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Nitroreductive metabolic activation of some carcinogenic nitro heterocyclic food contaminants in rat mammary tissue cellular fractions

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ABSTRACT

Several nitrofurans and nitroimidazoles have been widely used in veterinary medicine. Some of these compounds are breast carcinogens in rodents and their mechanism of action is hypothesized to be related to reactive metabolites generated by nitroreduction and/or via oxygen-dependent redox cycling. The present work describes the nitroreductive metabolism of nitrofurazone, nitrofurantoin, furazolidone, and metronidazole by the cytosolic and microsomal fractions of mammary tissue from female Sprague–Dawley rats. The data obtained were compared with those obtained with nifurtimox and benznidazole, two well-known rodent carcinogen/mutagens nitroheterocycles. The nitroreductase activity of pure milk xanthine-oxidoreductase (XOR) was evaluated for screening purposes. All the nitrofurans were nitroreduced either by the pure XOR or the cytosolic fraction in the presence of hypoxanthine, and these activities were inhibited by allopurinol. Furthermore, they were nitroreduced by the microsomal fraction in the presence of NADPH, except for the nitrofurazone, suggesting the participation of cytochrome P450 reductase. Nitrofurans metabolism was significantly more intense than that of NFX. No equivalent nitroreductase activity was observed in either subcellular fraction using nitroimidazolic compounds as substrates. These results suggest that the nitroreductive metabolism of nitrofurans and the subsequent redox cycling might be involved in the associated mammary tissue carcinogenic effects.

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1. Introduction

Several nitrofurans and nitroimidazolic chemicals have been used in veterinary medicine and by bee-keepers in honey-producing hives. As a consequence, residues of theses nitro heterocycles could result in animal-derived foods and honey (European Council, 1990; Federal Register, 2002; Khong et al., 2004; Tribalat et al., 2006; Lopez et al., 2007; Zhou et al., 2007). Nitrofuran and nitroimidazolic residues in honey have been reported in several studies (Khong et al., 2004; Zhou et al., 2007; Lopez et al., 2007). These may pose a risk to human health, because of their potential toxicological effects, including mutagenic and carcinogenic properties (Ertürk et al., 1970; Rustia and Shubik, 1979; IARC, 1983, 1987, 1990a,b; Cavaliere et al., 1984; Wang et al., 1984; Kari et al., 1989). Some of the nitrofurans and nitroimidazolic compounds used as veterinary antimicrobial drugs are: nitrofurazone (NF), nitrofurantoin (NFT), furazolidone (FZ), and metronidazole (MTZ) (Fig. 1). The mammary tissue is a target for the carcinogenic effect of these toxicants. Different testing procedures demonstrated that these compounds require an enzymatic nitroreductive step to lead to toxic or mutagenic metabolites (IARC, 1983, 1987, 1990a,b; Hiraku et al., 2004). Nifurtimox (NFX) and benznidazole (BZ), a nitrofuran and nitroimidazole compound, respectively, are employed for the treatment of Chagas' disease (Fig. 1). Their enzymatic nitro reduction has been exhaustively studied (Docampo and Moreno, 1985; Castro et al., 2006b). Both are toxic, mutagenic, and carcinogenic (Docampo and Moreno, 1985; Castro et al., 2006b) and NFX has also been shown to be a mammary tumorigenic compound (Steinhoff and Grundmann, 1972). These two chemicals were included for comparative purposes, on account of the fact that they are representative of two different metabolic activation processes, following the initial step of nitroreduction to their corresponding nitro anion radical (Fig. 2). In the case of NFX, the nitro anion radical may interfere with oxygen metabolism via a redox cycling process leading to superoxide anion radical. In contrast, BZ proceeds through an additional reductive process to yield the toxic reactive nitroso or nitrosyl radicals (Castro et al., 2006b). The enzymes, involved in those nitroreductive processes, which occur in the subcellular fractions of liver and other organs, were considered to be cytochrome P450 reductases; cytochrome P450, xanthine-oxidoreductase (XOR), and aldehyde oxidase (AO)

Abbreviations: NFX, nifurtimox; BZ, benznidazole; NFT, nitrofurantoin; NF, nitrofurazone; FZ, furazolidone; MTZ, metronidazole; XOR, xanthine-oxidoreductase; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; DMF, *N*,*N*-dimethylformamide.

