

ELECTROCHEMICAL, SPECTRAL AND MO INVESTIGATION OF THE REDUCTIVE ACTIVATION OF ANTITUMORAL DRUGS DOXORUBICIN AND EPIRUBICIN

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abstract: The electrochemical and chemical reduction of the antitumoral drugs doxorubicin and epirubicin is investigated in aprotic and protic media, in the presence and absence of oxygen by coupled electrochemical and spectral techniques. The cyclic voltammetry with stationary and rotating disc electrode (RDE) points out a quite similar electrochemical behaviour, consisting of two electron transfers and evidence as intermediate species the anion-radical ($A^{\cdot-}$), the dianion (A^{2-}) and the protonated species AH^{\cdot} , AH , AH_2 . EPR and optical spectra registered during the electrochemical reduction allow the identification of these species and suggest the possibility of back oxidation of the drug by electron transfer to molecular oxygen. AM1 semiempirical MO-calculations allow a rationalisation of the experimental results regarding the reactivity in redox processes and outline the dominant role of the anthraquinone moiety. Biological implications of the reductive activation of molecular oxygen are also discussed.

Introduction

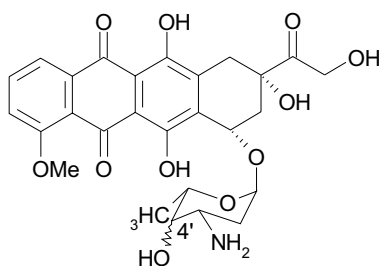
Doxorubicin and its epimer epirubicin are potent anthracycline antitumoral drugs which due their action to the intercalation of the anthracycline (aromatic) moiety between the DNA base-pairs, resulting in the inhibition of transcription by blockage of RNA polymerase [1÷3]. Their binding to DNA was intensively studied using absorption and emission spectroscopy [4÷9], and to a smaller extent and especially in analytical purposes, electrochemical methods [10,11]. However, besides their benefic action, these drugs possess also an undesirable toxicity. The generally accepted mechanism for this process implies the mono or bielectronic reduction of the drug, with the appearance of reactive reduction intermediates, radical species which may mediate electron transfer to molecular oxygen, with formation of superoxide anion radicals (and other reactive oxygen species), responsible for cellular damage and cardiotoxicity. However, the possibility of formation of an adduct between the anthracycline drug and singlet oxygen (AO_2) which is reduced to $AO_2^{\cdot-}$ and can release superoxide anion radical and recover the initial quinone, was also

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discussed [12,13]. This process, usually called “reductive activation”, is not completely understood, although several studies were devoted to this subject [14÷20]. It is the aim of the present paper to investigate the behaviour in reduction processes of doxorubicin and epirubicin, in both aprotic and protic media, in the presence or absence of oxygen, by coupled electrochemical and spectral techniques, including EPR and absorption spectroscopy, in order to identify the intermediate species and to propose a reaction mechanism. Semiempirical MO-calculations were performed to outline the electronic structural features implied in redox processes. The possibility of electron transfer from the different reduction intermediates to molecular oxygen and/or of formation of covalent adducts with ($^1\Delta_g$) O_2 was also analysed using model compounds.



Molecular formulae of doxorubicin (4' - OH - axial) and epirubicin (4' - OH - equatorial).

Experimental

Cyclic voltammetry experiments with both stationary and rotating disc electrode (RDE) were performed in phosphate buffer and dimethyl sulphoxide (DMSO) with 0.1 M tetra butyl ammonium tetrafluoroborate (TBABF₄) as supporting electrolyte, at a VOLTALAB-32 electrochemical laboratory, with platinum working and counter electrodes and Ag-quasi reference electrode [21]. All potentials are referred to a saturated calomel electrode (SCE), using for calibration the ferrocen/ferrocenium couple. To study the influence of dissolved oxygen, air was bubbled through all solutions for 15-60 min. The optical spectra were registered during the electrochemical and chemical reduction using *in-situ* techniques developed in our laboratory [22] on a C. Zeiss Jena and UNICAM-UV 4 spectrophotometers. The EPR spectra were recorded during the electrochemical reduction on a JES-3B spectrometer in the X-band frequency, using peroxyamine disulphonate as standard ($a_N = 1.3$ mT $g = 2.0055$). The semiempirical MO calculations were performed using the AM1 hamiltonian in the MOPAC program package and RHF (ROHF/UHF) formalism for closed and respectively open-shell structures.

