Quantitative structure activity relationships for the conversion of nitrobenzimidazolones and nitrobenzimidazoles by DT-diaphorase: implications for the kinetic mechanism

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Abstract Quantitative structure activity relationships (QSARs) for the conversion of nitrobenzimidazolones and nitrobenzimidazoles by rat liver DT-diaphorase (EC 1.6.99.2) are described. The parameter used for description of the QSARs is the energy of the lowest unoccupied molecular orbital (E(LUMO)) of the nitroaromatic compounds. Interestingly, correlations with E(LUMO) were observed for both the natural logarithm of k_{cat}, but also for the natural logarithm of k_{cat}/K_m. The minimal kinetic model in line with these QSARs is a ping-pong mechanism that includes a substrate binding equilibrium in the second half reaction.

Key words: DT-diaphorase; Nitrobenzimidazol(on)e; Kinetic model; Quantitative structure activity relationship; Energy of lowest unoccupied molecular orbital

1. Introduction

DT-diaphorase (NAD(P)H:quinone oxidoreductase) (EC 1.6.99.2) catalyses the two-electron reduction of quinones and nitroaromatics [1–3]. It is a cytosolic dimeric flavoprotein containing one molecule of FAD per subunit [4].

The enzyme is believed to be involved in detoxification of electrophilic redoxactive quinones, since the two-electron reduction competes with the one-electron reduction of these compounds by flavoenzyme oxidoreductases, the latter leading to formation of toxic redox cycling intermediates [5,6]. On the other hand the enzyme could be involved in the bioreductive activation of specific drugs which require a two-electron reduction to become active. The observation that the activity of DT-diaphorase is strongly increased in certain tumour and virus transformed cells [1], provides the basis for the possible use of its activity in bioreductive activation of certain antitumour alkylating quinones [1,7,8].

In addition to quinones, nitroaromatics can be reduced by DT-diaphorase. Nitroaromatic compounds are widely used as industrial intermediate reagents in the production of dyes, rubbers and pharmaceuticals or are used as agrochemicals such as pesticides or veterinary drugs [9–11]. Although nitroaromatics can be reduced by DT-diaphorase, the activity of the enzyme with nitroaromatics is considerably lower than that with quinones [2,12–14]. Due to the low nitroreductase activity of DT-diaphorase attempts to use this enzyme for the bioreductive activation of nitroaromatics, inducing for example the genotoxicity of dinitropyrenes [3] or the cytotoxicity of CB-1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide), have been hampered. In a recent study, a series of newly synthesised nitrobenzimidazolones and nitrobenzimidazoles proved to be efficient substrates for DT-diaphorase, although still being ca. 3 orders of magnitude less reactive than quinone compounds [13].

Attempts to correlate and quantitatively understand the relative effectiveness of the various nitroaromatics as substrates for conversion by DT-diaphorase were unsuccessful so far [12,13,15]. The objective of the present study was to investigate whether the use of quantum mechanical computer calculations might provide a basis to investigate the possible existence of quantitative structure activity relationships for the conversion of the nitrobenzimidazol(on)es by DT-diaphorase.

2. Materials and methods

2.1. Chemicals

Fig. 1 presents the structural formulas of the nitrobenzimidazol(on)e model compounds used in the present study. Compounds 1–5, 9 and 10 were prepared as previously described [13]. Compounds 6–8 were synthesised using the same method. All compounds were characterised by melting point, ^{1}H-NMR, and IR spectroscopy (data will be published elsewhere).

2.2. Enzymatic assay

Rat liver DT-diaphorase (EC 1.6.99.2) was purified as described previously [6]. The enzyme concentration was determined spectrophotometrically using $\varepsilon_{460} = 11\ mM^{-1}\ cm^{-1}$. All kinetic measurements were carried out in 0.1 M potassium phosphate pH 7.0 containing 1 mM EDTA, 0.01% Tween-20 and 0.25 mg/ml bovine serum albumin, the last two as DT-diaphorase activators [7], at 25°C. The rate of nitroreductase activity of DT-diaphorase was monitored spectrophotometrically following NADPH oxidation ($\varepsilon_{340} = 6.2\ mM^{-1}\ cm^{-1}$). To a 1 ml reaction mixture 10–20 μl of a desired stock solution of the nitrobenzimidazol(on)e substrate, dissolved in DMSO, was added to start the reaction. Final concentrations of the nitrobenzimidazol(on)e substrates varied between 0.025 and 4 mM. It was tested previously that 1–2% of DMSO in the enzyme reaction mixture has no influence on the enzyme activity. Final NADPH concentrations used in the kinetic experiments varied between 15–100 μM.

