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Metabolism of nilutamide in rat lung

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Abbreviations:
2′AMP, 2′ adenosine monophosphate
DPIC, diphenyliodonium chloride
eNOS, endothelial NOS
iNOS, inducible NOS
NADD, norandrostenedione
NMN, *N*-methylnicotinamide
NOS, nitric oxide synthase
nNOS, neuronal NOS
NR, nitroreductase
2-OHP, 2-hydroxypyrimidine
*p*HMB, *para*-hydroxymercuribenzoic acid
P450R, NADPH:cytochrome c (P450) reductase
R-NO_2, nitro

ABSTRACT

Nilutamide is a non-steroidal anti-androgen drug proposed in the treatment of metastatic prostatic carcinoma. Its therapeutic effects are overshadowed by the occurrence of adverse reactions, mediated by mechanisms that remain elusive. To elucidate possible mechanisms for nilutamide toxicity, we investigated the metabolism of nilutamide in rat lung homogenates, in subcellular fractions and in freshly isolated cells.

In whole lung homogenates, the nitro group of nilutamide was reduced to the amine and hydroxylamine moieties. These conversions occurred exclusively in the absence of dioxygen, were increased by the addition of FMN, FAD, or NADPH.

Reductive metabolism of nilutamide to the amine and hydroxylamine was further evidenced in subcellular fractions obtained by differential ultracentrifugation. It was found to take place mainly in the cytosol of rat lung and to be stimulated, strongly, upon co-addition of NADPH and FMN. Addition of inhibitors of enzymes involved in the reductive metabolism of nitroaromatic compounds indicated that reduction of nilutamide involved, mainly, soluble flavoproteins. Incubations with freshly isolated lung cells revealed that macrophages were the main players in nitroreduction of nilutamide whereas the epithelial type II cells and the non-ciliated Clara cells were less efficient in catalyzing this reaction.

Our results show that nilutamide is extensively reduced by lung tissues in the absence of oxygen, especially by enzymes found in alveolar macrophages. In accordance with recent findings, subcellular localization, oxygen sensitivity, cofactor requirements and inhibitor studies lead us to suggest the involvement of a soluble nitric oxide synthase in lung cytosolic nitroreduction.

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R-NO, nitroso
R-NHOH, hydroxylamine
R-NH_2, amine

1. Introduction

A number of chemicals administered via various routes have been reported to damage the lung in experimental models and several drugs containing nitroaromatic groups have been reported to induce lung diseases [1–5]. Both oxidative (ring oxidation) and nitroreductive metabolic pathways may be important in explaining their toxic effects. The amino derivative of the drugs nifurtimox [6], nitrazepam [7] nitrofurazone [8] have been detected after incubation with pulmonary tissue in vitro. Factors including species, strains, metabolism of the parent compound or of a metabolite, as well as reactive intermediate metabolites in cells or subcellular organelles also seem to play roles in pulmonary toxicity [2,3].

Nilutamide is a nitroaromatic compound used as an antiandrogen drug in the treatment of metastatic cancer of the prostate gland. However, nilutamide has been reported to cause interstitial pneumonitis in 1–2% of patients in Europe [9] and 12–18% in Japan [10]. It undergoes metabolic activation in lung and liver tissues of animals [11,12]. A previous study detected some reductive metabolism of nilutamide in an experimental model using isolated, perfused rat lungs [13]. However, detailed identification of the metabolites and enzymes involved in the reaction was not undertaken [13].

There are few enzymes known to have nitroreductive activity (for review see [1,3,14,15]). These enzymes can be distinguished by their oxygen sensitivity, subcellular localization as well as their requirements for electron donors [16]. Using lung microsomes, Berger and coworkers detected the nitro radical anion of nilutamide as well as covalent binding to proteins. This reductive activation required NADPH and the absence of oxygen, and it was inhibited by NADPH–cytochrome P450 reductase (P450R) inhibitors. The authors proposed P450R to be implicated in these processes [12]. However, several other enzymes share common properties with P450R (i.e. NADPH requirements, oxygen sensitivity and P450R inhibitor sensitivity). They include P450R-like proteins such as nitric oxide synthases (NOS), methionine synthase reductase and novel reductase I [17–20]. These proteins reduce cytochrome *c* under the same conditions as P450R [19,21,22] and also display nitroreductase activity. Moreover, we have recently demonstrated that recombinant NOSs can reduce nilutamide to its nitro anion radical with further selective reduction to the corresponding hydroxylamine [23]. This reduction of nilutamide was catalyzed by full length NOSs and by their isolated reductase domain. It was inhibited by dioxygen but unaffected by standard NOS inhibitors that bind to the heme domain [23].

