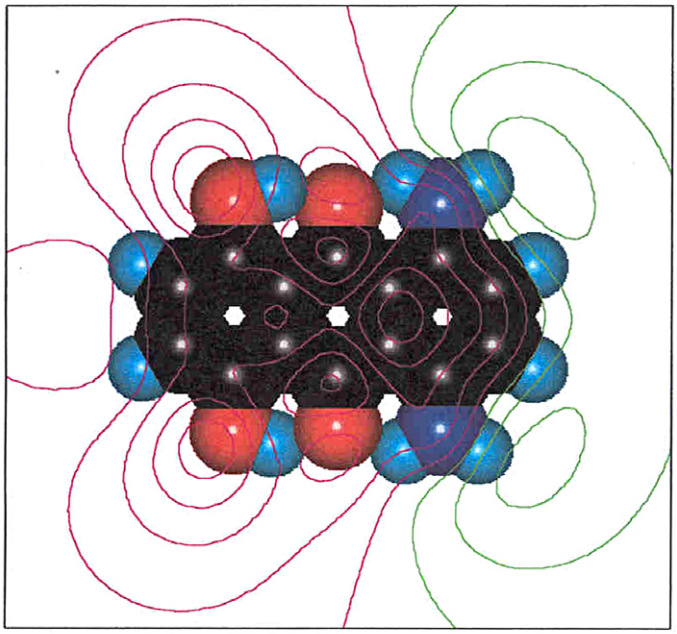


Fig. 3. MEP distribution for quinizarine and 1,4-diaminoquinizarine in a plane containing the quinone carbons and perpendicular to the ring system; magenta lines—negative potential, green lines—positive potential. Two neighbor isopotential lines are separated by 0.005 MEP units.



quinizarine



1,4-diaminoquinizarine

Fig. 4. MEP distribution for quinizarine and 1,4-diaminoquinizarine in a plane parallel to the ring system 2 Å over the quinone carbons; magenta lines—negative potential, green lines—positive potential. Two neighbor isopotential lines are separated by 0.005 MEP units.

in part covers the quinone oxygen atoms. To determine a significance of these features we synthesized and examined several model compounds. However, compound A (Fig. 1) for which, due to the lack of hydroxyl groups such interactions are significantly limited, exhibited still low ability (comparable to the ability of MX) to stimulate reactive oxygen species production (Table 1). Totally different properties exhibited amide analogs of AMET-AD2 and AD7 (Fig. 1). These compounds, in contrast to anthracenediones having aromatic amine groups, were highly active in stimulating oxygen radical formation (Tables 1 and 2). The results of molecular dynamics simulations found for the amide derivatives AD2 (Fig. 2), clearly indicate that side chain conformations as well as the participation of quinone oxygen atoms in the network of hydrogen bonds do not decide about the ability of anthraquinones to stimulate oxygen radical formation. The

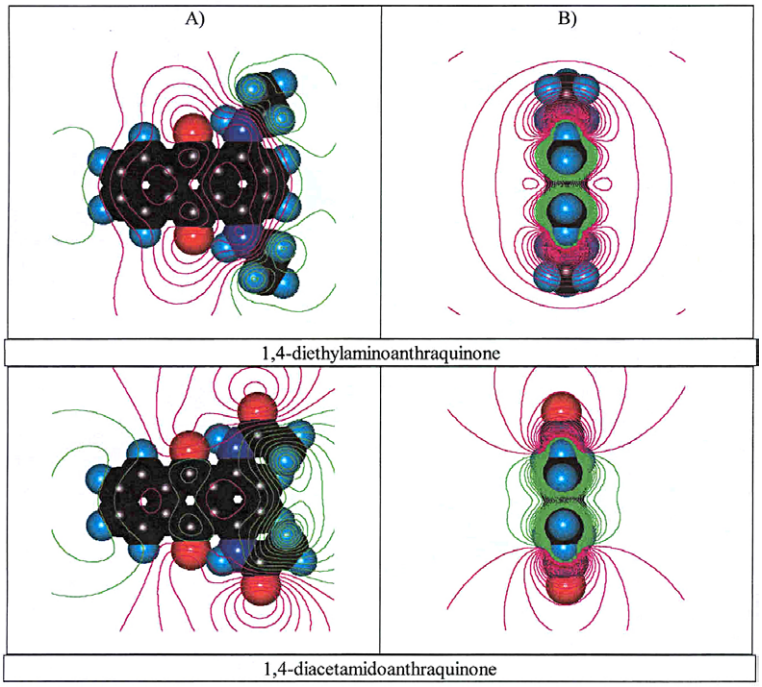


Fig. 5. MEP distribution for 1,4-diethylaminoanthraquinone and 1,4-diacetamidoanthraquinone in a plane (A) parallel to the ring system 2 Å over the quinone carbons, (B) containing the quinone carbons and perpendicular to the ring system; magenta lines—negative potential, green lines—positive potential. Two neighbor isopotential lines are separated by 0.005 MEP units.

results obtained for model compounds without side chains, quinizarine and 1,4-diaminoquinizarine, corroborate above hypothesis (Table 1).