Assuming ping-pong reaction kinetics [14,15], the kinetic parameters of the DT-diaphorase reaction, i.e. the catalytic constant k_{cat} and the parameter $k_{cat}/K_{m(nitrobenzimidazol(on)e)}$, correspond to the reciprocal intercepts and slopes of the Lineweaver-Burk plots in which enzyme concentration/rate is plotted against 1/nitrobenzimidazol(on)e concentration. The parameter k_{cat} represents the number of NADPH molecules oxidised per active centre of enzyme per 1 s. Typically five or six

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concentrations of the nitrobenzimidazol(on)es were used for k_{cat} and $k_{cat}/K_{m(nitrobenzimidazol(on)e)}$ determinations and experiments were performed in duplicate or triplicate. The rates obtained were corrected for intrinsic NADPH:oxidase activity of the enzyme.

2.3. Quantum mechanical calculations

Quantum mechanical molecular orbital calculations were carried out on a Silicon Graphics Indigo^2 using Spartan (version 5.0) (Wavefunction, Inc.). Different levels of theory and methods were used. For semi-empirical molecular orbital calculations the Hartree-Fock method and the AM1 and PM3 Hamiltonian was used. Ab initio calculations were performed using a Hartree-Fock method with the 3-21G(*) basis set and density functional calculations were performed with the pBP method using the DN** basis set. For all calculations geometries were fully optimised.

In this study, the outcomes of quantum mechanical calculations on molecules in vacuum are related to the electronic characteristics of the substrates in the active site of DT-diaphorase. Due to solvation effects and a different dielectric constant, the intrinsic properties of the compounds might be influenced upon binding to this active site. However, it is assumed that this phenomenon will not have a substantial influence on the relative differences between parameters for a series of closely related compounds. The outcomes of the in vacuum computer calculations can thus be used as an approach to study relative differences within a series of related compounds [16–19] or within one molecule [20].

2.4. Linear and multiple linear regression analysis

Linear regression analysis was applied for determination of correlations between the calculated E(LUMO) and the natural logarithm of k_{cat} or k_{cat}/K_m for the enzyme catalysed conversions, using Kaleidagraph.

In additional QSAR studies hydrophobicity and steric factors were included by using the calculated log P and Van der Waals volume of the substrates together with the E(LUMO) in multiple linear regression analysis. This multiple linear regression analysis was performed using SPSS for windows release 7.0.

3. Results

3.1. Kinetic constants for reduction of nitrobenzimidazol(on)es by DT-diaphorase

Table 1 presents an overview of the kinetic constants for the DT-diaphorase catalysed reduction of the various nitrobenzimidazol(on)e model compounds. The k_{cat} and $k_{cat}/K_{m(nitrobenzimidazol(on)e)}$ values of compounds 1–5, 9 and 10 were taken from the literature [13]. To extend the number of data points for our QSAR study, k_{cat} and $k_{cat}/K_{m(nitrobenzimidazolone)}$ values of newly synthesised nitrobenzimidazolones (compounds 6–8) were determined (Table 1). From Table 1 it can be derived that the kinetic constants of the various nitrobenzimidazol(on)es vary by several orders of magnitude.

