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ORIGINAL ARTICLE

Flavoenzyme-catalyzed single-electron reduction of nitroaromatic antiandrogens: implications for their cytotoxicity

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ABSTRACT

The therapeutic action of nitroaromatic antiandrogens nilutamide and flutamide may be complicated by their cytotoxicity, whose mechanisms are still incomprehensively understood. In particular this concerns the enzymatic redox cycling of flutamide and its metabolites, and its impact on their cytotoxicity. In this work, we examined the single-electron reduction of nilutamide, flutamide, its metabolites 2-hydroxyflutamide and 4-nitro-3-trifluorormethyl-phenylamine, and a topical antiandrogen (3-amino-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl)-phenyl) propanamide by NADPH:cytochrome P-450 reductase and adrenodoxin reductase/adrenodoxin. The obtained steady-state bimolecular rate constants of oxidant reduction (k_{cat}/K_m) enabled to establish single-electron reduction midpoint potentials (E^1_7) of compounds, −0.377 – −0.413 V, which were in line with enthalpies of formation of their free radicals, obtained by quantum mechanical calculations. Using murine hepatoma MH22a cells, the obtained cytotoxicity *vs.* E^1_7 correlation based on the data of model nitroaromatic compounds shows that redox cycling and oxidative stress could be the main factor of cytotoxicity of nitroaromatic antiandrogens. Other minor cytotoxicity factors could be their redox metabolism involving NAD(P)H:quinone oxidoreductase (NQO1) and cytochromes P-450.

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Introduction

The therapeutic and/or cytotoxic effects of nitroaromatic compounds ($ArNO_2$) frequently stem from their single-electron reduction by flavoenzymes dehydrogenases-electrontransferases, for example, NADPH: cytochrome P-450 reductase (P-450R) ([1–4], and references therein). The redox cycling of formed free radicals of nitroaromatics ($ArNO_2^{-\bullet}$) with oxygen yields superoxide ($O_2^{-\bullet}$), H_2O_2 and hydroxyl radical ($OH^\bullet$), that is, causes oxidative stress. Frequently, oxidative stress plays a leading role in aerobic cytotoxicity of $ArNO_2$. This is indicated by an increase of compound cytotoxicity with their single-electron reduction midpoint potential at pH 7.0 (E^1_7) with a relationship of $\Delta\log cL_{50}/\Delta E^1_7 \sim -10\,V^{-1}$, where cL_{50} is compound concentration for 50% cell killing [1,5–8]. This dependence mirrors the log (rate constant) *vs.* E^1_7 relationships in single-electron reduction of $ArNO_2$ by P-450R or other single-electron transferring flavoenzymes [1–4,7–9]. Although these dependences are scattered because of the superposition of oxidative stress and other cytotoxicity factors, E^1_7 represents an important parameter in the assessment of $ArNO_2$ action mechanisms and prediction of their cytotoxicity [5,7,8]. The additional cytotoxicity mechanisms include slow two(four)-electron reduction of $ArNO_2$ into DNA-alkylating hydroxylamines (ArNHOH) by NAD(P)H: quinone oxidoreductase (NQO1) ([10], and references therein). In parallel, cytochromes P-450 perform the oxidative denitration of certain $ArNO_2$ [11], and also convert their final reduction products, aromatic amines ($ArNH_2$), into ArNHOH [12]. One may note, however, that the introduction of bioreductively activated alkylating or leaving groups into $ArNO_2$ moiety abrogates the relationship between cytotoxicity and E^1_7 [13,14].

Nilutamide and flutamide (Figure 1) act as competitive antagonists of the androgen receptor, and are used in the treatment of advanced prostate cancer. Their therapeutic action may be complicated by the side effects such as lung fibrosis and drug-induced hepatitis,

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Supplemental data for this article can be accessed here.

