

# Protection Against Nitrofurantoin-Induced Oxidative Stress by Coelenterazine Analogues and their Oxidation Products in Rat Hepatocytes

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Coelenterazine (3,7-dihydro-2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-8-benzylimidazo[1,2-*a*]pyrazin-3-one) is a substrate for the bioluminescence reaction in many marine animals. Recent work showed that CLZn, its synthetic analogue CLZm, and their common oxidation product coelenteramine (CLM) have strong antioxidative properties in acellular lipid peroxidation systems as well as in rat hepatocytes subjected to tert-butyl hydroperoxide (t-BHP). Here, we analyzed the ability of CLZm and several imidazopyrazinone (IMPZs) analogues to protect primary cultures of rat hepatocytes against a nitrofurantoin (NF)-induced oxidative stress. Comparison of protection capabilities with reference antioxidants yielded the following ranking: CLZm >>> BHT > Trolox C<sup>®</sup> > probucol >  $\alpha$ -tocopherol. The comparison of CLZm with analogues lacking the phenol group in R<sub>1</sub> revealed no differences although the presence of this phenol conferred superior protection against t-BHP. CLM, as well as its methoxylated analogue mCLM which lacks chain-breaking properties, were equally potent in preventing cellular damage caused by NF. mCLM and  $\alpha$ -naphthoflavone, an inhibitor of cytochrome P450 (CYP450) IAI, similarly protected cells against NF-induced mortality and also equally inhibited EROD activity in methylcholanthrene-induced hepatocytes. The inhibition of EROD by CLZm and

CLM was less pronounced. We suggest that the extent of protection conferred by IMPZs against NF-toxicity reflects both the occurrence of antioxidative properties detoxifying ROS produced within cells and inhibitory actions on CYP450 isoforms involved in the bioreduction of NF.

**Keywords:** Imidazopyrazinones, Antioxidant, Nitrofurantoin, Redox Cycling, Cytochrome P450, Hepatocytes

## INTRODUCTION

Coelenterazine (CLZn, fig 1.) is an imidazopyrazinone compound found in the tissues of many marine organisms. Coelenterazine's only known physiological role is its participation in photogenic reactions occurring in many marine bioluminescent organisms<sup>[1,2]</sup>. Recently we have proposed that CLZn could also be part of the antioxidative arsenal of marine organisms thriving in surface waters<sup>[3]</sup>. Indeed CLZn and syn-

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thetic analogues harboring the fused imidazolopyrazinone ring have chain-breaking properties in acellular as well as in cellular models<sup>[5]</sup>. Besides the high reactivity of the fused imidazolopyrazinone ring with many reactive oxygen species (ROS), a significant part of the cellular protection conferred by IMPZs on hepatocytes subjected to lethal concentrations of t-BHP could be attributed to their oxidative conversion into coelenteramine (CLM, fig 1.)<sup>\*</sup>. The phenol group of CLM appears essential to the antioxidative action and IMPZs analogues yielding an aminopyrazine lacking this phenol are less powerful cytoprotectors.

Nitrofurantoin (N-(5-nitro-2-furfurylidine) 1-amino-hydantoin; NF) is an antibiotic agent commonly used in the treatment of urinary tract infections. Its long-term use in humans has been associated with considerable liver damage<sup>[6;7]</sup>. This hepatic toxicity has been ascribed to NF metabolic activation in hepatocytes during a series of redox cycling reactions. These reactions have been shown to generate ROS, such as the radical anion superoxide, hydrogen peroxide, and hydroxyl radical<sup>[8;9]</sup>. The large range of ROS which can be scavenged by IMPZs<sup>[10-15]</sup> prompted us to test whether the previously observed antioxidative properties of IMPZs in peroxide-treated hepatocytes submitted to t-BHP could be extended to this cellular oxidative stress model.

Data indicated that IMPZs and their oxidation products efficiently protect cells against NF. However, comparisons of activities among IMPZs analogues and oxidation products sug-

gest that antioxidative properties are not solely responsible for their protective effects. In some instances, interactions with cytochromes P450 (CYP450s) may be responsible for their observed protective effects.<sup>†</sup>

## MATERIALS AND METHODS

### Chemicals

Nitrofurantoin (NF), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 6-hydroxy-2,5,7,8-pentamethylchroman-2-carboxylic acid (Trolox C<sup>®</sup>),  $\alpha$ -tocopherol, probucol, tert-butyl hydroperoxide (t-BHP), collagenase (type IV, EC 3.4.24.3),  $\beta$ -glucuronidase (EC 238-550-0), aryl-sulfatase (EC 3.1.6.1), dicoumarol and methylcholanthrene (MC) were purchased from Sigma Chemical Co. Lactate dehydrogenase (LDH) assay kit and ethoxyresorufin were purchased from Boehringer. Butanol was bought from Fluka while dimethylsulfoxide (DMSO) was obtained from Acros organics. IMPZs and their aminopyrazine precursors were synthesized according to published methods<sup>[16;17]</sup>. <sup>1</sup>H- and <sup>13</sup>C-NMR spectra were used to assess their purity. Their structures are presented in figure 1. Williams' medium E (WE), Dulbecco's modified Eagle's medium (DMEM) F12 with HEPES and glutamax I (L-Alanyl-L-Glutamine) and RPMI 1640 medium lacking phenol red were purchased from Life Technologies.