In order to more fully investigate nilutamide metabolism, we incubated it with rat lung homogenates, subcellular fractions and isolated cells from rat lung. Beside the present work on a possible local pulmonary metabolic transformation of nilutamide, subcellular fractions of other rat tissues and organs were also tested to detect other possible, distant metabolic activation of nilutamide [24]. Here, we show that in rat lung, nilutamide is transformed mainly to the corresponding hydroxylamino and amino derivatives. This nitroreduction is oxygen-sensitive, localized in the cytosolic fraction, and dependent upon NADPH and FMN administration. The addition of standard inhibitors of known nitroreductases was ineffective. Maximal nitroreduction was observed in alveolar macrophages whereas the Clara cells and type II epithelial cells were much less active. Our results suggest the involvement of soluble P450R-like flavoprotein(s) and reinforce the hypothesis that NOS could be involved in the nitroreduction of nilutamide in the lung.

2. Materials and methods

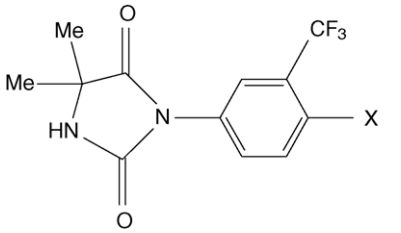
2.1. Animals

Lung homogenates and subcellular fractions were prepared from male, 250–350 g Sprague–Dawley rats (Iffa, L'Arbresle, France). They were housed under a 12-h dark/12-h light cycle and given food and water ad libitum. Lung cells were isolated from WKY male rats (200–250 g, Møllegaard, Ejby, Denmark). The animals were given Ewos R3 standard pelleted laboratory chow from Astra Ewos AB (Södertälje, Sweden) and water ad libitum.

2.2. Chemicals

Nilutamide and its amino derivative (RU-43866, Fig. 1) were gifts from Aventis (Romainville, France). The hydroxylamino derivative of nilutamide (R-NHOH, Fig. 1) was synthesized and fully characterized as described previously [23].

Norandrostenedione (internal standard for the HPLC analysis), β-NADPH, β-NADH, *N*-methylnicotinamide (NMN), 2-hydroxypyrimidine (2-OHP), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), diphenyleniodonium chloride (DPIC), *p*-hydroxymercuribenzoic acid (*p*HMB), adenosine 2′-monophosphate (2′-AMP), aminoguanidine hemisulfate (AG), N^{ω}-nitro-L-arginine methyl ester (L-NAME), allopurinol, menadione and dicoumarol were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Acetonitrile, metha-



1: X = NO_2, nilutamide
2: X = NHOH, hydroxylamine, R-NHOH
3: X = NH_2, amine,R-NH_2

Fig. 1 – Structure of nilutamide and its reduced metabolites.

nol and dichloromethane (HPLC-grade) were supplied by Carlo Erba (Milano, Italy).

For lung cell isolation studies, protease (type 1: crude), DNase I (type III), dimethyl sulfoxide (DMSO), trypan blue and proteinase K (KP0390) were obtained from Sigma–Aldrich. RPMI 1640 cell culture medium with L-glutamine and bovine serum albumin (BSA) were purchased from Gibco BRL (Paisley, Scotland, UK).

2.3. Preparation of lung homogenates

Animal surgery was performed according to the guidelines of the Ministère de la Recherche et de la Technologie, France. Rats were anesthetized with sodium pentobarbital (70 mg/kg, i.p.). The anterior cervical region and chest were cut, and a tracheal cannula (PE 14G) was inserted. Heparin (1500 I.U.) was delivered by direct injection to the right ventricle. A PE 14G cannula was then inserted into the pulmonary artery and the lung was flushed with 10 ml 0.9% saline. The lungs were then excised en bloc, freed from extrapulmonary airways, heart and extraneous tissues, and weighed. The lungs were then homogenized in 2 ml of ice-cold 50 mM Tris/HCl 150 mM KCl buffer with a Polytron (setting 9 × 1 min). This homogenate was diluted by adding 4 ml of ice-cold buffer and nilutamide metabolism was directly investigated using 500 μl aliquots of this preparation.

2.4. Preparation of subcellular fractions

Subcellular fractions were obtained from lung homogenate as described previously [25]. Five milliliter of homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C (Beckman J21C, Villepinte, France). The pellet (P) was resuspended in 5 ml ice-cold 50 mM Tris/HCl 150 mM KCl buffer and recentrifuged as described above. This washed pellet was taken as the nuclear (P_1) pellet.

The supernatant (S) was centrifuged further to obtain the following fractions: P_9 (mitochondrial: 9000 × *g* × 20 min), S_9 (post-mitochondrial), P_{105} (microsomal 105,000 × *g* × 60 min), and S_{105} (cytosol). During this stepwise centrifugation procedure, all pellets were washed as described above for P_1 [25]. Proteins concentrations were determined using the P5656 kit from Sigma.

All subcellular fractions were kept at 4 °C, in the dark, and were used within 24 h of preparation.