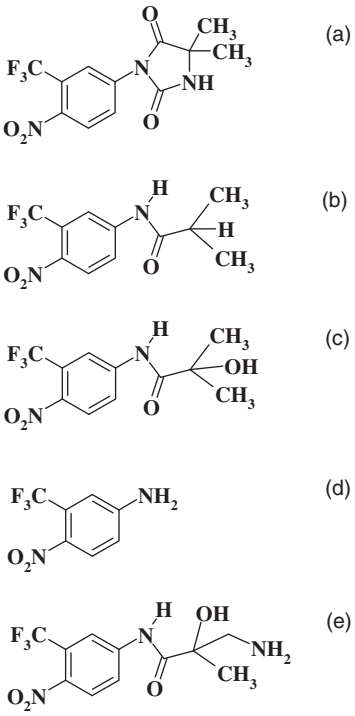

Figure 1. Formulae of examined nitroaromatic antiandrogens and their metabolites.

whose mechanisms are still incomprehensively understood [15–17]. The cytotoxicity of nilutamide in mammalian cells was attributed to redox cycling and oxidative stress [15]. However, the data on enzymatic redox cycling of structurally similar flutamide and its impact on cytotoxicity are controversial [16,18]. It is supposed that flutamide acts mainly through its metabolites 2-hydroxyflutamide and 4-nitro-3-trifluoromethyl-phenylamine (Figure 1), whose products of cytochrome P-450-catalysed oxidation react with reduced glutathione (GSH) and/or bind to cellular proteins [19–23]. Another possible cytotoxicity mechanism of nilutamide and flutamide is the inhibition of mitochondrial Complex I [24,25].

In this context, one may note that the enzymatic reduction and electron-transfer properties of nilutamide and flutamide were scarcely investigated [18,26,27], and their possible correlation with cytotoxicity was not examined. In this work, we characterized the range of E^1_7 values of nilutamide, flutamide, and its derivatives (Figure 1) by combining the kinetic studies of flavoenzyme-catalyzed reduction and quantum mechanical calculations. The obtained cytotoxicity *vs.* E^1_7 correlation based on the data of model nitroaromatic compounds pointed to redox cycling and oxidative stress as the main factor of their cytotoxicity.

Materials and methods

Enzymes and reagents

Recombinant rat P-450R, bovine adrenodoxin reductase (ADR) and adrenodoxin (ADX) were prepared as described in [28], their concentrations were determined according to $\varepsilon_{456} = 21.4\,mM^{-1}\cdot cm^{-1}$, $\varepsilon_{450} = 11.0\,mM^{-1}\,cm^{-1}$ and $\varepsilon_{414} = 10.0\,mM^{-1}\,cm^{-1}$, respectively. NQO1 was prepared from rat liver according to Prochaska [29], its concentration was determined according to $\varepsilon_{460} = 11.0\,mM^{-1}\,cm^{-1}$. (3-Amino-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl)phenyl) propanamide was obtained from Clearsynth (Mississauga, ON, Canada). Nilutamide, flutamide and its metabolites, NADPH, cytochrome *c* and its partly acetylated form, and other enzymes and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), and used as received.

Enzymatic assays

The kinetic measurements were carried out spectrophotometrically using a PerkinElmer Lambda 25 spectrophotometer (PerkinElmer, Waltham, MA, USA) in 0.1 M K-phosphate buffer (pH 7.0) containing 1 mM EDTA at 25 °C. The enzyme activities, expressed as number of moles of cytochrome *c* reduced per mole of the enzyme active center per second, were determined according to the rate of reduction of 50 μM cytochrome *c* ($\Delta\varepsilon_{550} = 20\,mM^{-1}\,cm^{-1}$). At NADPH concentrations indicated below, they were close to those reported previously [30]: $39\,s^{-1}$ (P-450R, [NADPH] = 100 μM), $7.5\,s^{-1}$ (ADR, [ADX] = 0.5 μM, [NADPH] = 50 μM), and $1750s^{-1}$ (NQO1, [NADPH] = 150 μM, [menadione] = 10 μM). In this case, 0.01% Tween 20 and 0.25 mg/mL bovine serum albumin were added as NQO1 activators. In all the cases the native form of cytochrome *c* was used unless specified otherwise.

The initial rates of enzymatic NADPH-dependent reduction of nitroaromatics were determined according to the rates of NADPH oxidation ($\Delta\varepsilon_{340} = 6.2\,mM^{-1}\,cm^{-1}$) using NADPH concentrations as in cytochrome *c* reduction assays, 50 nM enzyme, and 6–7 oxidant concentrations not exceeding 250 μM. In the case of ADR, 0.5 μM ADX was added into reaction mixture. The order of addition of reactants was NADPH, nitroaromatic compound, and, if necessary, ADX, cytochrome c or its acetylated form, and superoxide dismutase. The reactions were started by the addition of corresponding flavoenzyme. The reactions of NQO1 were examined in the presence of activators. The stock solutions of oxidants were prepared in DMSO (dilution

