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 Sn^{2+} or Fe^{2+} as oxidant. The addition of H_2O_2 enhanced DNA breakage induced by metal ions, particularly Fe^{2+}. Under these conditions, norbixin started to protect plasmid DNA against single- and double-strand breakage at a metal:norbixin ratio of 1:1 (Sn^{2+}) and 1:10 (Fe^{2+}). However, at lower ratios to Sn^{2+}, norbixin enhanced Sn^{2+}-induced DNA breakage ($P < 0.05$). The ability of norbixin to protect genomic DNA against oxidative damage was assessed in murine fibroblasts submitted to H_2O_2-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 H_2O_2-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 H_2O_2. 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 ($C_{24}H_{28}O_4$) is an unusual dicarboxylic water-soluble 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 ($C_{25}H_{30}O_4$), 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 anti-cancer 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

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 cytocrome 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 $^1O_2$ and as a scavenger of O_2^-, peroxynitrite 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 Sn^{2+} or Fe^{2+} and H_2O_2 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 H_2O_2-induced oxidative stress.

Materials and methods

Chemicals

Dulbecco's minimal essential medium (DMEM), penicillin, streptomycin, trypsin, Triton X-100, $SnCl_2.2 H_2O$, $Fe(NH_4)_2(SO_4)_2.6 H_2O$ 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). Ca^{2+}- and Mg^{2+}-free Hanks Balanced Salt Solution, dimethylsulphoxide (DMSO), EDTA acid and agarose were from GibcoBRL (Gaithersburg, MD). H_2O_2 (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 $CH_3CH_2OH:H_2O$ (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 $CHCl_3$ 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 μM) column (250 × 4·6 mm I.D.). The mobile phase consisted of CH_3CN–0·08 % CF_3CO_2H 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-$SnCl_2$ or 50 μM-$Fe(NH_4)_2(SO_4)_2$, in the absence or presence of 50 μM-H_2O_2, in a total volume of 20 μl. Stock solutions of 1 M $SnCl_2$ and 0·1 M $(Fe(NH_4)_2(SO_4)_2$ 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 × TAE buffer (40 mM-Tris-acetate, 1 mM-EDTA), followed by ethidium bromide staining (0·5 μg/ml) and visualization by u.v. transillumination. 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).

Antimutagenicity 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 (10^9 cells/ml) were incubated with 50 µl of different amounts of norbixin in water (up to 5 mg/plate), 50 µl of H_2O_2 (200 µg) and 500 µl of 0·1 M-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 incubation for 72 h at 37°C, the number of reverting *his*⁺ bacteria colonies was scored. The assay was performed in triplicate and the data shown correspond to the mean of two independent determinations. The standard error of the mean did not exceed 15 %.

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 % CO_2 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 (6×10^4 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 BPB. 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 BPB was determined at 570 nm (Microplate reader, BioRad, Hercules, CA, USA).

To determine DNA synthesis, cells were plated onto a 24-well multidish (Nunc), 2×10^4 cells/well, in DMEM/ 10 % FBS, and 24 h later treated with norbixin as described above. DNA synthesis was evaluated by ³H-methylthymidine

(³H-TdR, 0·92 MBq/well (Amersham)) 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 H_2O_2 treatment. For this, cells in complete medium were plated onto a 6-well multidish (Nunc), $3–4 \times 10^4$ 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 H_2O_2 (10 µM or 25 µM), for 30 min, at 37°C. To verify DNA damage, cells were processed for the comet assay, immediately after H_2O_2 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, 10 % 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
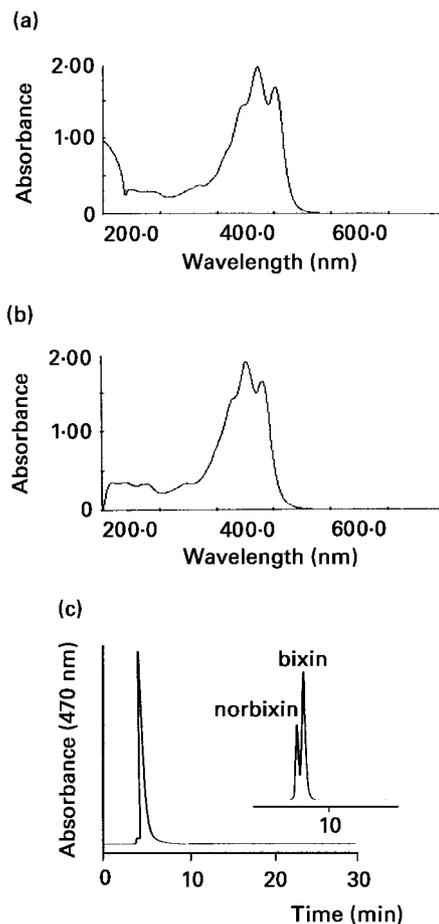
Fig. 1. Absorption spectra (a and b) and HPLC chromatograms (c and insert) of bixin (a and c) and norbixin (b and insert) after isolation from *Bixa orellana* seeds. (a) Bixin in chloroform; (b) Norbixin in water; (c) Bixin after purification from annatto seeds. Insert shows both bixin and norbixin in a sample taken from the saponification reaction.