2.5. Isolation of primary lung cells

The methods for isolating epithelial type II cells and Clara cells from rat lung have been described previously [26]. Briefly, the lungs from six rats were perfused via the pulmonary artery with phosphate buffer. Alveolar macrophages were removed/isolated with EGTA buffer by lavage. Epithelial cells were isolated by enzymatic digestion of the perfused lung with protease followed by centrifugal elutriation of the crude cell suspension. Type II cells and Clara cells were collected and the cell populations were purified by differential attachment. Cell viability was assessed by trypan blue exclusion and exceeded 90% for both cell populations. Purity of type II cells was approximately 90%. In the Clara cells population, purity was in the range of 40–60% with type II cells the predominant contaminant.

To prepare cell sonicates, 2–6 × 10^6 cells were suspended in cold PBS buffer without Ca^{2+} and Mg^{2+} (PBS^-) and sonicated on ice. For intact cells, 2–6 × 10^6 cells were suspended in glass vials with a sealable septum in 15 mM HEPES buffered RPMI 1640 cell culture medium supplemented with 1% BSA and placed on ice.

2.6. Nilutamide metabolism in lung homogenates, subcellular fractions and isolated lung cells

Incubations were performed in air (aerobic) or under a gentle flow of N_2 (anaerobic). Following preparation, aliquots of lung homogenates or subcellular fractions were deoxygenated by a slow flow of N_2 for 15 min on ice. Then, the cofactors FMN, NADPH, NADH, or FMN and NADPH (500 μM each), were added to samples of lung homogenate (H; 150 mg lung or 1 mg protein as described in the figure legends) or subcellular fraction (P_1, P_9, S_9, P_{105}, S_{105}, all corresponding to 1 mg protein). Nilutamide (500 μM) was then added and the total volume of the incubation mixture was brought to 500 μl using 50 mM Tris/HCl 150 mM KCl buffer pH 7.4. Incubations were run for 3 h at 37 °C in the dark with gentle, horizontal shaking.

Sonicated or intact cells were kept on ice during preparation of the incubation mixtures. Nilutamide (500 μM) was added to the samples and a combination of NADPH + FMN (both 500 μM) was incubated with the sonicates in a total volume of 500 μl. Oxygen was removed by a gentle flow of N_2 for 15 min before the vials were sealed. The samples were brought to 37 °C and incubated for 3 h in the dark with gentle, horizontal shaking.

2.7. Kinetic analysis

The rate of amine formation as a function of nilutamide concentration was examined in rat lung cytosol. Incubations with nilutamide (10 μM–1 mM) were conducted under N_2 with the addition of FMN and NADPH (500 μM each) as described above. Data was analyzed using Sigma Plot enzyme kinetic module 1.0.

2.8. Effects of inhibitors

Each inhibitor (allopurinol, dicoumarol, 2′AMP, *p*HMB, menadione, DPIC, aminoguanidine, *S*-methyl-L-thiocitrulline) was tested at six concentrations (25–500 μM) and was added to the lung cytosol sample immediately before FMN, NADPH and nilutamide. Incubations were conducted under N_2 for 3 h. Control cytosol samples, from the same rat, were incubated without inhibitor.

2.9. Analysis of nilutamide and metabolites

After 3 h, internal standard (norandrostenedione, 100–2000 ng in 100 μl of MeOH), was added to the incubation mixtures and extractions were performed using 4 ml dichloromethane as described previously [23]. After centrifugation, the organic layers were transferred to clean glass tubes, and evaporated under N_2 at 37° until dry. The residues were re-dissolved in 1.0–4.0 ml methanol, depending on nilutamide concentration, and

vortex-mixed. Ten microliter were injected for HPLC analysis. Mean extraction ratio for amine recovery was 86 ± 3% (S.E.).

The HPLC system consisted of two Waters 501 (Milford, MA) reciprocating pumps, with manual (Waters U6K) and automatic (Gilson 231, Villiers-Le-Bel, France) sample injectors, UV detector (Waters 484; setting 230 nm), and integrator (Waters 745). The stationary phase was a C18 column (3.8 mm × 150 mm, Nova-Pak, Waters), and the mobile phase was a mixture of acetonitrile and methanol in water (30:5:65, v/v/v) run, isocratically, at a flow rate of 1 ml/min. Spectra of nilutamide metabolites were recorded by Waters Millenium software using a Waters 940 PDA detector. Nilutamide, its amino- and hydroxylamino metabolites exhibited retention times of 25.0, 5.8 and 4.4 min, respectively. Calibration curves were achieved by injecting known amounts of nilutamide and metabolites (5–200 ng each), and were repeated once every 20 HPLC runs. Where appropriate, extracts of homogenates or subcellular fractions were spiked with known amounts of the authentic amino- or hydroxylamino metabolites.

