Biochemical behaviour of norbixin during in vitro DNA damage induced by reactive oxygen species

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Naturally occurring antioxidants such as carotenoids are extensively studied for their potential in reducing the risk for cancer and other chronic diseases. In the present study, the radical-scavenger activity of the food additive norbixin, a water-soluble carotenoid extracted from Bixa orellana seeds and commercialized as annatto, was evaluated under conditions of DNA damage induced by reactive oxygen species, particularly by hydroxyl radicals. The cell-free scavenger activity of norbixin was evaluated using plasmid DNA as target molecule and Sn2+ or Fe2+ as oxidant. The addition of H2O2 enhanced DNA breakage induced by metal ions, particularly Fe2+. Under these conditions, norbixin started to protect plasmid DNA against single- and double-strand breakage at a metal:norbixin ratio of 1:1 (Sn2+) and 1:10 (Fe2+). However, at lower ratios to Sn2+, norbixin enhanced Sn2+-induced DNA breakage.

The ability of norbixin to protect genomic DNA against oxidative damage was assessed in murine fibroblasts submitted to H2O2-induced oxidative stress and the results were evaluated by the comet assay. Under low serum conditions (2 % fetal bovine serum (FBS)), a protective effect of norbixin against H2O2-induced DNA breakage was inversely related to its concentration, a protection ranging from 41 % (10 μM) to 21 % (50 μM). At higher concentrations of norbixin, however, oxidative DNA breakage was still enhanced, even in the presence of a high serum concentration (10 % FBS). Under normal conditions, norbixin per se has no detectable genotoxic or cytotoxic effects on murine fibroblasts. The antimutagenic potential of norbixin against oxidative mutagens was also evaluated by the Salmonella typhimurium assay, with a maximum inhibition of 87 % against the mutagenicity induced by H2O2. Although plasmid DNA and Ames data indicated that norbixin can protect DNA against oxidative damage, it seems to be a risky guardian of genomic DNA as it can also increase the extent of oxidative damage.

Norbixin: Annatto: Soluble carotenoid: Antioxidant: Pro-oxidant

Norbixin (C24H28O4) is an unusual dicarboxylic watersoluble carotenoid present as a minor component in the pericarp of the seeds of Bixa orellana L. (from the Bixaceae family), a tropical shrub commonly found in Brazil. It is derived from the hydrolysis of a methyl ester group of bixin (C25H30O4), the major carotenoid present in those seeds, and both bixin and norbixin form a colourant material known as annatto, utilized particularly in the food industry. As the use of annatto in man is based only on its colourant property, most of the published studies about annatto pigments have concentrated on the improvement of extraction protocols or on the determination of annatto in food. Little attention has been given to the biological properties of bixin and norbixin, particularly to their antioxidant and anticarcinogenic potential.

Carotenoids have always been believed to have anticancer properties until results from the ATBC (α-tocopherol β-carotene) study were published, showing that high doses of β-carotene supplements may increase the risk for lung cancer among smokers (Heinonen & Albanes, 1994; Omenn et al. 1996). Since then, several hundred studies about carotenoids have been published in

Abbreviations: BPB, bromophenol blue; DMEM, Dulbecco’s minimal essential medium; DMSO, dimethylsulphoxide; FBS, fetal bovine serum; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TCA, trichloroacetic acid.

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an effort to clarify their role in cancer prevention. However, the procarcinogenic effect of β-carotene (Paolini et al. 1999) seems to be restricted to this carotenoid and to a particular type of cancer (Nishino, 1998; Wang & Russell, 1999). The protective role of carotenoids against cancer emergence has been credited to their antioxidant properties, although antioxidant activity alone is not sufficient to inhibit the carcinogenic process (Bertram et al. 1991; Zhang et al. 1991, 1992). In a comparative study on the ability of different carotenoids to function as anticarcinogenic substances, bixin showed negative results, in contrast to α- and β-carotene and canthaxanthin (Bertram et al. 1991). In addition, bixin did not show any substantial antimutagenic potential when assayed in the Salmonella assay (Rauscher et al. 1998). On the other hand, it has been recently demonstrated that dietary bixin is able to enhance cytochrome P450 enzyme activity in different tissues (Jewell & O'Brien, 1999), raising the possibility that this food colorant might influence carcinogenic events through modulation of the activities of some of the xenobiotic metabolizing enzymes. As an antioxidant, bixin has confirmed activity as a quencher of O2·- and as a scavenger of O3·-, peroxy nitrite and ‘OH (Dimascio et al. 1990; Zhao et al. 1998).

