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Protection of Peroxide-treated Fish Erythrocytes by Coelenterazine and Coelenteramine

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European seabass (*Dicentrarchus labrax*) erythrocytes treated with *tert*-butyl hydroperoxide (*t*-BHP) showed decreasing levels of reduced glutathione, increased lipid peroxidation and DNA damage, and ultimately underwent haemolysis. The addition of the marine luciferin coelenterazine (CLZn) markedly delayed the onset of the haemolytic process induced by *t*-BHP as well as lipid peroxidation and glutathione oxidation. CLZn also protected the red blood cells' DNA against *t*-BHP-triggered damage. CLZn's oxidation product coelenteramine (CLM) also delayed the lysis of the cells as well as the occurrence of oxidative stress indicators but it did not offer protection against DNA damage. Both compounds proved more efficient than the vitamin E analogue Trolox C® at similar doses. These results demonstrate the ability of CLZn and CLM to protect fish cells against oxidative stress, providing further support to the evolutionary model suggesting that CLZn's first physiological role was that of an antioxidant in fish thriving in surface layers of the ocean, later evolving into its light-emitting function in deep-sea species.

Keywords: Coelenterazine; Peroxide; Erythrocytes; Lipid peroxidation; Glutathione; DNA damage

INTRODUCTION

Bioluminescence, the emission of ecologically functional light by living organisms, is a common phenomenon among deep-sea animals.[1,2] Its widespread occurrence in deep-sea organisms has been linked to the successful utilisation of coelenterazine (CLZn) serving as chemiluminescent substrate (luciferin) for many luciferases and photoproteins.[3] The wide distribution of this imidazopyrazinone compound in the tissues of bioluminescent and non-bioluminescent marine organisms led to the proposal that CLZn pre-existed the development of bioluminescence and played other physiological roles in animal's tissues. Considering the high reactivity with reactive oxygen species (ROS) of CLZn and analogues,[4] it was suggested that this compound could scavenge ROS, and in doing so, help cells coping with oxidative stress.[5] The antioxidative properties of CLZn have been established in human fibroblasts subjected to *tert*-butyl hydroperoxide (*t*-BHP).[3] The oxidation product coelenteramine (CLM) also demonstrated powerful chain-breaking properties in cellular and acellular lipid peroxidation systems.[6,7] CLZn analogues and CLM were shown to efficiently protect rat hepatocytes submitted to *t*-BHP.[7] Results obtained on mammalian cells suggest that CLZn and CLM could constitute an antioxidative cascade: the antioxidative activity of the mother compound generates a new antioxidant activity which prolongs the protection of cellular targets.[6]

In order to test the antioxidant function of CLZn in fish, we investigated its ability as well as that of its oxidation product CLM to protect marine fish cells at concentrations close to physiological conditions. Since CLZn in fish is most likely obtained from the diet and transported to all tissues through the blood,[5] we have used oxidant-treated marine fish erythrocytes as models for investigating antioxidant protection by CLZn and CLM.

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MATERIALS AND METHODS

Animals

Adult sea bass, *Dicentrarchus labrax* (200–250 g weight) were obtained from the aquaculture farm AquaNord, Gravelines (France). The fish were held in glass tanks filled with 20°C artificial seawater at 35‰ salinity (*Aqua Marin Salt*) and fed commercial fish pellets.

Chemicals

Coelenterazine (CLZn; 3,7-dihydro-2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-8-benzylimidazo[1,2-a]pyrazin-3-one), coelenteramine (CLM; 2-amino-3-benzyl-5-(*p*-hydroxyphenyl)-1,4-pyrazine) and methyl-coelenteramine (mCLM; 2-amino-3-benzyl-5-(*p*-methoxyphenyl)-1,4-pyrazine) were synthesized as previously described.[8] Benzocain, heparin, *t*-BHP, thiobarbituric acid (TBA), trichloroacetic acid (TCA), 4′,6-diamine-2-phenylindole dihydrochloride (DAPI), reduced and oxidised glutathione (GSH and GSSG) were purchased from Sigma Chemical Co. Trolox C® (6-hydroxy-2,5,7,8-pentamethylchroman-2-carboxylic acid) and malondialdehyde (MDA) were obtained from Aldrich Chem. Co. Butanol was from Fluka while dimethylformamide (DMF) was obtained from Acros organics. Agarose was purchased from Gibco-BRL. HPLC grade methanol came from SDS (Labconsults, Brussels).