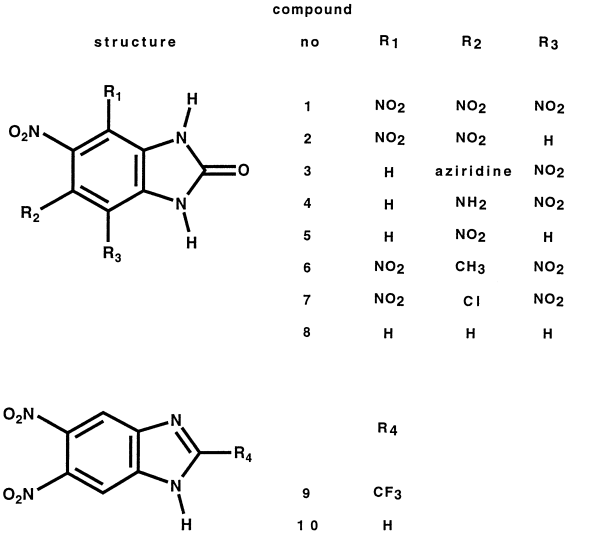

Fig. 1. Structural formulas of the nitrobenzimidazolone (1–8) and nitrobenzimidazole (9, 10) model compounds used in the present study.

3.2. Molecular orbital calculations

Table 2 presents the energies of the lowest unoccupied molecular orbital, E(LUMO), of the different nitrobenzimidazol(on)e model compounds of the present study, as calculated by different quantum mechanical methods at different levels of theory.

Although the absolute outcomes vary from one method to another, the relative orders are consistent going from one method to another. This is corroborated by the fact that the data calculated with one method correlate with those obtained by another method ($r = 0.975$–0.996 in all cases).

3.3. Quantitative structure activity relationships (QSARs)

First, the natural logarithm of the $k_{cat}/K_{m(nitrobenzimidazol(on)e)}$ for the reduction of nitrobenzimidazol(on)es by DT-diaphorase was plotted against the calculated E(LUMO) values of these compounds (Fig. 2a). A clear correlation is obtained. Fig. 2a presents the data as obtained with the semi-empirical PM3 method ($r = 0.965$). Similar correlations are found with the other methods, namely $r = 0.951$, $r = 0.932$, and $r = 0.938$ for respectively the semi-empirical AM1, the ab initio 3-21G(*) and the density functional DN** method.

In addition, in Fig. 2b the natural logarithm of the k_{cat} is plotted against the calculated E(LUMO) of the nitrobenzimi-

Table 1
Kinetic constants for the conversion of the nitrobenzimidazol(on)e model compounds, as taken from the literature or measured in the present study

Compound	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	Reference
1	102 ± 7.0	$5.0 \pm 1.0 \times 10^6$	[13]
2	2.4 ± 0.3	$2.6 \pm 0.5 \times 10^4$	[13]
3	1.5 ± 0.1	$8.6 \pm 0.9 \times 10^3$	[13]
4	1.1 ± 0.2	$8.0 \pm 1.5 \times 10^2$	[13]
5	0.7 ± 0.1	$5.0 \pm 0.8 \times 10^3$	[13]
6	91 ± 9	$6.2 \pm 1.5 \times 10^6$	present study
7	117 ± 11.5	$6.6 \pm 1.5 \times 10^6$	present study
8	0.04 ± 0.01	$1.0 \pm 0.2 \times 10^1$	present study
9	1.5 ± 0.2	$6.0 \pm 1.0 \times 10^3$	[13]
10	0.12 ± 0.01	$2.9 \pm 0.2 \times 10^3$	[13]

Structural formulas are presented in Fig. 1. K_m refers to $K_{m(nitrobenzimidazol(on)e)}$.

Table 2
Calculated E(LUMO) values for the nitrobenzimidazol(on)e model compounds

Compound	E(LUMO) in eV			
Method:	HF/AM1	HF/PM3	HF/3-21G(*)	pBP/DN**
1	−3.276	−3.120	−1.185	−5.689
2	−2.644	−2.517	−0.795	−4.911
3	−1.854	−1.971	0.450	−4.251
4	−1.886	−1.869	−0.056	−4.491
5	−2.096	−2.028	−0.050	−4.486
6	−2.875	−2.834	−1.013	−5.187
7	−3.002	−2.897	−1.175	−5.388
8	−1.264	−1.305	1.067	−3.801
9	−2.242	−2.232	0.066	−4.551
10	−1.862	−1.806	0.280	−4.275

Structural formulas are presented in Fig. 1. All energies are presented in eV.

dazol(on)es. Again, a clear correlation is observed for all methods used, i.e. $r = 0.935$ for AM1, $r = 0.957$ for PM3, $r = 0.919$ for 3-21G(*) and $r = 0.944$ for the DN** method, respectively.