Although bixin is by far the predominant pigment present in annatto extract, a recent study indicated that there is a considerable conversion of bixin into norbixin in the intestinal tract and in the bloodstream, after ingestion of a single dose of annatto food color (Levy et al. 1997). As the antioxidant potential of norbixin has been insufficiently explored, the present work was designed to evaluate its biochemical behaviour under conditions of DNA damage induced by different generation systems of reactive oxygen species (ROS). The ROS-scavenging activity of norbixin was evaluated in a cell-free system of DNA damage composed of Sn2+ or Fe2+ and H2O2 as oxidizing agents and plasmid DNA as targeting molecule. In addition, the antimutagenic and antigenotoxic potentials of norbixin were also evaluated in Salmonella typhimurium and in murine fibroblasts respectively submitted to H2O2-induced oxidative stress.

### Materials and methods

#### Chemicals

Dulbecco’s minimal essential medium (DMEM), penicillin, streptomycin, trypsin, Triton X-100, SnCl2·2 H2O, Fe(NH4)2(SO4)2·6 H2O and bromophenol blue (BPB), were purchased from Sigma (St Louis, MO, USA). Fetal bovine serum (FBS) was from Cultilab (Campinas, Brazil). Ethidium bromide was supplied by Serva (Heidelberg, Germany). Ca2+- and Mg2+-free Hanks Balanced Salt Solution, dimethylsulphoxide (DMSO), EDTA acid and agarose were from GibcoBRL (Gaithersburg, MD). H2O2 (Perhidrol 30 %) was from Merck (Brazil). All other reagents were of the highest purity purchased from Merck (Brazil).

### Isolation of bixin and its chemical conversion to norbixin

Fresh seeds were collected from the annatto bush (Ilheus, Bahia, Brazil) and stored in the dark, at room temperature, until used. Bixin was purified by the following procedure. Pigments present in the pericarp of the annatto seeds were extracted two to three times with three volumes of CH3CH2OH:H2O (93:7, v/v), at 37°C for several hours, with vigorous shaking. Most of the extracted bixin molecules were thus rendered insoluble by the extraction solution. The insoluble bixin was separated from gross particulate material by filtration through gauze, followed by filtration through filter paper. The bixin retained on the filter paper was removed and washed exhaustively by being stirred in hexane, to eliminate contaminant molecules. Residual hexane was evaporated at 50°C, leaving purified bixin in a crystallized form. By this protocol, the final yield of bixin ranged between 1 and 2 g for each 100 g of annatto seeds. To prepare the water-soluble norbixin, bixin was saponified by agitation in aqueous NaOH solution (bixin:NaOH, 1:3 (mol/mol)) for several hours at 37°C. Samples of the saponification reaction were taken for analysis by HPLC until the conversion of bixin to norbixin was close to 100 %. Water was evaporated to dryness at 50°C and crystallized norbixin was kept at −20°C, until further use. Bixin and norbixin were identified by spectrophotometry (by maximum absorption in CHCl3 and water respectively). The u.v. visible spectra were recorded on a Shimadzu UV-160A spectrophotometer (Tokyo, Japan). Analyses of the carotenoids were performed on a Shimadzu HPLC system, equipped with SCL-10A system controller, LC-10AD pump set at a flow rate of 1.0 ml/min, a 7125 syringe-loading injection port with a 20 μl loop, a Shimadzu SPD-10AV UV-VIS detector set at 470 nm, a C-R6A Chromatopac integrator and a Supelco LC-8 (10 μl) column (250 × 4.6 mm I.D.). The mobile phase consisted of CH3CN–0.08 % CF3CO2H at a ratio of 85:15 (v/v).

### Oxidative damage of plasmid DNA by stannous or ferrous iron ions

The procedure described by Dantas et al. (1999) was used, with minor modifications. Briefly, 500 μg of plasmid DNA (pZEM3-EJ-Ras, a recombinant DNA containing the entire open reading frame of human EJ-ras oncogene subcloned in the pZEM3 vector; unpublished construct) was treated for 30 min, at room temperature, with either 50 μM-SnCl2 or 50 μM-Fe(NH4)2(SO4)2, in the absence or presence of 50 μM-H2O2, in a total volume of 20 μl. Stock solutions of 1 M SnCl2 and 0.1 M (Fe(NH4)2(SO4)2 were freshly prepared in HCl 2.4 N and HCl 0.05 N respectively. Different amounts of norbixin (up to 2 mM) in water were added to the oxidation reaction. After oxidative treatment, DNA was immediately submitted to electrophoresis in 0.8 % agarose gel, in 1 x TAE buffer (40 mM-Tris-acetate, 1 mM-EDTA), followed by ethidium bromide staining (0.5 μg/ml) and visualization by u.v. transilumination. The resulting single- and double-stranded breaks (relaxed and linear forms respectively) as well as the residual supercoiled form were subsequently estimated by
gel densitometry. Images were captured and analysed using the EDAS 120 system from Kodak. Results from four to six individual DNA oxidative reactions were used for the calculation of the densitometric data.