Red Blood Cell Suspension

Red blood cell suspension was obtained as described earlier.[9] Fish were anaesthetised in a seawater solution to which benzocain (10% in acetone) had been added (1:1000). After a 3 min immersion in the anaesthetising solution, heparin (300 μl, 1000 U/ml) was injected in the caudal vein, and the fish held in the anaesthetic solution for further 2 min. The blood (4 ml average) was taken from the caudal vein and immediately centrifuged (Biofuge 15R, Heraeus Sepatech) at 11,000*g* for 5 min at 4°C. The plasma was removed and replaced by the same volume of saline buffer (NaCl 176 mM, KCl 5.4 mM, $MgSO_4$ 0.81 mM, KH_2PO_4 0.44 mM, $NaHCO_3$ 5 mM, $CaCl_2$ 1.5 mM, Hepes-Na 10 mM; pH 7.6). The red blood cells (RBC) were then resuspended and washed three times following the same procedure. Finally, they were resuspended in the saline containing 10 mM glucose and kept at 4°C for a maximum of 48 h.

Experimental Conditions

Before each experiment, 50 μl of the RBC suspension were mixed with a trypan blue solution prepared in the saline buffer (1:10) and RBC counted under the microscope on a Fuchs–Rosenthal cell. The suspension is then diluted to a final RBC concentration of 70×10^6 cells/ml. All experiments were carried out at 25°C. RBC were incubated with 0.5 mM *t*-BHP prepared in the saline buffer in the presence or absence of the tested antioxidants. Before its addition into saline buffer, CLZn was first solubilized in ethanol (final concentration $<0.05\%$), while CLM, mCLM and Trolox C®, the hydrophilic analogue of vitamin E, were first solubilized in dimethylformamide (DMF, final concentration $<0.05\%$). Control experiments indicated that the solvent neither influenced the cellular survival, the peroxidation level of cellular lipids, the integrity of DNA nor the redox status of the cells.

Haemolysis Assay

The extent of RBC haemolysis was followed continuously by the decrease in absorbance at 540 nm recorded every 90 s for a 2-h period in a wavelength tunable microplate spectrophotometer (SpectraMax 190, Molecular Devices). In each well, 100 μl of the RBC stock solution (70×10^6 cells/ml) were incubated with 50 μl *t*-BHP and 50 μl of saline, some containing various concentrations of the tested antioxidants. Controls included untreated cells, cells incubated with the antioxidants alone, and cells lysed with distilled water (100% haemolysis). Before each reading the plate was agitated in the spectrophotometer. The time of half-haemolysis (THH), which is the time at which 50% of the RBC are haemolysed, was calculated.

Lipid Peroxidation Assay

The extent of lipid peroxidation was determined by quantifying the amount of TBARS (Thiobarbituric Acid Reactive Substances) present in the saline and in the cells.[10] Treatments were carried out in tubes containing RBC (35×10^6 cells/ml), *t*-BHP and the tested antioxidants when required. Samples (200 μl) were taken every 5 min for 1 h, then added to a mixture of 200 μl trichloroacetic acid (TCA) 15% and 400 μl thiobarbituric acid (TBA) 0.67% (w/v), and finally heated (95°C) for 25 min in a water bath. After cooling on ice, 1.5 ml of butanol were mixed thoroughly into the solution and tubes were centrifuged (3 min, 720*g*, 25°C). The butanol phase (100 μl) was transferred into a 96-well plate, and the fluorescence of the MDA–TBA chromogen (excitation: 515 nm; emission: 555 nm) was measured on a microplate fluorimeter (Fluoroskan Ascent FL, Labsystem). A standard curve was constructed using malondialdehyde (MDA) and the signal converted into MDA equivalents per well. Control tests indicated that none of the tested antioxidants interfered with the assay.