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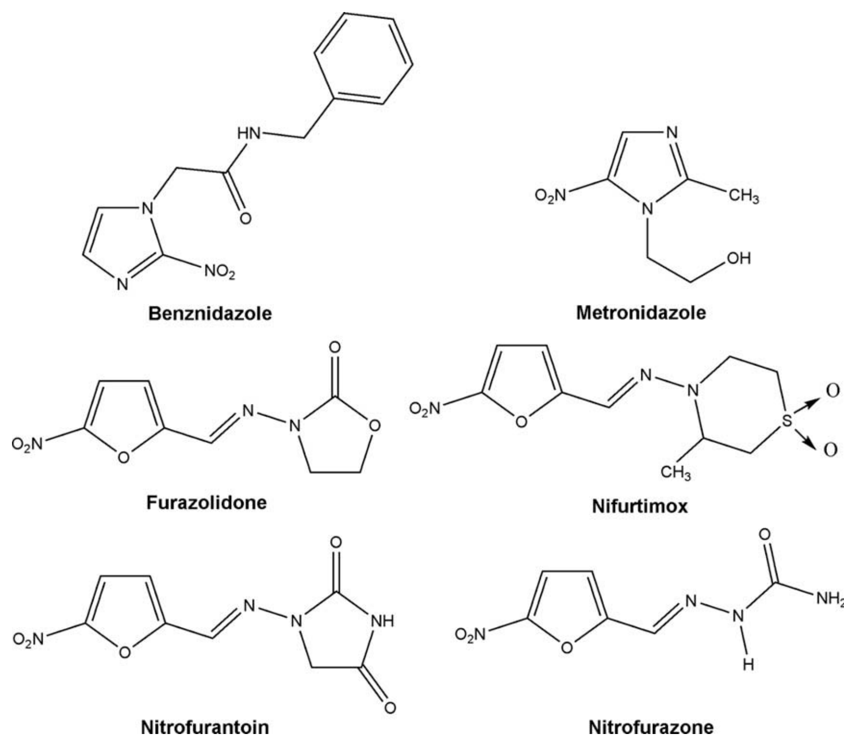

Fig. 1. Chemical structures of nitroimidazole and nitrofuran compounds.

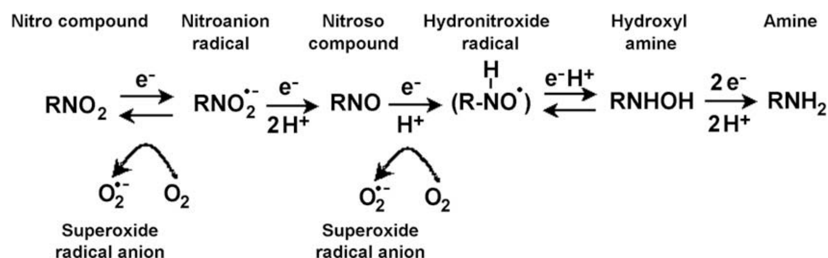

Fig. 2. Nitroreductive metabolic pathways.

(Masana et al., 1984; Docampo and Moreno, 1985; Castro et al., 2006b). These enzymes, but not AO, proved to be present in the mammary tissue and the activity of XOR was particularly high in the epithelial cells (Castro et al., 2001, 2006a; Maciel et al., 2004).

In the present study, we describe the nitroreductase activity exhibited by the microsomal and cytosolic fractions of rat mammary tissue in response to some carcinogenic nitroheterocyclic substrates. We aimed to compare these with NFX and BZ.

2. Materials and methods

2.1. Chemicals

Nifurtimox (3-methyl-*N*-[(5-nitro-2-furanyl)-methylene]-4-thiomorpholin-amine-1,1-dioxide) was a gift from Bayer Laboratories (Argentina) and benznidazole (*N*-benzyl-2-nitroimidazole-1-acetamide) from Roche (Argentina); nitrofurazone (5-nitro-2-furaldehyde semicarbazone) [59-87-0], nitrofurantoin (*N*-(5-nitro-2-furfurylidene)-1-amino-hydantoin) [67-20-9], furazolidone (3-(5-nitrofurfurylideneamino)-2-oxazolidinone) [67-45-8], metronidazole (2-methyl-5-nitroimidazole-1-ethanol) [443-48-1], and xanthine-oxidoreductase from buttermilk (E.C.1.1.3.22) were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals employed were of analytical grade.