factor 100). The values of turnover rate, k_{cat}, which reflect the maximal number of moles of NADPH oxidized or oxidant reduced per mole of the enzyme active center per second, and k_{cat}/K_m, the bimolecular rate constant of oxidant reduction (or catalytic efficiency constant), correspond to the inverse intercepts and slopes in Lineweaver-Burk coordinates, [E]/v vs. 1/[oxidant], where [E] is enzyme concentration, and v is reaction rate. The reaction rates were corrected for intrinsic NADPH oxidase activities of enzymes, 0.05 s^{-1} (P-450R), 0.1 s^{-1} (NQO1), and 0.12 s^{-1} (ADR + 0.5 μM ADX). The rates of NQO1-catalyzed reactions were additionally corrected for 340 nm absorbance changes due to disappearance of nitroaromatic compounds. The latter were obtained under the same conditions in the presence of NADPH regeneration system, 10 mM glucose-6-phosphate and 3 μg/mL glucose-6-phosphate dehydrogenase. The values of k_{cat} and k_{cat}/K_m were obtained by fitting the experimental data to the parabolic or linear expression using the SigmaPlot 2000 (version 11.0, Systal Software, San Jose, CA, USA). Oxygen consumption during the reactions was monitored using a Digital Model 10 Clark electrode (Rank Brothers Ltd., Bottisham, UK) assuming that the initial O_2 concentration at 25 °C is 250 μM.

Quantum mechanical calculations

The calculations of heat of formation of compounds (Hf) were performed on nitroaromatic compounds and their single-electron reduced forms using the Gaussian09 program [31]. In order to obtain the most stable conformers of compounds corresponding to equilibrium state, Berny optimization procedure was performed using three approaches: B3LYP/cc-pVTZ, B3LYP/cc-pVTZ with the inclusion of empirical dispersion D3, and wB97XD/cc-pVTZ. The empirical dispersion D3 was included in the calculation because it plays an important role in the formation of larger molecules and systems possessing weak intra- or intermolecular contacts, and are crucial to obtain their equilibrium state [32]. The achievement of equilibrium state was verified by vibrational analysis. The enthalpies of free radical formation ($\Delta Hf(ArNO_2^{-\bullet})$) were calculated according to the Equation (1):

$$\Delta Hf(ArNO_2^{-\bullet}) = Hf(ArNO_2^{-\bullet}) - Hf(ArNO_2). \quad (1)$$

Cell culture and cytotoxicity assays

Murine hepatoma MH22a cells obtained from the Institute of Cytology of the Russian Academy of Sciences (St. Petersburg, Russia), were grown and maintained at 37 °C in DMEM medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, as described in [30]. In the cytotoxicity experiments, 3.0×10^4/mL cells were seeded in 5-mL flasks on microscope cover slides either in the presence or in the absence of compounds and were grown for 24 h. The slides were subsequently washed twice with phosphate buffer saline, and the adherent cells were counted under a light microscope. In the absence of compounds, cells reached 35–45% confluence. The viability of attached cells determined by Trypan blue accumulation, was 98.5–99.3% [30]. Cell viability after the compound treatment was expressed as the percentage of remaining adherent cells with respect to control. Stock solutions of compounds were prepared in DMSO. Its concentration in cultivation media did not exceed 0.2% and did not affect cell viability. The experiments were conducted in triplicate.

Statistical analysis and calculations

The statistical analysis was performed using Statistica (version 4.3, Statsoft, Toronto, CA). Octanol/water distribution coefficients at pH 7.0 (log D) were calculated using LogD Predictor (https://chemaxon.com). The structures of compounds were obtained using Molden software package (https://www.its.hku.hk/services/research/hpc/software/molden).

Results

Kinetics of enzymatic reactions of nitroaromatic antiandrogens

In this work, we used nilutamide, flutamide, its metabolites 2-hydroxyflutamide and 4-nitro-3-trifluorormethylphenylamine, and a topical antiandrogen (3-amino-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl)phenyl) propanamide [33] (Figure 1). We studied their reactions with P-450R, which possibly plays the most important role in the single-electron reduction and redox cycling of quinones and $ArNO_2$ in the mammalian cell [1,2]. As an additional model reaction, we studied the reduction of compounds by Fe_2S_2 redox protein adrenodoxin (ADX). Flavoenzyme NADPH:adrenodoxin reductase (ADR) reduces $ArNO_2$ in single-electron way, however, these reactions are relatively slow and complicated by the NADPH substrate inhibition [9]. On the other hand, ADX stimulates nitroreductase activity of ADR, eliminating the inhibition by NADPH and providing an alternative more efficient electron-transfer pathway *via* ADX.

