Cancer Chemother Pharmacol (2010) 65:267–276
DOI 10.1007/s00280-009-1031-8

ORIGINAL ARTICLE

Celecoxib prevents tumor growth in an animal model by a COX-2 independent mechanism

Amanda Leite Bastos-Pereira · Daiana Lugarini · Adriana de Oliveira-Christo · Thiago Vinicius Ávila · Simone Teixeira · Amanda do Rocio Andrade Pires · Marcelo Nicolás Muscará · Sílvia Maria Suter Correia Cadena · Lucélia Donatti · Helena Cristina da Silva de Assis · Alexandra Acco

Received: 7 January 2009 / Accepted: 5 May 2009 / Published online: 9 June 2009
Springer-Verlag 2009

Abstract
Purpose Nonsteroidal antiinflammatory drugs (NSAIDs) have been shown to reduce cell growth in several tumors. Among these possible antineoplastic drugs are cyclooxygenase-2 (COX-2)-selective drugs, such as celecoxib, in which antitumoral mechanisms were evaluated in rats bearing Walker-256 (W256) tumor.
Methods W256 carcinosarcoma cells were inoculated subcutaneously (10^7 cells/rat) in rats submitted to treatment with celecoxib (25 mg kg^{-1}) or vehicle for 14 days. Tumor growth, body-weight gain, and survival data were evaluated. The mechanisms, such as COX-2 expression and activity, oxidative stress, by means of enzymes and lipoperoxidation levels, and apoptosis mediators were also investigated.
Results A reduction in tumor growth and an increased weight gain were observed. Celecoxib provided a higher incidence of survival compared with the control group. Cellular effects are probably COX-2 independent, because neither enzyme expression nor its activity, measured by tumoral PGE_2, showed significant difference between groups. It is probable that this antitumor action is dependent on an apoptotic way, which has been evaluated by the expression of the antiapoptotic protein Bcl-xL, in addition to the cellular changes observed by electronic microscopy. Celecoxib has also a possible involvement with redox homeostasis, because its administration caused significant changes in the activity of oxidative enzymes, such as catalase and superoxide dismutase.
Conclusion These results confirm the antitumor effects of celecoxib in W256 cancer model, contributing to elucidating its antitumoral mechanism and corroborating some recent literature about its effect on other types of cancer.

Keywords Cancer · Walker-256 · Celecoxib · Apoptosis · Oxidative stress · Rat

Abbreviations

CAT	Catalase
COX-2	Cyclooxygenase-2
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FDA	Food and Drug Administration
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
IP	Intraperitoneal
NSAID	Nonsteroidal antiinflammatory drug
$O_2^{\cdot}$	Superoxide anion
PGE_2	Prostaglandin E_2
PMSF	Phenylmethylsulfonyl fluoride
ptn	Protein

A. L. Bastos-Pereira · D. Lugarini · A. de Oliveira-Christo · T. V. Ávila · H. C. da Silva de Assis · A. Acco (✉)
Department of Pharmacology,
Federal University of Paraná, Biological Science Sector,
81530-990 Curitiba, Brazil
e-mail: aleacco@ufpr.br

S. Teixeira · M. N. Muscará
Department of Pharmacology, Biological Sciences Institute,
São Paulo University, São Paulo, Brazil

A. R. A. Pires · S. M. S. C. Cadena
Department of Biochemistry and Molecular Biology,
Federal University of Paraná, Biological Science Sector,
81530-990 Curitiba, Brazil

L. Donatti
Department of Cell Biology, Federal University of Paraná,
Biological Science Sector, 81530-990 Curitiba, Brazil

RNS Reactive nitrogen species
ROS Reactive oxygen species
SOD Superoxide-dismutase
TBS-t Tris-buffered saline Tween-20
W256 Walker-256 tumor

