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Substrate Selectivity of Human Aldehyde Oxidase 1 in Reduction of Nitroaromatic Drugs

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Abstract

Human aldehyde oxidase 1 (AOX1) catalyzes the oxidation of various drugs and endogenous compounds. Recently, we found that AOX1 catalyzed the reduction of drugs such as nitrazepam and dantrolene. In this study, we aimed to clarify the substrate selectivity of human AOX1 for the reduction of nitroaromatic drugs to obtain helpful information for drug development. We investigated whether 11 nitroaromatic drugs were reduced by AOX1 using recombinant AOX1 and human liver cytosol (HLC) in the presence of N^1-methylnicotinamide, an electron donor to AOX1. We found that clonazepam, flunitrazepam, flutamide, nilutamide, nimesulide, and nimetazepam were substantially reduced by recombinant AOX1 and HLC, whereas azelnidipine, nifedipine, and nimodipine were slightly reduced and metronidazole and tolcapone were not reduced. Via structural analysis, we observed that nitroaromatic drugs reduced by AOX1 possessed a relatively electron-deficient nitro group. Since the addition of NADPH to human liver microsomes (HLM) did not increase the reductase activities of the drugs that were reduced by recombinant AOX1, it was determined that NADPH-dependent enzymes in microsomes, such as cytochrome P450, were not involved in this process. Inhibition studies using known AOX1 inhibitors supported the role of AOX1 in the reduction of drugs in HLC. In conclusion, this provides new information related to the substrate selectivity of human AOX1 for the reduction of nitroaromatic drugs.

1. Introduction

Drug-metabolizing enzymes metabolize over 70% of the drugs in our body to increase their hydrophilicity and promote their excretion. Drug metabolism reactions are classified into phase I and phase II reactions, and phase I reactions include oxidation, reduction, and hydrolysis. The aldo-keto reductase (AKR) and short-chain dehydrogenase/reductase (SDR) families are well-studied enzyme families that cause drug reduction. For example, AKR1C3 and carbonyl reductase 1 (CBR1) catalyze the reduction of doxorubicin [1,2] to detoxify it, and CBR1 reduces loxoprofen to form a pharmacologically-active metabolite [3].

Aldehyde oxidase 1 (AOX1) is known to catalyze the oxidation of drugs such as allopurinol and zaleplon in humans [4–6]. In recent years, AOX1 has received attention for its role in drug metabolism [7,8]. We recently found that AOX1 catalyzes the reduction of the aromatic nitro groups of nitrazepam and dantrolene to form arylamine metabolites [9,10]. In this reduction process, hydroxylamines, which are known to cause hepatotoxicity via binding to DNA or proteins [11] or reactive oxygen species [12], are likely formed as intermediates [9,10] (Fig. 1). Additionally, hydroxylamines are produced via oxidation of arylamines by enzymes, including cytochrome P450s [13] (Fig. 1). Therefore, the reduction of nitroaromatic drugs can increase hepatotoxicity, and some nitro group-containing drugs have been known to cause liver injury as a side effect [11].

Many nitroaromatic drugs are available on the market. Some of these drugs appear to be reduced at their nitro group in the human body [14,15], prompting us to speculate that nitroaromatic drugs other than nitrazepam and dantrolene can also be reduced by AOX1. In this study, we investigate the potential of human AOX1 to reduce nitroaromatic drugs and discuss the chemical structures of drugs reduced by AOX1.

2. Materials and methods

2.1. Materials

Human liver cytosol (HLC) (pooled, n = 150) and human liver microsomes (HLM) (pooled, n = 50) were obtained from Corning (Corning, NY). Azelnidipine, clonazepam, dantrolene sodium salt, flunitrazepam, flutamide, nilutamide, nimetazepam, nitrazepam, and nifedipine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Aminoflunitrazepam, aminonitrazepam, nimesulide, and nimodipine were purchased from Sigma-Aldrich (St. Louis, MO). Tolcapone and metronidazole were purchased from Toronto Research Chemicals (Toronto, Canada) and LKT Laboratories (St. Paul, MN), respectively. N^1-Methylnicotinamide (MNA) was purchased from Cosmo Bio (Tokyo, Japan). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) were purchased from Oriental Yeast (Tokyo, Japan). Aminodantrolene, a reduced metabolite of dantrolene, was synthesized previously [10]. Recombinant AOX1 expressed in *Escherichia coli* was prepared previously [16]. All other chemicals and solvents were of the highest grade commercially available.

2.2. Synthesis of reduced metabolites of nitroaromatic drugs

2.2.1. Aminoclonazepam, aminoflutamide, aminonilutamide and aminonimetazepam

Clonazepam (10.8 mg, 34.2 μmol), flutamide (20.0 mg, 72.9 μmol), nilutamide (30.2 mg, 95.2 μmol), or nimetazepam (4.6 mg, 15.6 μmol) dissolved in ethanol (5 mL) was incubated for 12-87 hr with $SnCl_2$ (29.5-200.4 mg), a reducing agent. After we confirmed that these compounds were completely reduced via thin-layer chromatography (TLC), the reaction was quenched with saturated $NaHCO_3$ (aq., 3 mL). The formed amine was then extracted with ethyl acetate. The organic phase was dried at 37˚C under a nitrogen gas stream and evaporated with an oil pump. ^{1}H NMR analysis was performed in $CDCl_3$ with a JEOL JNM ECA400 (400 MHz) spectrometer to determine the structure of the obtained amine. The chemical shifts

were shown in supplemental Table S1.

2.2.2. Aminonimesulide and aminotolcapone

Nimesulide (26.0 mg, 84.3 µmol) or tolcapone (10.7 mg, 39.2 µmol) were dissolved in ethyl acetate (5 mL), which was mixed with 10% activated Pd/C (2.6 mg or 2.0 mg) and stirred for 1 hr or 5 min in a hydrogen atmosphere. After we confirmed that these compounds were completely reduced via TLC, the mixture was filtered through a pad of Celite to remove the catalyst and dried at 37˚C under a nitrogen gas stream. The residue was dissolved in ethyl acetate, and hexane was added to the ethyl acetate solution to form a precipitate. The precipitate was collected, washed with diethyl ether, and dried under reduced pressure. ^{1}H NMR analysis was performed in $CDCl_3$ with a JEOL JNM ECA400 (400 MHz) spectrometer to determine the structure of the obtained amine.