The steady-state bimolecular rate constants (k_{cat}/K_m) of reduction of examined compounds by P450R and

Table 1. The rate constants of reduction of 4-nitro-3-trifluorormethyl-phenylamine derivatives by NADPH:cytochrome P-450 reductase (P-450R), adrenodoxin reductase/adrenodoxin (ADR/ADX) and NAD(P)H:quinone oxidoreductase (NQO1), 0.1 M K-phosphate, pH 7.0, 25 °C.

	Single-electron reduction			Reduction by NQO1	
Compound	P-450R k_{cat}/K_m ($M^{-1}s^{-1}$)	ADR/ADX k_{cat}/K_m ($M^{-1}s^{-1}$)	log k_{cat}/K_m(avge)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Nilutamide	$7.0 \pm 0.5 \times 10^3$	$6.4 \pm 0.3 \times 10^3$	3.83	0.20 ± 0.03	$8.2 \pm 0.7 \times 10^2$
Flutamide	$4.0 \pm 0.4 \times 10^3$	$1.9 \pm 0.3 \times 10^4$	3.94	0.30 ± 0.04	$1.4 \pm 0.1 \times 10^3$
2-Hydroxyflutamide	$7.5 \pm 0.6 \times 10^3$	$1.3 \pm 0.2 \times 10^4$	4.0	<0.05	–
4-Nitro-3-trifluorormethyl-phenylamine	$3.8 \pm 0.4 \times 10^3$	$9.0 \pm 0.7 \times 10^3$	3.76	0.17 ± 0.03	$3.8 \pm 0.2 \times 10^3$
(3-Amino-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl)phenyl) propanamide	$5.1 \pm 0.2 \times 10^3$	$5.4 \pm 0.5 \times 10^4$	4.22	0.20 ± 0.04	$5.9 \pm 0.2 \times 10^2$

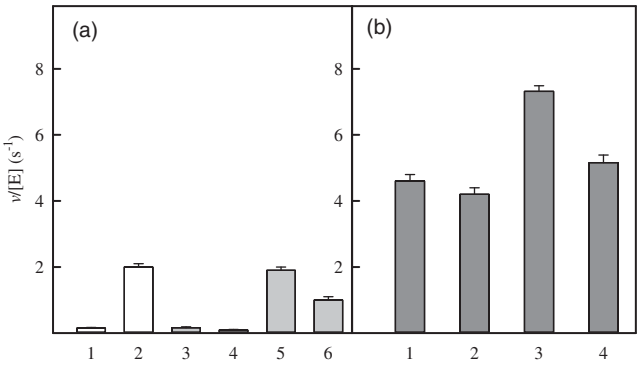

Figure 2. NADPH oxidation, oxygen consumption, and reduction of acetylated cytochrome *c* during the reduction of flutamide by ADR/ADX. (a) Rates of NADPH oxidation (1,2) and oxygen consumption (3-6) in the presence of 300 μM NADPH, 75 nM ADR and 0.5 μM ADX. Additions: none (1,3), 100 μM flutamide (2,5), 100 U/ml catalase (4), 100 μM flutamide and 100 U/ml catalase (6), $n = 3$. (b) Rates of reduction of 5 μM acetylated cytochrome *c* in the presence of 50 μM NADPH, 12.5 nM ADR and 0.5 μM ADX. Additions: none (1), 20 U/ml superoxide dismutase (2), 100 μM flutamide (3), 100 μM flutamide and 20 U/ml superoxide dismutase (4), $n = 3$, $p < 0.002$ for 3 vs. 1,2,4, $p < 0.05$ for 4 vs. 1,2. Reaction rates are expressed as moles of NADPH or O_2 consumed (A), or acetylated cytochrome *c* reduced per mole of ADR active center per second.

