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CYP1A1 and Cnr nitroreductase bioactivated niclosamide *in vitro*

Evelyn Beristain-Castillo, Mariano Martínez-Vázquez[1], Rafael Camacho-Carranza and Javier J. Espinosa-Aguirre*

Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas and [1]Departamento de Productos Naturales, Instituto de Química, Universidad Nacional Autónoma de México, A.P.70–228, Ciudad Universitaria, México DF 04510, México

* To whom correspondence should be addressed. Tel: +52 55 56229114; Fax: +52 55 56229182; Email: jjea@biomedicas.unam.mx

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Niclosamide produces genotoxic effects, such as point mutations in *Salmonella* sp., sperm-head abnormalities in mice and clastogenic effects in human lymphocytes *in vitro* and *in vivo*. As cytochrome P450 could be involved in the bioactivation of niclosamide, we investigated which subfamily was involved. We used liver microsomal fractions from rats treated with phenobarbital/β-naphthoflavone (PB/β-NF), benzo[a]pyrene (BaP) or cyclohexanol, which are known to induce different cytochrome P450 subfamilies, such as CYP2B, CYP1A1, CYP1A2 and CYP2E1. We also inhibited CYP1A and CYP2E using α-NF and diethyldithiocarbamate to identify the cytochrome P450 involved. Liver-S9 fractions obtained from PB/β-NF- and BaP-treated rats significantly increased the number of revertants induced by niclosamide, while the CYP1A1 inhibitor α-NF decreased the number of revertants. The incubation of niclosamide with CYP1A1 Supersomes™ increased the number of revertants, suggesting that CYP1A1 is responsible for the bioactivation of niclosamide. Nitroreduction is also involved in niclosamide bioactivation, as the nitroreductase-deficient strain YG7132 did not respond to the niclosamide treatment. Our findings indicated that a metabolite, derived from the action of CYP1A1 and a nitroreduction-reaction process, has a key role in the bioactivation of niclosamide.

Introduction

Niclosamide (5-chloro-*N*-[2-chloro-4-nitrophenyl]-2-hydroxybenzamide) has a broad use ranging from pest management to medicine. It is commonly used in treating infections produced by trematodes and cestodes in human and non-human organisms (1,2) and for pest control, including molluscs (3). Niclosamide is also used as an effective treatment against intestinal tapeworm infections (4) and as an effective drug to inhibit the synthesis of coronavirus proteins in severe acute respiratory syndrome in Vero cells (5). Niclosamide is also of interest to researches because it might be used as a treatment for cancer. This interest is supported by the finding that niclosamide inhibited S100A4-induced metastasis formation in a mouse model of colon cancer (6) and inhibited Wnt/Frizzled1 signalling in human colon cancer cell lines and colorectal cancer cells (7,8). In addition, niclosamide induced apoptotic and autophagic cell death in HeLa cells (9). Moreover, some evidence has indicated that niclosamide damages DNA. For example, niclosamide-treated mice showed an increase in the number of sperm-head abnormalities (10). Niclosamide also produced clastogenic effects in human lymphocytes *in vitro* and *in vivo* (11) and frame-shift mutations in *Salmonella* sp. (12). Importantly, the mutagenic effect of niclosamide *in vitro* depends on the presence of the liver-S9 fraction, which incorporates phase I enzymes (13), including cytochrome P450 (CYP). In most cases, a loss in the biological activity of the substrate results from the participation of phase I enzymes. These metabolic reactions are considered detoxification pathways (14). However, the biotransformation of some xenobiotics leads to the formation of reactive metabolites involving oxidation and reduction reactions (15,16).

CYPs play a crucial role in the biosynthesis and metabolism of a number of endogenous substrates (e.g. steroid hormones and lipophilic signal molecules) and in the detoxification or activation of a variety of xenobiotics (17). CYPs have been implicated in the bioactivation of many substances, including pesticides (18). CYP2E1, CYP2B1, CYP1A1/2 and CYP2C11 are important CYPs that are involved in the bioactivation of xenobiotics in rat lung and liver tissues (19). Other CYPs are known to play relatively minor roles in the metabolic activation of procarcinogens and promutagens.

Nitroreduction is also involved in the bioactivation of various compounds, such as nitroaromatic hydrocarbons or nitroarenes (20,21). This process forms nitrenium ions, which might be responsible for the mutations involved in carcinogenesis (22,23), such as DNA-strand breaks (24). Niclosamide mutagenicity seems to depend on nitroreduction (12), and there is evidence suggesting that this compound could even interact with DNA following reductive activation (25).

