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4-Amino-2-chlorophenol: Comparative In Vitro Nephrotoxicity and Mechanisms of Bioactivation

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Abstract

Chlorinated anilines are nephrotoxicants both in vivo and in vitro. The mechanism of chloroaniline nephrotoxicity may occur via more than one mechanism, but aminochlorophenol metabolites appear to contribute to the adverse in vivo effects. The purpose of this study was to compare the nephrotoxic potential of 4-aminophenol (4-AP), 4-amino-2-chlorophenol (4-A2CP), 4-amino-3-chlorophenol (4-A3CP) and 4-amino-2,6-dichlorophenol (4-A2,6DCP) using isolated renal cortical cells (IRCC) from male Fischer 344 rats as the model and to explore renal bioactivation mechanisms for 4-A2CP. For these studies, IRCC ($\sim 4 \times 10^6$ cells/ml) were incubated with an aminophenol (0.5 or 1.0 mM) or vehicle for 60 min at 37° C with shaking. In some experiments, cells were pretreated with an antioxidant or cytochrome P450 (CYP), flavin monooxygenase (FMO), peroxidase or cyclooxygenase inhibitor prior to 4-A2CP (1.0 mM). Lactate dehydrogenase (LDH) release served as a measure of cytotoxicity. The order of decreasing nephrotoxic potential in IRCC was 4-A2,6-DCP > 4-A2CP > 4-AP > 4-A3CP. The cytotoxicity induced by 4-A2CP was reduced by pretreatment with the peroxidase inhibitor mercaptosuccinic acid, and some antioxidants (ascorbate, glutathione, N-acetyl-L-cysteine) but not by others (α -tocopherol, DPPD). In addition, pretreatment with the iron chelator deferoxamine, several CYP inhibitors (except for the general CYP inhibitor piperonyl butoxide), FMO inhibitors or indomethacin (a cyclooxygenase inhibitor) failed to attenuate 4-A2CP cytotoxicity. These results demonstrate that the number and ring position of chloro groups can influence the nephrotoxic potential of 4-aminochlorophenols. In addition, 4-A2CP may be bioactivated by cyclooxygenase and peroxidases, and free radicals appear to play a role in 4-A2CP cytotoxicity.

Keywords

Nephrotoxicity; In Vitro; Rat; 4-Amino-2-chlorophenol

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest with this work.

1. Introduction

Aniline (aminobenzene) and its halogenated derivatives have been widely used to manufacture drugs, cosmetics, dyes, pesticides, photographic chemicals, polyurethane and a wide range of industrial compounds [1–3]. Exposure to these chemicals can occur in the industrial setting, from in vivo metabolism of aniline derivatives, and from environmental exposure. Chloroanilines are considered priority pollutants in environmental risk assessments because of their adverse health effects and release into the environment in agricultural areas following degradation of pesticides [1,4].

The toxicity induced by aniline and the chloroanilines includes hemotoxicity (hemolytic anemia, methemoglobinemia), hepatotoxicity, splenic toxicity and nephrotoxicity [1,3, 5–10]. The role that metabolites play in the nephrotoxicity induced by aniline and chloroaniline compounds remains to be fully determined. In vivo, phenyl ring oxidation (phenol formation), N-oxidation and N-acetylation are the three primary routes of biotransformation for anilines, and aminophenol and aminochlorophenol metabolites and their conjugates are the major metabolites for this series of compounds [11,12]. The role that aminophenols play in the nephrotoxicity of parent compounds is an area of ongoing investigation, but 4-aminophenol (4-AP) and many aminochlorophenols are nephrotoxics in vivo and/or in vitro [13–19]. Also, in vivo and in vitro studies with 4-AP have suggested the need for oxidative metabolism and possible conjugation of 4-AP metabolites with glutathione for the full development of 4-AP-induced nephrotoxicity [20–22]. In vitro, Yan et al. [23] found that Sprague-Dawley rat hepatocytes extensively metabolized 4-AP with the major route of biotransformation resulting from formation of 1,4-benzoquinoneimine followed by conjugation with glutathione and subsequent catabolism to mercapturate metabolites of 4-AP (Fig. 1). This pathway accounted for ~83% of 4-AP metabolites. Thus, a potential pathway for organ-directed toxicity of aniline compounds could involve formation of aminophenol and aminophenol-derived metabolites. In addition, for 4-AP, oxidative stress appears to play a role in nephrotoxic mechanisms [13,24,25]. However, the ultimate nephrotoxic metabolite(s), mechanisms of bioactivation, and mechanisms of nephrotoxicity for 4-AP and the phenolic metabolites of the chloroanilines remain to be fully determined.