2.10. LC–MS

To further identify the metabolites, the HPLC system was connected to an Esquire-LC (Bruker Daltoniks, Bremen, Germany) mass spectrometer (MS), equipped with an electrospray ionization (ESI) source. Spectra were recorded after optimization in the negative and positive ionization modes for the detection of nilutamide and its amino derivative. The authentic hydroxylamino metabolite was detected in the negative mode. The MS parameters were optimized by flow injection at a rate of 800 μl/h. All spectra were recorded in full scan mode (*m*/z range: 50–500), and maximum accumulation time was set at 200 ms.

2.11. Statistical analysis

Results are presented as mean ± S.E. Statistical analysis relied on ANOVA, post hoc one-tailed Dunnett test, and p values ≤ 0.05 were considered as significant.

3. Results

3.1. Nilutamide metabolism in rat lung homogenates

Regardless of the concentration of nilutamide or the addition of cofactors, no trace of reductive or oxidative metabolism of nilutamide was evidenced when the incubations were carried out under air or in the presence of boiled homogenates (data not shown). In contrast, lung homogenates under an atmosphere of nitrogen metabolized nilutamide. This is illustrated by the presence of two new peaks (retention times 4.4 and 5.8 min) on the chromatograms, in addition to nilutamide (Fig. 2). Spiking the samples with authentic hydroxylamino and amino derivatives of nilutamide increased both the height and the area of peaks 1 and 2, respectively, and the associated UV spectra of these new peaks were identical to those obtained for the authentic standards (Fig. 2). Furthermore, LC–MS analysis confirmed that these two metabolites had molecular masses corresponding to the hydroxylamine (*m*/z = 303) and the amine (*m*/z = 287) of nilutamide, respectively (data not shown). The presence of the hydroxylamine (peak 1) was reproducible and was never observed under experimental conditions associated with the absence of the amino metabolite (peak 2, e.g. air exposure). However, a large variability could be observed when aliqoutes of same subcellular fraction were incubated at the same time. Due to the irreproducible

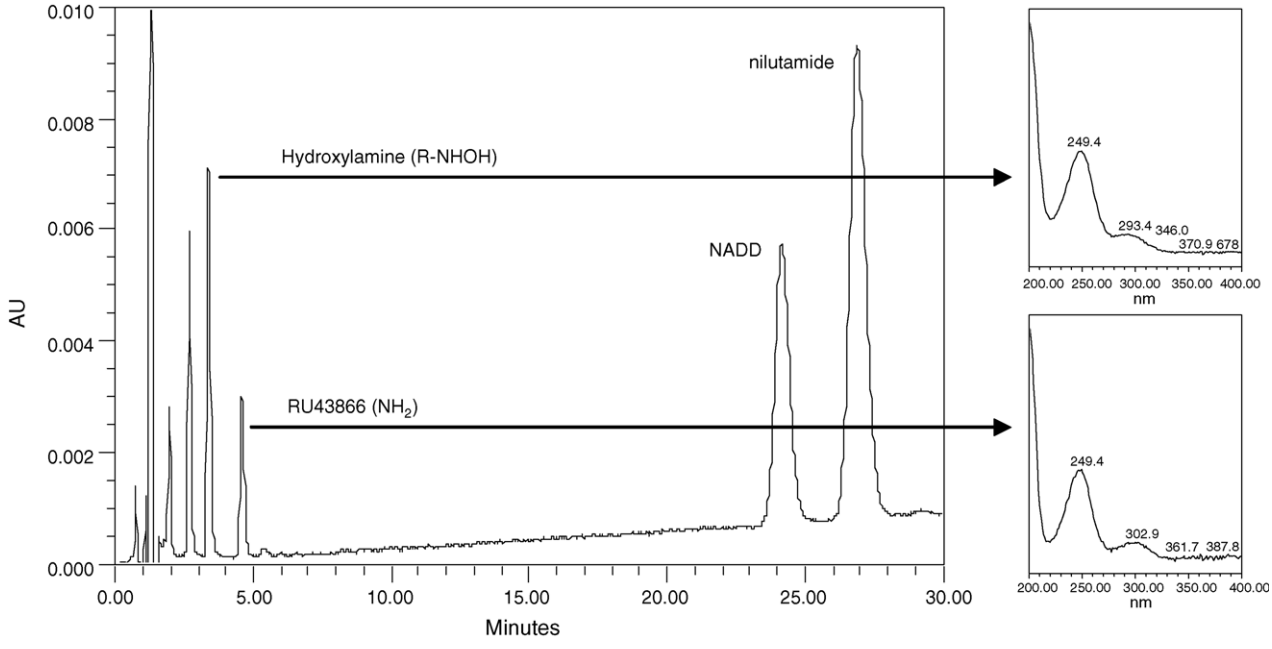


Fig. 2 – Reduction of nilutamide to the amine and hydroxylamine by alveolar macrophages. A representative HPLC chromatogram and associated UV spectra of the hydroxylamino and amino metabolites of nilutamide obtained after incubation of alveolar macrophages under N_2 atmosphere for 3 h in the presence of nilutamide, FMN and NADPH (500 μM of each compound). Reaction mixture was extracted and analyzed by RP-HPLC as indicated in Section 2.