2.2. Animals and treatment

Non-inbred female Sprague–Dawley rats (18 weeks, 300 ± 20 g bw) were used; these were post-lactation mothers (2 weeks after weaning their pups). Procedures used for the breeding, housing, and handling animals were in accordance with guidelines set by the Administración Nacional de Medicamentos, Alimentos y Tecnología (ANMAT), Buenos Aires. They were kept in a controlled room on a 12 h light: 12 h dark cycle (light phase from 7 to 19 h), the room temperature was 23 (±2) °C, and the relative humidity was 45–65%. Food and water were available ad libitum. Animals were fasted for 12–14 h with free access to water before being sacrificed by decapitation in a Harvard guillotine and bled, to minimize potential interferences by haemoglobin in cellular fractions. Mammary tissue was rapidly excised and processed at 4 °C.

2.3. Isolation of cellular fractions

Mammary tissue from a pool of rats ($n = 25$) was homogenized (1/4 w/v) in a Teflon–Glass–Potter–Elvehjem homogenizer with 0.25 M sucrose, 5 mM EDTA, 20 mM Tris/HCl, pH 7.4. The homogenate was passed through a cotton cloth and vacuum to exclude the fat and the not homogenized tissue. The homogenate was centrifuged for 20 min at 9000*g*, and the supernatant was centrifuged for an additional 1 h at 100,000*g* to obtain the microsomal pellets. The supernatant fraction was then dialyzed overnight in a cold room with 2 l of the same buffer to obtain the cytosolic fraction. All procedures were performed at 0–4 °C (Masana et al., 1984).

2.4. Nitroreductase activity

All incubations were anaerobically (N_2) run in 20 ml septum sealed flasks shaking at 150 oscillations/min in a covered Dubnoff shaker (to ensure darkness) at 37 °C. The duration of shaking depended on the cellular fraction and the nitro compound tested.

The incubation mixtures contained the corresponding drug, dissolved in DMF (final concentration 2%) and 20 mM phosphate buffer (pH 7.4) in a final volume of 2.5 ml.

In the pure XOR assay the incubation mixture contained the enzyme (46 μg/ml final concentration), 0.25 mM hypoxanthine, and 50 μM of each nitrofuran or 100 μM of each nitroimidazole.

In order to evaluate the cytosolic nitroreductase activity, the incubation mixture contained: cytosol (5.0 mg/ml protein concentration), 0.25 mM hypoxanthine, and 100 μM of each nitrofuran. To inhibit this activity, 0.15 mM allopurinol was also added.

To determine the microsomal nitroreductase activity, the incubation media contained the microsomes resuspended in the incubation buffer (2.1 mg/ml protein concentration), 0.5 mM NADPH-generating system and 100 μM of each nitro compound.

After incubation, the reaction was interrupted by the simultaneous addition of 1 ml of 15% (w/v) $ZnSO_4$. A fraction of each incubation mixture was poured over 1 g of NaCl and extracted with 4 ml of ethyl acetate. The absorbance of the organic layer was spectrophotometrically determined at 375 nm (NF), 370 nm (NFT), 367 nm (FZ), 400 nm (NFX), and 320 nm (MTZ and BZ). Calibration curves were made with standards ranging from 1 and 35 μM ($r^2 \sim 0.998$), which were treated identically.

The nitroreductase activity was calculated as the difference between the nitro compound concentration at the beginning and at the end of the reaction from the decrease in substrate over time. Recoveries of known additions of each drug to incubation mixtures were more than 95%.

2.5. Protein concentration

Protein concentration was determined according to the method of Lowry (Lowry et al., 1951), using bovine serum albumin as the standard.

2.6. Statistical analysis

The significance of the differences among the mean values was assessed by analysis of variance test and Tukey–Kramer posttests (Gad, 2001). Calculations were performed using Graph Pad software (GraphPad Instat). For all analyses, the criterion of significance was set at $p < 0.05$.

3. Results

3.1. Nitroreductase activity

The XOR assay showed that all nitrofurans were nitroreduced by the pure buttermilk enzyme in the presence of hypoxanthine serving as the co-substrate. However, that was not the case for the nitroimidazolic compounds, which were not metabolized under similar conditions (Table 1). NFT was the most intensively nitroreduced nitrofuran. In fact, NFT nitroreductase activity was as much as 2-fold higher than any other nitrofurans.

The cytosolic fraction of mammary tissue possesses a marked ability to metabolize all tested nitrofurans in the presence of hypoxanthine as co-substrate (Table 2). The potent inhibitory effect of allopurinol that was observed with each nitrofuran indicates the possible role of XOR in the process. According to the results obtained with the pure XOR assay, NFT was more significantly metabolized than the other nitrofurans. Furthermore, NF and FZ exhibited no appreciable differences in reduction potential and both were approximately metabolized half of NFT. NFX was the least metabolized compound. Nitroimidazolic compounds were not determined in this cellular fraction, because of their undetectable activity with the pure XOR enzyme.