Although there are isolated reports concerning the nephrotoxicity induced by a number of aminophenols and aminochlorophenols, there are few comparative studies between these compounds using the same in vitro model (e.g. [8]). In general, 4-aminophenols appear to be more potent nephrotoxics than 2-aminophenols [8,14,26]. However, comparative studies examining the effect of chloro groups on 4-AP nephrotoxicity in vitro are limited to a comparison of 4-AP and 4-amino-3-chlorophenol (4-A3CP) in rat renal cortical slices [8,27].

The purpose of this study was to compare the nephrotoxic potential of three 4-aminochlorophenols with 4-AP in the Fischer 344 rat isolated renal cortical cell model to begin to explore in vitro structure-nephrotoxicity relationships among the 4-aminochlorophenols (Fig. 1). In addition, using 4-amino-2-chlorophenol (4-A2CP) as a

model aminochlorophenol, the role of renal biotransformation and potential toxic mechanisms in 4-A2CP cytotoxicity was examined. The male Fischer 344 rat was selected as the animal model because our previous *in vivo* studies with chloroanilines and their metabolites were conducted in male Fischer 344 rats.

2. Materials and methods

2.1 Experimental animals

Male Fischer 344 rats (200–250 g) were obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA). All animals were kept in a controlled environment with a regulated light cycle (on 06.00 h, off 18.00 h), temperature (21–23°C), and humidity (40–55%). Food (Purina Rat Chow) and water were available *ad libitum*. Rats were allowed at least one week to acclimate to the facilities prior to use in any experiments. Animal use for these experiments was approved by the Marshall University Institutional Care and Use Committee. All studies were performed in an AAALAC-accredited facility (Association for the Assessment and Accreditation of Laboratory Animal Care International). Rat care and use was in accordance with the American Association for Laboratory Animal Science (AALAS) Policy on the Humane Care and Use of Laboratory Animals (<http://www.aalas.org>).

2.2 Chemicals

4-A2CP was synthesized and purified as previously described [14]. 4-AP, 4-A3CP, 4-amino-2,6-dichlorophenol (4-A2,6DCP), N-octylamine, metyrapone and N,N'-diphenyl-*p*-phenylenediamine (DPPD), piperonyl butoxide and mercaptosuccinic acid were obtained from the Aldrich Chemical Co., (Milwaukee, WI). All other compounds were obtained from Sigma Co. (St. Louis, MO). For each compound, the highest purity level available was used.