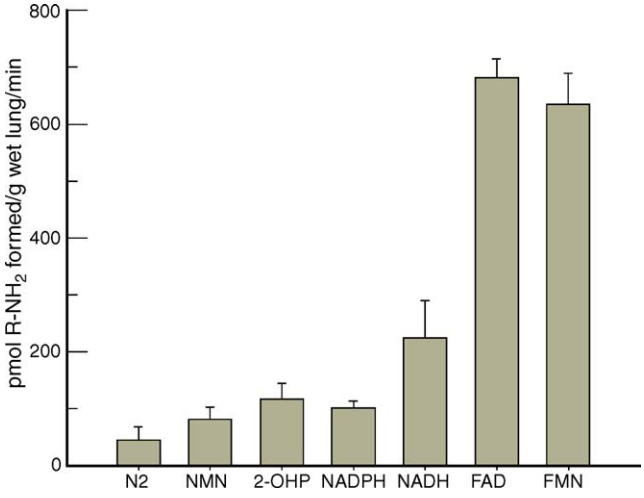


Fig. 3 – Cofactor requirements of the oxygen-sensitive nitroreduction of nilutamide by lung homogenate. Nilutamide (100 μM) was incubated under hypoxic conditions in the presence of lung homogenate alone (NCA; no cofactor addition) and after addition of different cofactors (500 μM). Results are expressed as nmol of amine/g wet lung/min.

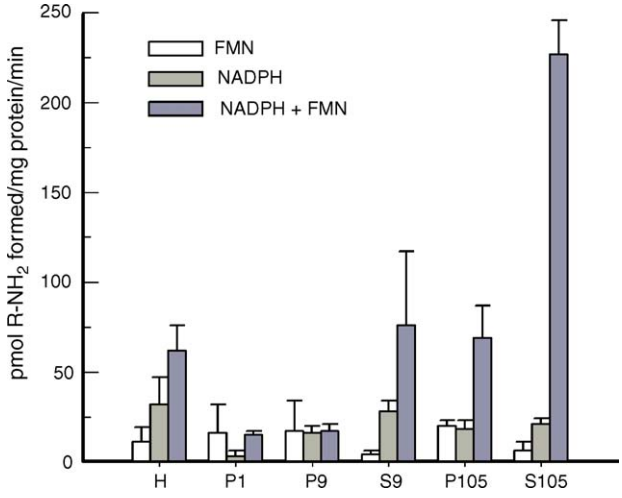


Fig. 4 – Subcellular localization of oxygen-sensitive nilutamide reductase. Lung subcellular homogenate (H) and fractions (P1; nuclear, P9; mitochondrial, S9; post-mitochondrial, P105; microsomal, S105; cytosol) were incubated in the presence of nilutamide (500 μM) and FMN, NADPH or FMN + NADPH (500 μM each) in hypoxic conditions for 3 h. Results are expressed as pmol of amine formed/mg protein/min. See Section 2 for further details of incubation, extraction and RP-HPLC analysis.

quantitation of hydroxylamine between samples, the reductive metabolism of nilutamide was expressed only as the amount of amine R-NH_2 formed.

Fig. 3 shows that at a starting concentration of 100 μM nilutamide in lung homogenates, 45 ± 23 pmol of amino-metabolite were formed/g wet lung/min. The metabolic rate increased when usual cofactors of oxidoreductases were added (Fig. 3). Incubations performed in the presence of 500 μM FAD led to a 17-fold higher value than without addition of cofactor (Fig. 3). Among the cofactors tested, FMN and FAD were the most potent at increasing the rat of nitroreduction of nilutamide, followed by NADH, NADPH, 2-OH-P, and NMN (Fig. 3). As the incubations were performed with relative high amount of proteins (1/12th of total lung) and incubated without storage or freezing, we cannot exclude the presence of endogenous electron donors.

3.2. *Nilutamide metabolism in subcellular rat lung fractions*

In order to further characterize the reductive metabolism of nilutamide subcellular fractions were prepared as described under materials and methods. Due to the multiple samples that had to be incubated, we chose to incubate nilutamide with approximately 2 mg of proteins and within 24 h of preparation. As observed previously with rat lung homogenates, no consistent oxidative and reductive metabolism of nilutamide could be detected in the nuclear, mitochondrial, microsomal or cytosolic fractions when incubations were performed under normal atmospheric conditions, with or without the addition of cofactors (data not shown). When incubations were performed under anaerobic conditions, in the absence of exogenous cofactors, only minute amounts of the amino metabolite were detected in all subcellular fractions. The greatest extent of nitroreductive metabolism was observed when FMN and NADPH were added together. The largest increases in the nitroreduction of nilutamide were observed in the microsomal fraction and, to an even greater extent, in the cytosolic fraction, as compared to the nuclear or mitochondrial fractions (Fig. 4). Accordingly, all further incubations were carried out with rat lung cytosol in the presence of FMN and NADPH and under an atmosphere of nitrogen.