Despite the insight that we have gained into the effects of niclosamide, its bioactivation pathway has not been fully elucidated. Studying the bioactivation pathway of niclosamide will add to the understanding of the mechanism of action and its possible interaction with other xenobiotics including drugs, food components or environmental contaminants. Previous reports have suggested that CYPs and nitroreductases participate in the bioactivation of niclosamide; therefore, this study aimed to identify the enzymes involved in this bioactivation. First, we confirmed the mutagenic and toxic effects of niclosamide using the Ames test. Second, we examined (i) the effect of different liver-S9 fractions prepared from rats treated with known CYP inducers; (ii) the effect of CYP inhibitors and recombinant CYPs on the bioactivation of niclosamide; and (iii) the participation of Cnr nitroreductase in the bioactivation of niclosamide.

Materials and methods

Animals

For CYP induction, 15 male Wistar rats aged 9–10 weeks were used. The rats were obtained from the animal facility of the Instituto de Investigaciones Biomédicas and housed in groups of four in plastic cages. The rats were placed in a light- and temperature-controlled environment with *ad libitum* access to food and water. The experiments, which were conducted in accordance with the ethical guidelines for investigation with laboratory animals, were approved by an ethical committee for animals at the Instituto de Investigaciones Biomédicas.

Bacterial strains

We used the following *Salmonella typhimurium* strains for the Ames test: TA98, YG1020, YG1021, YG1024 and YG7132 whose genotypes were previously described (26–29). Each strain was designed to be responsive to frame-shift mutations. The strains were maintained as described by Maron and Ames (30).

Chemicals

We used the following chemicals for the experiments: niclosamide, β-nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate, L-histidine, D-biotin, D-glucose, potassium phosphate, magnesium sulphate, benzo[a]pyrene (BaP), 1-nitropyrene, α-naphthoflavone (α-NF) and dimethylsulfoxide (DMSO). These chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). We also used nutrient broth No. 2 (Oxoid Inc., Ogdensburg, NY, USA), 2-aminoanthracene (2-AA; Chemical Company, Milwaukee, WI, USA), nitrofurantoine, N-nitrosopyrrolidine and diethyldithiocarbamate (DDTC; Sigma-Aldrich). The solvents used in this study were of a high analytical grade. Supersomes™ were obtained from Becton Dickinson (Gentest Co. Woburn, MA, USA). Supersomes™ are microsomes from insect cells transfected with complementary DNAs encoding for rat CYP1A1 and CYP2B1, along with NADPH-CYP reductase and cytochrome b5.

Animal treatment

The procedure for treating the rodents with enzyme inducers to increase the expression of CYP1A, CYP2B and CYP2E is described elsewhere (31,32). For this purpose, the rats were randomly assigned to three groups (five rats/group) and treated with either BaP, phenobarbital (PB)/β-NF or cyclohexanol (CH). The enzyme inducers were diluted in corn oil and administered intraperitoneally in a volume of 200 µl, except for CH, which was diluted in drinking water. The first group of rats received 60 mg/kg of PB for 3 days followed by 30 mg/kg of PB and 80 mg/kg of β-NF on the fourth day. The second group received a single dose of 50 mg/kg of BaP. Finally, the third group received 2.5% vol/vol of CH in drinking water, which was available *ad libitum* for 5 days. The rats were euthanised 24 h after the last treatment day, and their livers were obtained aseptically.

Mutagenicity test

Experiments of niclosamide bioactivation by S9 fractions prepared from rats treated with known CYP inducers were conducted using the standard plate incorporation method of Ames test as described elsewhere (30,33). Niclosamide was dissolved in DMSO. 1-Nitropyrene (0.01 µg/plate), BaP (5 µg/plate), N-nitrosopyrrolidine (200 µg/plate) and 2-AA (5 µg/plate) were used as positive controls and were dissolved in DMSO or water, depending on their solubility.

In the following experiments, we used the pre-incubation method (30), (i) inhibition of niclosamide bioactivation; (ii) bioactivation of niclosamide with Supersomes™; (iii) involvement of nitroreduction in niclosamide bioactivation and (iv) genotoxicity of aminoniclosamide in the presence of liver-S9 fraction. All reaction mixtures were incubated for 30 min at 37°C in a shaking bath, and 2 ml of molten top agar were added and distributed in the minimal agar plates. The plates were incubated at 37°C for 48 h, and the number of revertant colonies was counted.