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† Abbreviations: AMP, Aminopyrazine; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CLM, Coelenteramine; CLZn, coelenterazine; CLZm, 3,7-dihydro-2-methyl-6-(*p*-hydroxyphenyl)-8-benzylimidazo[1,2-*a*]pyrazin-3-one; CLZp, 3,7-dihydro-2-phenyl-6-(*p*-hydroxyphenyl)-8-benzylimidazo[1,2-*a*]pyrazin-3-one; Cyt P450, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium F12 with HEPES and L-Alanyl-L-Glutamine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; FCS, fetal calf serum; IMPZ, imidazolopyrazinone; LDH, lactate dehydrogenase; mCLM, methoxycoelenteramine; mCLZm, 3,7-dihydro-2-methyl-6-(*p*-methoxyphenyl)-8-benzylimidazo[1,2-*a*]pyrazin-3-one; mCLZp, 3,7-dihydro-2-phenyl-6-(*p*-methoxyphenyl)-8-benzylimidazo[1,2-*a*]pyrazin-3-one; MDA, malondialdehyde; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NADH, nicotinamide adenine dinucleotide, reduced form; NF, nitrofurantoin;  $\alpha$ -NF,  $\alpha$ -naphthoflavone; PBS, phosphate buffer saline; ROS, reactive Oxygen Species; S.E., standard error of the mean; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; t-BHP, tert-butyl hydroperoxide; t-BHP, tert-butyl hydroperoxide; TCA, trichloroacetic acid; WE, Williams' medium E.

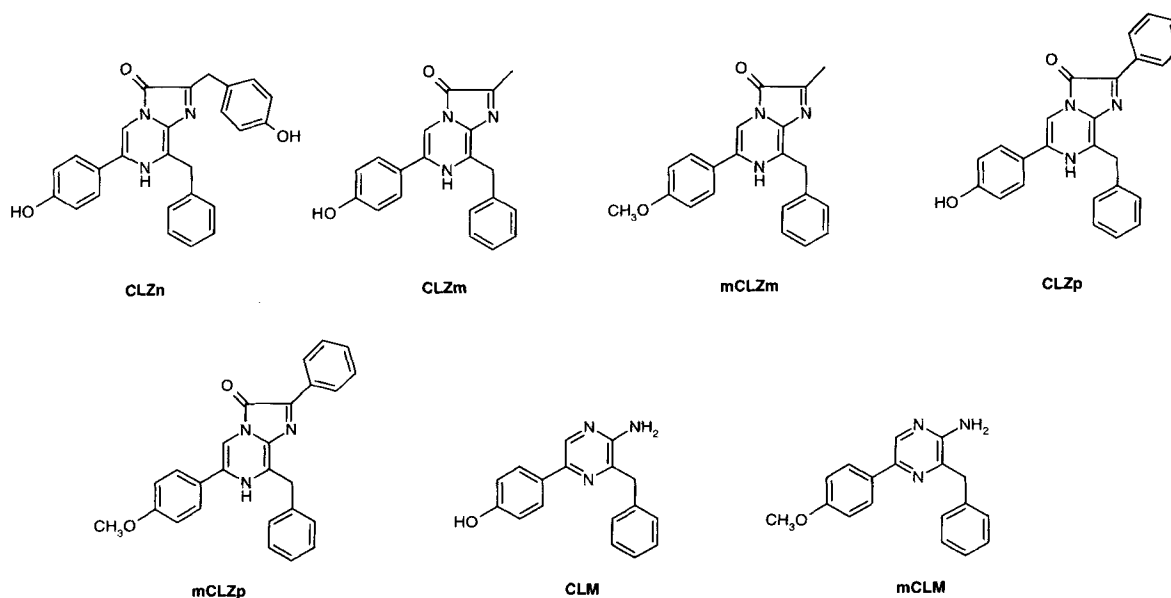


FIGURE 1 Chemical structures of the imidazolopyrazinones and aminopyrazines used in this study

## Methods

### Cell Culture

Male Wistar-Yops rats (averaging body weight, 200 g) were obtained from Iffa Credo (Belgium). Hepatocytes isolated according to a modified *in situ* collagenase liver perfusion technique of Seglen<sup>[18]</sup> were transferred to collagen-precoated culture plates (Cellon Bovine dermal Collagen, 60 µg/ml). Cells were seeded either on 96-well microplates (20,000 cells/well) for MTT and LDH-based experiments or on 6-well plates (1,000,000 cells/well) for the measurements of TBARS. Hepatocytes were first cultured for 4 h at 37 °C (95 % humidity, 5 % CO<sub>2</sub>) in WE medium containing 10 % fetal calf serum (FCS) and 1% penicillin and streptomycin. WE medium supplemented with antibiotics and additional factors (10 nM Insulin, 0.1 µM Dexamethasone, 10 µg /ml of a linoleic acid-BSA complex, 0.1 mM ascorbic acid and 50 ng/ml EGF) was then used.