**Transformation efficiency of Escherichia coli cells by plasmid DNA**

*Escherichia coli* K12 strain AB1157 (wild-type to DNA repair lesions) competent cells were transformed with 125 ng of plasmid DNA treated as described above, and the number of resulting colonies was evaluated 24 h later. Bacterial competence induction followed the procedure described by Nishimura *et al.* (1990). Bacterial transformation was performed using the standard procedure described by Sambrook *et al.* (1989).

**Antimitagenticity assay**

The *Salmonella typhimurium* strain TA102 was used in this assay. The experimental protocol described by Ames *et al.* (1995) was adopted, with minor modifications (Gomes *et al.* 1995). Briefly, 100 μl of exponentially growing bacterial cells (10⁹ cells/ml) were incubated with 50 μl of different amounts of norbixin in water (up to 5 mg/plate), 50 μl of H₂O₂ (200 μg) and 500 μl of 0.1 mM-sodium phosphate buffer, pH 7.4, for 30 min, at 30°C, with shaking. At the end of incubation, 2 ml of top-agar (46°C) was added and the mixture poured onto minimal glucose agar plates. After end of incubation, 2 ml of top-agar (46°C) was added and the mixture poured onto minimal glucose agar plates. After end of incubation, 2 ml of top-agar (46°C) was added and the mixture poured onto minimal glucose agar plates. After end of incubation, 2 ml of top-agar (46°C) was added and the mixture poured onto minimal glucose agar plates. After end of incubation. Bacterial competence induction followed the procedure described by Nishimura *et al.* (1990). Bacterial transformation was performed using the standard procedure described by Sambrook *et al.* (1989).

**Mammalian cell culture and cytotoxic assays**

The cytotoxicity of norbixin was evaluated by determining the growth kinetics and DNA synthesis of Balb/c 3T3 fibroblasts (clone A31, ATCC CCL 163), in the presence of this carotenoid. Cells were routinely grown in DMEM supplemented with 10 % FBS and antibiotics (penicillin and streptomycin), and maintained in 10 % CO₂ at 37°C, using standard cell culture techniques. For the determination of growth kinetics, cells were plated onto 35 mm diameter culture dishes (Nunc) in DMEM–10 % FBS (6 × 10⁴ cells/dish, in duplicate) and 24 h later growth medium was renewed and norbixin added at different concentrations (up to 250 μM). Adherent cells were collected daily by *in situ* fixation with 5 % trichloroacetic acid (TCA) and their number determined by protein staining with PB. For this, fixed cells were stained for 30 min with 1 % BPB in 1 % acetic acid, rinsed quickly three times with water and stain extracted for 15 min with 10 mM-unbuffered Tris base. The absorbance of extracted PB was determined at 570 nm (Microplate reader, BioRad, Hercules, CA, USA).

To determine DNA synthesis, cells were plated onto a 24-well multidish (Nunc), 2 × 10⁴ cells/well, in DMEM/10 % FBS, and 24 h later treated with norbixin as described above. DNA synthesis was evaluated by ³H-methylthymidine incorporation. ³H-TdR was added after 36 h of treatment and cells collected 12 h later, by 5 % TCA fixation. Cells were solubilized with 200 μl of 0.2 N NaOH, absorbed into 3 MM paper, and macromolecules fixed with 5 % TCA. Radioactivity was determined by liquid scintillation counting (Beckman LS-6500 Scintillation System, Palo Alto, CA, USA).

**Oxidative stress induction on Balb/c 3T3 fibroblasts**

Oxidative stress was induced in A31 fibroblasts by H₂O₂ treatment. For this, cells in complete medium were plated onto a 6-well multidish (Nunc), 3–4 × 10⁴ cells/well, and 24 h later cells were treated with norbixin under low or high serum conditions. Norbixin (up to 450 μM) was added in DMEM supplemented with either 2 % or 10 % FBS, and 2 h later, cells were challenged *in situ* with H₂O₂ (10 μM or 25 μM), for 30 min, at 37°C. To verify DNA damage, cells were processed for the comet assay, immediately after H₂O₂ treatment. For this, adherent fibroblasts were harvested by trypsin–EDTA treatment, centrifuged for 3 min at 1000 g, and re-suspended in ice-cold DMEM–10 % FBS.