Single Cell Gel Electrophoresis (SCGE) Assay

Detection of DNA damage was accomplished with the SCGE, also known as the COMET assay, which employs alkaline unwinding to quantify DNA strand breaks in individual cells. In this assay, electrophoresis under alkaline conditions induces the migration of DNA strands in the direction of the positive electrode, producing nuclei with increasing length or "tailing" proportional to DNA damage.[11] Erythrocytes were collected under yellow light in order to avoid light-induced DNA damage, and left for 24 h before the experiment in the glucose-added saline buffer at 4°C. All subsequent steps of this assay were carried out either under yellow light or in darkness, at 4°C except when otherwise mentioned. Cells (35×10^6 cells/ml) were incubated with *t*-BHP in the absence or presence of the tested antioxidant during 5 min at room temperature, then centrifuged (11,000*g*, 3 min, 4°C) and washed twice with ice-cold saline buffer. Cells (90 μl) were then resuspended in 1 ml low melting point agarose 0.9% (w/v) at 42°C and 80 μl transferred onto a frosted glass microscope slide pre-coated with a layer of normal agarose 1% (w/v) in a phosphate saline buffer (pH 7.4) which was kept at 4°C for 15 min. Upon cooling, the preparation consisted of 5×10^4 cells embedded in a thin, uniform layer of gel. Slides were immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, NaOH to pH 10.0, and 10% (v/v) Triton X-100) for 1 h, then placed in a Sub-Cell GT horizontal electrophoresis tank (Bio Rad, Inc.) containing 300 mM NaOH and 1 mM EDTA, where left for 20 min. Electrophoresis was then performed for 30 min at 25 V. Slides were finally washed three times for 5 min each with ice-cold 0.4 M Tris–HCl (pH 7.5) and stored at 4°C in darkness and under high humidity to avoid desiccation. Slides were dyed with 50 μl DAPI stain (5 μg/ml) just before observation with a fluorescence microscope equipped with a 362 nm (exc.) monochromator (Reichert–Jung). Three slides per treatment were scored by a computerised image analysis of 50 randomly selected nucleoids per slide using Komet 4.0.2 (Kinetic Imaging, Ltd., Liverpool, UK).

Glutathione Assay

The redox status of the RBC was estimated by their contents in reduced (GSH) and oxidised (GSSG) glutathione. These were measured simultaneously by reversed-phase high-performance liquid chromatography equipped with a post-column coulometric electrochemical detector as previously described.[12]

Treatments were carried out at 25°C in tubes containing RBC (35×10^6 cells/ml), *t*-BHP and the tested antioxidants. Samples (400 μl) were taken every 10 min over a 30-min period and immediately centrifuged (11,000*g*, 4°C, 5 min). The supernatant was discarded and the pellet washed twice with the same volume of cold saline buffer. The RBC were then resuspended in 100 μl cold NaCl 0.9% (w/v) and stored at −80°C until analysis. On the day of analysis, thawed samples were mixed with 400 μl of $HClO_4$ (5% w/v) and left to stand at room temperature for 15 min. Samples were then centrifuged (12,000*g*, 5 min, 4°C). Supernatants were thoroughly mixed with 20 μl KOH (10 M) and centrifuged at 12,000*g* for 2 min. The resulting supernatants were 10-fold diluted in the mobile phase, then filtered (0.22 μm). The ready-to-inject samples were kept at 4°C in the autosampler tray before injection (100 μl) into the HPLC (Thermoquest®, Belgium). Chromatographic separations were performed in isocratic conditions (flow-rate of 1 ml/min) on a 150 mm × 4.6 mm (i.d.) 3 μm reversed phase Nucleosil C120-3 column (Macherey–Nagel, Filter Service, Belgium) maintained at 40°C. The mobile phase consisted of 10 mM NaH_2PO_4 adjusted to pH 2.7 with 85% (w/v) phosphoric acid, and containing 5% HPLC grade methanol (v/v). Electrochemical detection was carried out with a ESA Coulochem II detector (Intersciences, Louvain-la-Neuve, Belgium). The electrochemical parameters were as follows: first and analytical electrodes were set at 350 and 1000 mV, respectively. Analytical cell sensitivity of 100 μA was shifted to 5 μA at 2.35 min as to detect GSH, and to 500 nA at 2.85 min for detecting GSSG, then set to the initial voltage at 4 min until the end of the 6-min run. Electroactive contaminants from the mobile phase were eliminated by oxidising the eluent at +1150 mV with a guard cell placed before the column. GSH and GSSG were quantified against pure standards.

Statistical Analyses

Significance of differences between treatments was tested by one-way analysis of variance (ANOVA) coupled with Dunnett's post-test at a 99% confidence interval.