### Treatments with oxidative stress inducers

Hepatocytes were incubated for 6 hours (37°C, 95 % humidity, 5 % CO<sub>2</sub>) with NF  $3 \times 10^{-4}$  M or t-BHP  $2.5 \times 10^{-4}$  M in the presence or absence of the tested antioxidants. Antioxidants were solubilised in ethanol (final concentration < 1%) or dimethylformamide (DMF) (final concentration < 0.03%), before addition into the culture medium. Control experiments indicated that none of these solvents influenced cellular viability parameters.

### Survival measured by the MTT assay

Cells were incubated for 6 hours in serum-free DMEM supplemented with 0.2-mg/ml insulin and 0.25 mg/ml BSA containing NF or t-BHP and the tested antioxidants. Microplates were then rinsed with phosphate buffered saline (PBS) and their viability estimated by MTT assay<sup>[19]</sup>.

### Lactate dehydrogenase (LDH) release

Cells in 96-well plates were incubated for 6 hours with either NF or t-BHP and the antioxi-

dants in serum-free RPMI medium without Phenol Red. The release of LDH in the supernatant was then quantified following the previously described method<sup>[20]</sup>. Controls included untreated cells (no mortality) and cells lysed with triton 2% in the medium (100 % LDH). Cellular mortality was calculated according to this scale.

#### **Lipid peroxidation assay**

The extent of lipid peroxidation was determined by quantifying the amount of thiobarbituric reactive substances (TBARS), mainly malondialdehyde (MDA), released in the supernatant of cells incubated for 6 hours with NF  $3 \times 10^{-4}$  M as previously described<sup>[51]</sup>. Previous work indicated that most of the MDA produced by hepatocytes is being released in the culture medium<sup>[51]</sup>.

#### **Ethoxyresorufin O-dealkylase assays (EROD) in cultured rat hepatocytes**

Determination of EROD activity in methylcholanthrene (MC)- treated hepatocytes was carried out on intact cells according to [19]. The induction of CYP P450IA was started 4 hours after the seeding of the hepatocytes on 96-well plates by adding MC 200  $\mu$ M into the culture medium. After one night of induction, cells were transferred in phenol red-lacking RPMI medium with the inhibitors to be tested. After 40 minutes, monolayers were washed twice with PBS and the assay was started by the addition of 100  $\mu$ l/well of culture medium containing 8  $\mu$ M 7-ethoxyresorufin and the tested compounds. Dicoumarol (10  $\mu$ M) was added to the assay medium as to prevent further metabolism of the resorufin formed by the cytosolic enzyme diaphorase. After a 30-min incubation at 37°C, a 75- $\mu$ l aliquot of cell medium was withdrawn from each well and transferred to another 96-well plate. Fifteen Fishman units of  $\beta$ -glucuronidase and 120 Roy units of aryl-sulfatase dissolved in 25  $\mu$ l of sodium acetate buffer, pH 4.5, were added to each well containing 75  $\mu$ l of sample. The plate was incubated at 37°C in order to hydrolyze resorufin conjugates. After 2 hours,

200  $\mu$ l of ethanol was added to each well and plates were centrifuged at 3000 rpm for 10 min. Fluorescence of supernatants was measured using a fluoroscan Ascent FL (Labsystems) with 530 nm excitation and 590 nm emission filters.

#### **Statistical analysis**

Significance of differences between treatments was tested by one-way analysis of variance (ANOVA).

## **RESULTS**

#### **Effect of CLZm $10^{-5}$ M on the dose-dependent cytotoxicity of NF**

Hepatocytes submitted for 6 hours to increasing NF concentrations (ranging from  $3.9 \times 10^{-5}$  M to  $10^{-3}$  M) presented decreasing viability levels (figure 2). CLZm  $10^{-5}$  M significantly shifted the cytotoxicity curve towards higher NF concentrations. No protection was observed when low ( $< 2 \times 10^{-4}$  M) NF doses were administered. The protection was statistically significant only at NF concentrations ranging  $2 \times 10^{-4}$  M –  $6.7 \times 10^{-4}$  M. At NF  $3 \times 10^{-4}$  M, the survival was increased from  $11.4 \pm 2.1$  % in controls to  $48.7 \pm 9.4$  % in cells treated in the presence of CLZm  $10^{-5}$  M. This NF concentration was chosen for the following experiments. The shift of t-BHP dose-response induced by CLZm  $10^{-5}$  M is illustrated in figure 2. Although cytotoxicity curves were similar for both stressor, the shift induced by CLZm markedly differs as the protection against t-BHP is apparent at the lowest cytotoxic doses of the peroxide.