The microsomal fraction was able to nitroreduce all tested nitrofurans in the presence of NADPH, except for NF. Nitroimidazolic compounds were not nitroreduced, as shown by the undetectable activity under the same conditions (Table 3). FZ and NFT had no significant differences between them as the most metabolized of this group with 2-fold higher reductions than NFX.

4. Discussion

These results demonstrate that several nitrofurans, which were previously reported as contaminants present in food and honey, can be nitroreduced by the cytosolic and microsomal fractions of rat mammary tissue. In the case of NF, no nitroreductase activity could be detected in the microsomal fraction. This could be related to a lack of sensitivity in the method employed and/or the determination of substrate disappearance as an indirect measure for enzymatic activity. Furthermore, other nitrofuran tumorigenic drugs like NFX (Steinhoff and Grundmann, 1972; Castro et al., 2006b), which was used as a positive control, behave similarly. However, the nitroreduction of nitroimidazolic derivatives was not observed (MTZ and BZ). The evidenced in situ ability of mammary tissue to nitroreduce these mutagenic and carcinogenic nitrofurans might

Table 1
XOR nitroreductase activity.

Experimental conditions	XOR Nitroreductase activity (nmol/mg protein min)					
	NFX	NF	NFT	FZ	BZ	MTZ
Hypoxanthine	40.0 ± 0.5	29.1 ± 5.5	90.1 ± 0.4	36.7 ± 0.5	ND	ND
Activity (%)	100	73	225	92	–	–

Incubation times: NFX and FZ 30 min; NFT 15 min; NF, MTZ and BZ 20 min. Values are mean ± DS of quadruplicate determinations. ND: not detected. See details in Section 2.

Table 2
Cytosolic fraction nitroreductase activity.

Experimental conditions	Nitroreductase activity (pmol/mg protein min)			
	NFX	NF	NFT	FZ
Hypoxanthine	67.0 ± 20.3	476.9 ± 38.6	809.6 ± 43.4	402.4 ± 46.4
Hypoxanthine + allopurinol	ND	ND	ND	ND
Activity (%)	100	712	1208	601

Incubation times: NFX 90 min, NF 30 min, NFT 20 min, FZ 45 min. Values are mean ± DS of quadruplicate determinations. ND: not detected. See details in Section 2.

Table 3
Microsomal fraction nitroreductase activity.

Experimental conditions	Nitroreductase activity (pmol/mg protein min)					
	NFX	NF	NFT	FZ	BZ	MTZ
Without NADPH	ND	ND	ND	ND	ND	ND
NADPH	118.4 ± 10.6	ND	251.1 ± 17.5	294.1 ± 43.4	ND	ND
Activity (%)	100	ND	212	248	–	–

Incubation time: 60 min. Values are mean ± DS of quadruplicate determinations. ND: not detected. See details in Section 2.

be significant (Ertürk et al., 1970; Rustia and Shubik, 1979; IARC, 1983, 1987, 1990a,b; Cavaliere et al., 1984; Wang et al., 1984; Kari et al., 1989). NFT or NF as well as the positive control NFX have the potential to produce either reactive metabolites derived from the initial drug (Boyd et al., 1979), reactive oxygen species (ROS), or both during the nitroreductive process (Hoener et al., 1989; Hiraku et al., 2004; Letelier et al., 2004). These highly reactive species would not be able to travel from distant sites (e.g., the liver) to the mammary gland via the blood. Consequently, the herein reported in situ nitroreductive activation might offer a reasonable alternative to understand the occurrence of a mutagenic event resulting from the effect of those reactive metabolites on DNA that initiate a carcinogenic process. This hypothesis was previously put forth by Hiraku et al., 2004 in studies of estrogen-sensitive breast cancer MCF-7 and non-tumorigenic mammary epithelial MCF-10A estrogen receptor negative cells that were exposed to NF. In a separate experiment, NF-induced oxidative damage to DNA was found to occur only when Cu(II) and cytochrome P450 reductase were present with NF. Also, ESR studies under in vitro conditions indicated that pure cytochrome P450 reductase led to the formation of free radicals in the presence of NADPH.