Introduction

Tumor cells have high viability and defense mechanisms against apoptosis. These characteristics contribute to its resistance to chemotherapic agents. For this reason, new and alternative therapies against cancer have been required. Celecoxib, a nonesteroidal antiinflamatory drug (NSAID) that potentially inhibit the cyclooxygenase (COX)-2 enzyme, has been approved by the Food and Drug Administration (FDA) in 1999 as a chemopreventive drug [1, 2]. It is an effective agent that can act at several stages of carcinogenesis [3]. Apart from carcinogenesis, celecoxib can also act on established tumors. Its possible antineoplastic mechanism involves blockade of both cellular growth and angiogenesis, stimulation of apoptosis, and induction of the immune response [2]. The mechanisms of these effects, in addition to the toxicity and dosage needed to attain them, are not yet well defined for the different types of cancer. Mechanisms of tumor inhibition that are both dependent on and independent of COX-2 have been proposed [4]. COX-2 is regulated in many types of tumor cells. The antineoplastic properties of NSAIDs have been proven to occur by this mechanism through epidemiologic studies in some experimental models. One of the most important COX-2 metabolites is prostaglandin E2 (PGE_2), which is produced in large quantities in some tumors, and can induce angiogenic factors in many tissues [5].

There is deliberation about COX-2-independent antineoplastic actions of celecoxib; one suggested the possibility being the antioxidant mechanism [6]. The involvements of reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implied in the pathogenic process of carcinogenesis. Another possibility is the drug's involvement with apoptosis, which is characterized by the expression of specific genes that increase the apoptotic process (Bax, Bcl-x) and other genes that inhibit cellular death (Bcl-2 and *Bcl-xL*) [7].

W256 is a fast-growing carcinosarcoma, and its implantation in rats has been considered an appropriate model for studying the syndrome of cachexia and anticancer treatments because it is specie-specific and easily transplantable [8]. Within a short time after its inoculation, there is a reduction in body weight, anorexia and difficulty in the catabolism of proteins, carbohydrates, and lipids. Fourteen days after implant, the tumor mass may represent a considerable fraction of the body weight of the animal, and its death may occur right after (or at) this period [9].

The objective of this report is to evaluate the antineoplasic mechanisms of celecoxib using rats bearing the W256 model of cancer. The incidence of cancer is high and so is the morbidity and mortality associated with it; therefore, new therapies to combat it are constantly needed. This is why information about the cellular effects of celecoxib in neoplasms assumes importance, regardless of the real potential of this drug as an anticancer agent.

Materials and methods

Animals

Male Wistar rats (200–300 g) were obtained from the Central Animal House of the Federal University of Paraná (PR, Brazil). The animals were housed at 22 ± 1°C under a 12-h light–dark cycle, and had free access to standard laboratory food (Purina®) and tap water. The experiments were conducted following the recommendations of the Brazilian Law 6638, 05/11/1979 for the scientific management of animals, and the procedures were approved by the Institutional Animal Ethics Committee (CEEA-UFPR, certificate number 153).

Cell maintenance

The maintenance of W256 cells was carried out by weekly passages achieved by intraperitoneal (IP) inoculation. The cells were maintained aseptically in saline buffer of pH 7.4. After five to seven days of growth in the ascitic fluid, the liquid collected was centrifuged for 10 min at 1,126*g*, at a temperature of 4°C. The supernatant was discarded, and the precipitate was suspended in 1.0 ml of PBS buffer (16.5 mM phosphate, 137 mM NaCl, 2.7 mM KCl), and subsequently the viability of tumor cells was checked by the Trypan blue-exclusion method in a Neubauer camera.

For use in experimental animals, the cells of carcinosarcoma were injected subcutaneously (10^7 cells per rat), in the right pelvic limb of animals. The treatments began one day after implant and continued for 14 days. Celecoxib (Celebra®—Pfizer Laboratory) was dissolved in Tris buffer (pH 8.6) and administered by gavage in dose of 25 mg kg^{-1} daily. The Control group animals received only Tris buffer. For some parameters, another group (baseline) was added, which was composed of individuals not inoculated with tumor and treated only with Tris buffer.

Animals undergoing treatment had their body weight checked daily. The weight of the tumor at the end of the period of treatment was measured in an analytical balance to assess the actual change in weight during the treatment

period. The volume of the tumor was calculated by measuring the diameters (with a ruler), according to the description of Mizuno et al. [10].

In addition to observing and weighing, the tumor samples were collected and stored in a freezer at −70°C for further analyses. Samples of liver were also submitted to the same procedure for such purposes.