#### **Protective effects afforded by CLZm and reference antioxidants on the survival of NF or t-BHP-treated rat hepatocytes**

The protection by CLZm of NF-treated hepatocytes was compared with that conferred by com-

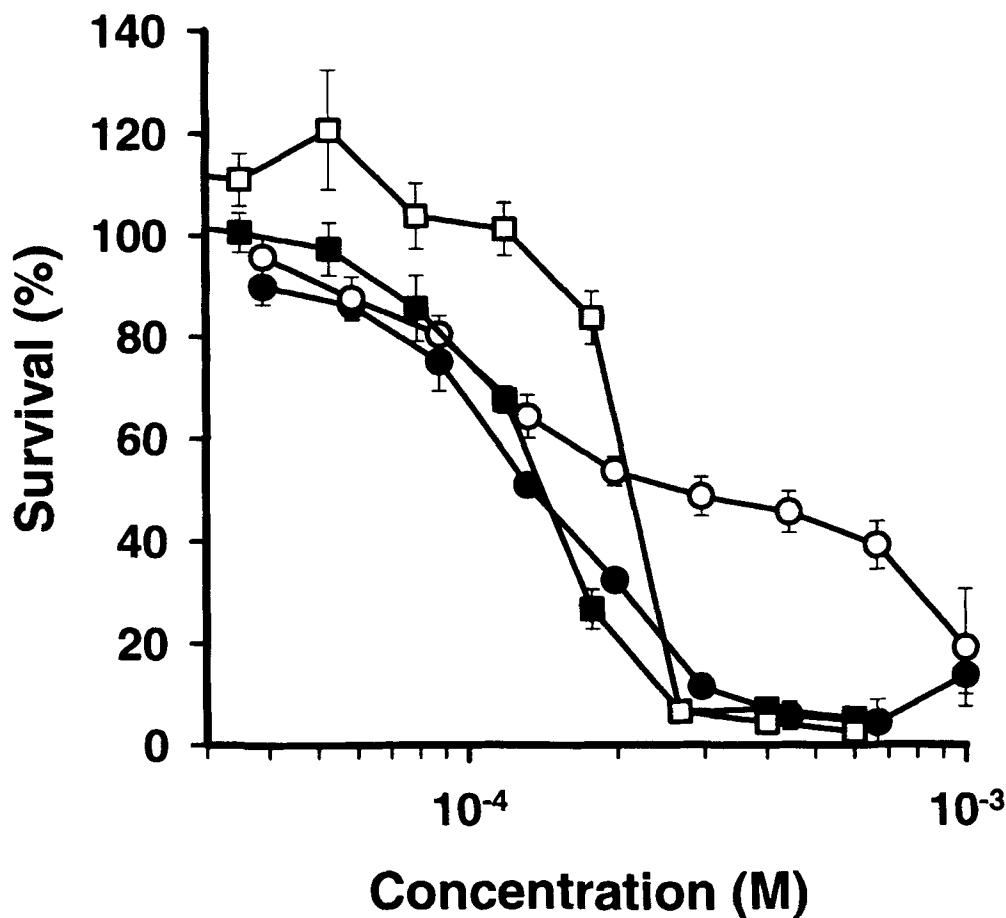


FIGURE 2 Effect of CLZm on the dose-dependent cytotoxicity of NF and t-BHP. NF (circles) and t-BHP (squares) were applied for 6 hours on hepatocytes either alone (filled symbols) or in the presence of CLZm  $10^{-5}$  M (open symbols). The cellular survival was assayed by the MTT test. Values are expressed as means  $\pm$  S.E. (n=6). Total survival (100 %) represents cells treated with culture medium only

monly used antioxidants namely  $\alpha$ -tocopherol, BHT, probucol, BHT and Trolox C<sup>®</sup>. Dose-responses curves (not shown) for protection against NF cytotoxicity were established with CLZm and all the reference antioxidants, within their respective limits of solubility. The protection extent and optimal concentration were determined for each tested compound (table I). These were compared to values determined from dose-response curves previously established on t-BHP-treated hepatocytes<sup>[5]</sup>. Cellular resistance

was estimated by the release of LDH from damaged cells into the culture medium.

CLZm, BHT and Trolox C<sup>®</sup> provided the best protection against NF, with maximum survival of 80.9, 80.6, and 83.6 %, respectively. However, CLZm optimal concentration was as low as  $10^{-5}$  M while Trolox C<sup>®</sup> and BHT only reached their maximal efficiency at  $10^{-3}$  M and  $5 \times 10^{-4}$  M, respectively. While significantly improving cellular viability probucol and  $\alpha$ -tocopherol were not as efficient as the first three compounds as

they decreased LDH levels to 49 and 59 %, respectively. Quite remarkably, the relative protective potencies of the five antioxidants were almost identical to those observed in the case of a t-BHP-induced oxidative stress, suggesting that similar protective mechanisms and constraints could be at work with both stressors.

### Effects of IMPZs analogues on the survival of hepatocytes submitted to NF

In order to verify this hypothesis the efficiencies of various IMPZs analogues, some lacking the phenol group essential for the antioxidative activity of the oxidation product CLM, were compared.

IMPZs (CLZm and CLZp) and mIMPZs (mCLZm and mCLZp) analogues (see figure 1 for structures) were coincubated with NF  $3 \times 10^{-4}$  M. Interestingly, all compounds similarly improved the cellular survival which increased from 15 % in cells treated with NF alone to about 50 % in cells treated in the presence of the IMPZs applied at  $5 \times 10^{-5}$  M (table II). Quite remarkably, no differences were found between IMPZs and mIMPZs. Moreover, the concentration-dependence of the observed protection conferred by IMPZs and their corresponding methoxylated analogs were almost identical. This is well shown in figure 3 where the protective effects of increasing CLZm and mCLZm concentrations are compared. Similar observations were made with CLZp and mCLZp (not shown).