RESULTS

Protection of *t*-BHP-induced Haemolysis of RBC by the Tested Compounds

The oxidative stress induced by 0.5 mM *t*-BHP led to a complete haemolysis of the cells within 30 min. CLZn, CLM or Trolox C® dose-dependently delayed the onset of the RBC haemolysis (Fig. 1). Interestingly, CLM mostly modified the kinetic of the haemolysis itself, slowing down the rate of haemolysis, while

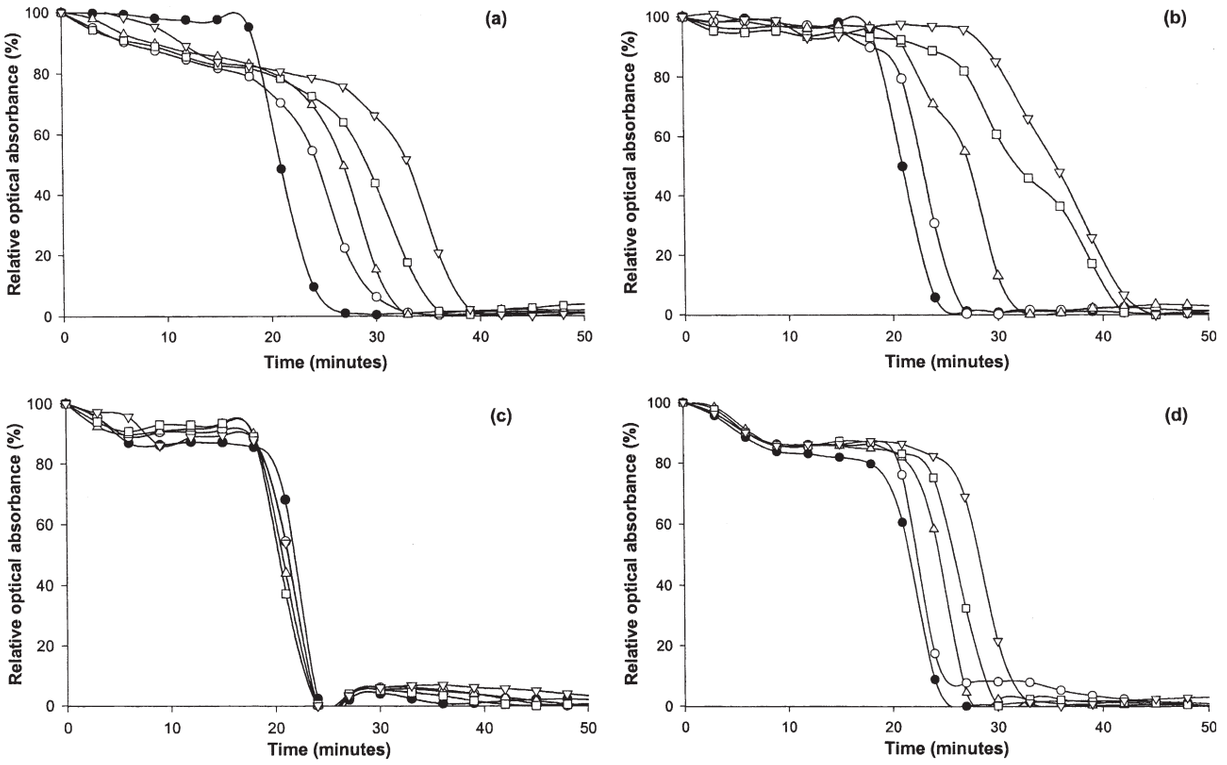

FIGURE 1 RBC (35×10^6 cells/ml) haemolysis measured in absorbance at 540 nm during 2 h (only first 60 min shown), triggered by *t*-BHP 0.5 mM (•), in presence of (a) CLZn, (b) CLM, (c) mCLM and (d) Trolox C® at the following concentrations: 2.5 μM (○), 5 μM (△), 10 μM (□), 20 μM (▼).

CLZn and Trolox C® had no influence on it. On the other end, mCLM, the inactive analogue of CLM, did neither delayed the onset nor the rate of the haemolytic process (Fig. 1). A significant delay was observed from the lowest concentration (2.5 μM) tested for CLZn, CLM and Trolox C® (Table I). At the concentration of 10 μM, CLM and CLZn significantly increased the time required for the lysis of half the RBC population (Time of Half-Haemolysis, THH) by 51.6 and 42.3%, respectively (Table I). The addition of the reference antioxidant Trolox C® also significantly increased the THH, by 23.5%, but significantly ($p < 0.01$) less than the two pre-cited molecules. During the 2 h experiments, no haemolysis occurred either in the untreated or antioxidant-treated cells.