Survival rate

An individual treatment group slightly different from that previously described was used to assess the survival rate of animals, with the objective of comparing the mortality of the celecoxib-treated animals with those left untreated, to check the viability of the tumor cells used in the W256 experiment, and to assess whether the animals were responding homogeneously to the experimental model. The subcutaneous inoculation and the beginning of treatment were carried out as previously described, with seven animals per group. However, the treatment time was carried out for 30 days instead of 14 days. Animals that died during this period were counted. At the end of 30 days, the animals that survived underwent euthanasia, with the use of thiopental for anesthesia followed by beheading.

Isolation of rat liver mitochondria

The animals were starved for 12 h before being killed by decapitation. Mitochondria were immediately isolated from the rat liver by differential centrifugation [11], using an extraction medium consisting of 250 mM D-mannitol, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid-potassium hydroxide (better known as HEPES-KOH, pH 7.2), 1 mM ethylene glycol tetraacetic acid, and 0.1 g% bovine serum albumin. Disrupted mitochondria, obtained by a freeze-thaw treatment, were used to determine the activities of the enzymes NADH oxidase and succinate oxidase.

COX-2 expression

Western blotting was used to verify possible changes in the COX-2 enzyme expression in both tumor and liver. Samples of tissue (tumor or liver) were weighed and homogenized in 50 mM Tris–HCl buffer, containing 1 mM PMSF, a serine–protease inhibitor. Subsequently, samples were centrifuged in an Eppendorf centrifuge model 2412 (Eppendorf, California, USA) at 1,000*g* for 5 min, and the supernatants were used for COX-2 analysis. Total protein concentration in tissue homogenates was determined by the Bradford method [12]. The primary antibody used was anti-COX-2 (mice polyclonal IgG—Santa Cruz Laboratory), at a dilution of 1,000 ng ml^{-1} in TBS-t, applied for 16 h. The secondary antibody used was the antimouse antibody conjugated with alkaline phosphatase at a 1:3000 dilution in TBS-t, incubated for two hours. The membranes were then washed with the same buffer and subjected to visualization of the immunoreactive bands. The bands detected by chemiluminescence were captured in a ChemiImager-5500 system (Alpha Innotech Corporation, California). From these images, the bands were analyzed by densitometry.

Quantification of prostaglandin E2

Determination of PGE_2 concentration was carried out using an enzyme-linked immunosorbent assay kit following the manufacturer's instructions (Cayman Chemical, USA). The tumor tissue samples were collected after euthanasia with thiopental and stored as instructed in the kit.

Oxidative damage

In vitro free radical-scavenging activity (DPPH)

The reactivity of celecoxib (concentrations of 10–200 μg ml^{-1}) with the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined according to the method described by Chen et al. [13], with some modifications. The system consisted of 750 μl of the test solution (celecoxib) and 250 μl of a methanolic solution of DPPH (1 mg in 25 ml). After 5 min, the decrease of absorbance was measured. A solution of the reducing agent, ascorbic acid (50 μg ml^{-1}), was used as the positive control and distilled water was used as the negative control.

Determination of catalase, superoxide dismutase, and glutathione-S-transferase activities

For biochemical analyses of these enzymes, liver and tumor samples were homogenized in phosphate buffer pH 6.5. Catalase activity was measured according to the procedure described by Aebi [14]. The reaction was monitored for 60 s for liver samples and for 90 s for tumoral tissue, at 240 nm in a spectrophotometer Model Ultrospec 4300 Pro (Amersham Biosciences).

Superoxide dismutase (SOD) activity was measured by the ability of this enzyme to inhibit pyrogallol autooxidation [15]. The reaction was monitored in a microplate reader Sunrise Remote model (Tecan Deutschland GMBH) at 440 nm. The amount of enzyme that inhibited the reaction by 50% (IC_{50}) was defined as one unit of SOD, and the enzyme activity was expressed in units of SOD per milligram of total protein (U SOD mg ptn^{-1}).

GST activity was measured following the method of Habig et al. [16] using a Sunrise Remote microplate reader

(Tecan Deutschland GMBH), which assessed the linear increase in extinction at 340 nm.