2.2.3. Aminoazelnidipine, aminonifedipine, and aminonimodipine

Azelnidipine (5.9 mg, 10.1 µmol) or nimodipine (8.9 mg, 21.3 µmol) were dissolved in ethyl acetate (5 mL), and nifedipine (14.2 mg, 41.0 µmol) was dissolved in methanol (5 mL); these compounds were mixed with 10% activated Pd/C (2.0 mg) and stirred for 5 min-31 hr in a hydrogen atmosphere. After we confirmed that these compounds were completely reduced via TLC, the mixture was filtered through a pad of Celite to remove the catalyst and dried at 37˚C under a nitrogen gas stream. The formed amine was dried under reduced pressure. ^{1}H NMR analysis was performed in $CDCl_3$ with a JEOL JNM ECA400 (400 MHz) spectrometer to determine the structure of the obtained amine.

2.2.4. Aminometronidazole

Fifty microliters of 4 M HCl in ethyl acetate was added to metronidazole (25.0 mg) dissolved in 1,4-dioxane (5 mL). The mixture was stirred for 10 min and concentrated *in vacuo*. The residue was dissolved in methanol (1 mL) and stirred with 10% activated Pd/C (25.0 mg) in a hydrogen atmosphere. After we confirmed that these compounds were completely reduced via TLC, the mixture was filtered through a pad of Celite to remove the

catalyst and mixture was dried at 37˚C under a nitrogen gas stream. The formed amine was dissolved in methanol, and the precipitate that was formed by adding ethyl acetate was dried under reduced pressure. ^{1}H NMR analysis was performed in D_2O with a JEOL JNM ECA400 (400 MHz) spectrometer to determine the structure of the obtained amine.

2.3. Reductase activities of nitroaromatic drugs by recombinant human AOX1, HLM, and HLC

To measure the reductase activities exhibited toward the 11 nitroaromatic drugs (azelnidipine, clonazepam, flunitrazepam, flutamide, metronidazole, nifedipine, nilutamide, nimesulide, nimetazepam, nimodipine, and tolcapone), a typical incubation mixture (final volume of 0.2 mL) containing 25 mM or 100 mM potassium phosphate buffer (pH 7.4), 67.5 nM recombinant human AOX1 or 0.5 mg/mL HLM or HLC, and in some cases, an NADPH-generating system (0.5 mM glucose-6-phosphate, 5 mM $MgCl_2$, and 1 U/mL glucose-6-phosphate dehydrogenase) or 2 mM MNA was prepared. After preincubation at 37˚C for 2 min, reactions were initiated by addition of a substrate dissolved in DMSO at a final concentration of 100 μM. The final concentration of DMSO was 1%. After a 3 hr incubation at 37˚C, the reaction was terminated by the addition of 200 μL of ice-cold acetonitrile. The mixture was centrifuged at 20,380 g for 5 min, and 5 μL of the supernatant was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using an LCMS-8040 (Shimadzu, Kyoto, Japan) equipped with an LC-20AD HPLC system. The column used was a Develosil ODS-UG-3 (2.0 × 150 mm, 3 μm; Nomura chemical, Seto, Japan). The flow rate was 0.2 mL/min, and the column temperature was 40˚C. The mobile phase was (A) 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. Nitrogen was used as the nebulizer gas and drying gas. The operating parameters were optimized as follows: nebulizer gas flow, 3 L/min; drying gas flow, 15 L/min; desolvation line temperature, 300 ˚C; and heat block temperature, 400 ˚C. The LC-MS/MS was operated in the positive

electrospray mode. Each arylamine, a reductive metabolite of nitroaromatic drug, was monitored in multiple reaction monitoring (MRM) mode. The conditions of the mobile phase and *m/z* values for the detection and collision energies (CE) are summarized in Table 1. Analytical data were processed using LabSolutions (version 5.82.1; Shimadzu). Reductase activities toward dantrolene and nitrazepam, which have been demonstrated to be substrates of AOX1 in our previous studies [9,10], were also determined.

2.4. Kinetic analyses

Kinetic analyses were performed with the conditions summarized in supplemental Table S2. The assay conditions were determined to maintain linearity with respect to protein concentration and incubation time in advance. The kinetic parameters were estimated from a curve fitted using a computer program designed for nonlinear regression analysis (KaleidaGraph; Synergy Software, Reading, PA). The following equations were used:

Michaelis-Menten equation: $V = V\mathrm{max} * [S] / (K\mathrm{m} + [S])$

$$CL\mathrm{int} = V\mathrm{max} / K\mathrm{m}$$

where V is the velocity of the reaction, S is the substrate concentration, Km is the Michaelis-Menten constant, and Vmax is the maximum velocity.

2.5. Inhibition studies

The reductase activities of nitroaromatic drugs in HLC were measured in the presence of 50 μM estradiol, estrone, and raloxifene (potent inhibitors of AOX1, 93 – 100% inhibition or IC_{50} values 0.0057 – 0.43 μM against phthalazine oxidation in HLC), ketoconazole, promazine, and simvastatin (moderate inhibitors of AOX1, 79 – 87% inhibition), diclofenac, metronidazole, and naloxone (non-inhibitors of AOX1, 0 – 9% inhibition) [17,18], or allopurinol [xanthine oxidoreductase (XOR) inhibitor]. The final concentration of nitroaromatic drugs were set at approximately the Km values obtained using HLC as follows;

clonazepam, nitrazepam: 25 μM, flunitrazepam, dantrolene: 60 μM, flutamide: 50 μM, nilutamide: 200 μM, nimesulide: 250 μM, nimetazepam: 40 μM. The final concentration of DMSO in the incubation mixture was 2%. The other conditions were same as described above.

2.6. Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey's test. A value of $P < 0.05$ was considered statistically significant.

3. Result and Discussion

3.1. Reduction of nitroaromatic drugs by recombinant human AOX1

We investigated whether nitroaromatic drugs other than nitrazepam and dantrolene could be reduced by AOX1. The nitroaromatic drugs azelnidipine, clonazepam, flunitrazepam, flutamide, metronidazole, nifedipine, nilutamide, nimesulide, nimetazepam, nimodipine, and tolcapone were used. We chemically synthesized the corresponding reduced amino forms of these drugs as standards. These 11 nitroaromatic drugs were incubated with recombinant human AOX1 in the presence of MNA, which is a substrate of AOX1 to be oxidized and transfers electrons to AOX1 by its oxidation to enhance reduction of nitroaromatic compounds (Fig. 2). We found that 6 drugs (clonazepam, flunitrazepam, flutamide, nilutamide, nimesulide, and nimetazepam) were reduced by AOX1, whereas 3 drugs (azelnidipine, nifedipine, and nimodipine) were slightly reduced and metronidazole and tolcapone were not reduced. We confirmed that dantrolene and nitrazepam were also reduced by AOX1 as positive controls [9,10]. Thus, it was demonstrated that not all nitroaromatic drugs were reduced by AOX1.