**Single-cell gel electrophoresis (alkaline comet assay)**

To detect single- and double-stranded DNA breaks (Fairbairn *et al.*, 1995), 10 μl of the cell suspensions was mixed with 120 μl of 0.5 % low-melting-temperature agarose in phosphate-buffered saline (PBS) and added to microscope slides precoated with 1–5 % normal-melting-temperature agarose in PBS. Slides were covered with a microscope coverslip and refrigerated for 5 min to gel, followed by immersion in alkaline lysing solution (2.5 M NaCl, 10 mM-Tris, 100 mM-EDTA, 1 % DMSO, 1 % Triton X-100, final pH >10.0), for at least 1 h. Slides were then incubated for 20 min in ice-cold electrophoresis solution (0.2 M NaOH, 1 mM-EDTA), followed by electrophoresis at 25 V/300 mA, for 25 min. After electrophoresis, slides were rinsed with water, allowed to dry at 37°C and stained with 20 μg/ml ethidium bromide. The DNA of individual cells was viewed using an epifluorescence microscope (Olympus), with 516–560 nm emission from a 50 W mercury light source, and quantitated as described.

**Quantitation of DNA lesions**

Quantitation of DNA breakage was achieved by visual scoring of fifty randomly selected cells per slide, classifying them into five categories representing each different degree of damage, ranging from no comet (type 0, undamaged cells) to maximum length comet (type 4, maximally damaged cells). Comets of type 1 are representative of cells with a minimal detectable frequency of DNA lesions, while comets of types 2 and 3 are representative of cells with moderately low to moderately high frequency of DNA lesions respectively. Slides were analysed by investigators blinded to the experimental conditions used for the treatment of fibroblasts from which the samples were prepared. A score of total damage was arbitrarily assigned for each treatment by multiplying the number of cells...
allocated to each category of DNA damage by the numeric value of the corresponding category and summing over all categories, giving a maximum possible score of 200. Results from three to five independent experiments were pooled and the mean and standard error calculated. Statistical analysis was performed by Student’s t test.

**Results**

**Norbixin isolation from Bixa orellana seeds**

Norbixin was isolated from annatto seeds by an indirect method (see Materials and methods, p. 432). The typical absorption spectra of bixin in chloroform and of norbixin in water are shown in Fig. 1(a and b), with maximum absorptivity at 471 and 502 nm for bixin, and at 453 and 482 nm for norbixin. The grade of purity by HPLC analysis was greater than 98 % (Fig. 1c).

**Influence of norbixin on the oxidative damage of plasmid DNA by metal ions and hydrogen peroxide**

Plasmid pZEM3-EJ-Ras was incubated with either 50 μM Sn²⁺ or 50 μM Fe²⁺, without and with 50 μM H₂O₂, and the resulting DNA oxidative damage estimated by gel electrophoresis. As shown in Figs. 2 and 3, in the absence of H₂O₂, Sn²⁺ (Figs. 2a and 3a) was a much stronger oxidant than Fe²⁺ (Figs. 2b and 3b). Sn²⁺ introduced numerous breaks into DNA strands that occasioned a final 30 % reduction in the total amount of DNA recovered at the end of the reaction (Figs. 2a and 3a). DNA damage induced by this metal ion was not considerably enhanced by the addition of H₂O₂ (Figs. 2c and 3c), although an additional loss of 25 % in total DNA was observed. On the other hand, Fe²⁺ in the absence of H₂O₂ introduced relatively few single- and double-stranded breaks into DNA (Figs. 2b and 3b). In contrast, Fe²⁺ plus H₂O₂ induced a full oxidative degradation of the plasmid DNA (Figs. 2d and 3d). The addition of increasing amounts of norbixin (up to 2 mM) to any of the four oxidative reactions conferred a concomitant protection on DNA against oxidative strand breakage (Figs. 2 and 3). Norbixin started to decrease the formation of the relaxed and open DNA forms at a metal:norbixin ratio of 1:1 (Sn²⁺) and 1:10 (Fe²⁺). However, oxidative DNA damage induced by Sn²⁺ (in the absence of H₂O₂) was significantly enhanced by norbixin concentrations below 100 μM (P < 0.05) (Fig. 3a), as demonstrated by the appearance of increasing amounts of DNA fragments with different lengths (Fig. 2a). This pro-oxidant behaviour of norbixin was also confirmed by estimating the ability of the SnCl₂-treated DNA plasmid to transform E. coli AB1157 competent cells, as shown in Fig. 4. There was a significant reduction in the final number of transformants induced by DNA treated with SnCl₂ and low amounts of norbixin (up to 100 μM), when compared to DNA treated with SnCl₂ only. Norbixin concentrations above 100 μM, however, induced a progressive protection of the DNA transformation activity against the damaging effects of SnCl₂.