Reduction of niclosamide nitro group

The nitroreduction method for niclosamide was adapted from the procedure used by Khier *et al.* (34). Briefly, the reduction was obtained by dissolving niclosamide in a 1 N hydrochloric acid solution to which 2 g of zinc dust was added; the solution was occasionally shaken. The reaction was allowed to stand for 1 h at room temperature, and then it was filtered; its product was recrystallised. The synthesised aminoniclosamide was determined by thin-layer chromatography (TLC) and infrared (IR) spectroscopy, which was used to follow the formation of aminoniclosamide. TLC was utilised as a first approach to identify the aminoniclosamide molecule. The product of the reaction was spotted on plates with 60F silica gel (EM Science) and developed in hexane/ethyl acetate (1:2, vol/vol). The plates were dried following TLC development, and the areas to which the substances had migrated were visualised with a UV lamp at a wavelength of 254 nm.

IR spectroscopy was used as a second approach to identify aminoniclosamide. IR spectra were recorded using a FT-IR Bruker tensor 27. Potassium bromide disks were prepared from powdered aminoniclosamide mixed with dry potassium bromide. The spectra were recorded in transmittance mode from 300 to 4000 cm^{-1} at a resolution of 4 cm^{-1}.

Experimental design

We used randomised block designs for the experiments (35). Each experiment consisted of the application of treatments to plates with bacteria. As we could only run three replicates per trial in a given day, two groups (blocks) of three replicates each were run for each experiment; however, for two of the experiments, which had sample sizes of three, only one block was used. We allocated treatments independently from block to block and randomly within each block provided that each treatment occurred three times in each block.

Statistical analysis

To satisfy the standards for normality and homogeneity of variance, the Shapiro–Wilk test was applied. If normality was attained, the block was included as a random-effects variable, and linear mixed models were applied. Conversely, if the data were not normally distributed, the block was assigned as a random-effects variable, and generalised linear mixed models were run. Alternatively, generalised linear models with negative binomial errors were used (36). In addition, we used model simplification to obtain minimal adequate models (36), which are those for which all terms are significant. We used the G^2 statistic (i.e. −2 ln [likelihood ratio]), which has a sampling distribution close to that of a χ^2 distribution, for the model selection and either Wald t or Wald Z for the hypothesis testing. The results were expressed as the mean ± standard error of the mean (SEM) for each experiment. Two-tail tests were used, and P values <0.05 were considered statistically significant. All statistical analyses were performed using *R* (37).

Results

Mutagenic effect of niclosamide in the presence of the liver-S9 fraction

The liver-S9 fraction contributed significantly to the total variation in the numbers of revertant colonies (Table I). The average number of revertants in the absence of the liver-S9 fraction (8.58 ± 2.41, n = 24) was statistically lower than in the presence of the liver-S9 fraction (51.83 ± 10.44, n = 24, Wald Z = 3.18, P = 0.001). Niclosamide concentrations >5 µg/plate totally reduced the average number of revertants in the absence of the liver-S9 fraction (Table II). Conversely, the liver-S9 fraction allowed the number of colonies to grow, although the concentrations of niclosamide >15 µg/plate did not hinder the liver-S9 fraction from protecting the bacteria (Table II). The general difference in the manner the bacteria responded to niclosamide in the absence and presence of the liver-S9 fraction was explained by the interaction term (Table I).

Cytochrome P450 contributes to the bioactivation of niclosamide

As the liver-S9 fraction incorporates most of phase I enzymes, we investigated whether CYPs were involved in the bioactivation of niclosamide. Both the niclosamide and liver-S9 fractions from rats previously treated with either CH, PB/β-NF or BaP significantly affected the average number of revertants (Table I), but as the exclusion of the interaction term did not change the variance of the model (Table I), the slopes (i.e. mutagenic potential) did not differ among the enzyme inducers (Figure 1). However, the average size of the effect varied. The effect of CH (111.72 ± 8.74, n = 18) was significantly smaller than that of PB/β-NF (177.88 ± 16.64, n = 18; Z = −4.72, P = 0.000) and BaP (201.61 ± 18.13, n = 18; Z = 4.46, P = 0.000). Altogether, these results suggested that the CYP1A and most likely CYP2B subfamilies might be involved in the bioactivation of niclosamide.