Sn^{2+} or 50 μM Fe^{2+}, without and with 50 μM H_2O_2, and the resulting DNA oxidative damage estimated by gel electrophoresis. As shown in Figs. 2 and 3, in the absence of H_2O_2, Sn^{2+} (Figs. 2a and 3a) was a much stronger oxidant than Fe^{2+} (Figs. 2b and 3b). Sn^{2+} 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 H_2O_2 (Figs. 2c and 3c), although an additional loss of 25 % in total DNA was observed. On the other hand, Fe^{2+} in the absence of H_2O_2 introduced relatively few single- and double-stranded breaks into DNA (Figs. 2b and 3b). In contrast, Fe^{2+} plus H_2O_2 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 (Sn^{2+}) and 1:10 (Fe^{2+}). However, oxidative DNA damage induced by Sn^{2+} (in the absence of H_2O_2) 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 $SnCl_2$-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 $SnCl_2$ and low amounts of norbixin (up to 100 μM), when compared to DNA treated with $SnCl_2$ only. Norbixin concentrations above 100 μM, however, induced a progressive protection of the DNA transformation activity against the damaging effects of $SnCl_2$.

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 H_2O_2 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 H_2O_2-induced mutagenic activity of 87 %. By omitting H_2O_2, 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
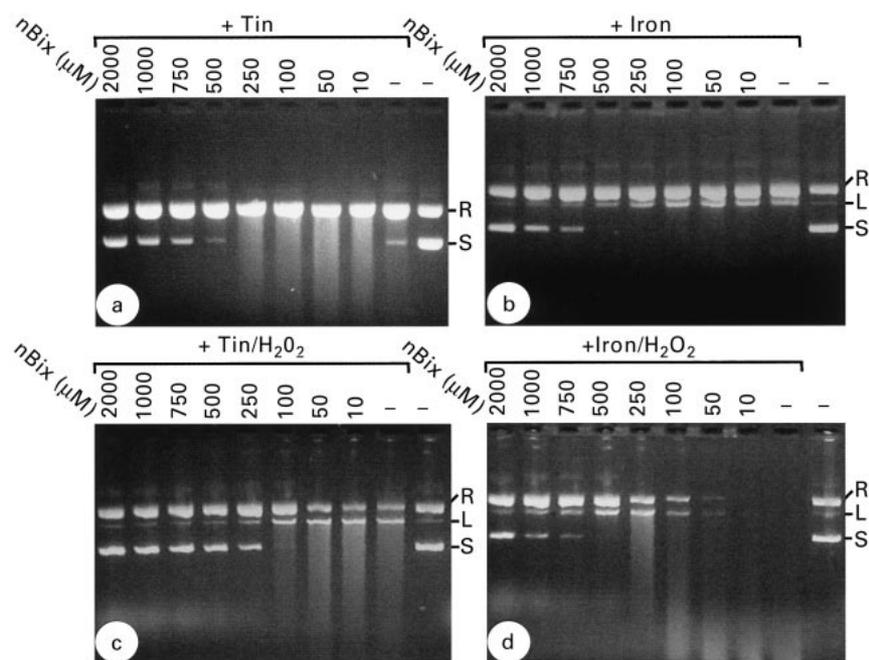
Fig. 2. Antioxidant evaluation of norbixin (nBix) by plasmid DNA as target molecule and stannous tin and ferrous iron as oxidants, in the presence and absence of H_2O_2: 500 μg of plasmid DNA (pZEM3-EJ-ras) was treated with either 50 μM $SnCl_2$ (a and c) or 50 μM $Fe(NH_4)_2(SO_4)_2$, in the absence (a and b) or in the presence (c and d) of 50 μM H_2O_2. Different concentrations of norbixin (10–2000 μM) were tested, and after 30 min incubation, reactions were submitted to gel electrophoresis. Results were evaluated by ethidium bromide staining and u.v. transillumination. Representative electrophoretograms are shown to illustrate each type of oxidative reaction. R, relaxed form; L, linear form; S, supercoiled form.