Results and Discussion

A. Cyclic voltammetry

Both doxorubicin and epirubicin exhibit in deaerated DMSO two reduction waves in the range 0 to -1.5 V/SCE (Fig. 1a), which were analysed separately according to the usual electrochemical criteria. It may be stated from the very beginning that the electrochemical

behaviour of both drugs is quite similar (identical) and therefore the results are reported either for doxorubicin or for epirubicin. The relevant electrochemical data are presented in Table 1. The first process is characterised by a well shaped anodic counter part, and the difference between the cathodic and anodic peak potentials allows an estimate of the standard electron transfer rate, k_s , using Nicholson's

formula: $\Psi = \frac{\gamma^{1/2} (RT)^{1/2} k_s}{(nFD_0\pi\nu)^{1/2}} = 28.8 \frac{k_s}{\nu^{1/2}}$, where the constant value was obtained considering

$\gamma = D_O/D_R \sim 1$, $D_O = 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, $n = 1$ and $RT/F = 25.67 \text{ mV}$ at 25°C . A value of $k_s = 2.3 \times 10^{-2} \text{ cm/s}$ was obtained, attesting to a rapid electron transfer.

Therefore, this wave was assigned to the reversible mono-electronic reduction of the drug to its anion-radical, $A + e^- \rightleftharpoons A^{\cdot-}$. However, the ratio of the peak currents i_{pa}/i_{pc} is much smaller than unity, with an increasing tendency with the sweep rate, and the ratio $i_{pc}/\nu^{1/2}$ decreases slightly with the scan rate, which are indications of a follow-up chemical reaction, consuming the anion-radical formed in the first reduction step. The most commonly encountered follow-up reaction in electrochemical experiments in standard aprotic solvents, is the protonation of the anion-radical by the protic impurities present in the solvents in amounts of the order of the substrate concentration: $A^{\cdot-} + H^+ \rightarrow AH^{\cdot}$. The pseudo first-order rate constant was evaluated from the dependence of the peak current ratio with the sweep rate [23] and a value of about 0.15 s^{-1} was obtained. Support for this assignment is furnished by the cyclic voltammograms registered when water is added in DMSO, up to a concentration of 1: 6 (v/v). Analysis of the 1-st wave in these conditions according to the electrochemical criteria already discussed, reveals a greater estimated rate for the chemical following step, $k = 1.1 \text{ s}^{-1}$, in agreement with the enhancement of the protonation reaction of the anion-radical (Fig. 1b).

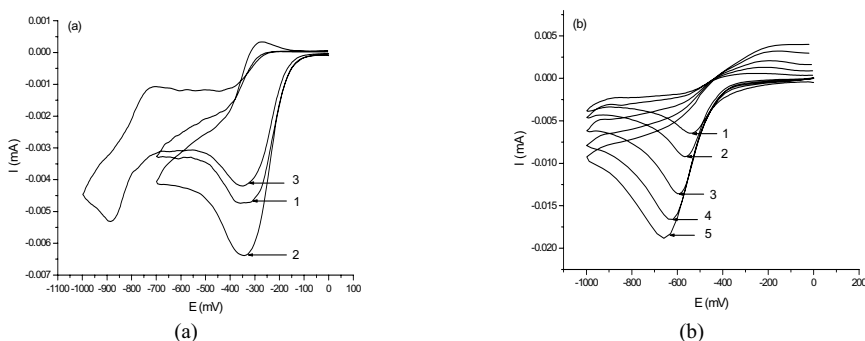


Fig. 1: (a) Cyclic voltammetry of doxorubicin ($C = 1.22 \times 10^{-3} \text{ M}$) in DMSO/0.1 MTBAPF₄: 1 – potential switched after the 1st wave, $\nu = 50 \text{ mV/s}$; 2 – $\nu = 100 \text{ mV/s}$; 3 – potential switched after the 2nd wave, $\nu = 50 \text{ mV/s}$.
(b) 1st wave of doxorubicin ($C = 3.94 \times 10^{-4} \text{ M}$) in DMSO: water (6:1) (v/v) at different sweep rates: 1 – $\nu = 50 \text{ mV/s}$, 2 – $\nu = 100 \text{ mV/s}$, 3 – $\nu = 200 \text{ mV/s}$, 4 – $\nu = 400 \text{ mV/s}$, 5 – $\nu = 600 \text{ mV/s}$.