2.3 IRCC preparation and treatments

Untreated rats were anesthetized (pentobarbital sodium, 75 mg/kg, ip) and isolated rat renal cortical cells (IRCC) were obtained via the collagenase perfusion method of Jones et al. [28]. Initial cell viability was typically 85–95% as determined by trypan blue (2% w/v) exclusion and initial lactate dehydrogenase (LDH) release (~5–10%). The yield of renal cortical cells for these experiments generally ranged between 35–45 million cells/two kidneys. IRCC were resuspended at a concentration of ~4 million cells/ml in Krebs-Henseleit buffer pH 7.4 containing 25 mM Hepes and 2% (w/v) bovine serum albumin. Toxicity experiments were conducted as previously reported [29]. Briefly, 3 ml of the IRCC re-suspension was placed in a 25 ml polycarbonate Erlenmeyer flask. The flask was placed in a shaking incubator (37°C water temperature) and sealed with a serum bottle stopper containing an inlet and outlet for gas flow. The atmosphere within the flask was equilibrated with 95% O₂/5% CO₂ with shaking for 5 min. An aminophenol compound (0.5 or 1.0 mM) or vehicle (dimethyl sulfoxide [DMSO], 30 µl) was then added, and the incubations continued for 1 h. At the end of the 1 h incubation period, the flasks were removed, and a 0.5 ml aliquot was taken from each flask for determination of LDH content. Each aliquot was centrifuged (3,000 × g, 5 min), and the supernatant was decanted and saved. Pelleted cells were then re-suspended in 1 ml of Triton X-100 (10% solution) to release the remaining cellular LDH activity. LDH activity was then determined in each fraction as

previously described [30] using a kinetic assay based on the amount of NADH produced from NAD. LDH released into the supernatant, as a measure of cytotoxicity, was expressed as % of total (LDH in supernatant plus pellet).

In some experiments, cells were pretreated with an antioxidant or enzyme inhibitor prior to the addition of an aminochlorophenol. Pretreatments were based on previously published studies with these compounds [16,31–40]. Pretreatment concentrations and pretreatment times are shown in Table 1.

2.4 Statistics

Data are presented as the mean $\pm$ S.E. for at least four separate experiments. In each experiment, samples were run as duplicates with the average of the two values serving as an N=1. The data were analyzed using a one-way analysis of variance (ANOVA) test followed by a Dunnett's or Newman-Keuls analysis. All statistical tests were run at a 95% confidence interval and significance was denoted as $P < 0.05$.

3. Results

3.1 Structure-cytotoxicity studies

Cytotoxicity of 4-AP, 4-A2CP, 4-A3CP and 4-A2,6DCP was determined at 0.5 and 1.0 mM (Fig. 2). At 0.5 mM, marked cytotoxicity was observed with 4-A2CP and 4-A2,6-DCP. Cytotoxicity was not observed with 4-AP or 4-A3CP at a concentration of 0.5 mM. When concentrations of the aminophenols were increased to 1.0 mM, cytotoxicity was observed with all test compounds, except 4-A3CP. These results indicate that the direct nephrotoxic potential of the aminophenols in decreasing order was 4-A2,6DCP > 4-A2CP > 4-AP > 4-A3CP.

3.2 Effect of antioxidants on 4-A2CP cytotoxicity

Previously, we reported the effects of various antioxidants and inhibitors of biotransformation enzymes on 4-A2,6DCP cytotoxicity in a renal cortical slice model from male Fischer 344 rats [18]. To determine if other aminochlorophenols responded similarly to the pretreatments on 4-A2,6DCP cytotoxicity, another structurally-related model compound, 4-A2CP, was selected for study.

To examine the role of free radical mechanisms, the effects of several antioxidants on 4-A2CP cytotoxicity were determined. Pretreatment with ascorbate (1 mM) attenuated 4-A2CP (0.5 or 1.0 mM) cytotoxicity (Fig. 3, Panel A). However, pretreatment with the antioxidants α -tocopherol (1.0 or 2.0 mM) or DPPD (0.05 mM) did not reduce 4-A2CP effects (data not shown). Since ascorbate was effective in reducing 4-A2CP cytotoxicity, studies were conducted with the iron chelator deferoxamine to determine if iron might play a role in free radical generation. However, deferoxamine (0.1 mM) pretreatment did not alter 4-A2CP cytotoxicity (data not shown). Thus, while free radical mechanisms may contribute to 4-A2CP nephrotoxicity in vitro, generation of free radicals is not iron dependent in IRCC.