Inhibition of the bioactivation of niclosamide

To determine which CYP was associated with the bioactivation of niclosamide, we used α-NF, which inhibits both CYP1A1 and CYP1A2. The results showed that concentrations of 100–150 µM of α-NF significantly reduced the average number (~50%) of BaP-induced revertants (Z = 9.08, n = 18, P = 0.000; Figure 2a), and concentrations of 175–200 µM reduced this number even more (~65%; Z = 12.44, n = 12, P = 0.000; Figure 2a). Similarly,

Table I. Statistical summary of model simplification of the fit of generalised linear mixed models to the data of the bioactivation of niclosamide as a function of the number of revertants

Step*	Simplification	*df*	logLik	χ^2	*P*-value
a)					
Maximal model	Niclo:S9 (PB/β-NF)	39	−268.67		
	Niclo+S9 (PB/β-NF)	32	−184.88	83.790	0.000
b)					
Maximal model	Niclo:S9 (PB/β-NF, BaP, CH)	7	−271.29		
	Niclo+S9 (PB/β-NF, BaP, CH)	5	−273.95	5.304	0.070
	Niclo	3	−289.37	30.842	0.000
c)					
Maximal model	α-NF (a, b, c, d, e, f)	8	−200.20		
	α-NF (a, b, cd, e, f)	7	−200.20	0.004	0.949
	α-NF (a, bcd, e, f)	6	−200.79	1.186	0.276
	α-NF (a, bcd, ef)	5	−201.59	1.582	0.208
	Intercept	3	−231.84	60.518	0.000
d)					
Maximal model	α-NF (a, b, c, d, e, f)	8	−194.50		
	α-NF (a, bc, d, e, f)	7	−194.53	0.660	0.797
	α-NF (a, bcd, e, f)	6	−194.74	0.416	0.519
	α-NF (a, bcde, f)	5	−195.18	0.884	0.347
	Intercept	3	−216.56	42.746	0.000
e)					
Maximal model	Supersome™ 1A	6	−97.71		
	Intercept	3	−130.51	65.606	0.000
f)					
Maximal model	Supersome™ 2B	7	−107.21		
	Intercept	3	−133.53	52.630	0.000
g)					
Maximal model	Strain (YG1021, YG7132):Niclo	8	−152.56		
	Strain (YG1021, YG7132)+Niclo	6	−179.83	54.534	0.000
h)					
Maximal model	Aminoniclo (g, h, i, j)	6	−87.83		
	Aminoniclo (g, hi, j)	5	−88.13	0.590	0.442
	Aminoniclo (g, hij)	4	−88.51	0.760	0.382
	Intercept	3	−95.39	13.750	0.000

Plus and colon denote, respectively, inclusion of an explanatory variable and interaction in the model. *P*-values refer to the increase in deviance when the respective variable in the model was removed. Lowercase letters refer to treatment levels (a, 0 µM; b, 100 µM; c, 125 µM; d, 150 µM; e, 175 µM; f, 200 µM; g, 0 µg; h, 5 µg; i, 7.5 µg; j, 10 µg) and those that appear together were combined because they did not differ from each other.
*(a) Presence or absence of the liver-S9 fractions, (b) different liver-S9 fractions, (c) inhibition of BaP mutagenicity, (d) inhibition of niclosamide mutagenicity; the effect of (e) CYP1A1 Supersome™, (f) CYP2B1 Supersome™, (g) strains and (h) aminoniclosamide.

Table II. Mutagenicity[a] of niclosamide in *S.typhimurium* TA98 without or with liver-S9 fraction

Niclosamide (µg/plate)	Revertants/plate without S9 (mean ± SEM)	Revertants/plate with S9 (mean ± SEM)
0	26.66±3.17	52±6.08
1	23±4.16	38±7.36
5	19±1	105.3±9.35
10	T[b]	121.3±7.05
15	T	98±27.53
20	T	T
25	T	T
30	T	T

[a]Plate-incorporation method.
[b]Total absence of background lawn. BaP (5 µg/plate) was used as positive control with S9 (415 revertants/plate).