Analysis of the second wave using the same criteria points out a quasireversible electron transfer, the standard rate constant being about an order of magnitude lower, $k_s \sim 4.4 \times 10^{-3} \text{ cm s}^{-1}$. This process was assigned to the reduction of anion-radical to a dianion, $A^{\cdot-} + e^- \rightleftharpoons A^{2-}$.

Table 1. Relevant electrochemical data for doxorubicin ($C = 1.22 \times 10^{-3}$ M) in deaerated DMSO, in the potential range 0 to -1.5V/SCE.

v (V/s)	$-E_{pc}$ (V)	$-E_{pa}$ (V)	ΔE_p	i_{pa}/i_{pc}	$i_{pc}/v^{1/2} \times 10^3$
I st – wave					
0.050	0.568	0.500	0.068	0.429	0.671
0.100	0.573	0.509	0.064	0.455	0.640
0.200	0.579	0.518	0.061	0.488	0.625
0.400	0.584	0.520	0.064	0.507	0.630
0.600	0.592	0.494	0.098	0.486	0.580
II nd – wave					
0.050	1.116	0.937	0.179	0.695	0.750
0.100	1.046	0.928	0.113	0.848	0.557
0.200	1.046	0.902	0.139	0.827	0.498
0.400	1.054	0.928	0.126	0.847	0.528
0.600	1.069	0.934	0.135	0.839	0.538

Again the electrochemical criteria discussed above indicate a follow-up chemical step, *i.e.* an EC – process; as the dianion is a stronger base than the anion-radical, the protonation reaction probably occurs more rapidly. The results obtained with the rotating disc electrode (RDE) confirm that both waves are monoelectronic. Plots of E vs. $\ln((i_1-i)/i)$ (eq. 1, $r = 0.950$, $n = 11$) allow to determine $E_{1/2}$ values.

The plot of the cathodic limiting current in function of the square root of the rotating rate (eq. 2, $r = 0.961$, $n = 11$) allows the determination of the diffusion coefficients, D_O . The plot of the reciprocal of the current vs. the reciprocal square root of the rotating rate (eq. 3, $r = 0.995$, $n = 9$) allows the determination of the electron transfer rate [21]. The results are presented in Table 2.

$$E = E_{1/2} + \frac{RT}{\alpha nF} \ln \frac{i_k - i}{i} \quad (1)$$

$$i_k = 0.620nFAC_0D_0^{2/3}\omega^{1/2}v^{-1/6} \quad (2)$$

$$\frac{1}{i} = \frac{1}{i_k} + \frac{1}{0.620nFAC_0D_0^{2/3}\omega^{1/2}v^{-1/6}} \quad (3)$$

Table 2. RDE results of the investigated compounds.

	$E_{1/2}$ (V)	$k_s \times 10^2$ cm/s	$D_O \times 10^5$ cm ² /s	α	i_k (mA)
Ist wave	-0.550	2.9	1.58	-	0.109
II nd wave	-1.026	1.7	2	0.46	0.065

The influence of dissolved oxygen on the first reduction wave of doxorubicin is presented in Fig. 2a. It may be observed that the wave is shifted towards negative potentials and has no anodic counterpart, which is indication of an irreversible follow-up reaction consuming

the anion-radical; this could be assigned to the electron transfer from the anion-radical to molecular oxygen, besides the protonation reaction already discussed. At the same time a new wave is apparent at more negative potentials, in the range of the reduction potential of molecular oxygen in DMSO. This wave could be assigned either to the reduction of molecular oxygen in DMSO, or to the reduction of a complex formed between the drug and singlet oxygen (AO_2), as stated for other anthraquinone derivatives in literature [13]. The fact that this wave has no anodic counterpart seems to be a support for the second hypothesis, as the reduction of oxygen in DMSO is known to be reversible. If this wave corresponds to the process $AO_2 + e^- \rightarrow AO_2^{\cdot-}$, the possible follow-up reaction would be: $AO_2^{\cdot-} \rightarrow A + O_2^{\cdot-}$, and would explain the formation of superoxide anions in the system. It is interesting to note that the influence of dissolved oxygen in neutral protic media is different (Fig. 2b), *i.e.* the new wave on the voltammogram being at positive potentials *vs.* the first wave of the drug in DMSO, which becomes irreversible. This can be explained by the fact that the reduction of molecular oxygen in protic media occurs at more positive potentials, the superoxide anion being thermodynamically stabilised in protic solvents, and therefore the new wave may be assigned to this process.