Protection of Cellular Lipids Against *t*-BHP-induced Oxidation

t-BHP-induced haemolysis has previously been ascribed to its ability to trigger the peroxidation of membrane lipids.[13] Therefore, the delaying of the lysis by CLZn, CLM and Trolox C® suggests that they could protect cellular lipids against *t*-BHP-induced oxidative damages, delaying lipid peroxidation. The kinetics of lipid peroxidation during the first 50 min of the exposure to *t*-BHP in both the absence and presence of these molecules were determined. The CLM analogue mCLM was included as negative reference and TBARS, which mainly represent malondialdehyde production from lipid peroxides, were used as peroxidation

TABLE I Dose-dependent effects of CLZn, CLM, mCLM and Trolox C® on the time of half-haemolysis (THH) of RBC treated with 0.5 mM *t*-BHP

Tested molecules concentration (μM)	CLZn	CLM	mCLM	Trolox
0	21.4 ± 0.2	21.0 ± 0.3	21.0 ± 0.3	21.7 ± 0.2
2.5	25.0 ± 0.3*	23.2 ± 0.3**	21.0 ± 0.1	22.7 ± 0.3**
5	26.8 ± 0.6*	27.0 ± 0.3*	22.1 ± 0.5	24.1 ± 0.2*
10	30.3 ± 0.1*	32.3 ± 1.1*	21.0 ± 0.3	26.3 ± 0.2*
20	33.3 ± 0.1*	33.6 ± 1.1*	20.4 ± 0.6	29.2 ± 0.3*

Results, expressed in minutes, are mean ± standard error of four replicates. *($p < 0.01$) and **($p < 0.05$) indicate significant differences with *t*-BHP-treated cells in the absence of the tested compound.

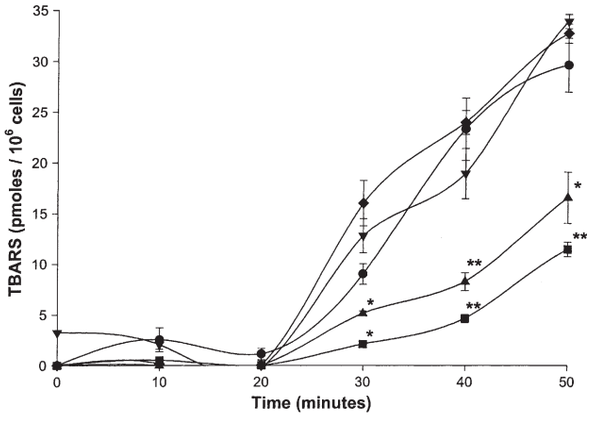

FIGURE 2 Time-dependent evolution of lipid peroxidation (TBARS, pmoles MDA/10^6 cells) in RBC (35×10^6 cells/ml) treated with 0.5 mM *t*-BHP (•) together with 10 μM of CLZn (■), CLM (▲), mCLM (▼) and Trolox C® (◆). Each value is the mean ± standard error of six replicates. **($p < 0.01$) and *($p < 0.05$) are significantly lesser than *t*-BHP-treated cells.

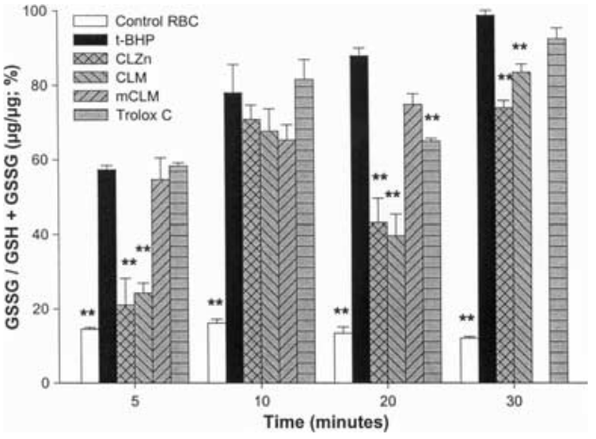

FIGURE 3 Time-dependent evolution of the RBC glutathione stores measured by the percentage of oxidised glutathione (GSSG) out of the total amount of glutathione (GSH + GSSG) in the cells treated with 0.5 mM *t*-BHP either with or without addition of the tested compounds (10 μM). Controls are RBC in saline without addition of *t*-BHP. Each value is the mean ± standard error of four replicates. **($p < 0.01$) are significantly lesser than *t*-BHP-treated cells.