3.2. Reduction of nitroaromatic drugs in HLM or HLC

We next investigated whether the 11 nitroaromatic drugs were reduced in HLC, where AOX1 is localized, or in HLM, which has other reductases present (Fig. 3). The reductase activities were detected in HLC for the 6 drugs that were reduced by recombinant human AOX1, and their reduction was significantly increased by the addition of MNA (Figs. 3A - F). Similar results were observed for the reduction of dantrolene and nitrazepam (Figs. 3G and H). These results suggest that the reductase activities in HLC are largely due to AOX1. Reductase activities were detected in HLC even in the absence of MNA, which may be due to the presence of a contaminant MNA or other endogenous electron donors for AOX1 in HLC.

The addition of NADPH in HLC did not increase the reductase activities toward clonazepam, flutamide, and nilutamide (Figs. 3A, C, and D), whereas it did increase the reductase activities toward flunitrazepam, nimesulide, and nimetazepam (Figs. 3B, E, and F). The increase in the activities of the latter group may imply the involvement of NADPH-dependent reductases, such as AKR enzymes, SDR enzymes, or NADPH-quinone oxidoreductase 1 (NQO1), although it remains unclear whether these enzymes can catalyze the reduction of nitro groups. The reductase activities related to clonazepam, flunitrazepam, flutamide, nilutamide, nimesulide, and nimetazepam in HLM were exceptionally lower than those observed in HLC and were not increased by NADPH (Figs. 3A - F), indicating that the contribution of cytochrome P450, a major drug-metabolizing enzyme that requires NADPH, is negligible.

Azelnidipine, metronidazole, nifedipine, nimodipine, and tolcapone, which were slightly reduced or not reduced by recombinant AOX1, were barely reduced in either HLC or HLM (Figs. 3I - M). Thus, it was demonstrated that for the nitroaromatic drugs analyzed in this study, their reduction in the human liver could be largely attributed to AOX1.

3.3. Kinetic analyses of the reductase activities in HLC

Kinetic analyses of the reduction of clonazepam, flunitrazepam, flutamide, nilutamide, nimesulide, and nimetazepam in HLC were performed in the presence or absence of MNA (Figs. 4A - F and Table 2). The kinetics of all of the examined drugs were fitted to a Michaelis-Menten kinetics curve. The *V*max values of all of the drugs were significantly increased by MNA, likely due to an enhancement of the AOX1 activity. A similar increase was observed in the reduction of dantrolene and nitrazepam (Figs. 4G and H, Table 2). The *K*m values of the flunitrazepam, flutamide, nimesulide, and nimetazepam reductase activities in the presence of MNA were similar to those in the absence of MNA, whereas the *K*m values of clonazepam and nilutamide were increased by MNA. The increase in the *K*m values may

be due to structural changes in the substrate binding site of AOX1 induced by MNA via an allosteric effect because the substrate binding site of AOX1 for reduction appears to be different from that observed for oxidation [19]. Collectively, the significant increase in the catalytic efficiency in the presence MNA supported the ability of AOX1 to reduce nitroaromatic drugs in the human liver.

3.4. Inhibitory effects of AOX1 inhibitors on the reductase activity of 6 nitroaromatic drugs in HLC

To investigate whether the reduction of 6 nitroaromatic drugs in the human liver was catalyzed by AOX1, not other enzymes that require MNA, an inhibition study was performed at *K*m values of nitroaromatic drugs in HLC. The used inhibitors (potent inhibitors, moderate inhibitors, and non-inhibitors of AOX1) were from papers by Obach (2004) [18] and Obach et al. (2004) [17]. If the inhibitory potency was observed as reported, the responsibility of AOX1 for the reduction of 6 nitroaromatic drugs in HLC could be explained. As a result, the reductase activities of all of the tested drugs were potently inhibited by estradiol, estrone, and raloxifene and were moderately inhibited by ketoconazole, promazine, and simvastatin, whereas the reductase activities were barely inhibited by diclofenac, metronidazole, and naloxone (Figs. 5A - F). Similar inhibitory potency has been observed for reduction of nitrazepam or dantrolene in our previous studies [9,10], and the almost same results have also been obtained in this study (Figs. 5G and H). We investigated the effects of allopurinol, a potent inhibitor of human XOR [20], because XOR has a similar substrate specificity and molecular structure to those of AOX1 [21] with a relatively high (approximately 50%) amino acid homology and requirement for a molybdenum cofactor [22]. The reductase activities of the 6 nitroaromatic drugs in addition to dantrolene and nitrazepam were not inhibited by allopurinol. Thus, the involvement of XOR was excluded. These results supported that AOX1 is the enzyme responsible for the reduction of these nitroaromatic drugs in HLC.

3.5. Structural characteristics of nitroaromatic drugs that can or cannot be reduced by human AOX1

The structures of the nitroaromatic drugs used in this study are shown in Fig. 6 and are categorized into two groups: those that were substantially reduced by human AOX1 or those that were barely by human AOX1. As shown in Fig. 6A, the nitroaromatic drugs that were reduced by human AOX1 tend to possess a relatively electron-deficient nitro group. For example, flutamide and nilutamide have a strongly electron-withdrawing trifluoromethyl group at the *ortho* position of the nitro group. Recently, it has been reported that 5-nitroquinoline, in which a nitro group is connected to an electron-deficient quinolone ring, is reduced by AOX1 [19]. This result is consistent with the above theory. However, as shown in Fig. 6B, the compounds that were not reduced by human AOX1 tend to have a relatively electron-rich nitro group. For example, the nitro group in metronidazole is likely to be relatively electron-rich due to electron donation from the N-1 atom in the imidazole ring. The nitro group in tolcapone would also be relatively electron-rich due to electron donation from the hydroxyl group at the *ortho* position of the nitro group. To definitively claim substrate selectivity of AOX1 for nitro reduction, further studies with an increased number of compounds are required. AOX1 can catalyze oxidation and hydrolysis [23]. AOX1 tends to oxidize electron-rich carbon atoms on heteroaromatic rings or hydrolyze amide bonds if an electron donating group is located at the *ortho* position of aniline [24]. The electron-rich carbon atom in AOX1 substrates loses electrons when it is oxidized, and the electron-deficient nitro group in AOX1 substrates receives electrons when it is reduced. These mechanisms are chemically reasonable. This study provides new knowledge about the reduction catalyzed by AOX1 in addition to oxidation and hydrolysis.