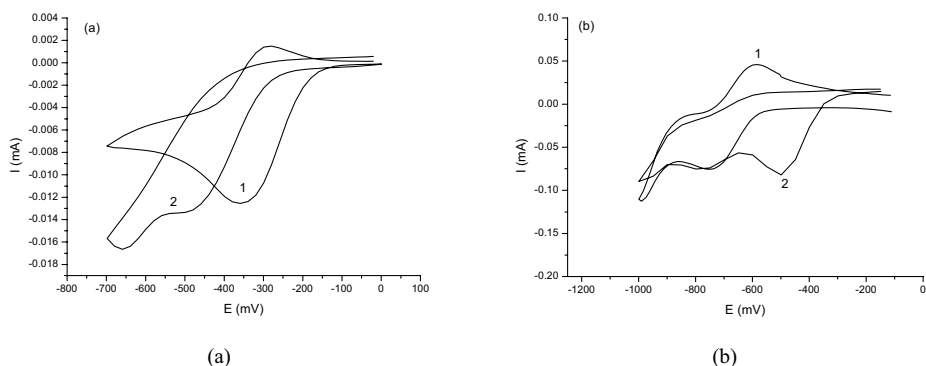


Fig. 2: Influence of dissolved oxygen on the 1st reduction wave of doxorubicin ($C = 1.22 \times 10^{-3} M$) (a) in DMSO, $v = 400 mV/s$: 1 – without oxygen, 2 – aerated; (b) in protic media (phosphate buffer, $C = 1 \times 10^{-2} M$), $v = 100 mV/s$: 1 – without oxygen, 2 – aerated.

If cycling is performed on an extended potential range (Fig. 3), it may be observed that new redox couples are apparent on the oxidation scan. At the same time, the intensity of the anodic peak of the first wave decreases, attesting for an enhanced reactivity of the anion-radical in aerated media. Possible reactions accounting for this behaviour are: $A^{\cdot-} + O_2 \rightarrow A + O_2^{\cdot-}$ and $2A^{\cdot-} \rightarrow A^{2-} + A$, the dismutation reaction explaining also the increase of the anodic peak of the second wave, corresponding to the oxidation of the dianion. The new redox couples on the positive scan were assigned respectively to the oxidation of the intermediate species AH^{\cdot} (couple III), as well as of the final reduction product, the dihydroquinone AH_2 (couple IV).

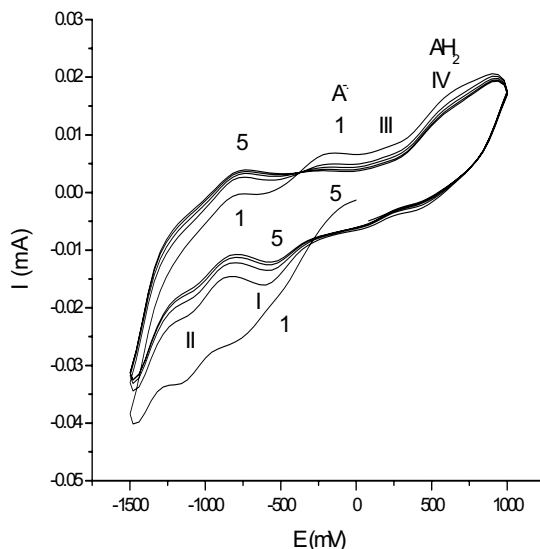


Fig. 3: Cyclic voltammetry of doxorubicin ($C = 1.22 \times 10^{-3} M$) in DMSO (aerated), extended potential range (+1 to -1.5V), successive scans ($v = 800 \text{ mV/s}$).