It was suggested earlier [15], that the reduction potential of the anthraquinone molecule might be an essential factor determining its ability to stimulate oxygen radical formation in oxidoreductase systems. In our previous works we have shown that, at least for anthracycline antibiotics, this factor is not crucial [10]. Thus, we determined also the reduction potentials for some compounds studied in this work (Table 3). The obtained results indicated that model compounds active in stimulating radical formation were characterized by slightly more positive reduction potential than non-stimulating derivatives. However, these relatively small differences in reduction potential cannot explain drastic differences in the ability to stimulate oxygen radical formation. The careful analysis of data presented in Tables 1 and 2 revealed an interesting fact, namely that all anthracenediones, inactive in stimulating oxygen radical formation, possess amino or alkylamino groups in positions 1 and 4 of the anthraquinone core. The acylation of these functional groups restored the affinity of obtained derivatives for NADH dehydrogenase. The structure–activity relationship strongly suggests that, in the case of NADH dehydrogenase and synthetic anthracenediones, electronic properties of anthraquinone ring system are crucial for their ability to stimulate formation of reactive oxygen species.

The results of quantum semi-empirical calculations revealed that energies of border orbitals, HOMO and LUMO, did not correlate with the ability of examined compounds to stimulate reactive oxygen species formation (Table 4). However, such correlation existed for the distribution of MEP around quinone system (Figs. 3–5). For anthracenediones, effective in stimulating oxygen radical formation, clouds of negative MEP covered the aromatic core together with the quinone system. Such potential distribution exists also around ubiquinone, the natural substrate of the enzyme (data not shown). In contrary, for non-stimulating anthracenediones the clouds of negative MEP were not continuous and the clouds of positive MEP covered the quinone carbon atoms. Up to now, it is not clear how MEP distribution around the quinone system of anthracenediones may determine their interaction with NADH dehydrogenase. It is possible that an active center of the NADH dehydrogenase recognize MEP distribution of the substrate preferring molecules with continuous negative clouds and that in the second step the substrate MEP distribution influence the electron transfer from the enzyme to the quinone system. The differences in V_{max} values found for NADH oxidation and $O_2^{-\bullet}$ production for stimulating and non-stimulating anthraquinones suggest that MEP distribution might, at least in part, affect this stage. We will try to solve this problem in our further studies.

4. Experimental protocol

4.1. Drugs and chemicals

DR was kindly provided by the Drug Development Branch, National Cancer Institute, Bethesda. Quinizarin and 1,4-diaminoquinizarin were purchased from Janssen Chemica, Beerse (Belgium). MX [16], AMET [16], compound A [16],

AD2 [17] and AD7 [17] were prepared in our laboratory according to the procedures described.

Cytochrome *c* (type VI from horse heart), NADH dehydrogenase and NADH were from Sigma Chemical Co., St. Louis, MO.

4.2. Enzymatic studies

Stock solutions (C_0 = 1 mM) were prepared just prior to use. Because of the insolubility of some model compounds (quinizarin and 1,4-diaminoquinizarin), they were dissolved in DMSO.

NADH dehydrogenase activity was determined using cytochrome *c* as the electron acceptor. It was examined by following cytochrome *c* reduction at 550 nm using an extinction coefficient for cytochrome *c* (reduced minus oxidized) of 19,600 M^{-1} cm^{-1}. Enzyme activity has been expressed in units, where 1 unit of activity is the amount of NADH dehydrogenase capable of reducing 1 μmol of cytochrome *c* per min at pH 7.4 at 25 °C.

NADH oxidation was measured at 340 nm using an extinction coefficient for NADH of 6220 M^{-1} cm^{-1}. Superoxide production was examined by following SOD-inhibitable cytochrome *c* reduction. Enzymatic reactions were initiated by the addition of NADH dehydrogenase. All absorption measurements were made on a Beckman 3600 spectrophotometer [12].

4.3. Cyclic voltammetry studies

The redox potential of compounds was examined by cyclic voltammetry method. Voltammograms were obtained at a glass-carbon electrode and recorded on Polarographic Analyzer PA-4 and X-4 recorder 4106, Praha. The electrode was prepared before each voltammograme using standard procedure. Conventional three-electrode electrochemical cells contained platinum counter electrode and saturated calomel reference electrode were used. Redox potential measurements were carried out in 5 ml of electrolyte contained 0.1 M KCl/0.05 M Tris–HCl buffer, pH 7.27 and DMSO in a ratio 40:60 (v/v). Compound concentration was equal to 1.2×10^{-4} M. Solutions were deoxygenated by passing argon for 10 min. The scan rate was 50 mV s^{-1}.

4.4. Molecular modelling calculations

The initial co-ordinates for the atoms of compounds studied were obtained from molecular modelling by HyperChem program [18] using standard bond lengths and angles.

Molecular dynamics simulations were performed by the GROMOS software package [19], according to the procedure described earlier [13]. Briefly, the integration of the classical equations of motion was done with a time step of 2 fs with all bonds lengths constrained within 10^{-4} relative to the reference lengths using the SHAKE method [20]. For all calculations, the standard GROMOS force field was used. No explicit hydrogen bond function was employed in this force field. No periodic boundary condition was employed. The list of non-bonded neighbors was updated every 10 MD steps. The systems were equilibrated for 10 ps to relax any artificial starting conditions. During the main dynamic runs the temperature was kept at 300 K by coupling the kinetic energy of the system to a heat bath with a relaxation time of 100 fs.

Quantum chemical calculations were done employing HyperChem semi-empirical module. In all calculations the AM1 parameterization was used.

Acknowledgements

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