The similar protection extents by the IMPZs analogues are rather surprising as phenol-lacking mIMPZs proved markedly less efficient for protecting hepatocytes against lethal effects of t-BHP<sup>[5]</sup>. With this stressor the higher potency of IMPZs was ascribed to their oxidative transformation into CLM. Since this aminopyrazine is endowed with antioxidative properties, it furnishes an additional protection to that firstly conferred by the parent compound. At the opposite, mCLM, the oxidation product of mIMPZs,

has no antioxidative properties and cannot prevent the lethal action of t-BHP on hepatocytes. Accordingly, the equal potencies of IMPZs and mIMPZs on NF-induced mortality either suggest that neither CLM nor mCLM confer any resistance to cells or, on the contrary, that they bear similar protective properties. This point was therefore investigated.

TABLE I Comparative effects of antioxidants on the release of LDH from hepatocytes treated during 6 hours with NF  $3 \times 10^{-4}$  M or with t-BHP  $2.5 \times 10^{-4}$  M. LDH values are expressed as means  $\pm$  S.E. (n=3). Each antioxidant was tested at his optimal concentration. Within each column data sharing the same letter are not significantly different

Treatment	LDH release (% of controls)	
	t-BHP <sup>a</sup>	NF
Control (stress alone)	100 $\pm$ 3.0 <sup>a</sup>	100 $\pm$ 2.7 <sup>a</sup>
+ CLZm $10^{-5}$ M	13.4 $\pm$ 0.4 <sup>b</sup>	19.1 $\pm$ 0.4 <sup>b</sup>
+ Trolox C <sup>®</sup> $10^{-3}$ M	12.9 $\pm$ 1.8 <sup>b</sup>	16.4 $\pm$ 1.0 <sup>b</sup>
+ $\alpha$ -tocopherol $10^{-3}$ M	67.7 $\pm$ 5.2 <sup>c</sup>	59.3 $\pm$ 2.9 <sup>c</sup>
+ Probucol $10^{-4}$ M	67.8 $\pm$ 11.6 <sup>c</sup>	49.0 $\pm$ 7.9 <sup>c</sup>
+ BHT $5 \times 10^{-4}$ M	13.0 $\pm$ 1.7 <sup>b</sup>	19.4 $\pm$ 2.2 <sup>b</sup>

a. Calculated from ref. 5.

TABLE II Protective effects of aminopyrazines and imidazolopyrazinones on NF-induced cytotoxicity in rat hepatocytes. Hepatocytes were treated for 6 hours with NF  $3 \times 10^{-4}$  M. Cell survival was estimated by MTT test and the release of LDH in the culture medium. Values are expressed as means  $\pm$  S.E. n=6 (MTT) or n=3 (LDH). In each column, data sharing the same letters are not significantly different

Treatment	Survival (%)	LDH release (%)
NF alone	15.5 $\pm$ 2.6 <sup>a</sup>	100 $\pm$ 10.7 <sup>a</sup>
+ CLZm $5 \times 10^{-5}$ M	48.4 $\pm$ 8.4 <sup>b</sup>	n.d.
+ mCLZm $5 \times 10^{-5}$ M	48.8 $\pm$ 2.2 <sup>b</sup>	n.d.
+ CLZp $5 \times 10^{-5}$ M	51.7 $\pm$ 1.4 <sup>b</sup>	n.d.
+ mCLZp $5 \times 10^{-5}$ M	54.8 $\pm$ 3.9 <sup>b</sup>	n.d.
+ CLM $10^{-5}$ M	35.2 $\pm$ 3.9 <sup>c</sup>	65.7 $\pm$ 9.7 <sup>a,d</sup>
+ CLM $5 \times 10^{-5}$ M	72.1 $\pm$ 0.9 <sup>d</sup>	23.9 $\pm$ 1.7 <sup>b</sup>
+ mCLM $10^{-5}$ M	36.5 $\pm$ 1.8 <sup>c</sup>	78.9 $\pm$ 7.3 <sup>a,d</sup>
+ mCLM $5 \times 10^{-5}$ M	56.3 $\pm$ 1.2 <sup>b</sup>	62.1 $\pm$ 4.9 <sup>c,d</sup>

N.d., not determined.