B. EPR results

In order to identify the paramagnetic intermediates in the reduction process of doxorubicin, EPR spectra on the electrochemical and chemical reduction were recorded. If the electrochemical reduction is performed in deaerated DMSO a fairly stable anion-radical is obtained with the spectrum in Fig. 4. The hfsplittings used to simulate the experimental spectrum were assigned to the protons in the benzenic ring of the anthraquinone moiety and to the methyl group in the methoxy group ($a_{\text{CH}_3} = 0.0936 \text{ mT}$). The value of the g-factor, 2.0038 and the spin distribution are in agreement with the quinone structure of the drug, attesting to a semiquinone type anion-radical, and point out that the main features of the electronic structure implied in reduction process are due to the anthraquinone moiety.

C. Absorption spectra

In order to get a deeper insight into the reduction mechanism of the investigated drugs, optical spectra were performed during the chemical and electrochemical reduction using *in-situ* techniques. The family of curves obtained at the electrochemical reduction of doxorubicin in DMSO at a potential after the first wave on the voltammogram is presented in Fig. 5. The absorption band of the starting compound, consisting of two overlapped bands located at 480 and 500 nm*, decreases in time and a new absorption range at 585 nm is apparent, with an isosbestic point around 550 nm. When the electrolysis is stopped and the solution is opened to air, the new band decreases and the absorption band of the initial compound is partially recovered. Therefore this band was assigned to the anion-radical ($A^{\cdot-}$), also evidenced by EPR spectroscopy in similar experimental conditions. A similar

behaviour was noted at the chemical reduction of doxorubicin with potassium *t*-butoxide, and, as the same EPR signal was obtained in these conditions too, the species absorbing at 585 nm was assigned to the anion-radical. At prolonged reduction, new absorption ranges are apparent at about 640 nm and 357 nm, the reduction process becoming irreversible. Therefore these bands could be assigned to the protonated reduction intermediate (AH⁻) and respectively to the reaction product, presumably the hydroquinone AH₂, also signaled by the electrochemical study (Fig. 3).

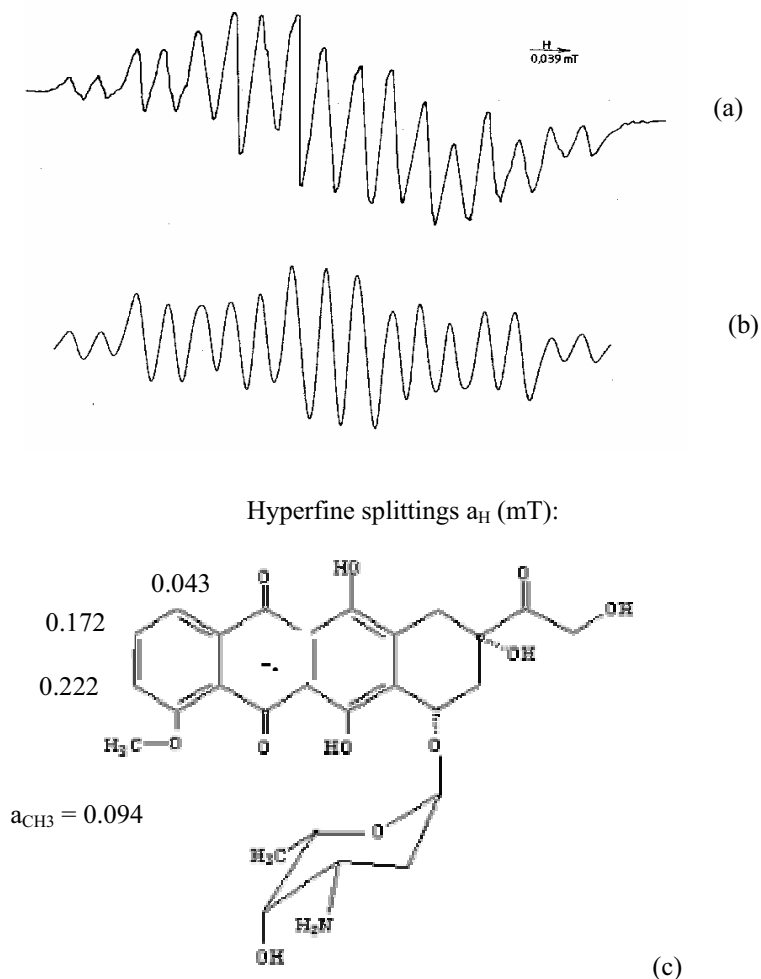


Fig. 4: EPR spectrum obtained at the electrochemical reduction of a 1.23×10^{-3} M solution of doxorubicin in DMSO/0.1 M TBABF₄ at the potential of the 1st wave, a) experimental; b) simulated; c) assignment of the hfsplitting constants to the different positions in the molecule.