The quantum mechanical molecular orbital calculations also provide many other parameters in addition to the E(LUMO) of the nitrobenzimidazol(on)es. Using the hydrophobicity parameter log P and/or the calculated Van der Waals volume of the substrates as a second parameter in addition to the E(LUMO), multiple linear regression analysis was performed. The results obtained do not show a substantial improvement of the correlations and do not point at a significant influence of these parameters in addition to the E(LUMO). Thus, it can be concluded that the main characteristic determining the DT-diaphorase catalysed conversion of the nitrobenzimidazol(on)es is their ease of reduction, reflected by their calculated E(LUMO) value.

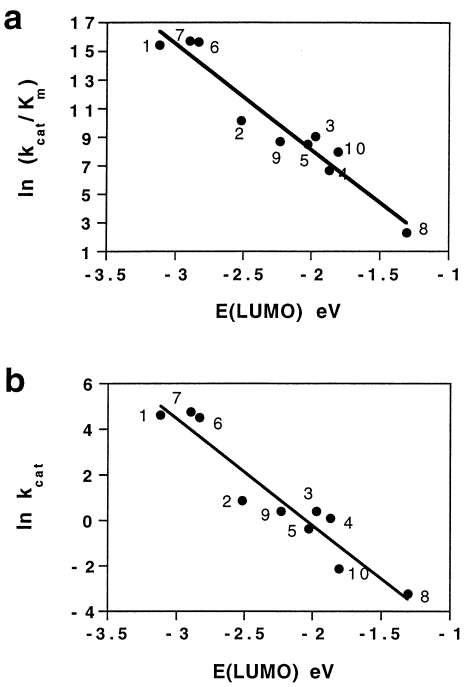

Fig. 2. Quantitative structure activity relationships describing the correlation between (a) the natural logarithm of $k_{cat}/K_{m(nitrobenzimidazol(on)e)}$ and E(LUMO) ($r = 0.965$), as well as between (b) the natural logarithm of k_{cat} and E(LUMO) ($r = 0.957$), for the reduction of a series of nitrobenzimidazol(on)es by DT-diaphorase. E(LUMO) data used were those calculated by the PM3 method.

3.4. Reevaluation of the kinetic model

Fig. 3a presents the kinetic model on which the idea of using the k_{cat}/K_m parameter for QSAR studies is based. In this model $k_{cat}/K_{m(nitrobenzimidazol(on)e)}$ represents the bimolecular rate constant for the reaction between the reduced enzyme and the nitrobenzimidazol(on)e substrate, k_5 in Fig. 3a. This mechanism can be characterised as an incomplete ping-pong kinetic mechanism in which the reversible formation of a complex between the reduced enzyme and the nitroaromatic substrate is ignored. For this model the following steady-state rate equation can be derived:

$$v = \frac{k_2[e]}{1 + \frac{(k_{-1} + k_2)}{k_1[NADPH]} + \frac{k_2}{k_5[nitrobenzimidazol(on)e]}}. \quad (1)$$

From this equation it follows that k_{cat} equals k_2, the $K_{m(NADPH)}$ for the first half reaction equals $(k_{-1}+k_2)/k_1$, and