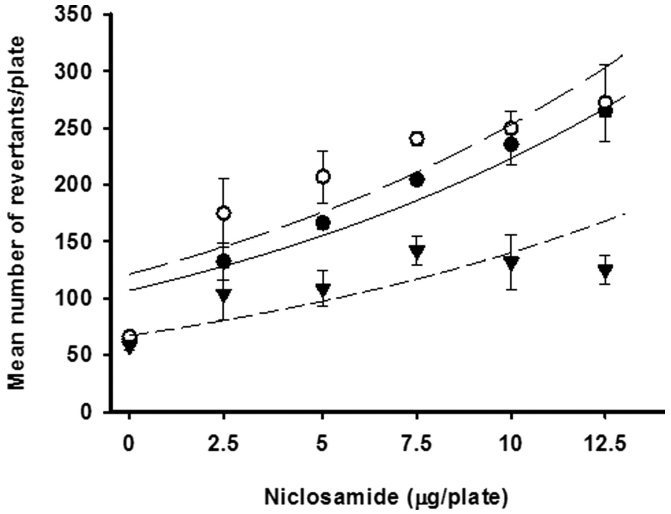

Fig. 1. Liver-S9 fractions from BaP- and PB/β-NF-treated rats promoted the bioactivation of niclosamide in the plate-incorporation method. YG1024 strain bacteria were incubated with niclosamide (2.5–12.5 µg/plate) and liver-S9 fractions from rats treated with PB/β-NF (filled circles), BaP (circles) or CH (filled triangles). BaP (5 µg/plate) was used as positive control (563 revertants/plate in the presence of S9 PB/β-NF). The data are shown as the means ± SEM of 3 trials/treatment.

concentrations of 100–175 µM of α-NF significantly reduced (~50%; Z = 7.48, n = 24, P = 0.000; Figure 2b) the average number of niclosamide-induced revertants, and the concentration of 200 µM caused a significant reduction of ~60% (Z = 8.20, n = 6, P = 0.000; Figure 2b) in the average number of niclosamide-induced revertants (168.16±38.88). The effect size of α-NF on the two mutagens (niclosamide and BaP) was similar in both cases, which suggested that CYP1A was one of the main CYPs involved in the bioactivation of niclosamide. No inhibition of niclosamide mutagenicity was found when DDTC was used (data not shown).

Contribution of a single cytochrome P450 in the bioactivation of niclosamide

The CYP1A1 Supersome™ contributed significantly to the total variation in the average number of revertants (Table I). Niclosamide incubated with 1 µg of CYP1A1 Supersome™ produced a significant increase (Z = 2.88, P = 0.004) in the average number of revertants (56.50±4.99, n = 6) compared to the average number of spontaneous revertants (41.83±2.94, n = 6; Figure 3a). As expected, 2.5 and 5 µg of CYP1A1 Supersome™ also increased the average number of revertants (113.66±10.17, n = 6, Z = 10.26, P = 0.000, and 198.83±29.72, n = 6, Z = 16.33, P = 0.000, respectively). In contrast, only 150 µg of CYP2B1 produced a significant increase (t-test, t_{10} = 3.23, P = 0.009) in the average number of revertants (64.16±6.20, n = 6) compared to the average number of spontaneous revertants (42.5±2.23, n = 6; Figure 3b).

Nitroreduction promotes the bioactivation of niclosamide in the presence of the liver-S9 fraction

The results showed that the *Salmonella* strain YG1021 increased the average number of revertants in response to the treatment with 5 µg (179.5±7.28, n = 6, Z = 13.93, P = 0.000) and 7.5 µg of niclosamide (280.83±16.82, n = 6, Z = 17.75, P = 0.000; Figure 4). In contrast, the nitroreductase-deficient strain YG7132 did not respond to the niclosamide treatment,