Lipid peroxidation

Lipid peroxidation rate was measured by the FOX-2 method [17], which quantifies the formation of lipid hydroperoxides during peroxidation. W256 tumor and liver samples were homogenized in methanol, at a ratio of 1:5, in a Polytron homogenizer at a speed of 25,000 rpm min^{-1}, centrifuged at 5,000*g* for 5 min at 4°C, and read using a spectrophotometer model Ultrospec 4300 Pro at 560 nm.

Measurement of enzymatic activities in mitochondria

NADH oxidase and succinate oxidase activities were assayed polarographically as described by Singer [18]. Oxygen uptake was evaluated at 28°C in a 1.3-ml closed, thermostatically controlled water-jacketed chamber under magnetic stirring. Oxygen consumption was measured polarographically using a Clark-type electrode, connected to a Gilson oxygraph and a standard medium containing 80 mmol l^{-1} phosphate buffer, pH 7.4, 50 μmol l^{-1} EDTA. The medium was supplemented either with 0.17 mmol l^{-1} NADH or with 10 mmol l^{-1} sodium succinate and 1.0 mg of mitochondrial protein. The results were expressed as nmol of oxygen consumed per min per mg of mitochondrial protein [19]. In these analyses, four groups of animals were used: (A) baseline: healthy rats; (B) celecoxib: healthy rats treated with celecoxib; (C) control: tumor-bearing rats treated with Tris buffer; and (D) tumor-bearing rats treated with celecoxib (25 mg kg^{-1}).

Bcl-xL expression

For the immunological detection of apoptotic proteins a standard method of western blotting was used, as described by Sambrook et al. [20]. Samples (50 μg) of protein tissue were applied to a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, which was subjected to reaction with specific antibodies against the protein *Bcl-xL*. Subsequently, with the appropriate secondary antibody in conjunction with peroxidase, the proteins were viewed by their chemiluminescence signals using a ChemiImager 5500 System (Alpha Innotech Corporation, California).

Transmission electron microscopy

For microscopy studies, pieces of tumor were collected after 14 days treatment. The tumor was fixed with a modified Karnovsky's fixative (paraformaldehyde 2,0%, glutaraldehyde 2,5% in 0.1 M cacodylic acid buffer) for 2 h, washed in 0.1 M cacodylic acid buffer pH 7.3, postfixed in 2% OsO_4 in 0.1 M cacodylic acid buffer pH 7.3 for 1 h, dehydrated with ethanol and acetone, embedded in Epon 812 resin [21]; contrasted with uranyl acetate and lead citrate [22], and examined with a JEOL-JEM 1200 EX II transmission electron microscope at an accelerating voltage of 80 kV (Peabody, MA, USA).

Statistical analysis

The data were analyzed statistically by variance analysis and the Student *t*-test for comparison of averages. Mean values ± standard error of mean (SEM) were calculated; values were considered significant when $P < 0.05$. For the survival rate, the Kaplan–Meier method was used, and the curves of survival were compared by Log-rank test. This test, when comparing two groups, generates a *P*-value that tests the null hypothesis, which in turn argues that the two curves are equal. Regarding histopathology, the analysis was descriptive.

Results

Tumor weight, tumor volume, body-weight gain, and survival rate

Important differences in tumor weight and volume were found in tumor-bearing rats treated with celecoxib. Lower significant values were found in the treated rats, both in tumor volume and tumor weight. The volumes were 146.60 ± 25.39 cm^3 in the control group and 22.72 ± 15.79 cm^3 in the celecoxib group. With relation to tumor weight, the results showed an average of 18.60 ± 3.20 g in the control group and 1.77 ± 0.87 g in the celecoxib group. Those results can be viewed in Fig. 1. Regarding tumor suppression in the celecoxib-treated rats, 84.5% reduction in volume and 90% reduction in weight were recorded. The body-weight gain during treatment is an important analyzed parameter, because the W256 tumor is an animal model characterized by cachexia, and the loss of weight or lack of weight gain is one of its main signals. The change in weight of the animals during treatment, discounting the final weight of the tumor, indicated a greater weight gain in the animals treated with celecoxib. In the control group, the average weight gain was 15.22 ± 3.72 g, and in the celecoxib group, 30.97 ± 2.41 g (Fig. 1). All animals from the Celecoxib group survived until the 30th day of treatment, whereas 75% of the animals in the control group died before the 20th day of treatment (Fig. 1).