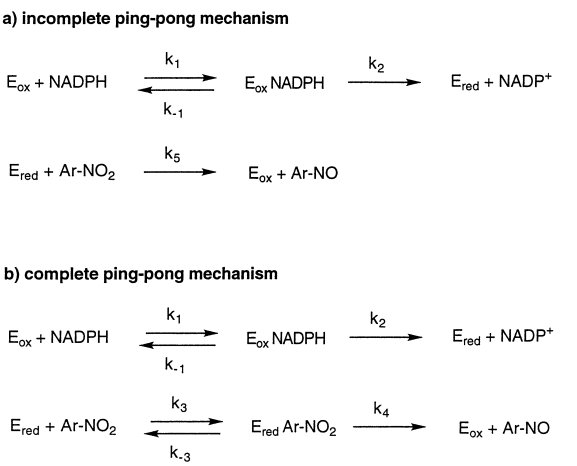

Fig. 3. Kinetic scheme and the corresponding rate equation for (a) an incomplete ping-pong mechanism, i.e. without a substrate binding equilibrium in the second half reaction and (b) a complete ping-pong mechanism, i.e. with a substrate binding equilibrium in the second half reaction. Ar-NO_2 and Ar-NO represent the oxidised and two-electron reduced form of the nitrobenzimidazol(on)e. The corresponding rate equations are discussed in the text. E_{ox} represents DT-diaphorase-FAD (oxidised form of the enzyme), and E_{red} represents DT-diaphorase-$FADH_2$ (reduced form of the enzyme).

the $K_{m(nitrobenzimidazol(on)e)}$ for the reduction of the nitroaromatic substrate in the second half reaction equals k_2/k_5. At saturating concentrations of NADPH, [NADPH] $\gg (k_{-1}+k_2)/k_1$, the $k_{cat}/K_{m(nitrobenzimidazol(on)e)}$ parameter equals k_5, which is the bimolecular rate constant for the reaction between reduced enzyme and the nitroaromatic substrate. This explains the correlation observed between the natural logarithm of $k_{cat}/K_{m(nitrobenzimidazol(on)e)}$ and the calculated E(LUMO) of the nitrobenzimidazol(on)es.

However, if the kinetic model presented in Fig. 3a would apply, a correlation between the natural logarithm of k_{cat} and the parameter describing the ease of reduction of the nitrobenzimidazol(on)es, E(LUMO), can not be explained. This can be derived from the fact that the rate equation for this model predicts that $k_{cat} = k_2$. Therefore, the observation of a correlation between the natural logarithm of k_{cat} and the E(LUMO) of the nitrobenzimidazol(on)es requires reevaluation of the kinetic model often used to describe the reduction of aromatic substrates by flavin dependent reductases [15,21–24], at least as far as the reduction of nitrobenzimidazol(on)es by DT-diaphorase is concerned.

In order to find a rationale for the observed correlation of the calculated E(LUMO) with the natural logarithm of both k_{cat} and $k_{cat}/K_{m(nitrobenzimidazol(on)e)}$, Fig. 3b presents the complete ping-pong kinetic scheme. The rate equation that can be derived for this model is as follows:

$$v = \frac{\dfrac{k_2 k_4 [e]}{(k_2 + k_4)}}{1 + \dfrac{k_4(k_{-1} + k_2)}{k_1(k_2 + k_4)[NADPH]} + \dfrac{k_2(k_{-3} + k_4)}{k_3(k_2 + k_4)[nitrobenzimidazol(on)e]}} \quad (2)$$

In this rate equation the k_{cat} is a function of the first order rate constants k_2 and k_4 for the first and second half reactions, $k_2k_4/(k_2+k_4)$. The apparent $K_{m(NADPH)}$ and $K_{m(nitrobenzimidazol(on)e)}$ equal $k_4(k_{-1}+k_2)/k_1(k_2+k_4)$ and $k_2(k_{-3}+k_4)/k_3(k_2+k_4)$, respectively. Thus, $k_{cat}/K_{m(nitrobenzimidazol(on)e)}$ now equals $k_3k_4/(k_{-3}+k_4)$, and contains the kinetic constants k_3 and k_{-3} for substrate binding in the second half reaction and is governed by the first order rate constant k_4 for conversion of the reduced enzyme-nitrobenzimidazol(on)e complex. So, when $k_{-3} \gg k_4$ and the association constants k_3/k_{-3} for the different nitrobenzimidazol(on)es do not vary substantially, this model can explain the correlation between natural logarithm of $k_{cat}/K_{m(nitrobenzimidazol(on)e)}$ and E(LUMO).

In contrast to the model presented in Fig. 3a, for which k_{cat} equals k_2, the model of Fig. 3b results in a $k_{cat} = k_4$ and, thus, can also explain the observation of a correlation between the calculated E(LUMO) of the nitrobenzimidazol(on)es and the natural logarithm of k_{cat}.