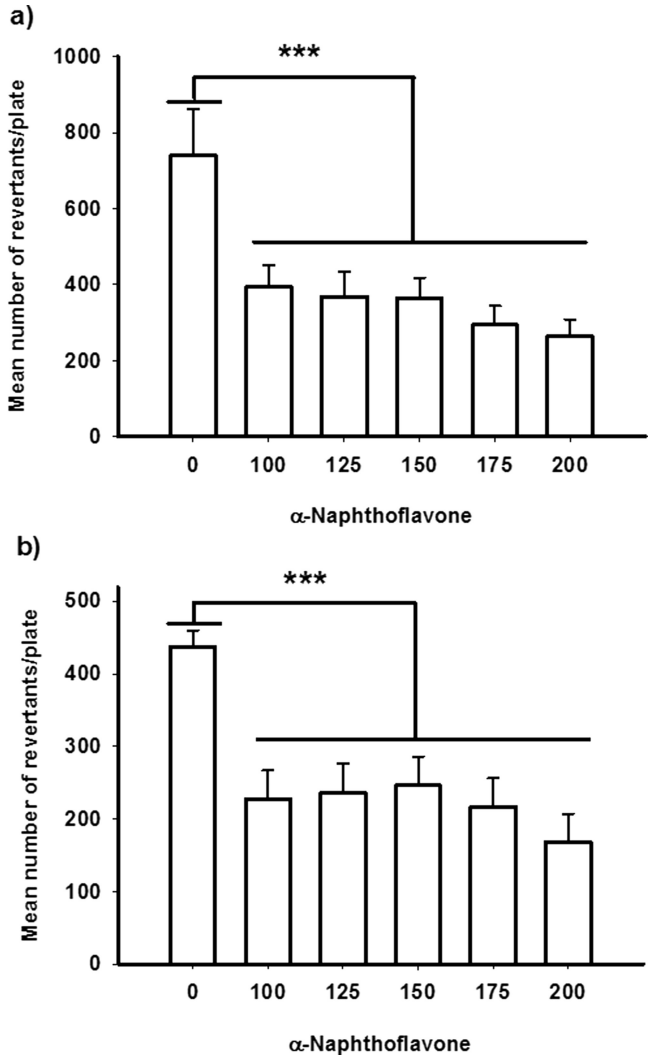

Fig. 2. α-NF inhibited the bioactivation of niclosamide in the pre-incubation method. YG1024 strain bacteria were incubated with 100, 150, 175 and 200 µg/plate of α-NF and 5 µg/plate of (**a**) BaP or (**b**) niclosamide. The data are shown as the means ± SEM of 6 trials/treatment. ***$P < 0.001$ compared with the control condition (0 µg/plate of α-NF).

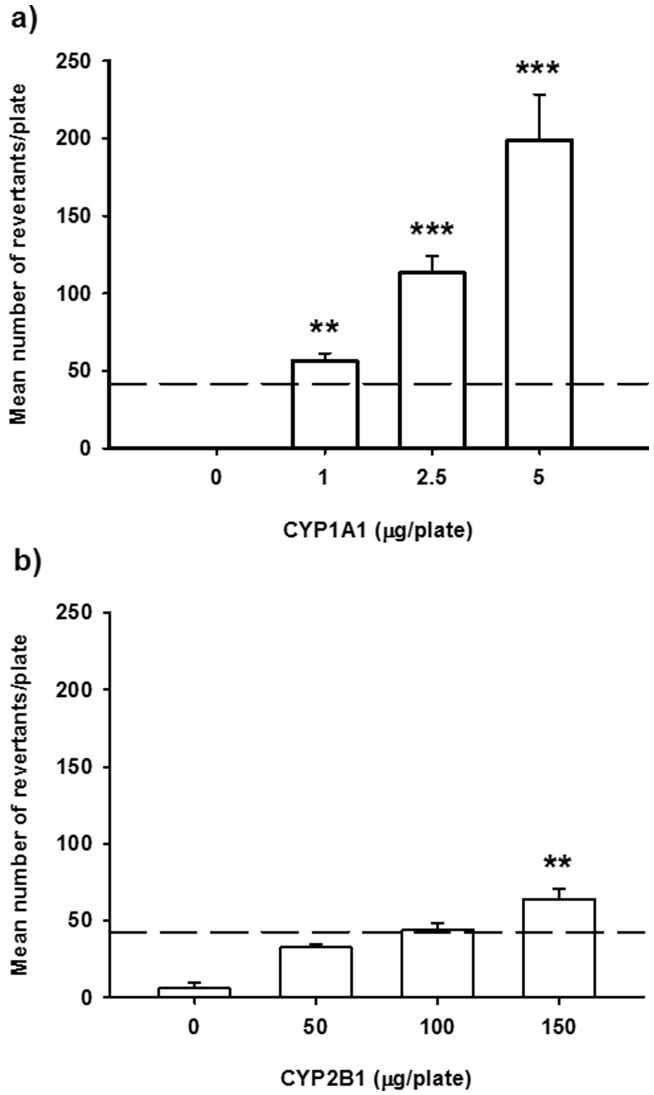

Fig. 3. CYP1A1 fosters the bioactivation of niclosamide in the pre-incubation method. YG1024 strain bacteria were incubated with 2.5 µg/plate of niclosamide and either (**a**) CYP1A1 Supersome™ or (**b**) CYP2B1 Supersome™. 2-AA (5 µg/plate) was used as positive control in the presence of 5 µg of CYP1A1 Supersome™ (4770 revertants/plate). The data are shown as the means ± SEM of 6 trials/treatment. **$P < 0.01$, ***$P < 0.001$ compared with the control condition (dashed line, spontaneous reversion).

indicating an important role for nitroreduction in the bioactivation of niclosamide.