**Evaluation of the antimutagenic potential of norbixin by the Salmonella assay**

The study was performed to evaluate whether norbixin could inhibit the mutagenicity induced by H₂O₂ in the Salmonella typhimurium strain TA102, as this strain is particularly responsive to oxidative and alkylating mutagens and detects active forms of oxygen. The results shown in Fig. 5 indicate that norbixin indeed has the ability to act as an antimutagenic factor, with a maximum inhibition of H₂O₂-induced mutagenic activity of 87 %. By omitting H₂O₂, the mutagenic activity of norbixin was also tested. As shown in Fig. 5, no mutagenic activity could be detected for norbixin.

**Influence of norbixin on the genotoxic effect of hydrogen peroxide in fibroblasts**

Balb/c fibroblasts were initially evaluated for a possible cytotoxic effect of norbixin under conditions of physiologically controlled cell growth. Exponentially growing fibroblasts were submitted for 4 d to different concentrations of norbixin in DMEM–10 % FBS, and the resulting growth kinetics are shown in Fig. 6a. No lethal effects were observed, with norbixin preferentially exerting a cytostatic...
effect on Balb/c 3T3 fibroblasts, with an IC\textsubscript{50} of 150 \SI{}{\mu M} (Fig. 6b).

To test the antigenotoxic potential of norbixin, Balb/c fibroblasts were treated in the short term with different amounts of norbixin, followed by subsequent \textit{H}\textsubscript{2}O\textsubscript{2} exposure and DNA breakage evaluation by the comet assay. As shown in Fig. 7 (tracks 2 to 5), none of the tested norbixin concentrations induced any significant genotoxic effect, with most of the treated cells displaying undetectable DNA breakage (comet type 0), as also observed in untreated cells (Fig. 7, track 1). Under low serum conditions (2 \% FBS), a 30 min treatment with 10 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2} caused lethal oxidative damage (grade 4) in 42 \% of the cells (Fig. 7, track 6), reaching an arbitrary damage score of 138.5 (from a possible maximum of 200) (Table 1). When the same oxidative treatment was applied to fibroblasts previously treated for 2 h with norbixin (10 to 450 \SI{}{\mu M}), a dual effect was observed. Norbixin concentrations up to 50 \SI{}{\mu M} were able to reduce the oxidative DNA breakage moderately by 41 \% (10 \SI{}{\mu M}) and 21 \% (50 \SI{}{\mu M}) (Fig. 7, tracks 7 and 8; Table 1). On the other hand, \textit{H}\textsubscript{2}O\textsubscript{2}-induced DNA breakage was markedly enhanced by norbixin concentrations above 50 \SI{}{\mu M} (\textit{P} < 0.05), with 62 \% and 83 \% of the cells reaching grade 4 of oxidative damage after treatment with 150 \SI{}{\mu M} and 450 \SI{}{\mu M} norbixin respectively (Fig. 7, tracks 9 and 10). Under conditions of more stringent oxidative damage (25 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}), after which 90 \% of the cells were lethally affected (Fig. 7, track 11).