However, NF was not found in the present study to be nitroreduced by the microsomal fraction in the presence of NADPH, which contradicts the general applicability of NF behaviour to all other nitrofurans as found in the previous study (Hiraku et al., 2004). In contrast, all other tested tumorigenic nitrofurans (NFX, NFT, FZ) were effectively nitroreduced by the microsomal fraction in the presence of NADPH. Further, the nitroreductase activity obtained in these cases was significantly higher than that obtained for NFX, which is a known mammary gland tumorigenic compound (Steinhoff and Grundmann, 1972) that leads to ROS formation and requires cytochrome P450 reductase for its activation to the nitro anion radical (Docampo and Moreno, 1985; Castro et al., 2006b).

Intriguingly, the cytosolic XOR-mediated ability of rat mammary tissue to nitroreduce all these carcinogenic nitrofurans was very intense. The cytosolic nitroreductive activity for NFT, NF, or FZ was significantly higher than the activity in the microsomal fraction. This may not be a surprise in light of the fact that XOR activity in mammary tissue epithelial cells is known to be one of the highest in the entire body (Castro et al., 2006a). Therefore, it is tempting to suggest a relevant role of the nitrofuranic food contaminants in the XOR cytosolic pathway of activation, which generates reactive metabolites that would be responsible for DNA attack. Also, other mammary gland carcinogens, such as 1-nitropyrene (Howard and Beland, 1982), are nitroreduced by XOR to reactive metabolites that covalently bind to DNA.

We recently reported other mammary tissue carcinogens, such as ethanol, can be activated by XOR to form hydroxyl radicals as well as the mutagen acetaldehyde (Castro et al., 2001). Therefore, XOR might be of relevance in the generation of reactive metabolites from some pro-carcinogens and thus be involved in the initiation step in the chemical carcinogenesis, which requires further investigation.

Nitroreductive and oxidative metabolic transformations may occur in the case of MTZ (Pérez-Reyes et al., 1980; Bergan et al., 1984). Those of reductive nature are widely known and provided a rational basis for the use of MTZ in the treatment of protozoal infections. Such transformations also provide evidence for MTZ-reported mutagenicity in bacterially-mediated tests (IARC, 1987). In mammals, this drug is extensively metabolized by the liver to oxidative metabolites and many of these compounds as well as the parent forms are excreted in urine and feces (Bergan et al., 1984).

In recent studies (Menéndez et al., 2002) using cell lines with different p53 functionality that determined the proliferation capacity an induction of micronuclei after treatment with MTZ or its hydroxy metabolite, the authors reported that the hydroxy metabolite induced a dose response increase of p53 and micronuclei, while MTZ increased proliferation in all the cell lines tested without inducing changes in the levels of p53 or micronuclei. Therefore, the overall carcinogenic effect of the drug might be attributable to a potential effect of the oxidative metabolite at the initiation steps plus a proliferative action that is mediated by MTZ at the promotion level. In light of these considerations, is not unexpected that MTZ has not been nitroreductively activated at either the cytosolic or the microsomal fractions. Most likely, hydroxylated MTZ mutagenic metabolites that are mainly produced in the liver would reach mammary tissue via the blood stream to interact with that DNA pool.

The case of our negative nitroimidazolic control (BZ) is less difficult to interpret. BZ was not found to be carcinogenic in mammary tissue (Teixeira et al., 1994; Castro et al., 2006b) and was shown to be anaerobically nitroreduced to reactive metabolites that attack DNA and nuclear proteins (Gorla et al., 1986). This process is inhibited by the presence of oxygen, but still occurs to some extent under in vivo conditions (Masana et al., 1984; Castro et al., 2006b). Consequently, the lack of BZ nitroreduction by mammary tissue cellular fractions, as observed in the present investigation, is in agreement with those previous observations.

In summary, our observations supported the hypothesis, evidenced in model systems for NF, that several nitrofurans present in food might be carcinogenic in mammary tissue prior to nitroreduction and redox cycling (Hiraku et al., 2004). Our studies suggest a more general XOR nitroreductive capacity of mammary tissue than the microsomal P450-mediated pathway. The lack of nitroreductive capacity evidenced by mammary tissue in the case of MTZ, plus the mutagenic capacity of the hydroxy metabolite produced in the liver, suggest that the carcinogenic capacity in mammary tissue is not due to the bioactivation in situ.

Also, our negative control BZ did not undergo nitroreductive activation in mammary tissue. That may suggest that certain chemicals, which during nitroreduction spark a redox cycling process, are more prone to be carcinogenic for mammary tissue than those requiring full reductive activation to reactive metabolites that covalently bind to DNA.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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