effect on Balb/c 3T3 fibroblasts, with an IC_{50} of 150 μM (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 H_2O_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 μM-H_2O_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 μM), a dual effect was observed. Norbixin concentrations up to 50 μM were able to reduce the oxidative DNA breakage moderately by 41 % (10 μM) and 21 % (50 μM) (Fig. 7, tracks 7 and 8; Table 1). On the other hand, H_2O_2-induced DNA breakage was markedly enhanced by norbixin concentrations above 50 μM ($P < 0.05$), with 62 % and 83 % of the cells reaching grade 4 of oxidative damage after treatment with 150 μM and 450 μM norbixin respectively (Fig. 7, tracks 9 and 10). Under conditions of more stringent oxidative damage (25 μM H_2O_2), after which 90 % of the cells were lethally affected (Fig. 7, track

Table 1. Effect of norbixin (nBix) against oxidative DNA damage induced by H_2O_2 in fibroblasts, under low (2 % fetal bovine serum (FBS)) and high (10 % FBS serum conditions

(Mean and standard error of the mean from 3–5 pooled independent experiments)

	Total score	
Treatment	Mean	SEM
Low serum conditions		
–	24·8	3·9
10 μM H_2O_2	138·5	15·7
10 μM H_2O_2+10 μM nBix	81·7	50·3
10 μM H_2O_2+50 μM nBix	109·2	24·8
10 μM H_2O_2+150 μM nBix	154·9	12·9[*]
10 μM H_2O_2+450 μM nBix	184·0	7·4[*]
25 μM H_2O_2	189·3	10·7
25 μM H_2O_2+10 μM nBix	126·5	8·5
25 μM H_2O_2+50 μM nBix	165·3	10·1
25 μM H_2O_2+150 μM nBix	173·0	6·4
25 μM H_2O_2+450 μM nBix	183·0	11·5
High serum conditions		
–	8·5	0·5
25 μM H_2O_2	113·5	16·0
25 μM H_2O_2+10 μM nBix	131·3	30·9
25 μM H_2O_2+50 μM nBix	159·5	5·5*
25 μM H_2O_2+150 μM nBix	121·7	12·0
25 μM H_2O_2+450 μM nBix	121·3	26·4