Table 1. Effect of norbixin (nBix) against oxidative DNA damage induced by \textit{H}\textsubscript{2}O\textsubscript{2} in fibroblasts, under low (2 \% fetal bovine serum (FBS)) and high (10 \% FBS serum conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low serum conditions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}</td>
<td>24.8</td>
<td>3.9</td>
</tr>
<tr>
<td>10 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+10 \SI{}{\mu M} nBix</td>
<td>138.5</td>
<td>15.7</td>
</tr>
<tr>
<td>10 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+50 \SI{}{\mu M} nBix</td>
<td>81.7</td>
<td>15.7</td>
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<tr>
<td>10 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+150 \SI{}{\mu M} nBix</td>
<td>109.2</td>
<td>24.8</td>
</tr>
<tr>
<td>10 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+450 \SI{}{\mu M} nBix</td>
<td>154.9</td>
<td>12.9</td>
</tr>
<tr>
<td>10 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+150 \SI{}{\mu M} nBix</td>
<td>184.0</td>
<td>7.4</td>
</tr>
<tr>
<td>25 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}</td>
<td>183.3</td>
<td>15.7</td>
</tr>
<tr>
<td>25 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+10 \SI{}{\mu M} nBix</td>
<td>126.5</td>
<td>8.5</td>
</tr>
<tr>
<td>25 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+50 \SI{}{\mu M} nBix</td>
<td>165.0</td>
<td>10.1</td>
</tr>
<tr>
<td>25 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+150 \SI{}{\mu M} nBix</td>
<td>173.0</td>
<td>6.4</td>
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<tr>
<td>25 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+450 \SI{}{\mu M} nBix</td>
<td>183.0</td>
<td>11.5</td>
</tr>
<tr>
<td>High serum conditions</td>
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<tr>
<td>25 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}</td>
<td>8.5</td>
<td>0.5</td>
</tr>
<tr>
<td>25 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+10 \SI{}{\mu M} nBix</td>
<td>131.3</td>
<td>16.0</td>
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<td>25 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+50 \SI{}{\mu M} nBix</td>
<td>159.5</td>
<td>5.5</td>
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<tr>
<td>25 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+150 \SI{}{\mu M} nBix</td>
<td>121.7</td>
<td>12.0</td>
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<tr>
<td>25 \SI{}{\mu M} \textit{H}\textsubscript{2}O\textsubscript{2}+450 \SI{}{\mu M} nBix</td>
<td>121.3</td>
<td>26.4</td>
</tr>
</tbody>
</table>

Total score was calculated as described in Materials and methods. Statistical analyses were performed by Student’s \textit{t} test.

* \textit{P} < 0.05.
Fig. 3. Gel densitometric analysis of single- and double-strand breaks. Results from 4–6 DNA oxidative reactions (Fig. 2) were used for the calculation of the densitometric data. Statistical analyses (Student’s t test) were performed by comparing the mean ± SEM of each control DNA form (oxidative reactions in the absence of norbixin) to the mean ± SEM of the experimental DNA form (oxidative reactions in the presence of norbixin). Images were captured and analysed using the EDAS 120 system from Kodak. Open circles, total; filled circles, relaxed linear forms; triangles, supercoiled form. a, Sn²⁺; b, Fe²⁺; c, Sn²⁺ + H₂O₂; d, Fe²⁺ + H₂O₂. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Fig. 4. Transformation ability of plasmid DNA after SnCl₂ treatment in the absence or presence of norbixin (nBix). Escherichia coli AB1157 competent cells were transformed with 125 ng of plasmid DNA treated as described in Fig. 2.

Fig. 5. Inhibition of the H₂O₂ mutagenic activity by norbixin in Salmonella typhimurium strain TA102. S. typhimurium cells were incubated with different amounts of norbixin (1–5 mg/plate) in the presence (filled circles) or absence (open circles) of 200 μg of H₂O₂, and processed as described in Materials and methods.
Results were similar to those observed under milder DNA oxidative conditions (10 mM H2O2). Similar experiments demonstrated by the complete oxidative destruction of DNA that was not observed when Fe2+ was used as oxidant (Fig. 2, c and d; Fig. 3, c and d). As Sn2+ and Fe2+ have different oxidative strengths on DNA, this could indicate that different oxygen radicals might be generated by each metal ion. At least for Sn2+, the identification of its mechanismic pathways will be important as these pathways largely determine the types of free radicals generated. It has been proposed that Sn2+ damages DNA by a Fenton-like chemistry (Caldeira-de-Araujo et al. 1996; Dantas et al. 1999; Yang et al. 1999; Liu & Jan, 2000) but hydrogen peroxide and transition metals such as Fe and Cu have been implicated in the generation of oxygen radicals, particularly hydroxyl radicals (via the Fenton reaction), which seriously damage DNA. Oxidative damage to DNA results in oxidized purine and pyrimidine bases and gross DNA changes such as strand breaks (Henle & Linn, 1997). Heavy metals such as Cd, Sn and Pb also induce oxidative damage to DNA (Caldeira-de-Araujo et al. 1996; Dantas et al. 1999; Yang et al. 1999; Liu & Jan, 2000) but the mechanisms involved are not well understood. By using a DNA plasmid as the target molecule and Sn2+ or Fe2+ ions as oxidants, we evaluated the capacity of norbixin to protect DNA against single- and double-strand breaks (Figs. 2 and 3). As visualized by gel electrophoresis, norbixin conferred a dose-related DNA protection against oxidative damage induced by both metal ions. However, Sn2+ ion was a much stronger oxidant than Fe2+ ion, as demonstrated by the complete oxidative destruction of DNA that was not observed when Fe2+ was used as oxidant (Fig. 2, a–b; Fig. 3, a–b), unless H2O2 was also present. Nevertheless, norbixin counteracted the oxidative effects of Sn2+ more efficiently than those of Fe2+. At a ratio of norbixin:metal ion of 1:1 and of 10:1 respectively. Even in the presence of H2O2, which markedly enhanced Fe2+-induced DNA breakage without much changing the DNA breakage induced by Sn2+, norbixin concentrations above 50 μM still reduced the oxidative effects with similar effectiveness to those observed in the absence of H2O2 (Fig. 2, c and d; Fig. 3, c and d). As Sn2+ and Fe2+ have different oxidative strengths on DNA, this could indicate that different oxygen radicals might be generated by each metal ion. At least for Sn2+, the identification of its mechanismic pathways will be important as these pathways largely determine the types of free radicals generated. It has been proposed that Sn2+ damages DNA by a Fenton-like chemistry (Caldeira-de-Araujo et al. 1996; Dantas et al. 1999). On the other hand, the role of Fe2+ has long been recognized in generating DNA damage from H2O2, by functioning as a Fenton oxidant (Meneghini, 1997). Since the Fenton chemistry generates oxidative DNA breakage, and in fact there was a predisposition to enhance the H2O2-induced genotoxic effect (Fig. 7, tracks 18 to 21).