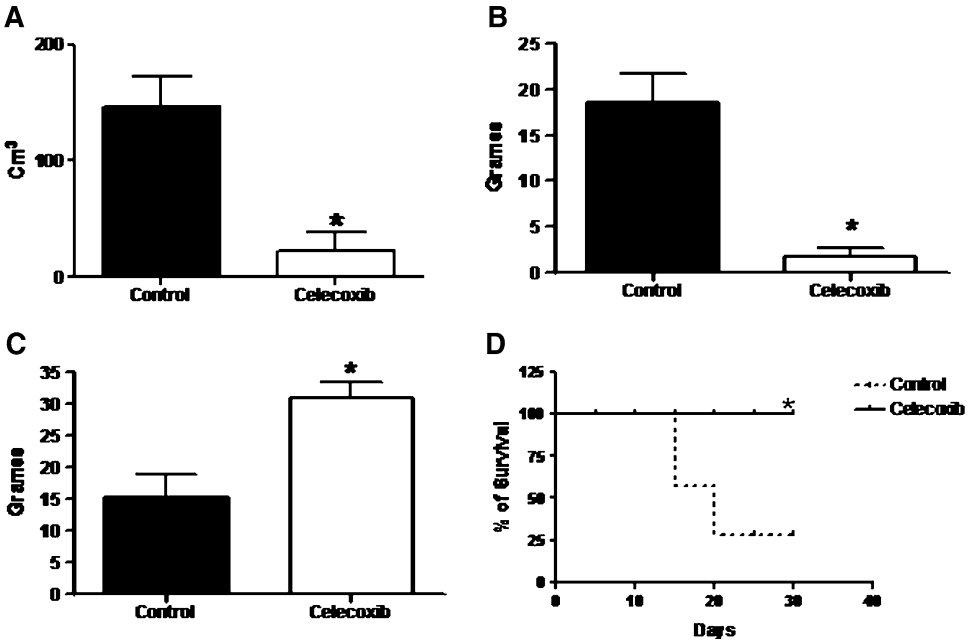

Fig. 1 Data after 14 days of treatment with celecoxib in rats bearing W256, indicating **a** Volume of tumor in control and celecoxib groups, in cm^3. **b** Tumor weight, in grams, of both control and celecoxib groups. **c** Body-weight variance of control and celecoxib rats during the period of treatment. This parameter was calculated by the difference between the body weights on the first and the 14th days of treatment, after discounting the final tumor weight. **d** Survival rate of the control and celecoxib groups during the 30 days of treatment. A, B, C: Data expressed as mean ± standard error of mean, Student *t*-test; D: Data expressed in percentage, Log-rank test. $*P < 0.05$ compared with control group. $N = 05$. 254 × 190 mm (72 × 72 DPI)