Total score was calculated as described in Materials and methods. Statistical analyses were performed by Student's t test.
* $P < 0.05$.
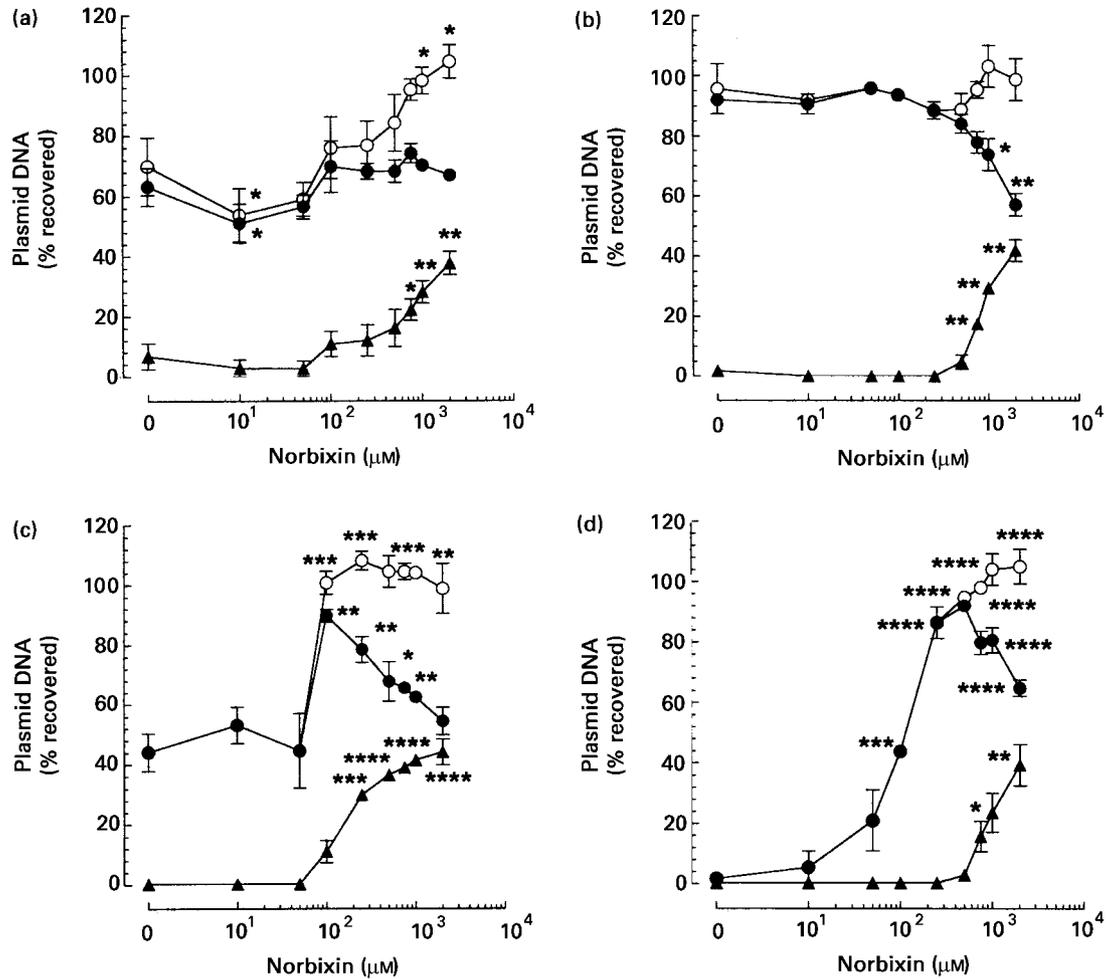
Fig. 3. Gel densitometric analysis of single- and double-strand breaks. Results from 4–6 DNA oxidative reactions (Fig. 2) were used for the calculation of the densitometric data. Statistical analyses (Student's *t* test) were performed by comparing the mean±SEM of each control DNA form (oxidative reactions 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, Sn^{2+}; b, Fe^{2+}; c, $Sn^{2+} + H_2O_2$; d, $Fe^{2+} + H_2O_2$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.
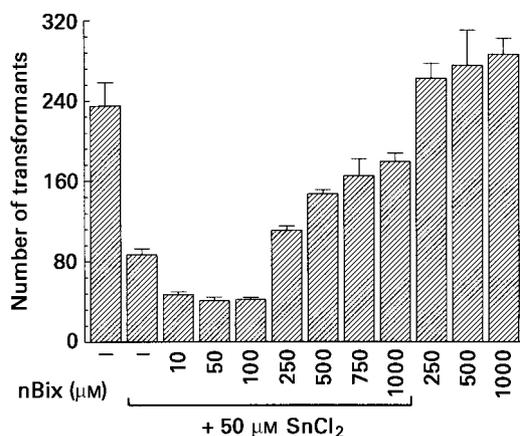
Fig. 4. Transformation ability of plasmid DNA after $SnCl_2$ treatment in the absence or presence of norbixin (nBix). *Escherichia coli* AB1157 competent cells were transformed with 125 ng of plasmid DNA treated as described in Fig. 2.
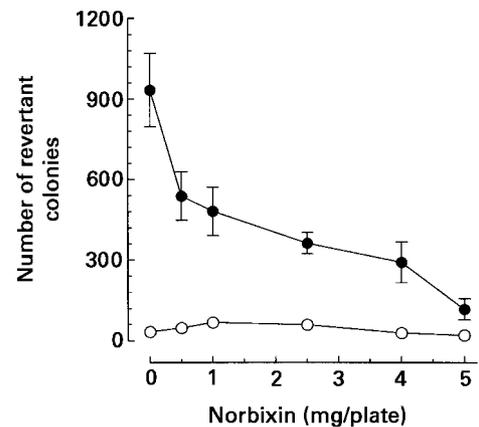