3.6. Significance of AOX1-mediated reduction of nitroaromatic drugs on pharmacokinetics

and toxicity

Interestingly, all of the nitroaromatic drugs that were found to be reduced by AOX1 have been reported to cause liver injury [25–32]. Because hydroxylamines formed via the reduction of nitroaromatic drugs may cause hepatotoxicity, AOX1-mediated reduction of nitroaromatic drugs is a concern because of their toxicity. Arylamine metabolites of the nitroaromatic drugs that were reduced by human AOX1 that were identified in this study were detected in blood [33–39]. Thus, the arylamine metabolites detected in humans would be produced by AOX1. By contrast, there are no reports of detectable reduced forms in human urine or blood for azelnidipine, metronidazole, nifedipine, and nimodipine, which were not reduced by AOX1. This study demonstrated that tolcapone, which is well-known to cause liver injury, was not reduced in HLM or HLC or by recombinant AOX1 (Figs. 2 and 3M), but its reduced form, aminotolcapone, was detected in urine [39]. Therefore, tolcapone can be reduced by intestinal bacteria, enzyme(s) in organs other than liver, or enzymes that require cofactors other than MNA and NADPH in the liver.

4. Conclusion

We examined the substrate selectivity of human AOX1 in the reduction of nitroaromatic drugs and found that compounds with a relatively electron-deficient nitro group were reduced by human AOX1. This study provides fundamental information about the substrate selectivity of human AOX1, and this information will be helpful to predict the possible toxicities of nitroaromatic drugs.

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Figure legends

Fig. 1. Possible metabolic pathways of nitroaromatic compounds in human.

Fig. 2. Reductase activities of 13 nitroaromatic drugs by recombinant human AOX1 in the presence of 2 mM MNA. Recombinant human AOX1 (67.5 nM) was incubated with nitroaromatic drugs (100 μM) for 3 hrs. Each column represents the mean ± SD of triplicate determinations. ND: Not detected.

Fig. 3. Reductase activities of 13 nitroaromatic drugs by HLM or HLC in the presence or absence of an NADPH-generating system or 2 mM MNA. HLM or HLC (0.5 mg/mL) were incubated with nitroaromatic drugs (100 μM) for 3 hrs. Each column represents the mean ± SD of triplicate determinations. ND: Not detected. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Fig. 4. Kinetic analyses of the reductase activities of 6 nitroaromatic drugs by HLC. Open and closed circles represent the activities in the absence and presence of MNA, respectively. Each point represents the mean ± SD of triplicate determinations.

Fig. 5. Inhibitory effects of several inhibitors on the reductase activities in HLC. The inhibitors used in this study were as follows: estradiol, estrone, and raloxifene: known as strong inhibitors of AOX1 [17,18]. Ketoconazole, promazine, and simvastatin: moderate inhibitors. Diclofenac, metronidazole, and naloxone: hardly inhibit AOX1. All compounds were used at the concentration of 50 μM. The control activities were 10.4 ± 0.4 pmol/min/mg protein (clonazepam: 25 μM), 2.96 ± 0.5 pmol/min/mg protein (flunitrazepam: 60 μM), 56.6 ± 7.1 pmol/min/mg protein (flutamide: 50 μM), 8.3 ± 0.6 pmol/min/mg protein (nilutamide: 200 μM), 33.3 ± 4.1 pmol/min/mg protein (nimesulide: 250 μM), 31.4 ± 1.6 pmol/min/mg

protein (nimetazepam: 40 μM), 24.4 ± 0.2 pmol/min/mg protein (dantrolene: 60 μM), and 11.7 ± 0.5 pmol/min/mg protein (nitrazepam: 25 μM). Each column represents the mean ± SD of triplicate determinations.

Fig. 6. Chemical structures of nitroaromatic drugs that (A) are or (B) are not reduced by human AOX1.

Table 1.

LS-MS/MS conditions for arylamines, reduced metabolites of nitroaromatic drugs.

Arylamine	Mobile phase	MRM	Collision energy
	B concentration (%)	*m/z*	V
Aminoazelnidipine	40-80 (0–4 min), 80 (4-5 min), 40 (5-7 min)	553.30 > 167.00	34.0
Aminoclonazepam	30-70 (0–2 min), 70 (2–6 min), 30 (6–8 min)	286.10 > 121.10	32.0
Aminodantrolene	10-90 (0-3 min), 90 (3-7.5 min), 10 (7.5-10 min)	285.15 > 186.00	18.0
Aminoflunitrazepam	50 (0–1 min), 95 (1-3 min), 50 (3-5 min)	284.00 > 135.05	28.0
Aminoflutamide	40-90 (0–2 min), 90 (2–6 min), 40 (6–8 min)	247.10 > 227.10	17.0
Aminometronidazole	20-70 (0–3 min), 70 (3-4 min), 20 (4-6 min)	141.90 > 98.05	19.0
Aminonifedipine	30-70 (0–2 min), 70 (2–4 min), 30 (4-6 min)	317.10 > 224.05	12.0
Aminonilutamide	30-70 (0–2 min), 70 (2–5.5 min), 30 (5.5–7.5 min)	288.10 > 268.05	18.0
Aminonimesulide	30-70 (0–2 min), 70 (2–6 min), 30 (6–8 min)	279.05 > 200.00	20.0
Aminonimetazepam	30-70 (0–2 min), 70 (2–6 min), 30 (6–8 min)	266.15 > 135.05	28.0
Aminonimodipine	40-80 (0–2 min), 80 (2–4 min), 40 (4-6 min)	389.20 > 313.05	9.0
Aminonitrazepam	30-70 (0–2 min), 70 (2–5 min), 30 (5–7 min)	252.30 > 121.20	28.0
Aminotolcapone	30-70 (0–2 min), 70 (2–5 min), 30 (5-7 min)	244.10 > 119.05	16.0

Table 2.

Kinetic parameters of each substrate of AOX1 in HLC in the absence or presence of MNA.

Substrate	MNA	*K*m	*V*max	*CL*int
		μM	pmol/min/mg protein	μL/min/mg protein
Clonazepam	-	6.7 ± 0.5	0.6 ± 0.1	0.1 ± 0.0
	+	27.9 ± 1.6	42.5 ± 1.5	1.5 ± 0.1
Flunitrazepam	-	46.0 ± 19.1	0.5 ± 0.1	0.0 ± 0.0
	+	62.3 ± 2.3	30.1 ± 0.8	0.5 ± 0.0
Flutamide	-	53.0 ± 0.9	4.0 ± 0.2	0.1 ± 0.0
	+	60.8 ± 6.7	148.9 ± 10.9	2.4 ± 0.1
Nilutamide	-	38.3 ± 1.4	1.2 ± 0.1	0.0 ± 0.0
	+	199.0 ± 10.9	41.2 ± 0.1	0.2 ± 0.0
Nimesulide	-	201.4 ± 22.1	1.8 ± 0.1	0.0 ± 0.0
	+	284.9 ± 28.7	65.0 ± 0.7	0.2 ± 0.0
Nimetazepam	-	18.5 ± 2.2	11.9 ± 2.0	0.6 ± 0.1
	+	41.2 ± 7.7	55.2 ± 2.1	1.3 ± 0.2
Dantrolene	-	45.4 ± 3.5	8.1 ± 0.2	0.2 ± 0.0
	+	55.1 ± 3.2	207.4 ± 4.3	3.8 ± 0.2
Nitrazepam	-	122.2 ± 16.5	10.1 ± 0.2	0.1 ± 0.0
	+	17.2 ± 0.4	40.2 ± 1.7	2.3 ± 0.2

Data are mean ± SD.