indicators. No TBARS were detected in the control red blood cells maintained in saline alone or containing 10 μM of the tested compounds. TBARS' formation started 20 min after the addition of 0.5 mM *t*-BHP and its level progressively increased to a maximum value of 29.62 ± 2.65 pmoles MDA/10^6 cells after 50 min (Fig. 2). The addition of mCLM did not affect the kinetics or the level of TBARS (33.91 ± 0.69 pmoles MDA/10^6 cells at 50 min) and neither did the addition of Trolox C® (32.73 ± 0.94 pmoles MDA/10^6 cells). On the other hand, the addition of CLM and CLZn significantly ($p < 0.05$ and $p < 0.01$, respectively) reduced the formation of TBARS throughout the experiment. Maximal TBARS levels were 16.55 ± 2.52 and 11.44 ± 0.71 pmoles MDA/10^6 cells, respectively, following 50 min of co-incubation with *t*-BHP.

Redox Status of the Red Blood Cells

In addition to damaging membranes, *t*-BHP may also induce the oxidation of reduced glutathione (GSH) and alter the overall reducing environment of the RBC.[14] In order to evaluate the redox status of cells and how it is affected by the antioxidants, RBC content in GSH and its oxidised form (GSSG) was monitored. Samples were taken at regular intervals during a 30-min incubation period with *t*-BHP, that is, before hemolysis and the subsequent release of both glutathione forms from disrupted cells. Results are expressed as a proportion (μg/μg; %) of the total glutathione present in its oxidised form. Untreated RBC presented a basal GSSG level equivalent to 14.0 ± 1.0% of their total glutathione content (Fig. 3). When the concentrations of glutathione are expressed in molar, this basal GSSG redox ratio is about 7.6 ± 0.5% (mol/mol), similar to what has been previously reported in horse and man.[12] This level was not significantly modified by the addition of the tested compounds alone (10 μM). After 5 min of incubation with 0.5 mM *t*-BHP, the proportion of GSSG increased to 57.3 ± 1.1% (μg/μg), and all glutathione was oxidised within 30 min. When CLZn was added, GSSG was maintained at a significantly ($p < 0.01$) lower level during the first 5 min. After 10 min the GSSG level increased to reach an amount similar to that obtained in the presence of *t*-BHP alone. Then, as if a new equilibrium was reached, after 20 min, GSSG decreased again to levels lower than reached in the treated cells ($p < 0.01$). After 30 min, the GSSG level (73.9 ± 2.0%, μg/μg) was still significantly ($p < 0.01$) lower than that observed in cells treated with *t*-BHP alone. The addition of CLM offered a similar protection against *t*-BHP-induced GSH oxidation, significantly ($p < 0.01$) reducing the formation of GSSG during a 30-min period (83.5 ± 2.6%, μg/μg, at 30 min), with a kinetic similar to that observed with CLZn. In the presence of Trolox C®, during the first 10 min, the GSSG level increased to levels similar to those reached in the absence of the antioxidant. Finally, after 20 min, it was brought back to a significantly ($p < 0.01$) lower level before reaching the maximal value (92.4 ± 2.9%, μg/μg) after 30 min, similar to that recorded in *t*-BHP treated cells. As expected, the addition of mCLM did not limit the depletion of GSH, all of it being oxidised within 30 min. No GSH or GSSG could be detected at that time, as all cells had been completely lysed.

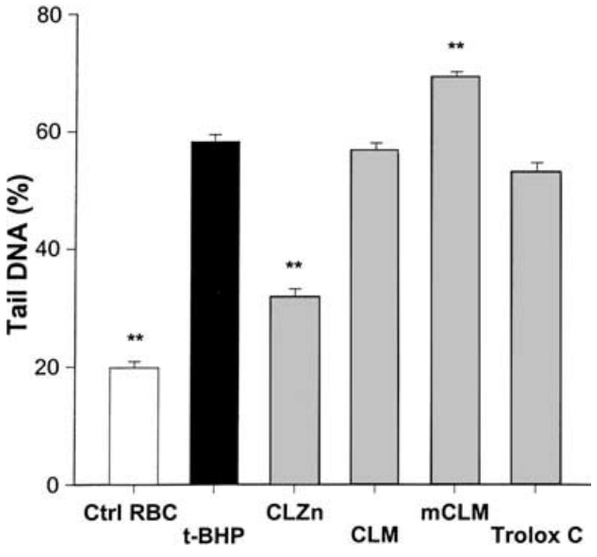

FIGURE 4 Evaluation by the COMET assay of oxidative DNA damages, represented by the percentage of DNA in the comet's tail of RBC nucleoids treated with 0.5 mM *t*-BHP (black) together with the tested compounds (10 μM) (grey). Controls are cells in saline (white). Results are mean ± standard error of 150 replicates. **($p < 0.01$) are significantly different from *t*-BHP-treated cells.