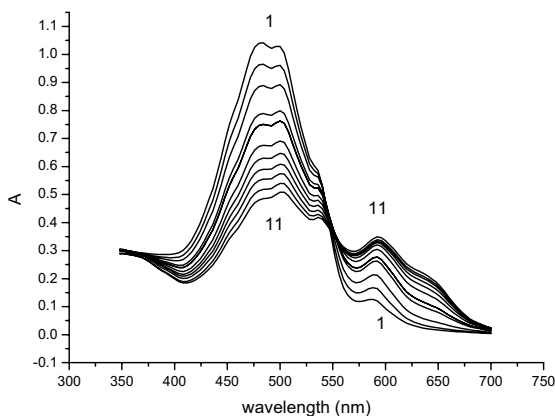
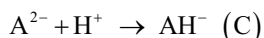
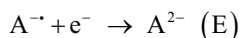
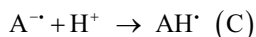
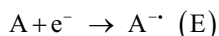


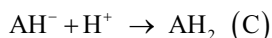
Fig. 5: Absorption spectra recorded at the electrolysis of an epirubicin solution ($C = 4 \times 10^{-3} \text{ M}$) in DMSO/0.1 M TBABF₄ at the potential of the 1st wave: 1–11 in time.

Corroborating all experimental data, the following reduction mechanism may be proposed for doxorubicin and epirubicin in deaerated aprotic solvents with low amounts of protons:



i.e. a complex sequence in which intermediate species like $A^{\cdot -}$, A^{2-} , AH^{\cdot} and AH^- were evidenced by cyclic voltammetry, EPR and/or optical spectroscopy. In aerated media the possibility of electron transfer to molecular oxygen competes with the protonation of the anion-radical in the chemical step, as pointed out by the dramatic decrease of the i_{pa}/i_{pc} ratio for the first wave on aeration.

In protic media further protonation of the dianion occurs:



leading to the final reduction product, according to a ECECC sequence.

D. MO calculations

The MO calculations were performed in order to rationalise the experimental data previously discussed and aim to answer the following questions:

- Which are the electronic structural features implied in the redox reactivity of the anthracycline drugs? and
- If the reductive activation of molecular oxygen by the reduction intermediates of doxorubicin or epirubicin is possible?

MO calculations were performed using the AM1 hamiltonian in the MOPAC program package and restricted Hartree-Fock (RHF) formalism for closed shell and both UHF/ROHF for open shell structures. As the experimental results have pointed out that the sugar ring exerts practically no influence on the behaviour of these molecules in redox processes, a model compound was considered, in which the sugar was replaced by an OH group (Model 1). The results were compared with 1,4-dihydroxy-5-methoxy anthraquinone, as reference compound (Model 2).

All structures were fully optimised using eigenvector-following (EF) optimisation algorithm. Relevant electronic parameters used in the discussion of reactivity in redox processes [18,19] are:

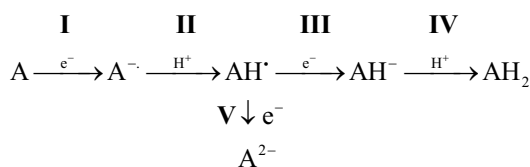
- the ionisation potential given by Koopman's theorem by: $IP = -\epsilon_{\text{homo}}$;
- the absolute electronegativity, $X_v = 1/2 (\epsilon_{\text{homo}} + \epsilon_{\text{lmo}})$;
- the adiabatic ionisation potential, IP_{ad} defined as ΔH for the reaction: $A \rightarrow A^+ + e^-$;
- the adiabatic electron affinity, EA_{ad} defined as the negative of ΔH for the reaction: $A + e^- \rightarrow A^-$;
- the adiabatic electronegativity, $X_{\text{ad}} = 1/2 (IP_{\text{ad}} + EA_{\text{ad}})$.

Table 3. AM1-calculated electronic parameters implied in redox processes.