3.3. *Kinetics of nilutamide metabolism in rat lung cytosol*

The rate of nitroreduction of nilutamide remained reasonably constant for the 3 h duration of the incubation (data not shown). Nitroreduction of nilutamide to the corresponding amine by rat lung cytosol was a saturable reaction with respect to nilutamide concentration and followed an apparent Michaelis–Menten kinetic with V_{max} and K_m values of 190 ± 6 pmol R-NH_2 formed/mg protein/min and 77 ± 10 μM (mean ± S.E.), respectively (Fig. 5).

3.4. *Effects of inhibitors on nilutamide metabolism in lung cytosol*

Known inhibitors of reductive metabolism of nitroaromatic compounds were examined for possible interference with nilutamide metabolism in rat lung cytosol. Compounds tested in this study and their respective target enzyme(s) were allopurinol (xanthine oxidase), dicoumarol (DT-diaphorase), 2′AMP (P450R), DPIC (P450R, NOS), *p*HMB (P450R, NADH-b5 reductase), menadione (aldehyde oxidase, NOS), L-NAME (NOS), aminoguanidine (NOS), and S-methyl-L-thiocitrulline (NOS) [21,25,27,28]. Anaerobic incubations of rat lung cytosol with

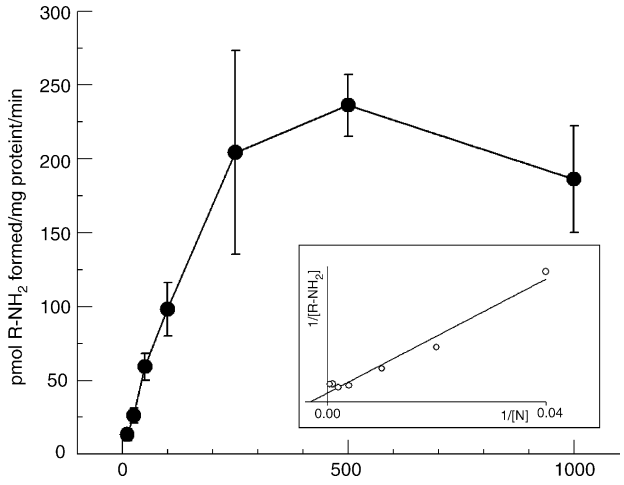


Fig. 5 – Kinetics of nilutamide in rat lung cytosol. Anaerobic incubations of rat lung cytosol were conducted in the presence of 10–1000 μM nilutamide, cofactors FMN and NADPH (both 500 μM) for 3 h. Results are presented as pmol of amine R-NH_2/mg protein/min and are mean ± S.E. from at least 5 incubations. Lineweaver–Burk plot is shown in inset.

nilutamide (500 μM) performed in the presence of 500 μM of the inhibitors allopurinol, dicoumarol, 2′-AMP, *p*HMB, L-NAME, aminoguanidine or S-methyl-L-thiocitrulline did not display a significant reduction in the amounts of the amine and hydroxylamine metabolites (data not shown). In contrast, addition of menadione or DPIC to the incubations inhibited formation of R-NH_2 in a concentration-dependent manner (Fig. 6).

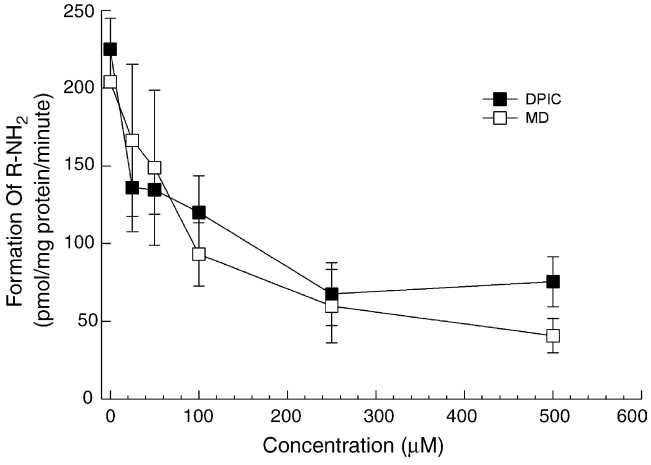


Fig. 6 – Inhibition of rat lung cytosolic reduction of nilutamide by DPIC and menadione. Anaerobic incubations of rat lung cytosol were conducted in the presence of 500 μM nilutamide, cofactors FMN and NADPH (both 500 μM), and increasing concentrations of DPIC or menadione (both 25–500 μM). Results are presented as pmol of amine R-NH_2/mg protein/min and are mean ± S.E. from three experiments.