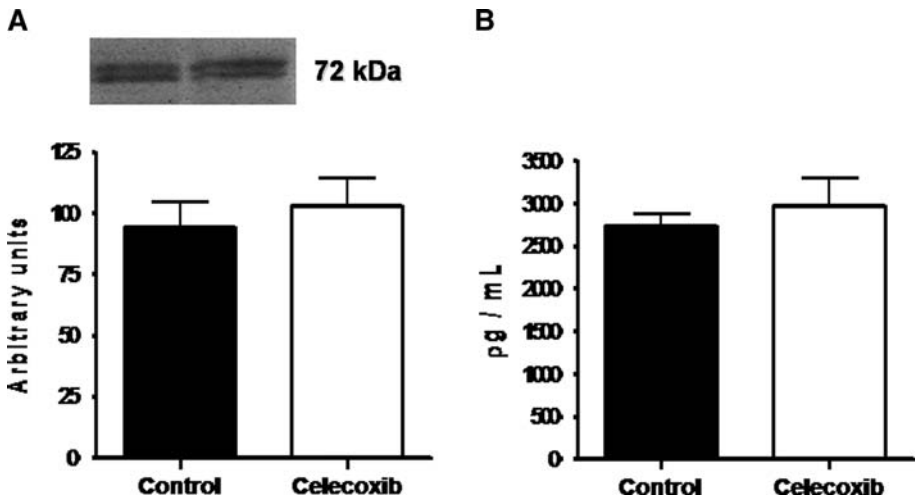

Fig. 2 **a** Expression of COX-2 by western blotting in tumor tissue, converted into arbitrary units evaluated by the densitometry technique in both control and celecoxib groups. **b** Prostaglandin E_2 levels (ρg ml^{-1}) in the tumor tissue in the same groups. Data expressed as mean ± standard error of mean, Student *t*-test. $N = 05$. 226 × 132 mm (72 × 72 DPI)

COX-2 expression and activity

As an attempt to clarify the antineoplastic mechanism of celecoxib, the COX-2 expression was assessed by western blotting. Figure 2 shows the expression of this protein in the tumor tissue, which was similar in both groups, without any statistical difference.

In addition to evaluating the expression of COX-2, its activity was tested by measuring one of its main products, PGE_2, in the tumor tissue. The results were also not statistically significant. The mean value of PGE_2 in the tumor tissue was 2725 ± 166.9 ρg ml^{-1} in the control group and 2966 ± 347 ρg ml^{-1} in the celecoxib group (Fig. 2).

Oxidative damage

In vitro study of the free radical-scavenging activity

The result of the assay that evaluated the capacity of celecoxib to scavenge reactive species, expressed as absorbance, verified that there was no antioxidant activity related to the molecule of celecoxib at the different concentrations

tested (10–200 μg ml^{-1}), compared with the positive control (ascorbic acid 50 μg ml^{-1}).

Catalase, SOD, and GST activities

Tables 1 and 2 show the effects of celecoxib on enzymes related to oxidative damage in tumor and liver, respectively. There was a statistical difference in the catalase activity in liver, which was lower in the control group samples. Indeed, there is no statistical difference between the celecoxib and baseline groups; the latter was composed of healthy animals, which were not inoculated with the tumor and were treated only with the vehicle (Table 2). It was observed that the activity of catalase in the tumor tissue was much lower than that in the liver tissue, showing that the enzyme is considerably more active in the liver than in the W256 cells. The treatment with celecoxib reduced CAT activity in the tumor (Table 1).

The activity of SOD was also evaluated in the tumor and liver tissues. Statistical difference can be observed among the groups. In liver, an increase in the values could be observed when compared with the baseline and control groups. In the treated animal samples, the values decreased in relation to control ($P < 0.05$), but their values were still higher than those in the baseline group. In the tumor tissue, SOD activity was higher in the celecoxib animals samples, compared to Control. However, the activity of SOD was higher in the tumor tissue than that in liver, contrary to what was evident for catalase.

The activity of GST in the liver showed no difference between the groups. When trying to evaluate the activity of the same enzyme in the tumor tissue, there was neither reaction nor any reading on the spectrophotometer, probably because W256 cells do not express this metabolic phase 2 enzyme.

Enzymatic activities in mitochondria

The activities of NADH oxidase and succinate oxidase were affected by celecoxib in the same pattern (Table 3). Their activities were reduced by treatment with celecoxib in animals without tumor, became more reduced in the presence of the tumor, and were not regained by celecoxib in animals with tumor.

Lipid peroxidation

The data of the lipid peroxidation assay showed no difference among the groups, both in the tumor tissue (Table 1) and in the liver (Table 2).

Table 1 Values of enzymatic activities evaluated in the tumor tissue after 14 days of treatment in control and celecoxib groups of rats bearing the W256 tumor

Parameter evaluated	Values		Unit	Statistical significance
	Control group	Celecoxib group		
CAT activity	36.84 ± 5.31	23.40 ± 0.78	μmol min^{-1} mg ptn^{-1}	$P < 0.05$
SOD activity	8.28 ± 0.16	9.11 ± 0.18	U SOD mg ptn^{-1}	$P < 0.01$
LPO	16.50 ± 0.34	16.30 ± 0.09	μmol mg ptn^{-1}	$P > 0.05$