Fig. 1.

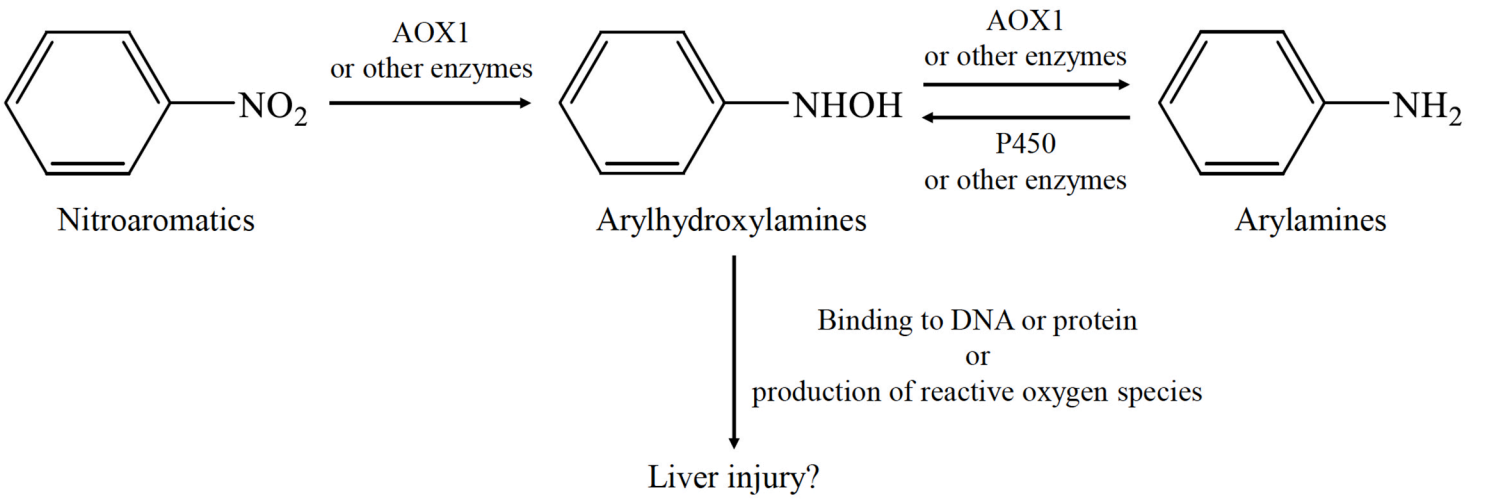

AOX1
or other enzymes
AOX1
or other enzymes
P450
or other enzymes
NO2
NHOH
NH2
Nitroaromatics
Arylhydroxylamines
Arylamines
Binding to DNA or protein
or
production of reactive oxygen species
Liver injury?

Fig. 2.