Table 1 – Comparison of the nitroreductase activities of rat lung and liver subcellular fractions, and of lung cell populations

Conditions	Activities (pmol R-NH_2/mg protein/min)
Lung homogenate	<5
Lung homogenate + FMN + NADPH	62 ± 14
Lung subcellular fractions (+FMN + NADPH)	
P1	15 ± 2
P9	17 ± 4
S9	76 ± 41
P105	69 ± 18
S105	227 ± 19
Alveolar macrophages	233 ± 22
Clara cells	50 ± 8
T II cells	20 ± 5
Liver cytosol + FMN + NADPH	132 ± 11
Liver microsomes + FMN + NADPH	34 ± 13

3.5. *Cellular localization of nilutamide nitroreduction in rat lung*

Freshly isolated alveolar macrophages, type II cells and Clara cells from WKY rats were sonicated and incubated in the presence of 500 μM nilutamide for 3 h to further define the role of the various cells in the reduction of nilutamide. As observed previously, no metabolism of nilutamide was detected from aerobic incubations, whereas, anaerobic conditions permitted the formation of the amine and hydroxylamine metabolites (data not shown). Alveolar macrophages reduced nilutamide to the corresponding amine and hydroxylamine with the highest rate (233 ± 22 pmol R-NH_2/mg protein/min (mean ± S.E.), whereas type II cells and Clara cells were 5- and 12-fold less active, respectively (Table 1).

Reduction of nilutamide in the presence of alveolar macrophages remained linear for at least 3 h and the observed nitroreduction was not associated with any apparent macrophage toxicity, as determined by trypan blue exclusion (data not shown).

4. Discussion

In a previous study, using an isolated perfused rat lung model, no evidence for accumulation of nilutamide was found but traces of the amino metabolite of nilutamide were detected [13]. In the present paper, we further characterized the pulmonary metabolism of this drug and investigated possible mechanisms involved in its toxic effects. We studied the metabolism of nilutamide in rat lung homogenates, subcellular fractions and in isolated lung cells and showed that under anaerobic conditions, nilutamide is reduced to its hydroxylamino and amino derivatives. These two metabolites were fully identified by UV–vis and mass spectroscopy, and co-elution with authentic compounds [23]. This reduction occurred only under an N_2 atmosphere, was enhanced by the co-addition of FMN and NADPH, and was associated predominantly with the cytosolic fraction of lung homogenate.

4.1. *Production of amine and hydroxylamine derivatives*

The toxicity of most nitroaromatics compounds is associated with their reductive metabolism, which forms reactive metabolites. The fully reduced amino derivatives are usually formed via the nitroso and hydroxylamine intermediates [29–32]. The hydroxylamino derivative of the nitro-compound often appears more reactive than the parent compound, as it is able to bind covalently to proteins or DNA. This is observed in the case of the hydroxylamino metabolite of procainamide that binds covalently to microsomal proteins to a higher degree than procainamide itself [33]. The hydroxylamino derivatives can be conjugated, e.g. by sulfatation or acetylation, and form ultimately reactive nitrenium ions [34]. Accordingly, the hydroxylamino derivative of nilutamide found in the present study may be important to the mechanism of toxicity of this nitroaromatic compound if this reduction were to occur in vivo.

4.2. *Cellular and cytosolic localization of pulmonary nilutamide nitroreduction*

Pulmonary cytosol has already been associated with oxygen-sensitive nitroreductase activity towards some nitroaromatic compounds [31]. A previous study showed that pulmonary cytosol reduced nitrofluoranthenes as extensively as the corresponding microsomes, and the co-addition of FMN and NADPH enhanced this reduction [35]. Here we show the specific requirement of both FMN and NADPH for nilutamide nitroreduction in the presence of pulmonary cytosol, with, respectively, 37- and 10-fold higher activities compared to the addition of FMN or NADPH alone. A small stimulatory effect of FMN and NADPH was also observed in the microsomal fraction and in the fractions containing both microsomes and cytosol, i.e. lung homogenate and post-mitochondrial fraction. These observations suggest that the cytosol may have been neglected by other investigators as the combination of FMN + NADPH cofactors has rarely been examined.

Furthermore, estimation of the amounts of cytosolic and microsomal proteins in whole rat lung indicated that cytosol contains about 2.5-fold more protein than microsomes. This may support the hypothesis that cytosolic nitroreduction of nilutamide is relevant to this drug's adverse effects.

Few studies have implicated alveolar macrophages as drug metabolizing sites since 1978, when Harris et al., showed that alveolar macrophages were able to metabolize benzo(*a*)pyrene to carcinogens [36]. Alveolar macrophages have also been found to reduce 1-NP to the corresponding amine [37] and to metabolites that binds covalently to DNA [38]. Furthermore, normal macrophage function may be directly impaired by in situ drug metabolism leading to adverse events.