Discussion

Oxidative stress results from an imbalance between ROS-generation systems and cellular antioxidant defences that may result in severe damage to lipids, proteins and nucleic acids. In particular, oxidative damage to DNA may contribute to the initiation of cancer or be involved in several chronic inflammatory and degenerative conditions (Wiseman & Halliwell, 1996). The carotenoids bixin and norbixin are widely used in foods for the unique purpose of conferring colour and very little information is found on the metabolism of both in animal and human tissues. As carotenoids have the intrinsic property of ROS scavengers, we decided to conduct in vitro studies with the purpose of investigating whether norbixin would have the capacity to protect DNA against oxidative damage, a biological property that has not been so far tested for this carotenoid.
hydroxyl radicals, one of the most powerful ROS that reacts quickly with almost anything, it might be argued that norbixin protected DNA against oxidative damage simply by working as an optional target molecule for hydroxyl radicals. Recently, it has been demonstrated that bixin is able to scavenge hydroxyl radicals generated by a cell-free reaction system of Fe$^{2+}$ with H$_2$O$_2$ (Zhao et al. 1998), although the authors did not establish the reaction mechanism. In the case of norbixin, given that it contains two negative charges, its protective action might rely on the formation of a complex between the carotenoid and the metal ions. At least for Fe$^{2+}$, it is well established that only Fe$^{2+}$ bound to DNA will significantly react with H$_2$O$_2$ and generate hydroxyl radicals in situ (Henle & Linn, 1997; Meneghini, 1997). There are also indications that Sn$^{2+}$ might mediate oxidative DNA damage through bonding to this molecule (Dantas et al. 1999). Therefore, norbixin might have sequestered metal ions in solution and/or displaced bound metal from DNA, and by these mechanisms protected DNA against oxidative damage, with norbixin having a stronger affinity for Sn$^{2+}$ than for Fe$^{2+}$. Metal sequestration by norbixin has been also confirmed by spectrophotometric and HPLC analyses, and metal ions can be displaced by 2-5 M EDTA (data not shown). By these assays, no chemical alterations of norbixin have been detected after incubation with either Fe$^{2+}$ or Sn$^{2+}$. Recently, it has been demonstrated that the antioxidant activity of some polyphenols rely on Fe chelation activity rather than on ROS scavenging activity (Sestili et al. 1998; Lopes et al. 1999).

On the other hand, when norbixin and Sn$^{2+}$ were present at ratios below 1:1, a condition that favours the presence of free Sn$^{2+}$ over norbixin molecules, there was indeed an enhancement of the oxidant effects of Sn$^{2+}$ on DNA, particularly in the absence of H$_2$O$_2$ (Fig. 2a). Under these conditions, norbixin preferentially behaved as a pro-oxidant molecule. This has also been confirmed by the results obtained from the transformation efficiency of the plasmid DNA treated with SnCl$_2$ and different amounts of norbixin (Fig. 4). The mechanism involved in the pro-oxidant effect of norbixin under conditions of oxidative stress induced by metal ions such as Sn$^{2+}$ has not yet been identified. The pro-oxidant effect of norbixin was not observed in the presence of Fe$^{2+}$.