ADR/ ADX are given in Table 1. In P-450R-catalyzed reactions, the reaction rates were proportional to the concentration of oxidants up to the limits of their solubility, ≥250 μM. The reduction rates of flutamide, 2-hydroxyflutamide and (3-amino-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl)phenyl) propanamide by ADR/ADX exhibited parabolic dependence on their concentration giving k_{cat} of 3.1–3.5 s^{-1}, which was close to 50% of ADX- mediated cytochrome *c* reduction rate. In ADR/ADX-catalyzed reaction, 100 μM flutamide undergoes redox cycling and oxidizes a significant excess of NADPH, 300 μM. The NADPH oxidation was accompanied by O_2 consumption proceeding at similar rate, which was decreased by two times in the presence of catalase (Figure 2(a)). The consumption of O_2 also took place in P-450R-catalyzed reactions, although with lower turnover rates (Supplementary Information, Table S1). The addition of catalase in the course of reaction caused an increase in oxygen concentration in the reaction mixture (data not shown). This shows that H_2O_2 is the final product of oxygen reduction. Because ADX and P-450R rapidly reduces cytochrome *c* directly, the formation of superoxide in the course of reaction was demonstrated using partly acetylated cytochrome *c*, which possesses lower affinity toward ADX and lower reduction rate [34,35]. The ADX-mediated reduction of acetylated cytochrome *c* was substantially increased by the presence of flutamide, whose effect was significantly abrogated by superoxide dismutase (Figure 2(b)). Our data demonstrate close to 1:1:2 stoichiometry of flutamide-stimulated NADPH oxidation, O_2 consumption, and acetylated cytochrome *c* reduction (Figure 2(a,b)). The same events took place in P-450R-catalyzed reactions (Supplementary Information, Table S2).

In P-450R- and ADR/ADX-catalyzed reactions, log k_{cat}/K_m of $ArNO_2$ linearly increase with E^1_7 [7–9]. This is attributed to an "outer-sphere" electron transfer mechanism, where the reactivity of compounds is insignificantly influenced by their structural peculiarities [36]. Therefore, log k_{cat}/K_m of homologous oxidants in single-electron enzymatic reduction reaction may serve as the parameter characterizing their E^1_7. The use of the geometric average of k_{cat}/K_m obtained in several enzymatic systems improves the prediction accuracy

[7,37,38]. The previously determined logarithms of geometric averages of k_{cat}/K_m of model $ArNO_2$ in P-450R- and ADR/ADX-catalyzed reactions (log k_{cat}/K_m (avge) $=0.5$ log k_{cat}/K_m (P-450R) $+$ 0.5 log k_{cat}/K_m (ADR/ADX)) [8] (Supplementary Information, Table S3) well correlate with their $E^1{}_7$ values:

$$\log\ k_{cat}/K_m\ \ (\text{avge}) = (8.66\pm0.33) + (11.56\pm1.03)$$
$$E^1{}_7,\ \ r^2 = 0.9059,\ \ \mathrm{F}(1,13) = 125.11. \qquad (2)$$

Using Equation (2) and log k_{cat}/K_m (avge) of 4-nitro-3-trifluoromethyl-phenylamine derivatives (Table 1), one obtains their calculated $E^1{}_7$ values ($E^1{}_{7\ (calc.)}$): −0.413 V (4-nitro-3-trifluorormethyl-phenylamine), −0.408 V (nilutamide), −0.399 V (flutamide), −0.394 V (2-hydroxyflutamide), and −0.377 V (3-amino-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl) phenyl) propanamide.

Flavoenzyme NAD(P)H:quinone oxidoreductase (NQO1) reduces quinones in two-electron way with k_{cat} in certain cases exceeding 1000s^{-1} ([10], and references therein). However, the reduction of $ArNO_2$ with the formation of ArNHOH is by 10^2–10^5 times slower than that of quinones [10,39]. The reactivity of nitroaromatics depends on $E^1{}_7$ and structural features in an ill-defined way [39]. The kinetic parameters of NQO1-catalyzed reduction of 4-nitro-3-trifluorormethyl-phenylamine derivatives are given in Table 1. These compounds did not increase the initial rate of cytochrome *c* reduction by NQO1 with a turnover number of 0.1 s^{-1}. This is in line with a net four-electron character of nitroreductase reactions of NQO1 without the formation of free radicals of nitroaromatics [10,39].

The enthalpies of free radical formation of 4-nitro-3-trifluoromethyl-phenylamine derivatives

There exists rough linear relationships between the enthalpies of free radical formation ($\Delta Hf(ArNO_2^{-\bullet})$) obtained in quantum mechanical calculations, and $E^1{}_7$ values of $ArNO_2$ [7]. According to calculated $\Delta Hf(ArNO_2^{-\bullet})$ values (Table 2), the derivatives of 4-nitro-3-trifluoromethyl-phenylamine are more efficient electron acceptors than nitrobenzene, and less efficient electron acceptors than 4-nitroacetophenone and 1,3- and 1,2-dinitrobenzenes. An important structural factor decreasing electron accepting potency of nitroaromatics is the nonplanarity of the nitro group and benzene ring [41]. Due to a sterical hindrance of trifluoromethyl group, the torsion angle of the nitro group of flutamide, 37°, is similar to that of 1,2-dinitrobenzene, 42° (Figure 3).