The last column indicates the statistical significance of each assay

Table 2 Values of enzymatic activities evaluated in the liver tissue after 14 days of treatment in baseline, control, and celecoxib groups of rats bearing the W256 tumor

Parameter evaluated	Values			Unit	Statistical significance
	Baseline group	Control group	Celecoxib group		
CAT activity	392.7 ± 50.09	82.48 ± 22.90	292.0 ± 31.03	μmol min^{-1} mg ptn^{-1}	$P < 0.001$ (baseline × control) $P < 0.01$ (control × celecoxib) $P > 0.05$ (baseline × celecoxib)
SOD activity	1.992 ± 0.16	4.377 ± 0.11	3.946 ± 0.089	U SOD mg ptn^{-1}	$P < 0.001$ (baseline × control) $P < 0.001$ (baseline × celecoxib) $P < 0.05$ (control × celecoxib)
LPO	7.980 ± 0.08	7.980 ± 0.06	8.060 ± 0.081	μmol mg ptn^{-1}	$P > 0.05$ (Comparing all groups)
GST activity	607.5 ± 29.52	588.8 ± 93.07	610.7 ± 108.5	ρmol min^{-1} mg ptn^{-1}	$P > 0.05$ (Comparing all groups)

The last column indicates the statistical significance of each assay

Table 3 Values of NADH oxidase and succinate oxidase activities obtained in isolated liver mitochondria from different groups of rats: (A) baseline: healthy rats; (B) celecoxib: healthy rats treated with tumors; (C) control: tumor-bearing rats treated with Tris buffer; and (D) tumor-bearing rats treated with celecoxib

Parameter evaluated	Values Groups				Unit	Statistical significance
	A	B	C	D		
NADH oxidase	47.31 ± 1.26	30.63 ± 1.61	14.76 ± 2.22	13.40 ± 0.69	Consume of O_2 (nmol min^{-1} mg ptn^{-1})	$P > 0.05$ (D *versus* C) $P < 0.001$ (Other groups compared one by one)
Succinato oxidase	28.32 ± 1.99	22.18 ± 2.10	11.97 ± 1.79	10.26 ± 0.79	Consume of O_2 (nmol min^{-1} mg ptn^{-1})	$P > 0.05$ (D *versus* C) $P < 0.05$ (B *versus* A) $P < 0.001$ (Other groups compared one by one)

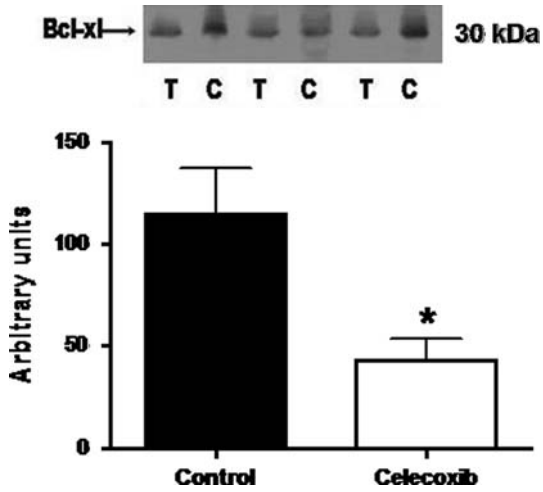

Fig. 3 Expression of the antiapoptotic enzyme *Bcl-xL* in the tumor tissue, whose values were converted into arbitrary units determined by densitometry, in control (C)- and celecoxib (T)-treated groups. Data expressed as mean ± standard error of mean, Student *t*-test. *$P < 0.05$. $N = 05$. 148 × 142 mm (72 × 72 DPI)

Bcl-xL expression

Regarding the influence of celecoxib in cellular apoptosis, its effect on the expression of *Bcl-xL* in the tumor tissue was determined through western blotting assay. Figure 3 shows that the expression of *Bcl-xL* was significantly lower in the tumors of treated animals, proving a relationship between treatment with celecoxib and a reduction in the levels of this antiapoptotic protein in W256 tissue.