It is of importance to stress that the model of Fig. 3b represents the minimal kinetic scheme able to explain our QSAR observations. This implies that transient kinetic studies may still reveal additional reaction steps of interest and lead to a more extended kinetic scheme. This is, however, beyond the scope of the present paper. In relation to this it is relevant to point out that literature data have reported bimolecular rate constants, obtained by transient kinetics, for the DT-diaphorase catalysed reduction of some quinones and ferricyanide in the second half reaction, which are almost identical to the k_{cat}/K_m values obtained in steady-state kinetics [15].

4. Discussion

The results obtained in the present study now, for the first time, show clear correlations between a parameter describing the relative ease of reduction of a series of nitroaromatic substrates, i.e. nitrobenzimidazol(on)es, and experimental data for their conversion by DT-diaphorase (EC 1.6.99.2). The parameter used for description of the quantitative structure activity relationships (QSARs) was the energy of the lowest unoccupied molecular orbital, E(LUMO), of the nitroaromatic compounds, calculated by quantum mechanical calculations. This E(LUMO) is representative for the relative ease of single- or two-electron reduction of the nitroaromatic model compounds. Especially for the nitrobenzimidazol(on)es, for which redox potentials for their single- or two-electron reduction are not known, the use of the computer calculation based approach proved valid. Although the E(LUMO) value is representative for the relative ease of both single- and two-electron reduction, previous results [13] showed that DT-diaphorase does not support redox cycling of nitrobenzimidazol(on)es, suggesting that the enzyme catalyses the two- and not the single-electron reduction of these substrates.

A correlation of E(LUMO) with the natural logarithm of k_{cat}/K_m was observed (Fig. 2a). This k_{cat}/K_m is a parameter previously often used to define QSARs for single-electron reduction of quinones and nitroaromatics by flavoenzymes [21–24]. However, in the present study it was also demonstrated that E(LUMO) correlates with the natural logarithm of k_{cat} itself (Fig. 2b).

Kinetic analysis shows that a possible minimal kinetic scheme able to explain the observed correlation between the natural logarithm of k_{cat}/K_m and E(LUMO), but also between the natural logarithm of k_{cat} and E(LUMO), includes a reversible substrate binding equilibrium in the second half reaction (Fig. 3b). Thus, it can be concluded that the QSARs and the kinetic analyses of the present study corroborate the existence of a substrate binding equilibrium between the reduced enzyme and the aromatic substrate in the case of nitroreduction by DT-diaphorase.

Essential in the kinetic ping-pong mechanism applied often to describe the reduction of quinones and nitroaromatics by flavin dependent reductases, is the supposed absence of the substrate binding equilibrium, because a second order reaction between the reduced enzyme and the substrate is supposed to lead directly to product formation [15,21–24]. However, in previous studies on the conversion of quinones and nitroaromatic substrates by DT-diaphorase, the difference in catalytic activity of DT-diaphorase with quinones and nitroaromatics has already been ascribed to the fact that nitroaromatics bind to the adenosine phosphate binding region of the NADPH binding site [13], whereas the quinones are supposed to bind in the close vicinity to the isoalloxazine ring of reduced FAD [13,25]. This explanation also includes complex formation between the aromatic substrate and DT-diaphorase.

For the other flavin dependent reductases only k_{cat}/K_m and not k_{cat} could be determined, due to the fact that the solubility of the nitroaromatic substrates is substantially below their respective K_m values. Therefore, the consequences of our present findings for the reduction of nitroaromatics and quinones by other flavin dependent reductases remains to be investigated. Furthermore, correlations reported by Orna et al.

[22] and O'Connor et al. [23] between the logarithms of V_{max} and V_{max}/K_m parameters and single- or two-electron reduction potential values, for the reduction of nitroaromatic compounds by other flavoenzymes, i.e. cytochrome P450 reductase, or xanthine oxidase, might as well be explained in the framework of a complete ping-pong kinetic scheme.

Finally, one may conclude, that the existence of quantitative structure activity relationships for the conversion of nitrobenzimidazol(on)es by DT-diaphorase may prove to be of interest for the prediction of DT-diaphorase activity with newly synthesised nitroaromatic compounds.

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