Aminoniclosamide is mutagenic for nitroreductase-deficient strain YG7132

As nitroreduction is necessary for the bioactivation of niclosamide, we nitroreduced niclosamide with zinc, and the product of the reaction, aminoniclosamide (Figure 5), was tested in the Ames assay with the liver-S9 fraction. The results showed that the nitroreductase-deficient YG7132 strain significantly increased the average number of revertants (55.44 ± 3.84, $n = 18$) in response to the treatment with 5–10 µg of aminoniclosamide with the liver-S9 fraction (Wald t, t_{19}= 4.18, $P = 0.000$; Figure 6).

Discussion

This study aimed to characterise some aspects of the bioactivation pathway of niclosamide. We hypothesised that CYP and nitroreductase enzymes were determinant factors in this bioactivation. Our results support the idea that a process of nitroreduction by Cnr, followed by an oxidation mediated by CYP1A1, generates mutagenic intermediates from niclosamide.

As expected, the bioactivation of niclosamide depended on the liver-S9 fraction (12). As CYPs are the main phase I enzymes in the liver-S9 fraction (13), it was likely that CYPs might be participating in the bioactivation of niclosamide. Therefore, we used the liver-S9 fraction from rats pre-treated with BaP, PB/β-NF or CH, as these compounds preferentially increase the activity of the CYP1A, CYP2B and CYP2E subfamilies, respectively (31,32). These CYP inducers did not differ from one another in their niclosamide mutagenic potential (slope of the dose–response curve), which is consistent with previous studies indicating that the mutagenicity of compounds is rarely determined by the activity of a single P450 enzyme and is instead dependent on several enzymatic pathways (38–40). The finding that the liver-S9 fraction obtained after treatment with either

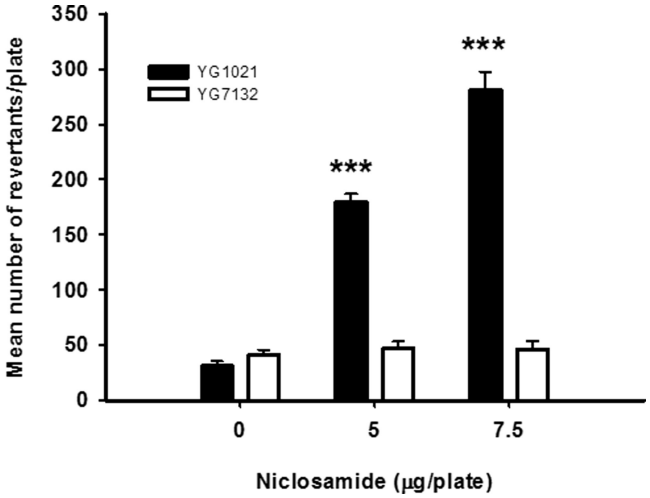

Fig. 4. Nitroreductase is a necessary enzyme for the bioactivation of niclosamide. Concentrations of 5 and 7.5 µg/plate of niclosamide were incubated with either YG1021 strain bacteria (overproducing Cnr nitroreductase enzyme) or YG7132 strain bacteria (disrupted Cnr nitroreductase gene). The pre-incubation method was used. 1-Nitropyrene (0.01 µg/plate) was used as positive control; YG1021 (113 revertants/plate), YG7132 (30 revertants/plate). The data are shown as the means ± SEM of 6 trials/treatment. ***$P < 0.001$ compared with the control condition (0 µg/plate of niclosamide).

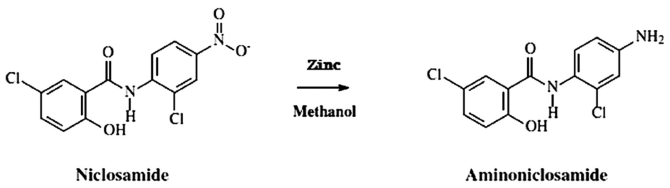

Fig. 5. Schematic representation of the formation of aminoniclosamide.