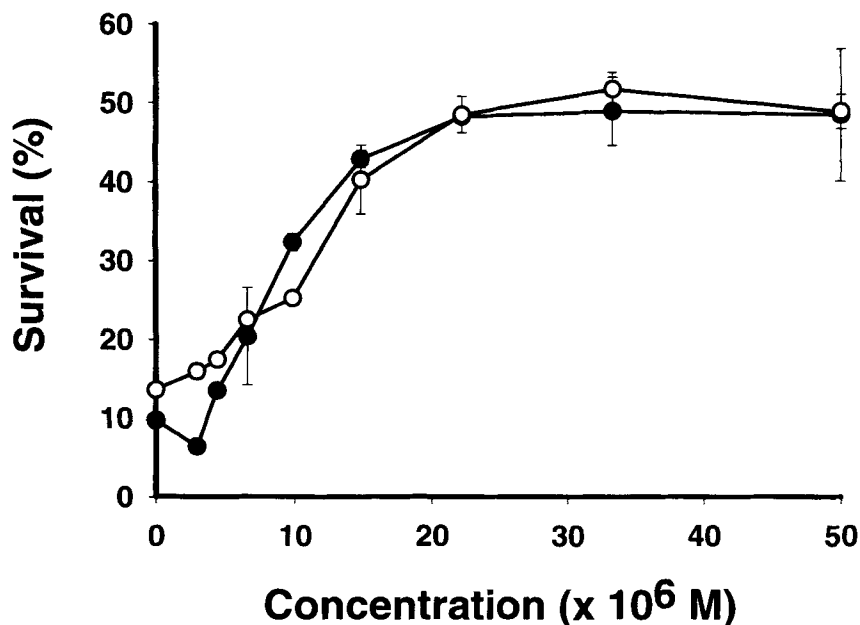


FIGURE 3 Protections by CLZm (filled circles) or mCLZm (open circles) on the survival of rat hepatocytes treated for 6 hours with NF  $3 \times 10^{-4}$  M. Survival was assayed by the MTT test. Values are means  $\pm$  S.E. (n=6). Controls (100% survival) were treated with culture medium (DMEM) only

### Protective properties of CLM and mCLM against NF

As expected, CLM was able to improve cellular resistance against NF  $3 \times 10^{-4}$  M. Survival, measured by the MTT test, increased from  $15.5 \pm 2.6$  % in NF-treated hepatocytes to  $35.2 \pm 3.9$  % and  $72.1 \pm 0.9$  % in the presence of CLM  $10^{-5}$  M and  $5 \times 10^{-5}$  M, respectively (table II). Although slightly less efficient than CLM, mCLM provided a significant protection as survival reached  $36.5 \pm 1.8$  and  $56.3 \pm 1.2$  at  $10^{-5}$  M and  $5 \times 10^{-5}$  M mCLM, respectively. LDH measurements confirmed the MTT results.

### NF-induced lipid peroxidation and protection by antioxidants

The protection provided by mCLM, which lacks antioxidative properties, suggests that the observed toxicity of NF could not necessarily involve oxidative stress. We thus examined

whether NF-treatment of hepatocytes was associated with increased lipid peroxidation levels and if the tested compounds could attenuate that peroxidation. Results (table III) indicated that NF induced a significant increase of TBARS in the culture medium. This surge in TBARS levels was efficiently suppressed by Trolox C<sup>®</sup>  $10^{-3}$  M. CLM  $5 \times 10^{-5}$  M also reduced the production of TBARS. Surprisingly, TBARS in CLZm-treated hepatocytes were higher than in NF-treated cells. This result was unexpected taking into account that CLZm strongly decreased NF-induced mortality and *t*-BHP-induced lipid peroxidation in hepatocytes<sup>[5]</sup>. Further tests indicated that this surprisingly high signal observed with CLZm results from the fluorescence of a product formed upon heating mixtures of NF, CLZm, and TBA. This fluorescence was not observed in the case of CLM and Trolox C<sup>®</sup>. As a consequence, TBARS levels generated by NF in the presence of CLZm could not be determined.

TABLE III Effect of Trolox C<sup>®</sup>, CLM and CLZm on lipid peroxidation induced by NF in rat hepatocytes. Hepatocytes were incubated for 6 hours with NF  $3 \times 10^{-4}$  M. Values are expressed as means  $\pm$  S.E. (n=3). Data sharing the same letter are not significantly different

Treatment	TBARS (% of control cells)
Untreated cells	13.2 $\pm$ 1.4 <sup>a</sup>
Control (NF alone)	100 $\pm$ 15.0 <sup>b</sup>
+ Trolox C <sup>®</sup> $10^{-3}$ M	16.7 $\pm$ 0.6 <sup>a</sup>
+ CLM $5 \times 10^{-5}$ M	34.8 $\pm$ 4.4 <sup>a</sup>
+ CLZm $5 \times 10^{-5}$ M <sup>a</sup>	-

a. TBARS produced by CLZm-treated hepatocytes could not be measured due to interferences with fluorescence measurements

TABLE IV Effect of the tested compounds on the EROD activity in rat hepatocytes induced by MC. Control consists of cells treated with culture medium alone. All compounds were tested at  $5 \times 10^{-5}$  M except for Trolox C<sup>®</sup> which was used at  $10^{-3}$  M. Values are expressed as means  $\pm$  S.E. (n=3). Data sharing the same letter are not significantly different ( $p < 0.05$ )

Treatment	EROD activity (% of control)
Control	100 $\pm$ 7.5 <sup>a</sup>
+ $\alpha$ -NF	23.2 $\pm$ 4.0 <sup>b</sup>
+ CLZm	35.5 $\pm$ 1.6 <sup>b</sup>
+ mCLZm	83.4 $\pm$ 10.6 <sup>a,d</sup>
+ CLZp	83.9 $\pm$ 2.8 <sup>a,d</sup>
+ mCLZp	95.7 $\pm$ 4.7 <sup>a</sup>
+ CLM	72.2 $\pm$ 6.7 <sup>c,d</sup>
+ mCLM	30.0 $\pm$ 1.4 <sup>b</sup>
+ Trolox C <sup>®</sup>	91.4 $\pm$ 7.4 <sup>a</sup>