The effects of two additional antioxidants, glutathione and N-acetyl-L-cysteine on 4-A2CP cytotoxicity were also examined. Glutathione (1.0 mM) completely attenuated 4-A2CP (1.0

mM) cytotoxicity, while N-acetyl-L-cysteine (2.0 mM) partially attenuated 4-A2CP-induced nephrotoxicity (Fig. 3, Panel B).

3.3 Effect of enzyme inhibitors on 4-A2CP cytotoxicity

The next set of experiments examined the ability of potential inhibitors/enhancers of four oxidative enzyme systems to attenuate 4-A2CP nephrotoxicity. Among the cytochrome P450 (CYP) inhibitors and flavin adenine dinucleotide monooxygenase (FMO) activity modulators, only piperonyl butoxide (1.0 mM) attenuated 4-A2CP cytotoxicity at 0.5 and 1.0 mM (Table 2). Metyrapone (1.0 mM) pretreatment potentiated 4-A2CP 0.5 mM cytotoxicity, but when the 4-A2CP concentration was increased to 1.0 mM, no potentiation or attenuation of cytotoxicity by metyrapone was observed (Table 2). No alteration of 4-A2CP cytotoxicity was observed with isoniazid, ketoconazole, methimazole or N-octylamine pretreatment (Table 2). Inhibition of cyclooxygenase (prostaglandin H synthase) with indomethacin (1.0 mM) or pretreatment with the peroxidase inhibitor mercaptosuccinate (0.1 mM) attenuated 4-A2CP nephrotoxicity (Fig. 3, Panel C), suggesting that prostaglandin H synthase and peroxidases may contribute to 4-A2CP bioactivation.

4. Discussion

A comparison of the in vitro nephrotoxic potential of 4-AP with three 4-aminochlorophenols revealed that the in vitro decreasing order of cytotoxicity was 4-A2,6DCP > 4-A2CP > 4-AP > 4-A3CP (Fig. 3). These results are in agreement with the previously reported in vivo nephrotoxic potential for these compounds in male Fischer 344 rats [8,14,17]. Thus, the in vitro and in vivo nephrotoxic potential for these compounds are in parallel and suggests that similar mechanisms of bioactivation and/or nephrotoxicity occur regardless of the nature of the exposure of the kidney to these compounds or their metabolites.

The position and number of the chloro groups present on an aminophenol had an important effect on nephrotoxic potential. Addition of one chloro group at the 2-position of 4-AP produces a compound (4-A2CP) with enhanced nephrotoxic potential, while addition of two chloro groups at the 2- and 6-positions (4-A2,6DCP) enhances nephrotoxic potential even further. However, addition of a chloro group adjacent to the amino group (4-A3CP) produces a compound with reduced cytotoxicity relative to 4-AP. These observations could be explained by several potential mechanisms including: (a) inhibition of the formation of detoxifying glucuronide or sulfate conjugates at the phenolic -OH when chloro groups are in the 2- or 2,6-positions due to chloro group-induced steric hindrance, (b) inhibition of oxidation at the nitrogen atom due to reduced binding of the aminophenol derivative to the oxidative enzymes' active sites due to steric hindrance by the 3-chloro group, and (c) alterations in oxidation potential of the aminophenol due to the addition of chloro groups in different positions on the ring. Of these possibilities, it is unlikely that the addition of the chloro groups is having a major impact on the oxidation potential of the 4-AP derivatives, since chloro groups, regardless of ring position, produce only minimal changes in the oxidation potential of anilines [41,42]. Thus, the number and position of the chloro groups are most likely important for altering nephrotoxic potential by affecting bioactivation (4-A3CP) or inactivation (4-A2CP and 4-A2,6DCP) metabolic pathways.