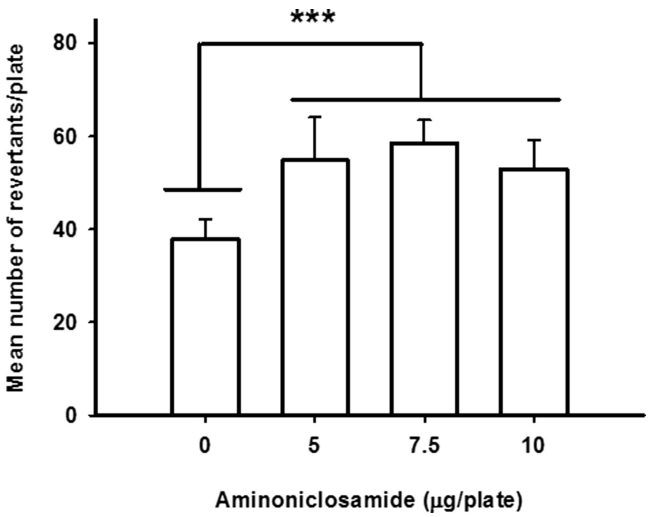

Fig. 6. Cytochrome P450 activates aminoniclosamide in the pre-incubation method. YG7132 strain bacteria (disrupted Cnr nitroreductase gene) were incubated with 5, 7.5 or 10 µg/plate of aminoniclosamide and the liver-S9 fraction from PB/β-NF-treated rats. The data are shown as the means ± SEM of 6 trials/treatments. ***$P < 0.001$ compared with the control condition (0 µg/plate of aminoniclosamide).

PB/β-NF or BaP produced the highest number of revertant colonies after niclosamide treatment (Figure 1) suggests that CYP1A1 and CYP2B1 were involved in this bioactivation. Further experiments with recombinant CYP1A1 and CYP2B1 revealed that the former promoted the highest mutagenic response to niclosamide (Figure 3). The results obtained with the liver-S9 fraction derived from CH-treated rats (Figure 1) and with the CYP2E1 inhibitor DDTC (data not shown) provided evidence that CYP2E1 plays an unimportant role in the bioactivation of niclosamide. The finding that CYP1A1 did play a key role in the mutagenicity of niclosamide agrees with previous studies, which have suggested that CYP1A1 is often involved in the bioactivation of several mutagens and carcinogens (41).

As CYP2B1 affected the mean number of revertants less than CYP1A1 (Figure 3), we focused our attention on confirming the importance of the latter. Accordingly, we used the inhibitor α-NF either with niclosamide or BaP, a positive control with a well-known mutagenic effect (42,43). The use of α-NF reduced equally the number of revertants produced by niclosamide and BaP (Figure 2). Furthermore, the observation that the incubation of niclosamide with the CYP1A1 Supersome™ increased the average number of revertants, which was similar to the results obtained with the liver-S9 fraction, confirmed the participation of CYP1A1 in the bioactivation of niclosamide. By contrast, a greater concentration of the CYP2B1 Supersome™ was required to obtain a slight increase in the number of revertants induced by niclosamide. While PB strongly induces CYP2B1 in rats, the human homologue, CYP2B6, has been only found in small amounts in the liver and does not seem to play an important role in the metabolism of xenobiotics (44).

The liver-S9 fraction protected the bacteria from niclosamide toxicity (Table II), most likely because the main xenobiotic function of CYP metabolism is to prevent toxicity (45). However, after niclosamide was reduced, CYP assumed a mutagenic capacity because of the release of genotoxic metabolite(s) (Figure 4). Chemicals containing nitro groups are known to require the reduction of these nitro groups to the corresponding hydroxylamines to achieve mutagenicity. We suggest that nitroreduction is the rate-limiting step in the bioactivation of niclosamide and that aminoniclosamide might be the reaction product of niclosamide nitroreduction. In addition, the finding that niclosamide interacts with DNA following reductive activation (25) supports the hypothesis that nitroreduction is a critical step in the bioactivation pathway. As we expected, aminoniclosamide became mutagenic in the presence of the liver-S9 fraction, although this compound only caused a slight increase in the number of revertants (Figure 6). This result might be due to differences between *in vitro* and *in situ* metabolism in living organisms.

Although a small quantity of niclosamide is absorbed in the gastrointestinal tract (46), results from *in vitro* and *in vivo* mutagenicity tests demonstrated that this quantity is sufficient to cause DNA damage (11,12). Niclosamide might be nitroreduced by intestinal bacteria before it enters the bloodstream. This process, in turn, would allow a portion of an administered dose to be absorbed and metabolised by CYP1A1, producing genotoxic metabolites. Such a process could take place in tissues in which CYP1A1 is a constitutive enzyme (47,48) or under conditions in which environmental xenobiotics could induce CYP1A1.

In conclusion, the main finding of our study indicates that the bioactivation of niclosamide relies on CYP1A1 and a nitroreduction mechanism to generate mutagenic metabolites. We cannot rule out the possibility that other enzymes are also involved

in this process, albeit to a lesser extent; thus, further study would be necessary to explain the precise mechanism of bioactivation.

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