Fig. 5. Inhibition of the H_2O_2 mutagenic activity by norbixin in *Salmonella typhimurium* strain TA102. *S. typhimurium* cells were incubated with different amounts of norbixin (1–5 mg/plate) in the presence (filled circles) or absence (open circles) of 200 μg of H_2O_2, and processed as described in Materials and methods.
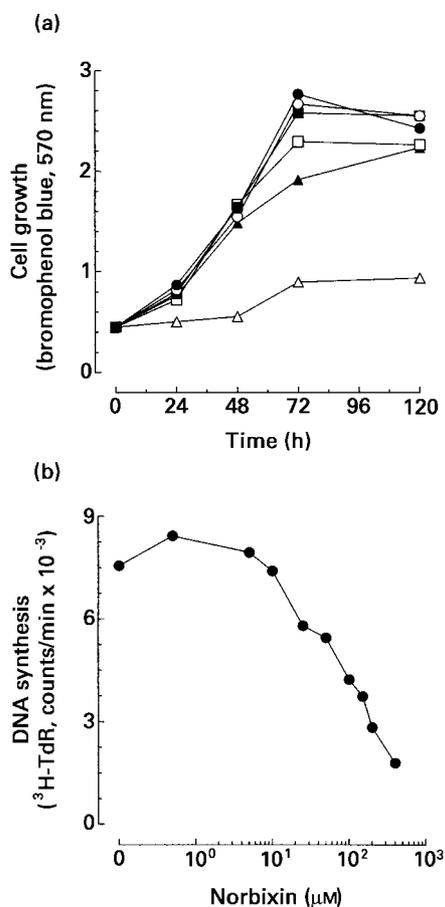
Fig. 6. Cytotoxic evaluation of norbixin in Balb/c 3T3 fibroblasts (clone A31) by growth kinetics (a) and by DNA synthesis (b) determinations. (a) Exponentially growing cells in Dulbecco's minimal essential medium (DMEM)−10 % fetal bovine serum (FBS) were grown for 4 d in the presence or absence of different concentrations of norbixin. At the indicated times, cells were collected by trichloracetic acid fixation and their number determined by staining with bromophenol blue, stain extracted with 10 mM Tris and absorbance read at 570 nm. Norbixin concentrations: filled circles, none; open circles, 10 μM; filled squares, 25 μM; open squares, 50 μM; filled triangles, 100 μM; open triangles, 250 μM. (b) Exponentially growing cells in DMEM−10 % FBS were treated with different concentrations of norbixin. At 36 h after treatment, ^{3}H-methylthymidine was added and cells collected 12 h later.