Cytotoxicity of nitroaromatic antiandrogens

In cytotoxicity studies, we determined the cL_{50} values of examined compounds in murine hepatoma MH22a

Table 2. The enthalpies of formation of free radicals of 4-nitro-3-trifluoromethyl-phenylamine derivatives and model nitroaromatic compounds ($\Delta Hf\ (ArNO_2^{-\cdot})$) obtained in quantum mechanical calculations. The values of $E^1{}_7$ taken from [40].

	$\Delta Hf\ (ArNO_2^{-\cdot})$ (kJ/mol)		
Compound	B3LYP/cc-pVTZ	B3LYP/cc-pVTZ including D3	wB97XD/cc-pVTZ
Nitrobenzene ($E^1{}_7 = -0.485$ V)	−95.94	−95.98	−83.30
4-Nitro-3-trifluoro-methyl-phenylamine	−102.38	−101.38	−89.37
Flutamide	−136.61	−135.27	−117.07
2-Hydroxyflutamide	−137.11	−136.40	−118.41
Nilutamide	−147.65	−145.77	−131.25
4-Nitroacetophenone ($E^1{}_7 = -0.355$ V)	−164.64	−164.72	−146.44
1,2-Dinitrobenzene ($E^1{}_7 = -0.287$ V)	−173.89	−173.76	−154.05
1,3-Dinitrobenzene ($E^1{}_7 = -0.345$ V)	−175.60	−175.64	−153.43

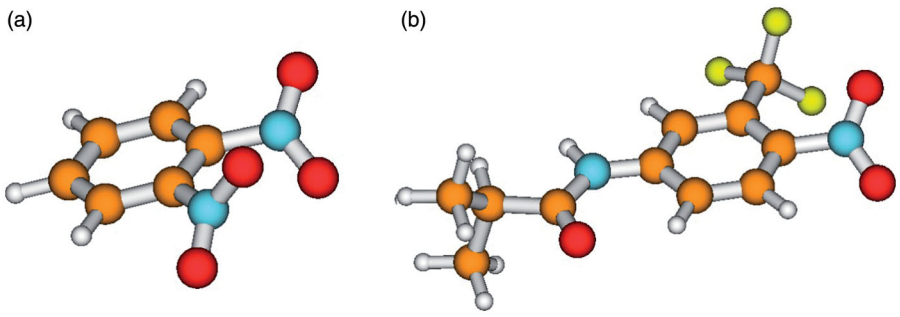

Figure 3. Structures of 1,2-dinitrobenzene (a) and flutamide (b) generated by Molden. Carbon atoms are shown in orange, nitrogen in aqua, oxygen in red, and fluorine in yellow.

Table 3. Concentrations of 4-nitro-3-trifluorormethyl-phenylamine derivatives causing 50% MH22a cell death (cL_{50}), their single-electron reduction midpoint potentials calculated according to Equation (2) ($E^1_{7(calc.)}$), and octanol/water partition coefficients at pH 7.0 (log *D*).

Compound	cL_{50} (μM)	$E^1_{7(calc.)}$ (V)	log *D*
Nilutamide	270 ± 30	−0.408	2.25
Flutamide	356 ± 42	−0.399	3.27
2-Hydroxyflutamide	277 ± 32	−0.394	2.21
4-Nitro-3-trifluorormethyl-phenylamine	220 ± 32	−0.413	1.96
(3-Amino-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl)phenyl) propanamide	266 ± 40	−0.377	−0.50

cells (Table 3). Previously we have shown that the cytotoxicity of a number of monofunctional nitrobenzenes, nitrofurans and nitrothiophenes in this cell line increased with their E^1_7 and octanol/water distribution coefficient (log *D*) [8]. The cytotoxicity data (Supplementary Information, Table S4) are described by Equation (3):

$$\log cL_{50} = -(0.22\pm0.35)-(8.45\pm1.03)\ E^1_7-(0.29\pm0.07)\log D,\ r^2 = 0.8818,\ F(2,12) = 44.78. \quad (3)$$