Since mutation events are required in carcinogenesis and other degenerative diseases, norbixin was also assayed for its potential to protect DNA against mutations induced by oxidative mutagens such as H$_2$O$_2$. In the Salmonella typhimurium assay, as little as 2 μmol norbixin was able to reduce the mutagenicity induced by 7 μmol H$_2$O$_2$ by 50 %, without any toxic side effect (Fig. 5). As bixin has no expressed inhibitory effect on the activity of several non-oxidative mutagens, in contrast to canthaxanthin, α- and β-carotene, lycopen and some other minor carotenoids (Rauscher et al. 1998), the antimutagenic effect of norbixin in Salmonella typhimurium strain TA102 seems specific to oxidizing mutagens. Since metal ions appear to be involved in the mutagenic effect of H$_2$O$_2$, the protective effect of norbixin against DNA mutagenicity might also be linked to its property of metal sequestration. The recent demonstration that bixin is able to influence the activity of several xenobiotic metabolizing enzymes on different rat tissues (Jewell & O’Brien, 1999) argues in favour of a putative in vivo modulation by norbixin of events belonging to the carcinogenesis and mutagenesis processes. Whether this enzymic modulation is mechanistically dependent on the antioxidant activity of bixin–norbixin remains to be clarified.
ROS-scavenging potential of norbixin

As norbixin demonstrated protective effects against DNA damage induced by oxygen radicals generated by metal ions and H₂O₂, studies were performed to evaluate how this property would be managed in a more complex cellular environment such as mammalian cells submitted to H₂O₂-induced oxidative stress. H₂O₂ is able to cross cell membranes and inside the cells it can react with Fe²⁺ or Cu²⁺ to form hydroxyl radicals via Fenton reactions (Nassil-Caló et al. 1989; Spencer et al. 1996; Meneghini, 1997). A 2 h pre-treatment of fibroblasts with different amounts of norbixin added to growth medium containing 2% FBS, either protected or enhanced DNA breakage induced by 10 μM H₂O₂, as detected by the alkaline version of the comet assay (Fig. 7). While low concentrations of norbixin (up to 50 μM) somewhat protected DNA against strand breaks, higher concentrations of norbixin concomitantly augmented the extent of DNA damage. Similar antioxidant–oxidative activities have been described for lycopene and β-carotene on adenocarcinoma cells (Lowe et al. 1999), and for β-carotene on hepatoma cells (Woods et al. 1999). These conflicting effects are not restricted to in vitro observations nor to carotenoid molecules, since recent studies on the major diet-derived antioxidants ascorbate and β-carotene indicated that both often increase DNA damage in humans, raising questions about their antioxidant roles (Carr & Frei, 1999; Halliwell, 1999a,b). Cumulative data show that several antioxidant molecules might work either as antioxidant or pro-oxidant, and that their final activity will depend on factors such as metal-reducing potential, chelating behaviour and solubility characteristics (Schartz, 1996; Decker, 1997). Both antioxidant and pro-oxidant effects of β-carotene, for example, seem to be strictly dependent on the oxygen tension (Burton & Ingold, 1984; Palozza et al. 1997). Low tensions of oxygen (up to 150 Torr) will favour the antioxidant effects of β-carotene while at higher oxygen partial pressures, pro-oxidant effects are preferentially exerted, particularly when high concentrations of β-carotene are present. No similar studies have been reported on bixin or norbixin.

In conclusion, our results demonstrated that norbixin has the potential to protect DNA against oxidative damage induced by H₂O₂ and metal ions. As hydroxyl radicals are so reactive that they cannot diffuse from their site of formation, those that damage DNA must be produced very close to the DNA. This raises the question of how norbixin is distributed within cells. Clarifying this question will help to elucidate the biochemical fate of norbixin during oxidative stress, particularly when maintenance of DNA integrity is involved. However, under non-physiological conditions, instead of interrupting the propagation sequence of the damaging oxidative signal, norbixin might generate conditions that amplify this signal unless some other antioxidant cellular defence comes into action.

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and beta-carotene protect against oxidative damage in HT29 cells at low concentrations but rapidly lose this capacity at higher doses. *Free Radical Research* **30**, 141–151.