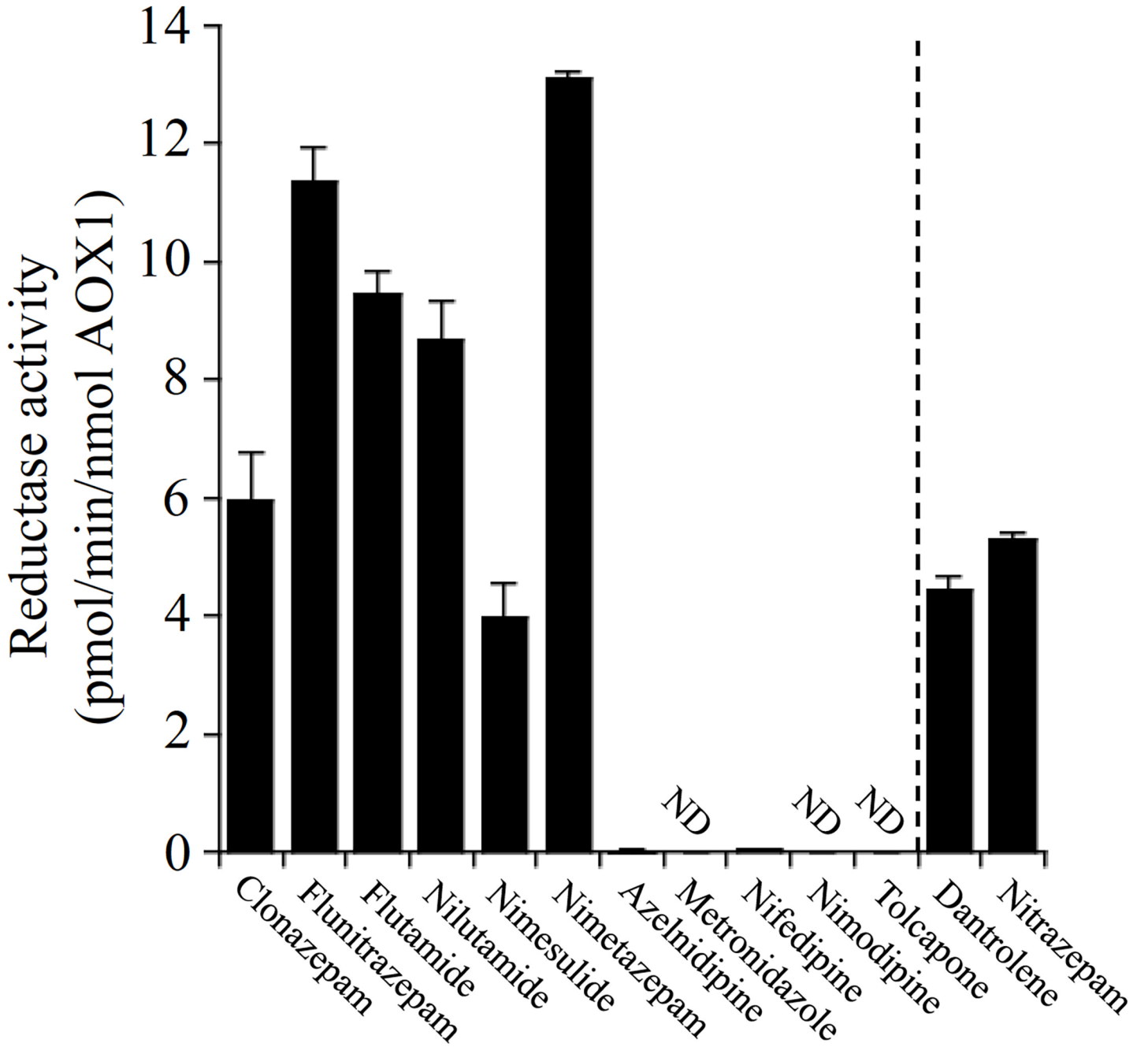

Reductase activity
(pmol/min/nmol AOX1)
14
12
10
8
6
4
2
0
ND
ND
ND
Clonazepam
Flunitrazepam
Flutamide
Nilutamide
Nimesulide
Nimetazepam
Azelnidipine
Metronidazole
Nifedipine
Nimodipine
Tolcapone
Dantrolene
Nitrazepam

Fig. 3.

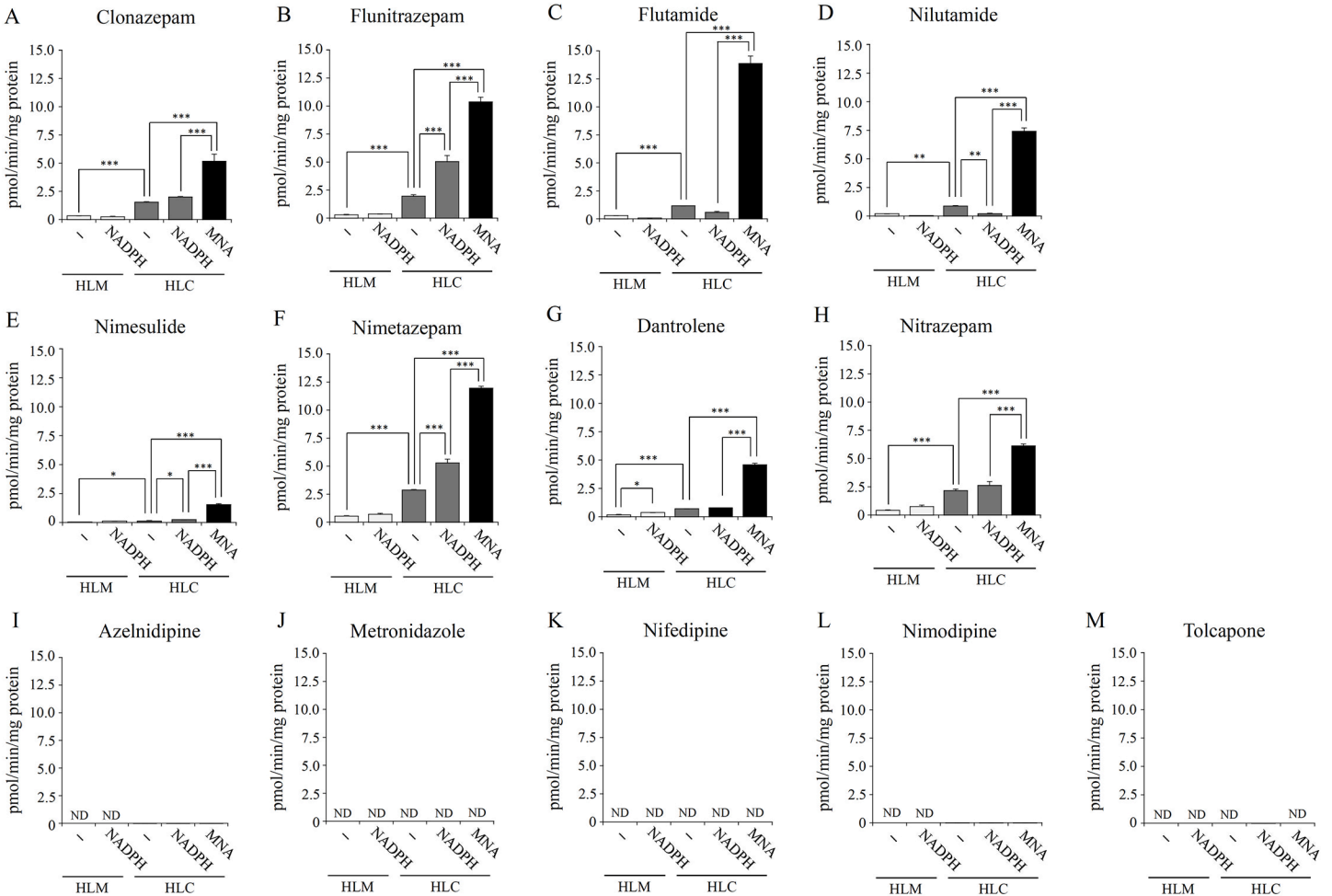

A
Clonazepam
B
Flunitrazepam
C
Flutamide
D
Nilutamide
E
Nimesulide
F
Nimetazepam
G
Dantrolene
H
Nitrazepam
I
Azelnidipine
J
Metronidazole
K
Nifedipine
L
Nimodipine
M
Tolcapone
pmol/min/mg protein
15.0
12.5
10.0
7.5
5.0
2.5
0
ND
–
NADPH
MNA
HLM
HLC
*
**
***

Fig. 4.