Model	ΔH (kcal/mol)	ϵ_{homo} (eV)	ϵ_{lmo} (eV)	X_v (eV)	IP_{ad} (kcal/mol)	EA_{ad} (kcal/mol)	X_{ad} (eV)
Model 1 (UHF)	-263.15	-8.91	-1.41	5.16	179.74	59.44	5.18
Model 2 (UHF)	-129.4	-8.98	-1.45	5.21	186.9	49.72	5.13

The results are presented in Table 3 and show low lying l.e.m.o orbitals and high absolute and adiabatic electronegativities, in agreement with the high reducibility of these compounds and with the experimental reduction potentials. Moreover, the adiabatic and absolute electronegativities are close to one another, indicating that no significant geometry modifications arise in the anion-radical as against the starting molecule. The presence of the saturated ring and the substituents on it in Model 1 (which actually differentiate the anthracene drugs) does practically not modify the energies of the frontier orbitals and the electronegativity values as against Model 2, which reflects the redox properties of the anthraquinone moiety. Comparison of the results of Model 1 with those obtained for the whole molecule (doxorubicin) shows only minor differences in the energies of the frontier orbitals (-8.92 eV for h.o.m.o and -1.45 eV for l.e.m.o.) and justifies the use of this simplified model in order to save computational time. Therefore only these results will be further discussed.

The reduction pathway as evidenced by experimental data may be considered as a sequence of electron and proton transfers. Therefore, the energetics of the following sequences was analysed, leading from the starting molecule to the final reduction product AH_2 , using the values of the formation enthalpies calculated for all intermediate species:



The results are contained in Table 4 and allow for the following comments: the first electron transfer leading to the anion-radical is energetically favourable; total reduction of the drug to AH₂ is exothermic; the formation of the dianion from the starting compound, although exothermic, seems to be less favourable than formation of the protonated anion-radical (AH⁻). The reaction of the reduced species AH₂ with the substrate A, leading to reactive species AH[·] is exothermic. Both the second electron transfer and the protonation of the anion-radical are endothermic.

Table 4. Energetics of the intermediate reduction steps for anthracycline drugs.

Step	Model 1 (UHF)
I	-59.44
II	41.68
III	-55.46
IV	58.30
Overall	-14.92
A + AH ₂ ⇌ 2AH	-20.60
A → A ²⁻	-18.82

As the reactivity towards molecular oxygen is concerned, the energetics of the ET-reactions in Table 5 was examined, for both ground (³Σ_g) and singlet (¹Δ_g) states of oxygen. For the oxygen species literature values [18] were employed.

Table 5. Energetics of electron transfer reactions to molecular oxygen.

Reaction	O ₂ [*]	Model 1
A ^{-·} + O ₂ → A + O ₂ ^{-·}	¹ Δ _g	27.74
	³ Σ _g	50.34
A ²⁻ + O ₂ → A ^{-·} + O ₂ ^{-·}	¹ Δ _g	-72.32
	³ Σ _g	-49.72
AH [·] + O ₂ → A + HO ₂ [·]	¹ Δ _g	-11.84
	³ Σ _g	10.76
AH ₂ + O ₂ → AH [·] + HO ₂ [·]	¹ Δ _g	-32.44
	³ Σ _g	-9.84

*For oxygen species values from [18] were employed:

For O₂ ΔH_f (¹Δ_g) = 18.4 kcal/mol;

 ΔH_f (³Σ_g) = -4.2 kcal/mol;

For O₂^{-·} ΔH_f = -13.3 kcal/mol;

For HO₂[·] ΔH_f = -11.2 kcal/mol.

Analysis of the data in Table 5 shows that the electron transfer from A^- is not energetically favourable for both states of oxygen, but from the dianion A^{2-} (*i.e.* after the second reduction step) is energetically favourable, even for the ground triplet state. Oxidation of the reduced species AH_2 by O_2 seems possible for both states of oxygen, whereas for AH^\cdot it is not favourable energetically.

This attempt to rationalise the experimental results by means of semiempirical calculations is only a simplified thermodynamic approach which does not take into account solvation effects, which are expected to be important especially in polar aprotic solvents, and give no indications about the rate and the real reduction mechanism. Therefore further study will be necessary to give a more complete understanding of the reductive activation of antitumoral drugs.

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