The corresponding regression plot is shown in Figure 3. It is important to note that according to their $E^1_{7(calc.)}$ and log *D* values (Table 3), cL_{50} of 4-nitro-3-trifluoromethyl-phenylamine derivatives closely follow this plot (Figure 4). The cytotoxicity of nilutamide and flutamide was decreased by desferrioxamine and the antioxidant *N,N′*-diphenyl-*p*-phenylene diamine (DPPD), and enhanced by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), the latter inactivating glutathione reductase and depleting GSH [42] (Table 4). This points to the prooxidant character of their cytotoxicity. Further, the cytotoxicity of nilutamide and flutamide was decreased by an inhibitor of NQO1, dicoumarol, and inhibitors of cytochromes P-450 1A1/1A2, 1B1 and 3A4, α-naphthoflavone, isoniazid, and miconazole (Table 4).

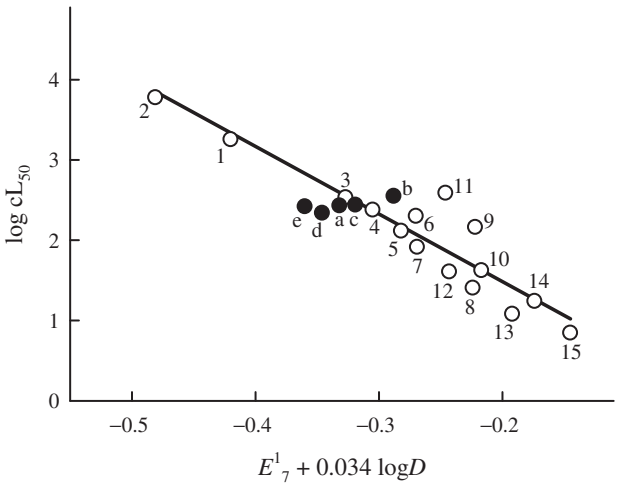

Figure 4. Cytotoxicity of nitroaromatic antiandrogens, their metabolites and model nitroaromatic compounds in MH22a cells. The regression line describing the dependence of log cL_{50} of model nitroaromatics (blank circles) on their E^1_7 and log *D* (Table S4) is drawn according to Equation (3). The numbers of model nitroaromatics correspond to those in Table S4. The log cL_{50} of nitroaromatic antiandrogens and their metabolites (solid circles, marked as in Figure 1), their $E^1_{7(calc.)}$ and log *D* are taken from Table 3.

Table 4. Modulation of cytotoxicity of nilutamide and flutamide in MH22a cells by antioxidants, dicoumarol, BCNU, and inhibitors of cytochromes P-450, $n = 3$, $p < 0.05^*$, $p < 0.02^{**}$, $p < 0.01^{***}$, $p < 0.005^{****}$ with respect to cell viability in the absence of additions.

	Cell *via*bility (%)	
Additions	+ 250 μM nilutamide	+ 360 μM flutamide
None	55.4 ± 5.2	47.3 ± 2.4
+ 3.0 μM DPPD	82.4 ± 5.4***	68.5 ± 5.5**
+ 1.0 mM desferrioxamine	96.6 ± 4.0****	78.3 ± 6.5**
+ 20 μM BCNU	35.5 ± 2.0***	29.4 ± 1.7***
+ 20 μM dicoumarol	85.0 ± 6.4**	67.4 ± 8.0*
+ 5.0 μM α-naphthoflavone	65.9 ± 5.0	63.4 ± 5.9*
+ 1.0 mM isoniazid	86.1 ± 1.9***	55.4 ± 4.4
+ 5.0 μM miconazole	93.8 ± 5.2****	58.8 ± 3.2*

Discussion

This study characterizes redox properties of nilutamide and flutamide and their relationship with their cytotoxicity. First, it extends the data on their reduction by isolated flavoenzymes [18,26], and demonstrates their similar reactivity in both P-450R and ADR/ADX-catalyzed processes (Table 1). In contrast to the evident single-electron reduction of nilutamide by P-450R [15], there were doubts about the ability of P-450R to reduce flutamide, which were based mainly on the absence of ESR signals under anaerobiosis [16]. However, this may be explained by other reasons, *e.g.* different dismutation rates of free radicals of flutamide and nilutamide. Besides, it has been shown later that under anaerobiosis, isolated P-450R reduced flutamide into its 4-amino metabolite [18]. Another relevant finding is the characterization of kinetic parameters of nilutinamide and flutamide reduction by NQO1 (Table 1). Although the reaction rates are low like in other nitroreductase reactions of NQO1 [10,39], they may contribute to cytotoxicity of above compounds (Table 4).