### Measurement of CYP1A1 activity (EROD) in intact hepatocytes incubated with the tested compounds

Whereas the above results suggest that ROS are involved in NF-induced mortality, some data, like the protective effect of mCLM, indicate that all observed protections could not be fully

ascribed to antioxidative mechanisms. As a possibility IMPZs, mIMPZs, or their oxidation products could inhibit the CYP450 activity responsible for the activation of NF. Since no information is available on which CYP450 isoforms are involved in this process, we have tested the possible interactions of imidazolopyrazinones and aminopyrazines with the IA1 isoform. Compounds were applied on MC-treated hepatocytes for the whole duration of the EROD activity test. As expected, the reference inhibitor of CYP1A1  $\alpha$ -naphthoflavone ( $\alpha$ -NF)  $5 \times 10^{-5}$  M reduced EROD activity by 77 % (table IV). Among imidazolopyrazinones, only CLZm inhibited the EROD activity significantly. Substantial but not statistically significant inhibitions were observed with mCLZm, CLZp, and mCLZp. Both aminopyrazines inhibited significantly CYP1A1 but mCLM was by far the most efficient inhibitor with inhibition reaching 70 % whereas that caused by CLM was only 28 %. Trolox C<sup>®</sup>  $10^{-3}$  M had no effect on EROD activity.

### Effect of $\alpha$ -naphthoflavone on the survival of NF-treated cells

The above data indicated that some inhibition of CYP450IA1 indeed occurs in our experimental conditions. However, this does not imply that this inhibition is responsible for the observed protections against NF toxicity. In order to verify that an inhibition of CYP450IA1 could indeed influence NF toxicity, the protection of NF-treated cells by  $\alpha$ -NF was tested. For this, hepatocytes were incubated for 6 hours with both  $\alpha$ -NF  $5 \times 10^{-5}$  M and NF  $3 \times 10^{-4}$  M, and their survival compared with that obtained in the absence of the CYP450 inhibitor. Results revealed that  $\alpha$ -NF  $5 \times 10^{-5}$  M afforded a significant cellular protection as the survival of NF-treated hepatocytes (10.9  $\pm$  1. %) was raised to 42.9  $\pm$  3.2 % in the presence of  $\alpha$ -NF  $5 \times 10^{-5}$  M.



## DISCUSSION

The present study clearly demonstrated that imidazo- and aminopyrazines increased the resistance of rat hepatocytes against NF-induced damages. Our results confirm the occurrence of oxidative damage in NF-treated hepatocytes as the increase in TBARS level was prevented by Trolox C<sup>®</sup>. The similar protection potencies of the antioxidants tested on NF- and t-BHP-treated hepatocytes indicate that ROS are at work with the two stressors. Imidazolopyrazinones are well able to protect rat hepatocytes against the lethal effects of NF. They improve cellular survival as measured by the MTT test and help to maintain the integrity of cellular membranes (LDH assay). Measurements of lipid peroxidation by the method of TBARS revealed that the enhanced cellular survival conferred by CLM and Trolox C<sup>®</sup> is associated with reduced lipid peroxidation levels. Unfortunately, interferences hampered the demonstration of lower lipid peroxidation levels in the presence of CLZm. It is likely though that this compound also limits lipid peroxidation, as seen on t-BHP-treated hepatocytes<sup>[5]</sup>. The shift of the dose-dependent cytotoxicity curve of NF by CLZm indicates that the protective effect is only observed at high ( $> 2 \times 10^{-4}$  M) NF concentrations. The mortality induced by lower NF doses or concentrations superior to  $6.7 \times 10^{-3}$  M could not be counteracted by CLZm. Interestingly the effects of CLZm on t-BHP dose-response curve are different as CLZm protects cells efficiently at the first cytotoxic t-BHP doses. This inability of CLZm to counteract the toxicity of low NF concentrations could not be explained by the need to generate oxidation products able to prevent the toxic effects of NF as Trolox C<sup>®</sup> and CLM shifted NF dose-response mortality curves similarly (data not shown). Rather this suggests the occurrence of two different mechanisms underlying NF toxicity. A first, ROS-independent, mechanism could be at work when low doses of this nitroaromatic compound are applied; the second

mechanism would involve ROS and be counteracted by exogenously applied antioxidants. The observation that protections by mCLZm and CLZm reach a plateau at 50 % protection supports this interpretation. Such a limitation in the protection extent was not observed when t-BHP was used as stressor<sup>[22]</sup>.