11; Table 1), the fibroblasts were still partially protected against DNA oxidative breakage (33 %), after previous treatment with 10 μM-norbixin (Fig. 7, track 12; Table 1). Higher norbixin concentrations did not allow a concomitant protective effect against 25 μM-H_2O_2 and the obtained results were similar to those observed under milder DNA oxidative conditions (10 μM-H_2O_2). Similar experiments were also performed in the presence of a high concentration of serum, under which the great majority of the cells showed an undamaged nucleus (98 %) (Fig. 7, track 16). At 10 % FBS, DNA breakage induced by 25 μM H_2O_2 was less deleterious than at 2 % FBS (Fig. 7, track 17 *v.* track 11), with 38 % of the cellular population showing lethal DNA breakage and an arbitrary damage score of 113·5 (Table 1). Under high serum conditions, norbixin (10–450 μM) was not able to product the fibroblasts against

oxidative DNA breakage, and in fact there was a predisposition to enhance the H_2O_2-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.

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 Sn^{2+} or Fe^{2+} 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, Sn^{2+} ion was a much stronger oxidant than Fe^{2+} ion, as demonstrated by the complete oxidative destruction of DNA that was not observed when Fe^{2+} was used as oxidant (Fig. 2, a–b; Fig. 3, a–b), unless H_2O_2 was also present. Nevertheless, norbixin counteracted the oxidative effects of Sn^{2+} more efficiently than those of Fe^{2+}, at a ratio of norbixin:metal ion of 1:1 and of 10:1 respectively. Even in the presence of H_2O_2, which markedly enhanced Fe^{2+}-induced DNA breakage without much changing the DNA breakage induced by Sn^{2+}, norbixin concentrations above 50 μM still reduced the oxidative effects with similar effectiveness to those observed in the absence of H_2O_2 (Fig. 2, c and d; Fig. 3, c and d). As Sn^{2+} and Fe^{2+} have different oxidative strengths on DNA, this could indicate that different oxygen radicals might be generated by each metal ion. At least for Sn^{2+}, the identification of its mechanistic pathways will be important as these pathways largely determine the types of free radicals generated. It has been proposed that Sn^{2+} damages DNA by a Fenton-like chemistry (Caldeira-de-Araujo *et al.* 1996; Dantas *et al.* 1999). On the other hand, the role of Fe^{2+} has long been recognized in generating DNA damage from H_2O_2, by functioning as a Fenton oxidant (Henle & Linn, 1997; Meneghini, 1997). Since the Fenton chemistry generates
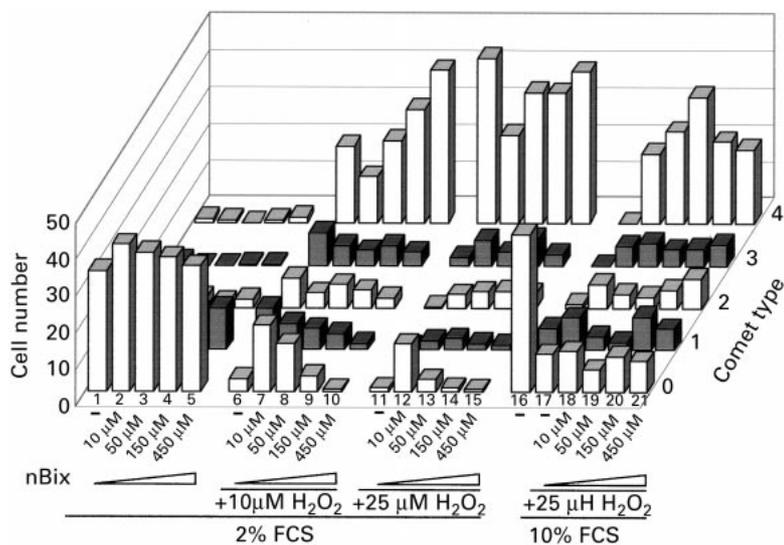
Fig. 7. Evaluation of DNA damage after H_2O_2-induced oxidative stress in Balb/c 3T3 fibroblasts pre-treated with norbixin (nBix) added to low- or high-serum medium. Exponentially growing fibroblasts were pre-treated for 2 h with norbixin (50, 150 or 450 μM) in Dulbecco's minimal essential medium (DMEM)−2 % fetal calf serum (FCS) or DMEM−10 % FCS, followed by 30 min treatment with 10 μM or 25 μM H_2O_2, at 37°C. H_2O_2 treatment was performed in the presence of norbixin, and DNA breakage was evaluated by the comet assay.

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_2O_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_2O_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+} that 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_2O_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_2O_2. In the *Salmonella typhimurium* assay, as little as 2 μmol norbixin was able to reduce the mutagenicity induced by 7 μmol H_2O_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_2O_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.

As norbixin demonstrated protective effects against DNA damage induced by oxygen radicals generated by metal ions and H_2O_2, studies were performed to evaluate how this property would be managed in a more complex cellular environment such as mammalian cells submitted to H_2O_2-induced oxidative stress. H_2O_2 is able to cross cell membranes and inside the cells it can react with Fe^{2+} or Cu^{2+} to form hydroxyl radicals via Fenton reactions (Nassi-Calo *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 H_2O_2, 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 antioxidative–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 H_2O_2 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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