Next, according to the best of our knowledge, the pulse-radiolysis studies and E^1_7 determination of

nilutamide and flutamide were not performed. The $E^1_{7.4}$ of −0.755 V vs. Ag/AgCl of flutamide was obtained from electrochemical studies in mixed solvent (40% water, 60% dimethylformamide) [27]. However, it is problematic to extrapolate this value to pure aqueous medium. On the other hand, the range of $E^1_{7(calc.)}$ of 4-nitro-3-trifluoromethyl-phenylamine derivatives, −0.377 – −0.413 V, obtained from the rates of their single-electron reduction (Equation (1)), Table 1), is in line with the data of quantum mechanical calculations (Table 2). According to the order of $\Delta Hf(ArNO_2^{-\bullet})$, the E^1_7 of examined compounds should be between −0.485 V and −0.355 V (Table 2). It is also interesting to compare the electron affinity of flutamide and *o*-dinitrobenzene that possess similar torsion angles of the nitro group (Figure 2) on the basis of electronic properties of their substituents. Flutamide should possess lower electron affinity than 1,2-dinitrobenzene ($E^1_7 = -0.287$ V), because the $-CF_3$ group is less electron accepting ($\sigma_p = 0.54$) than $-NO_2$ ($\sigma_p = 0.78$), besides, the electron affinity should be decreased by the presence of $-NHCOCH(CH_3)_2$ group ($\sigma_p = -0.12$) [43]. The presence of $-NH_2$ group ($\sigma_p = -0.66$) in 4-nitro-3-trifluoromethyl-phenylamine may further decrease the electron affinity. Taken together, these data show that the obtained $E^1_{7(calc.)}$ values are realistic. Typically, the values of a directly determined E^1_7 and $E^1_{7(calc.)}$ obtained from the data of enzymatic reduction differ by less than ±0.04 V [37].

Concerning the mechanisms of cytotoxicity of nilutamide and flutamide, it is accepted that nilutamide acts mainly through redox cycling [15]. In contrast, the enhanced production of $O_2^{-\bullet}$ by flutamide is supposed to be indirect, arising from the inhibition of Complex I and subsequent mitochondrial dysfunction [25]. Flutamide is supposed to act mainly through its deacylated product, 4-nitro-3-trifluorormethyl-phenylamine (Figure 1), which is converted by cytochromes P-450 into 1-hydroxylamine that depletes GSH and/or binds to proteins. This metabolite is also formed from flutamide hydroxylation product, 2-hydroxyflutamide (Figure 1) [19–23]. However, the reactivity of flutamide metabolites toward P-450R and ADR/ADX is similar to that of the parent compound (Table 1), which points to the possibility of their redox cycling-type cytotoxicity.

According to the best of our knowledge, the cytotoxicity of nilutamide and flutamide has not been quantitatively compared with other nitroaromatics. Our data show that they and their metabolites are cytotoxic like other $ArNO_2$ with similar redox cycling properties (Figure 3). Taken together with the protective effects of antioxidants (Table 4), this indicates that redox cycling could be the main factor of flutamide and nilutamide cytotoxicity. Although the inhibitor of NQO1, dicoumarol, and inhibitors of cytochromes P-450 protect against the cytotoxicity (Table 4), it seems that the roles of these enzymes are less important: i) the reactivity of nitroaromatics toward NQO1 does not depend on their E^1_7 values [39], whereas their cytotoxicity dependence on E^1_7 is well expressed (Figure 3), and ii) the examined cytochrome P-450 inhibitors also protect MH22a cells against nitroaromatics that do not possess 1-*N*-acylamino group [8]. Thus, the cytochrome P-450-catalyzed reactions of this substituent of flutamide [19–23] may be of little relevance to the cytotoxicity. It is possible that the cytotoxicity is exerted through a common mechanism, cytochrome P-450-catalyzed formation of hydroxylamines from the final nitroreduction products, amines [12], including 4-NH_2 metabolites of nilutamide and flutamide, whose minor amounts are formed under aerobic conditions [44,45].

In conclusion, this study helps to resolve a long lasting controversy about the mechanisms of cytotoxicity of nilutamide and flutamide, demonstrating a common role of redox cycling in their action.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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