Based on the biotransformation of 4-AP, chloroanilines and chloronitrobenzenes [12,23,43,44], aminochlorophenols can be predicted to undergo metabolism as shown in Fig. 1. Oxidation of the aminophenols to the 1,4-benzoquinoneimine metabolites would be expected to generate reactive oxygen species (ROS) and an arylating metabolite [24,25]. Conjugation of a 1,4-benzoquinoneimine metabolite with glutathione could produce a series of glutathione-derived metabolites that have the potential to redox cycle and generate oxidative stress as well as arylate cellular nucleophiles [45,46]. The ability of ascorbate to attenuate 4-A2CP nephrotoxicity in IRCC is not surprising and supports a free radical, and potential oxidative stress component to the mechanism of 4-A2CP cytotoxicity. Similar protection by ascorbate against 4-AP and 4-A2,6DCP nephrotoxicity have also been observed in vivo and in vitro [13,15,16,18,47], and ROS/oxidative stress plays a role in 4-AP and 4-A2,6DCP in vitro nephrotoxicity [18, 25]. The finding that not all antioxidants are protective against 4-A2CP cytotoxicity is also in line with findings using other aminophenol compounds. For example, BHT did not prevent 4-AP cytotoxicity in LLC-PK₁ cells [13] and α -tocopherol and DPPD were unable to attenuate 4-A2,6-DCP cytotoxicity in Fischer 344 rat renal cortical slices [18].

While free radical mechanisms may be a component of 4-A2CP nephrotoxicity, ascorbate was not able to fully protect the IRCC from 4-A2CP-induced nephrotoxicity, suggesting that other mechanisms are contributing to cell death. Foreman and Tarloff [25] came to a similar conclusion for 4-AP. They used several different inhibitors of ROS formation in an attempt to attenuate 4-AP cytotoxicity, and although they were successful in reducing ROS formation, only a small improvement in cell survival was observed. To explore this finding in more detail, the effects of two nucleophilic antioxidants, glutathione and N-acetyl-L-cysteine on 4-A2CP cytotoxicity were examined in the present study. Glutathione pretreatment was able to completely attenuate 4-A2CP cytotoxicity, while N-acetyl-L-cysteine was partially protective. Glutathione has the ability to protect cells through glutathione-S-transferase catalyzed conjugation with electrophilic species (e.g. 1,4-benzoquinoneimines) and as an antioxidant via the glutathione peroxidase mediated reaction and reduction of peroxidase-generated free radicals. Thus, glutathione may be protecting IRCC by inactivating reactive arylating metabolites of the aminochlorophenols, reducing ROS levels and neutralizing peroxidase-generated free radicals. Although the role that each protective mechanism plays in attenuating 4-A2CP cytotoxicity hasn't been established, Harman et al. [48] demonstrated that maintaining glutathione levels was an important mechanism for attenuating 4-AP nephrotoxicity. Additional studies are needed to clearly define the role of arylation and ROS-induced oxidative stress in 4-A2CP nephrotoxicity.

N-Acetyl-L-cysteine has been thought to primarily provide protection against electrophiles and ROS by providing cysteine for the formation of glutathione and to partially interact with electrophilic species [49,50]. Thus, the lack of full protective effects by N-acetyl-L-cysteine could be due to the time required to replenish glutathione. However, Zhang et al. [51] recently found that the cytoprotective effects of N-acetyl-L-cysteine against 2,3,5-tris(glutathione-S-yl)-hydroquinone, a nephrotoxicant metabolite of hydroquinone, were primarily due to scavenging ROS and in part via ERK1/2 activation rather than increasing glutathione levels in HK-2 cells. Therefore, N-acetyl-L-cysteine may also have multiple mechanisms for attenuating 4-A2CP cytotoxicity.