The previously reported superiority of CLZm over reference antioxidants such as BHT, Trolox C<sup>®</sup>, probucol and  $\alpha$ -tocopherol for t-BHP treated hepatocytes also occurs when NF is the stressor. This confirms the rather high protective efficiency of this IMPZ in rat hepatocytes. Several factors could account for this. At first, previous work showed that IMPZs readily diffuse into cells<sup>[22]</sup>, (Dubuisson, personal observation). Second, both IMPZs and mIMPZs have very high reaction rate constants with a large number of ROS, such as superoxide anions and hydroxyl radicals<sup>[9; 11-14]</sup>, two major species implicated in the NF-induced oxidative stress<sup>[23]</sup>. Although the ranking of protective efficiency on NF-induced mortality is almost identical to that observed for t-BHP-induced stress (CLZm > BHT > Trolox C<sup>®</sup> >  $\alpha$ -tocopherol), striking differences were found with IMPZs analogues. A previous study indicated that IMPZs are more effective cytoprotectors on t-BHP-treated hepatocytes than mIMPZs. Since CLM, but not mCLM, protects hepatocytes against t-BHP-induced mortality, and as CLM and mCLM are oxidatively generated from IMPZs and mIMPZs respectively, it was suggested that the better protection associated with IMPZs partly reflected the additional protection by their oxidation product. With NF-treated hepatocytes, all imidazolopyrazinone compounds lead to similar protections. Since IMPZ and mIMPZ show similar rate constants with superoxide anion (de Wergifosse, unpublished result), their equipotency could simply reflect the key role of superoxide in the cytotoxic effects of NF in hepatocytes. Their reaction with superoxide would protect cells while concomitantly generating either CLM or mCLM. These aminopyrazines

also protect hepatocytes when directly administered to cells, and it is thus likely that their production into cells could help cells coping with NF. However, since neither aminopyrazines react with superoxide anions (de Wergifosse, unpublished result), the protections they afford result from other mechanisms. CLM is an efficient chain-breaking agent and protects lipids against peroxidation<sup>[24]</sup>. The improved resistance of cells to NF conferred by CLM could thus be related to the recorded protection of lipid targets. That would slow the propagation of superoxide-initiated cellular damages. On the other hand, mCLM totally lacks the chain-breaking properties of the phenol-bearing aminopyrazine (de Wergifosse *et al.*, in preparation). Therefore, unless mCLM is converted into some derivatives with antioxidant activity by metabolic reactions associated to NF catabolism, the cellular protection it confers could not result from antioxidative properties. Other mechanisms are thus operating.

Among these, cytochrome P450-catalysed redox cycling reactions involved in the reduction of NF may be partially blocked. This could result from either inhibitions of NADPH-CYP450 reductase<sup>[8]</sup>, or direct inhibition of cytochromes P450. The strong inhibition of EPR signals when a CYP450 inhibitor like SK&F-525A is coincubated with quinonoid compounds in microsomes confirms the role of CYP450 in their bio-reduction<sup>[24]</sup>. Imidazole-containing compounds have often been described as inhibitors of several CYP450s<sup>[25;26]</sup>. Accordingly compounds inhibiting isoforms of the CYP450 complex involved in the activation of NF could protect cells against this stressor. These would be most effective because the NF-induced cascade of events leading to cellular death is very complex and protection will be most efficient if brought rapidly. Data obtained on EROD activity measured in intact cells support this hypothesis as CLZm, CLM, and remarkably mCLM inhibit CYP450IA1. Although no information is available from the literature on which CYP450 isoforms are involved in the reduction of NF, our

data suggest that the isoform IA1 may play a role. Indeed,  $\alpha$ -NF, the reference inhibitor of this isoform<sup>[27]</sup> improves the survival of NF-treated hepatocytes. Interestingly, the protective effect afforded by  $\alpha$ -NF is similar to that given by mCLM and so are their inhibitions of EROD activity. It could thus be that the protection by mCLM is totally related to CYP450 inhibition. On the other hand CLM, while reducing EROD to a lesser extent, proved more efficient in protecting the hepatocytes. In that case, the protection more likely results from its scavenging activity towards ROS produced by the redox-cycling of NF. The fact that mIMPZs very efficiently reduce NF toxicity although having apparently little influence on CYP1A1 could be due to both the efficient scavenging of superoxide anions and the generation of the potent CYP450 inhibitor mCLM upon reaction of mIMPZs with NF-generated ROS. In that case, the newly formed mCLM would inhibit CYP450 thus reducing the amount of ROS formed. Furthermore one should keep in mind that other CYP450 isoforms are likely to be involved in the reduction of NF and that these could also be potential targets for IMPZs and oxidation products. At last, recent work indicated that CYP450 inhibitors protect rat hepatocytes against endogenous oxidative stress following the impairment of antioxidant enzymes<sup>[26]</sup>. Therefore, the protection of NF-treated cells by IMPZs, AMPs and  $\alpha$ -NF could not necessarily indicate interferences with NF-activation mechanisms. They could simply result from reduced activities of any of the monooxygenases intensifying the effects of endogenous antioxidant depletion caused by NF redox cycling.

As a conclusion, the observed extent of protection would thus most likely result from actions involving inhibition of CYP450 isoforms by either the imidazolopyrazinones and/or their oxidation products, and antioxidative mechanisms directed towards superoxide anions and lipid radicals. The absence of antioxidative properties of the oxidation product of mIMPZs

would be compensated by a strong inhibitory effect of mCLM on CYP450 isoforms involved either in the activation of NF or in the propagation of NF-triggered oxidative reactions. CLM while being potentially less prone to act as an inhibitor of CYP450 would exert protection through its chain-breaking capabilities<sup>[24]</sup>. The balance of both mechanisms in IMPZs and mIMPZs would lead to the observed similar protection.

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