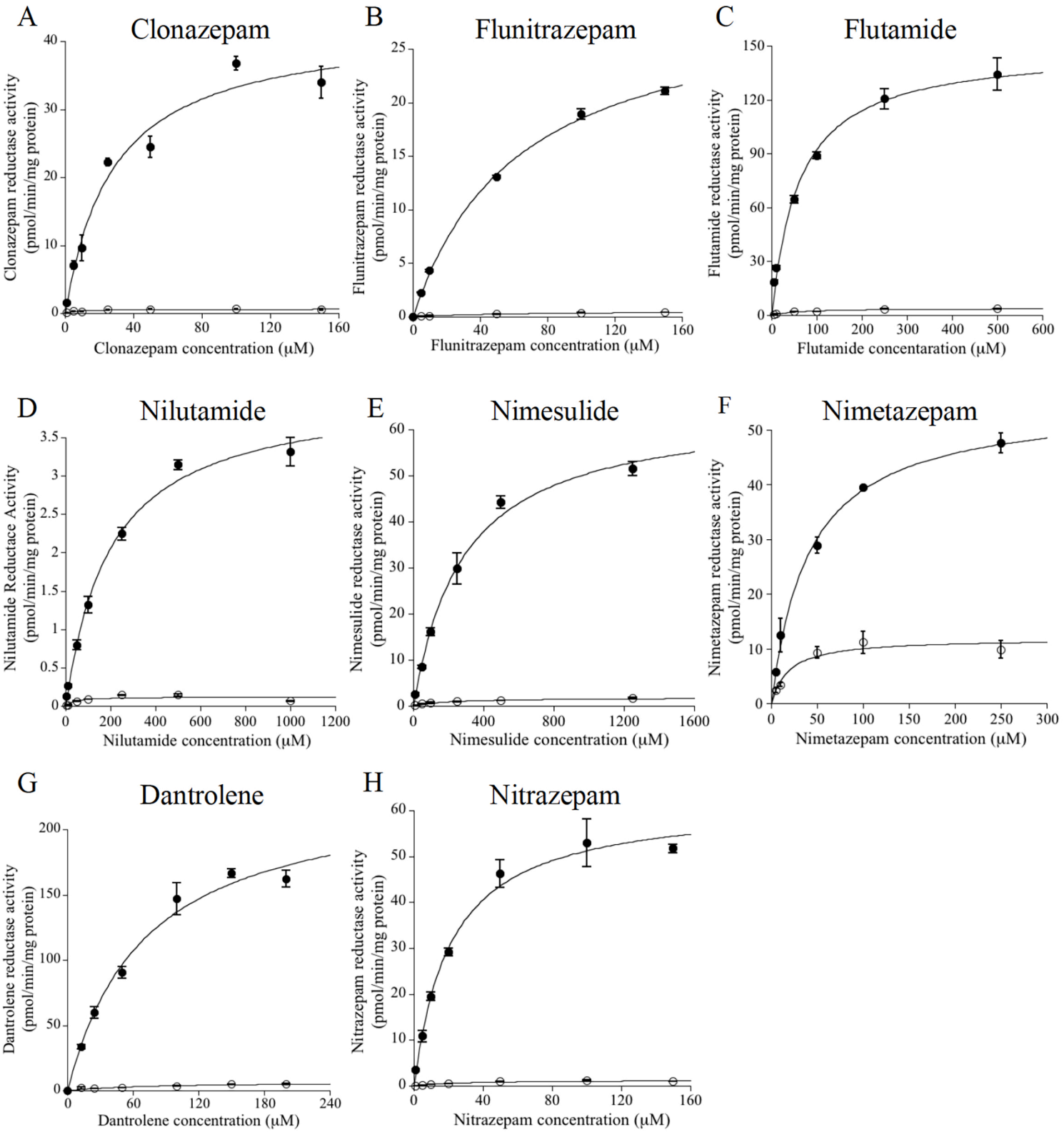

A
Clonazepam
Clonazepam reductase activity (pmol/min/mg protein)
Clonazepam concentration (μM)
B
Flunitrazepam
Flunitrazepam reductase activity (pmol/min/mg protein)
Flunitrazepam concentration (μM)
C
Flutamide
Flutamide reductase activity (pmol/min/mg protein)
Flutamide concentaration (μM)
D
Nilutamide
Nilutamide Reductace Activity (pmol/min/mg protein)
Nilutamide concentration (μM)
E
Nimesulide
Nimesulide reductase activity (pmol/min/mg protein)
Nimesulide concentration (μM)
F
Nimetazepam
Nimetazepam reductase activity (pmol/min/mg protein)
Nimetazepam concentration (μM)
G
Dantrolene
Dantrolene reductase activity (pmol/min/mg protein)
Dantrolene concentration (μM)
H
Nitrazepam
Nitrazepam reductase activity (pmol/min/mg protein)
Nitrazepam concentration (μM)

Fig. 5.

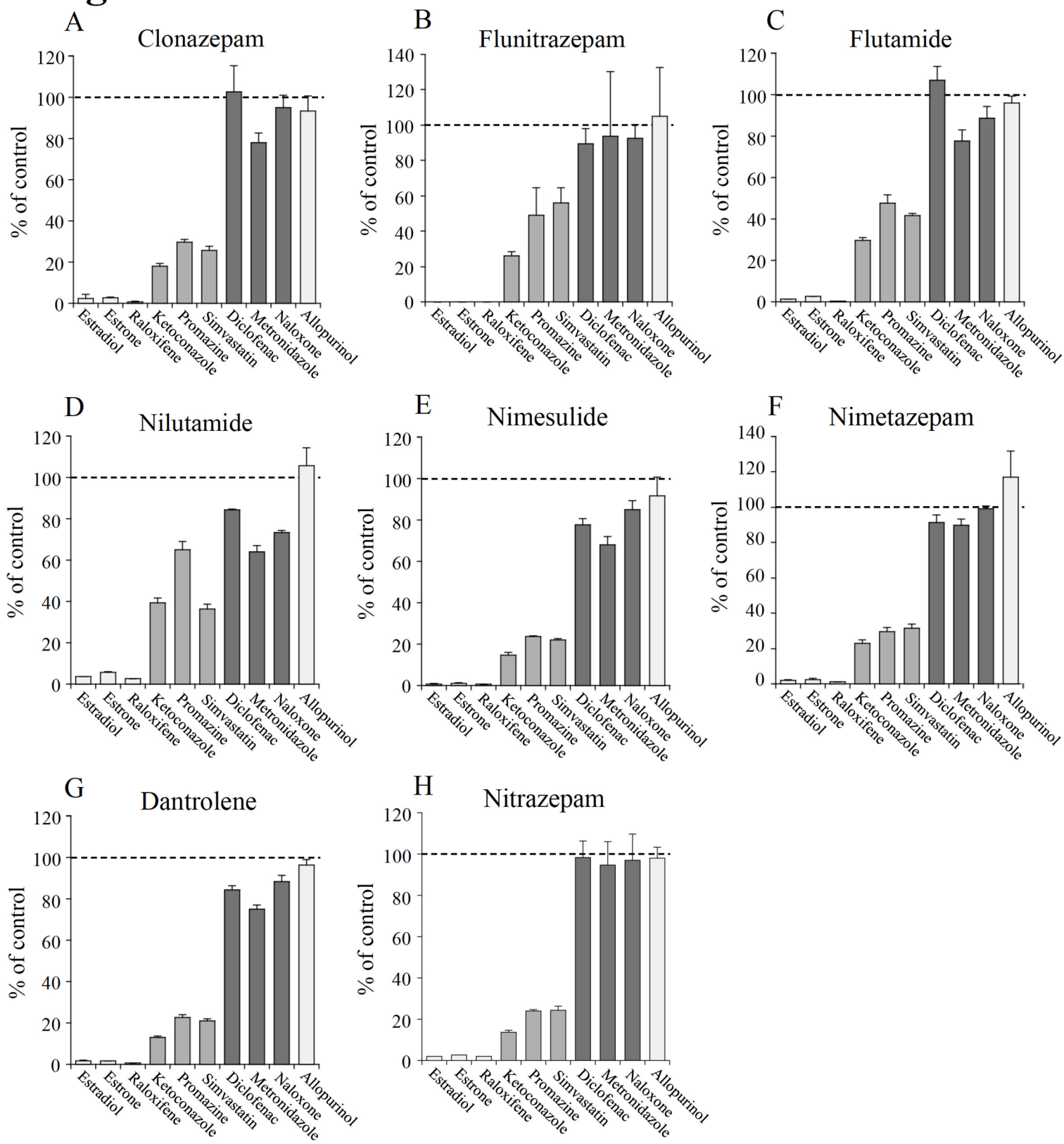
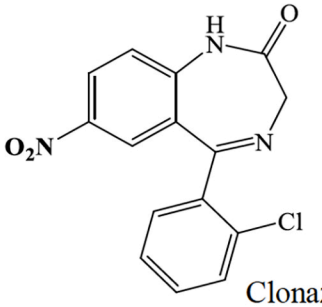