Oxidative Damage to DNA

In Contrary to mammalian red blood cells, fish erythrocytes retain their nucleus thus allowing protection against oxidative DNA damage to be tested. *t*-BHP is known to induce oxidative damage to DNA by either base methylation or strand breaks.[15,16] In fish, previous work indicated that *t*-BHP dose-dependently caused oxidative damages to erythrocytes DNA.[9] In this study, DNA-damaging effects of *t*-BHP and the possible protection by the tested compounds were quantified with the Comet assay. This is inferred by the amount of DNA present in the tail's nucleoid. Cells were treated with 0.5 mM *t*-BHP for only 5 min so as to obtain limited damage to their DNA. If untreated cells showed 19.8 ± 1.1% of the DNA in the tail of the nucleoid, this percentage increased to 58.3 ± 1.2% upon treatment with *t*-BHP (Fig. 4). Since previous experiments on other cell types (B. de Wergifosse, personal communication) indicated the need for higher antioxidant concentrations to observe protective effects, levels of all tested compounds were increased to 20 μM. The addition of CLZn significantly ($p < 0.01$) reduced DNA fragmentation with only 31.9 ± 1.4% of the DNA occurring in the tail of the nucleoid. Neither CLM nor Trolox C® prevented DNA damage and proportions of DNA in the tail reached 56.8 ± 1.2 and 53.1 ± 1.5%, respectively. As expected, mCLM offered no protection, and on the contrary exacerbated damages (69.3 ± 0.9% tail DNA) in cells exposed to *t*-BHP ($p < 0.01$).

DISCUSSION

Coelenterazine is well known for its role as luminescent substrate for bioluminescent enzymes and photoproteins in marine organisms.[17,18] Its widespread occurrence in both bioluminescent and non-bioluminescent marine organisms,[19] as well as the high reactivity of its imidazopyrazinone nucleus with ROS,[4] led us to the conclusion that CLZn's primitive function was that of an antioxidant useful for shallow water organisms.[3] Similarly, the luminescence in insects has recently been linked to some antioxidative activity of the luminescent system.[20] The model for CLZn-based luminescence suggests that a reduced oxidative threat accompanied the colonisation of the deep-sea water layers. In this dark environment, the chemiluminescent properties of CLZn could have served as a core for the development of highly beneficial bioluminescent communication and camouflage tools, while its implication in antioxidative protection was no longer essential. This hypothesis is supported by the very low levels of superoxide dismutase and glutathione peroxidase in tissues of deep-sea fish.[21] Furthermore, we have shown that CLM also possesses chain-breaking properties protecting cells subjected to oxidants.[6,7] This work brings further support to the proposed evolutionary model for CLZn-based luminescent systems, as it demonstrated that both CLZn and its etioluciferin CLM protect marine fish erythrocytes against peroxide-mediated haemolysis.

Peroxide-triggered RBC haemolysis has been shown to be an appropriate model for studying antioxidative mechanisms in fish cells.[9] In this model, a drop in GSH content and a concomitant appearance of lipid peroxidation products precede the haemolytic process whereas DNA damage occurs very early.

The above results demonstrate that both CLZn and CLM dose-dependently delayed fish RBC lysis. This action is accompanied by protection of GSH stores against oxidation and a delay of the lipid peroxidation process. mCLM, a CLM analogue lacking chain-breaking properties because of a substitution of a phenol by a methoxy-phenyl[6] is totally inactive on haemolysis and other oxidation indicators. This clearly shows that the CLM effect is linked to its antioxidant action. Interestingly enough, while CLZn and CLM had similar protective effects on GSH stores and lipid peroxidation, differences in the kinetics of the haemolytic process were observed. On one hand, CLZn markedly delayed the onset of the haemolysis while it only slightly modified the progression rate of the haemolytic process. On the other hand, the CLM increasing effect on the THH mainly resulted from a reduction of the rate of the haemolysis with

little effect on the lag-time. A further difference was found in the ability to prevent DNA damage: CLZn strongly protected the DNA, while CLM was completely inactive.