In the present study, we found that alveolar macrophages reduces nilutamide to a larger extent than the more known drug-metabolizing Clara cells and type II cells. Although the relative importance of these potential metabolizing cells in vivo has to be determined, the total number and state of the macrophage varies upon different pathological states, and has to be taken into account for the study of the toxicity of this compound. No major toxic effect could be observed under our conditions, as judged by trypan blue exclusion (data not shown). Further studies are required to evaluate fully the impact of reductive nilutamide metabolism by different cellular populations of the lung.

4.3. *Enzyme(s) implicated in nilutamide reduction*

The enzyme(s) responsible for nilutamide reduction have not yet been identified precisely. Involvement of P450 and P450R have been cited [12] and there is growing evidence that these membrane-bound enzymes participate in the reduction of several nitro-compounds [39–43]. The implication of a flavoprotein in nilutamide nitroreduction in rat lung is suggested by: (i) the requirement for both FMN and NADPH, and (ii) the inhibition by DPIC, a non-selective inhibitor of flavoproteins. Due to its subcellular localization, P450R does not seem to be implicated in the nitroreduction of nilutamide, which occurs predominantly in lung cytosol. Since their specific inhibitors failed to inhibit nilutamide nitroreduction, cytosolic enzymes, such as aldehyde oxidase and xanthine oxidase, probably do not account for the observed nilutamide reduction. Thus, we investigated another class of enzymes, the nitric oxide synthases, which exhibit several appealing properties.

First, NOSs displays a high percentage of amino acid sequence identity with P450R [17,44]. Second, NOS and P450R proteins have been implicated in the one electron reduction of cytochrome *c* [45], and of quinones, such as adriamycin, tirapazamine and doxorubicin [21]. Since the microsomal localization of P450R excludes its involvement in cytosolic nilutamide nitroreduction, some of the cytosolic NOS proteins, i.e. neuronal nNOS and inducible iNOS, are candidates for involvement in this reaction. Recently, we demonstrated the direct implication of the three NOS isoforms in nilutamide nitroreduction and their ability to selectively reduce nilutamide to the corresponding hydroxylamine [23]. The lack of inhibition by L-NAME was also observed with purified NOSs. L-NAME, aminoguanidine and S-methyl-L-thiocitrulline are all known ligands of the oxygenase domains of the NOSs and have limited effects on the activities of their reductase domains. Menadione is a competitive inhibitor of NOSs that interferes with the electron transfer from the reductase domain to the heme [46]. However, these elements do not exclude the involvement of other enzymes in nilutamide reduction, such as new members of the P450R family, which have been cloned recently [19,22].

4.4. *Pulmonary adverse reactions related to nilutamide metabolism*

Some elements correlate with our in vitro results and the observed patterns of nilutamide pneumonitis in vivo: (i) the amino derivative of nilutamide is the major metabolite of nilutamide excreted in humans, (ii) pulmonary adverse reactions after nilutamide treatment are reversible in man, thus, are probably related to metabolic events and (iii) toxicity is associated with severe hypoxemia ($PaO_2 = 6.6 \pm 0.54$ kPa, mean $\pm$ S.E., [9]). Therefore, it may be reasonable to speculate that the low oxygen tension permits nitroreduction of nilutamide in situ, with intermediate formation of the reactive nitroso or hydroxylamine derivatives that can be further conjugated to the

final toxic metabolite. While our results highlight a key role for alveolar macrophages in nilutamide reduction, further investigations are required to ascertain a link between nilutamide metabolism by macrophages and lung damage.

However, we must not exclude possible alternative nitroreductive and toxic pathways that could occur in both anaerobic as well as normoxic conditions. In addition to this study, nilutamide nitroreduction has also been studied in the presence of cytosolic and microsomal fractions of rat blood, brain, heart, liver, kidney, intestine and the corresponding intestinal content. Oxygen-sensitive nitroreductive activities seem predominant compared to oxygen-insensitive activities, but nilutamide appears to be reduced by nitroreductases present in liver, intestine and intestinal content that are not oxygen-sensitive [24]. This situation is reminiscent of reports on other lung toxicants that are metabolized in other tissues than the lung with intermediate metabolites transported via the circulation to the lung where they are further activated to the final reactive metabolite [47].

In conclusion, this study demonstrates that nilutamide is reduced in rat lung cytosol to its hydroxylamine and amino derivatives. This reduction is oxygen-sensitive and stimulated by the addition of FMN + NADPH. Alveolar macrophages reduce nilutamide more extensively compared to type II cells and Clara cells. This reduction is not governed by known cytosolic nitroreductases and we propose that nitric oxide synthase may be involved in this reaction. This may have implications for the understanding of the lung toxicity of nilutamide but another hypothesis remains possible, one in which nilutamide could be activated in other organs and that the reactive species are transported to the lung where they exert their toxic effects.

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