A
Clonazepam
B
Flunitrazepam
C
Flutamide
D
Nilutamide
E
Nimesulide
F
Nimetazepam
G
Dantrolene
H
Nitrazepam
% of control
Estradiol
Estrone
Raloxifene
Ketoconazole
Promazine
Simvastatin
Diclofenac
Metronidazole
Naloxone
Allopurinol

Fig. 6.

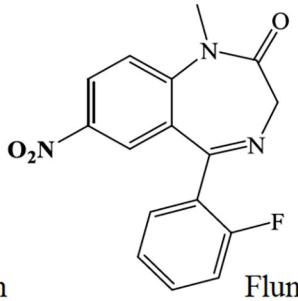
Nitroaromatic drugs

A

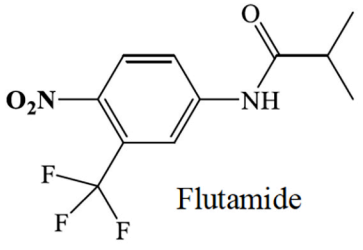
… that are reduced by AOX1



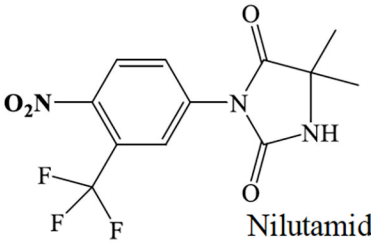
Clonazepam



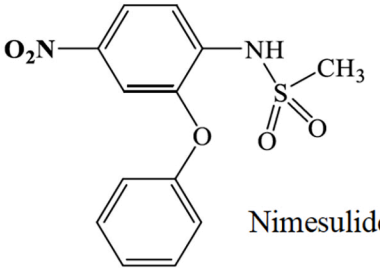
Flunitrazepam



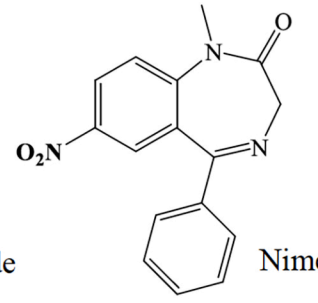
Flutamide



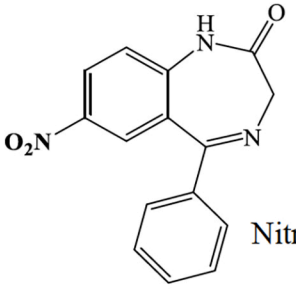
Nilutamide



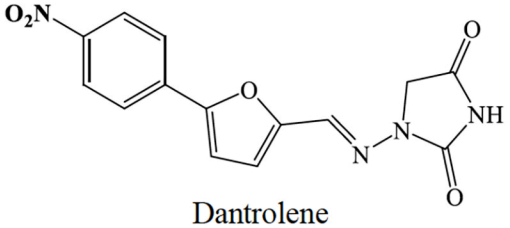
Nimesulide



Nimetazepam



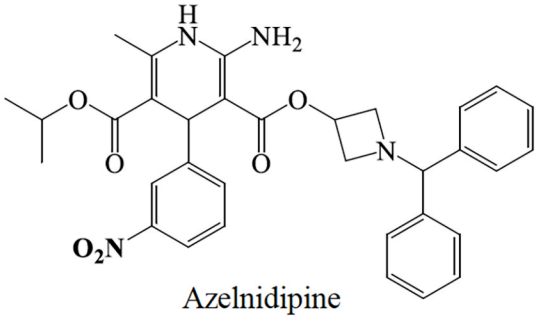
Nitrazepam



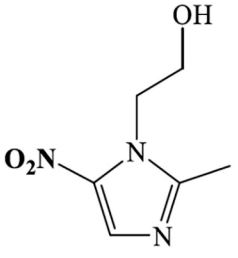
Dantrolene

B

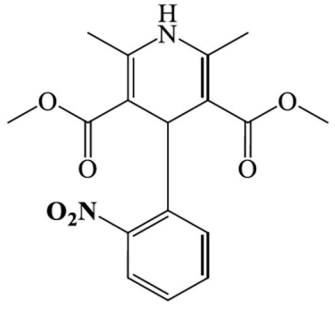
… that are "NOT" reduced by AOX1



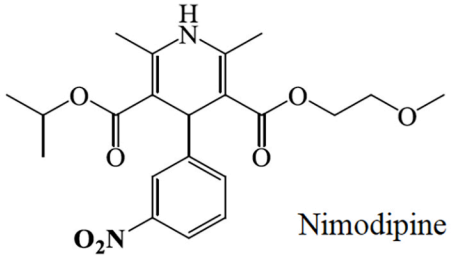
Azelnidipine



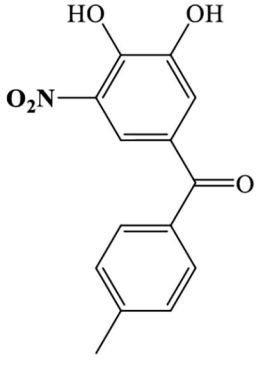
Metronidazole



Nifedipine



Nimodipine



Tolcapone

Highlights

- Among 11 nitroaromatic drugs, 6 drugs were reduced by recombinant AOX1 and HLC.
- Inhibition study using known AOX1 inhibitors showed the involvement of AOX1 in HLC.
- Drugs reduced by AOX1 possessed a relatively electron-deficient nitro group.