Similar observations have also been made on lipid micelles subjected to the free radical generator AAPH.[6] In this system, CLM induced no delay but markedly reduced the rate of the peroxidation, whereas CLZn generated a lag-time and a reduced propagation rate of the oxidative process. These differences between CLZn and CLM could reflect the differences in the scavenging activity of these two compounds with ROS involved in lipid peroxidation and DNA damage. Also, the relative efficiency of CLZn and CLM in preventing DNA damage matches observations made on rat hepatocytes subjected to *t*-BHP.[29]

Previous studies on erythrocytes revealed that the main ROS involved in the toxicity of *t*-BHP are peroxyl and alkoxyl radicals generated upon reaction between the peroxide and transition metals.[13,22] The alkoxyl radicals, detoxified by GSH, initiate and propagate lipid peroxidation in cells whose susceptibility towards oxidative stress is increased through the depletion of GSH by *t*-BHP catabolism.[22] The depletion in GSH and the consecutive lipid peroxidation appear as the determinant inducer of the haemolysis.[13] Also, *t*-BHP-derived peroxyl radicals are noted to be responsible for the induction of damage to DNA[16] through mechanisms different from those implicated in the haemolytic process.[23]

According to these observations, the difference between CLZn and CLM in the ability to protect DNA against oxidative damages could lie in their ability to react with peroxyl radicals. It is likely that CLZn has a high reactivity towards peroxyl radicals, therefore preventing oxidative damages to DNA molecules, while CLM's inability to protect DNA would reflect a low reactivity with these radicals. Even though peroxyl radicals are not pointed out as the major inducers of haemolysis, they are certainly implied in the haemolytic process. As CLZn and CLM offer a similar protection against RBS haemolysis, it is possible that the difference observed in its kinetics could be due to this discrepancy in reactivity towards this particular ROS. Confirmation of this hypothesis should be carried out through further experiments.

Results indicated that CLZn and CLM efficiencies are superior to that of Trolox C® in terms of protection against haemolysis, GSH depletion, lipid peroxidation and, in the case of CLZn, DNA damage. These results support those reported for rat hepatocytes subjected to *t*-BHP[7] suggesting that mechanisms underlying *t*-BHP toxicity in rat hepatocytes and fish erythrocytes are similar, in spite of the high iron content of the red blood cells, which might exacerbate erythrocytes' susceptibility to the peroxide. Since fish erythrocytes are far easier to collect and maintain, and thanks to the possibility of carrying out haemolysis assays on a microplate reader, this could be a very valuable model for testing new antioxidants. However, the ability of Trolox C® to delay haemolysis is intriguing because at the same concentration level, it seemed unable to postpone the consumption of GSH or the surge in TBARS. Our previous work carried out with higher Trolox C® concentrations (30–500 μM) indicated that TBARS and GSH depletion were delayed by this antioxidant.[9] This would indicate that low Trolox C® concentrations prevent the lysis of the cells through mechanisms independent of the chain-breaking effect on membrane lipids. The possibility that Trolox C® might associate with membranes therefore affecting their fluidity[24] and their susceptibility to haemolysis should be investigated.

Studies targeting coelenterazine-requiring photoproteins in mammalian cells indicated that CLZn readily accesses all cellular compartments such as the cytosol, the nucleus and the endoplasmic reticulum when applied externally on cells.[25,26] The efficiency of CLZn on fish RBC subjected to *t*-BHP oxidative stress suggests that is also true in these cells. HPLC-based assays on fish RBC incubated with CLZn indicated that CLZn rapidly associates with the erythrocytes, where its concentration remains stable during the 2-h incubation period (B.J. Janssens, unpublished results). This result is rather unexpected as CLZn is highly labile and spontaneously oxidises in saline, and suggests that CLZn association to fish RBC somehow stabilises this compound.

Similar observations were made in marine fish blood[27] incubated with *Vargula* luciferin, a luciferin sharing the same imidazolopyrazinone core as CLZn. In this study, red blood cells of different species of marine fish, both non and bioluminescent, protected the luciferin from autooxidation, suggesting that the protection of the luciferin is a non-specific property of fish erythrocytes. This result is very important, as most bioluminescent fishes seem to rely on dietary sources for their supply of CLZn.[5] Therefore such stabilisation of CLZn in the blood might protect the luciferin while it is being carried from the digestive system towards the luminous organs.

Due to the widespread occurrence of CLZn[28] in the tissue of marine organisms, it is likely that this compound, as well as its oxidation product CLM, could help tissues coping with ROS. However, their relative importance in the antioxidative arsenal of fish cell, particularly erythrocytes, remains to be determined.

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