The enzyme systems that are involved in bioactivating 4-aminophenols to their reactive and/or nephrotoxic metabolites have not been fully established. Calder et al. [52] could not determine if CYPs played a role in 4-AP nephrotoxicity in vivo. Shao et al. [53] noted that hepatic subcellular fractions incubated with rat renal proximal tubules potentiated 4-AP cytotoxicity and that 1-aminobenzotriazole, a general CYP inhibitor, did not alter 4-AP nephrotoxicity. They concluded that hepatic microsomal enzymes, other than CYPs, were responsible for this observation. Yan et al. [23] found that 1-aminobenzotriazole did not alter 4-AP metabolism by rat hepatocytes. Thus, at least for 4-AP, non-CYP enzymes appear to be responsible for 4-AP bioactivation. The current results generally support these findings in the rat IRCC model for 4-A2CP with only piperonyl butoxide, a general CYP inhibitor, providing partial protection from 4-A2CP cytotoxicity, while other CYP inhibitors were ineffective. FMOs don't appear to play an important role in 4-A2CP bioactivation, since methimazole and N-octylamine did not affect 4-A2CP nephrotoxicity. However, inhibition of the cyclooxygenase component of prostaglandin H synthase with indomethacin and inhibition of peroxidase activity with mercaptosuccinate provided significant protection from 4-A2CP cytotoxicity. These results suggest that in the kidney oxidation of 4-A2CP by cyclooxygenase and peroxidase-mediated metabolism of 4-A2CP are important bioactivation pathways for this compound. In addition, Martinkova et al. [54] recently demonstrated that piperonyl butoxide, but not metyrapone, has peroxidase inhibitor properties, which could explain the observation that this classical CYP inhibitor provided some attenuation of 4-A2CP cytotoxicity while other CYP inhibitors did not.

Conclusion

The results of the current study demonstrate that 4-AP nephrotoxicity was enhanced by addition of chloro groups at the 2- and 2-,6-positions and reduced by addition of a chloro group at the 3-position in the IRCC model. Studies with antioxidants and 4-A2CP supported a role for free radicals in aminochlorophenol cytotoxicity. In addition, CYPs and FMOs do not appear to play a role in 4-A2CP bioactivation, but oxidation of 4-A2CP via cyclooxygenase and biotransformation of 4-A2CP by renal peroxidase(s) do contribute to the formation of nephrotoxicant species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

4-AP	4-aminophenol
4-A2CP	4-amino-2-chlorophenol

4-A3CP	4-amino-3-chlorophenol
4-A-2,6DCA	4-amino-2,6-dichlorophenol
BHT	butylated hydroxytoluene
CYP	cytochrome P450
DPPD	N,N'-diphenyl- <i>p</i> -phenylenediamine
ERK 1/2	extracellular signal-regulated kinase 1/2
FMO	flavin adenine dinucleotide monooxygenase
IRCC	isolated renal cortical cells
LDH	lactate dehydrogenase
ROS	reactive oxygen species

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Highlights

1. Chlorine atoms next to the phenol of 4-aminophenol increase nephrotoxic potential.
2. Chlorine atoms next to the amine of 4-aminophenol decrease nephrotoxic potential.
3. Co-oxidation and peroxidase metabolism bioactivated 4-amino-2-chlorophenol.
4. Free radicals contribute to 4-amino-2-chlorophenol nephrotoxicity.

Figure 1.
Potential routes of biotransformation of 4-AP (x=0) and the aminochlorophenols (X=1–2) used in this study.

Figure 2.

Effect of aminophenol concentration on LDH release from IRCC. IRCC (~4 million/ml) were incubated with vehicle (control) or an aminophenol (0.5 or 1.0 mM) for 1 h, and LDH release (% of total) determined. Values are means $\pm$ S.E. from four separate experiments. An * indicates significantly different from the vehicle control group value, $P < 0.05$. A ♦ indicates that the value is significantly different from the corresponding 4-AP value, $P < 0.05$.

Figure 3.

Effect of pretreatments on 4-A2CP cytotoxicity. For these experiments, IRCC were pretreated with ascorbate (ASC)(Panel A), glutathione (GSH) (Panel B), N-acetyl-L-cysteine (NAC)(Panel B), indomethacin (Indo) (Panel C), mercaptosuccinate (MS)(Panel C) or vehicle, as described in Table 1, prior to the administration of 4-A2CP or 4-A2CP vehicle. Values are means $\pm$ SE for N=4 separate experiments. An * indicates significantly different from the control group value, $P < 0.05$. A ♦ indicates significantly different from the

pretreatment (Pre-TX) only group, $P < 0.05$. A ▲ indicates that the 4-A2CP + Pre-TX group value is significantly different from the corresponding 4-A2CP only value. $P < 0.05$.

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Table 1

Pretreatments Evaluated as Modifiers of 4-A2CP Cytotoxicity

Pretreatment	Concentration (mM)	Pretreatment Time (min)	Mode of Action
Ascorbate	1.0	5	Antioxidant
α -Tocopherol	1.0	5	Antioxidant
	2.0	5	
DPPD	0.05	30	Antioxidant
Glutathione	1.0	30	Antioxidant; nucleophile
N-Acetyl-L-cysteine	2.0	30	Antioxidant
Deferoxamine	0.10	5	Iron Chelator
Metynapone	1.0	5	CYP inhibitor
Piperonyl butoxide	1.0	15	CYP inhibitor
Isoniazid	1.0	5	CYP2E1 inhibitor
Ketoconazole	0.1	30	CYP inhibitor
Methimazole	1.0	5	FMO inhibitor
N-Octylamine	0.20	5	FMO enhancer
Indomethacin	1.0	15	Cyclooxygenase inhibitor
Mercaptosuccinate	0.1	15	Peroxidase inhibitor

Table 2Effects of CYP and FMO activity modulation on 4-A2CP cytotoxicity^a

Pretreatment (mM)	4-A2CP (mM)	LDH (% Release)		
		Control	4-A2CP	Pretreatment 4-A2CP + Pretreatment
CYP inhibitors				
Piperonyl butoxide (1.0)	0.5	21.1 ± 0.2	39.5 ± 0.6 ^b	25.0 ± 1.0
Metyrapone (1.0)	0.5	17.9 ± 1.0	43.0 ± 1.4 ^b	20.6 ± 0.9
Isoniazid (1.0)	0.5	18.7 ± 0.6	38.3 ± 4.9 ^b	20.7 ± 0.9
Ketoconazole (0.1)	0.5	22.5 ± 0.8	43.5 ± 3.2 ^b	26.1 ± 1.7
FMO modulators				
Methimazole (1.0)	0.5	20.7 ± 0.5	33.0 ± 2.7 ^b	22.0 ± 0.5
N-Octylamine (0.2)	0.5	21.5 ± 0.2	52.0 ± 2.5 ^b	27.5 ± 1.1
CYP inhibitors				
Piperonyl butoxide (1.0)	1.0	17.4 ± 0.9	42.6 ± 0.6 ^b	19.1 ± 2.5
Metyrapone (1.0)	1.0	19.5 ± 1.0	54.4 ± 2.3 ^b	21.3 ± 0.6
Isoniazid (1.0)	1.0	18.9 ± 1.5	51.3 ± 3.2 ^b	18.6 ± 0.7
Ketoconazole (0.1)	1.0	22.0 ± 1.1	51.4 ± 4.9 ^b	22.4 ± 0.9
FMO modulators				
Methimazole (1.0)	1.0	18.9 ± 1.4	53.9 ± 6.5 ^b	19.7 ± 2.2
N-Octylamine (0.2)	1.0	19.6 ± 0.9	49.9 ± 4.3 ^b	20.0 ± 0.9
				48.3 ± 4.3 ^b
				52.6 ± 3.4 ^b

^a Isolated renal cortical cells (IRCC) were prepared from untreated male Fischer 344 rats. IRCC were incubated with vehicles, 4-A2CP, pretreatment or 4-A2CP + pretreatment for 1 h post 4-A2CP or 4-A2CP vehicle. LDH release was determined at the end of the incubation period. Values are means ± S.E. for four to six separate experiments.

^b Significantly different from the control value, P < 0.05.

^c Significantly different from the 4-A2CP only value, P < 0.05.