Transmission electron microscopy

The analysis by electron microscopy (Fig. 4), which was carried out on the tumor tissue, showed major changes in the tumor cells of rats treated with celecoxib. These changes include heterochromatin decondensation, changes in the nuclear membrane, intense cytoplasmic vacuolization in spite of the presence of some mitochondria, vague cellular contours, and nuclei in the process of destruction. Furthermore, loss of cellular architecture was observed. It was not possible to identify several cytoplasmic organelles, which were easily observed in the pictures of tumors from the control group. These changes in the celecoxib group are strongly suggestive of cellular apoptosis.

Discussion

Several reports have shown the effects of celecoxib in neoplasms, proving a reduction in the severity and signs of cancer in various organs [23–26]. Harris et al. proved that celecoxib reduced the risk of breast cancer significantly (~71%) [23]. In addition, in mice inoculated with a carcinogen (1.2-MHD), the use of celecoxib in the diet, for a period up to 26–35 weeks after the carcinogenic stimulus, promoted a similar effect [24]. An in vitro assay, using some tumor cell lines from the human bladder, was carried out with two selective inhibitors of COX-2, NS-398 and celecoxib (100 μM), and both produced dose-dependent inhibition of growth [25].

The results presented here show the capacity of celecoxib to decrease the volume and mass of the W256. The dose used (25 mg kg^{-1} per day) caused ~84.5% inhibition of tumor growth, which was higher than the reduction previously reported by Acco et al. (~75%) with 12.5 and 25 mg kg^{-1} of celecoxib per day [26]. The current data reinforce the antineoplastic role of this drug, thus facilitating the addition of the animal model W256 to the list of cancers that are sensitive to celecoxib.

In addition to reducing the tumor, celecoxib has permitted the preservation of the weight gain of animals with W256 and retained the important metabolic pathways in the liver, at levels comparable to those in the normal rat. Glycolysis and

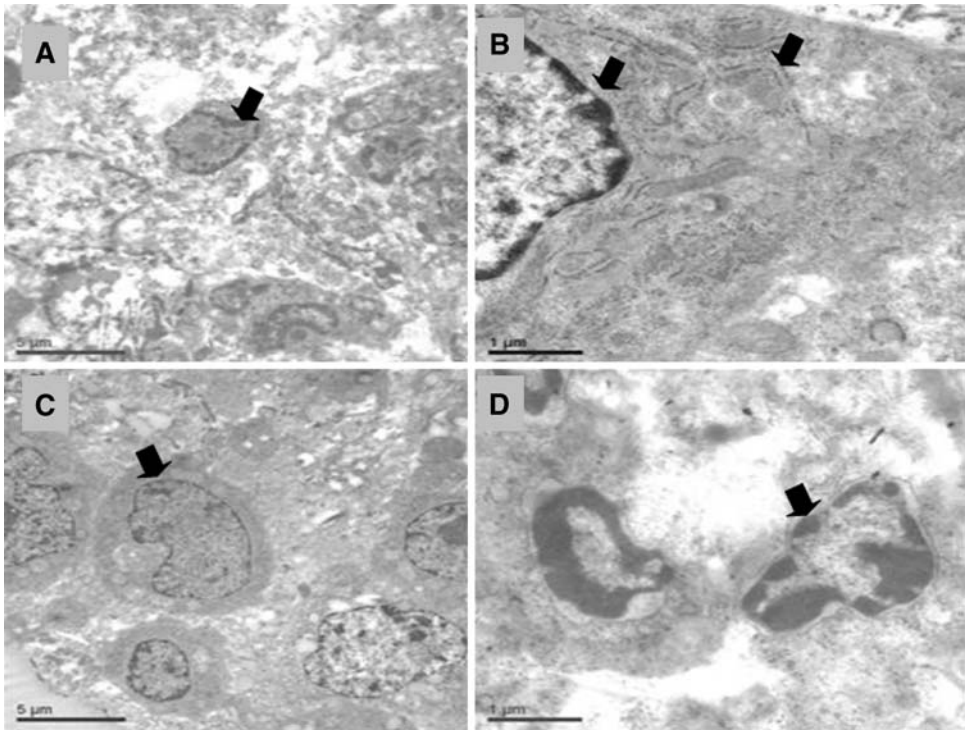

Fig. 4 Eletron microscopy of W256 cells. A = control group, showing euchromatic nuclei and peripheric heterochromatin, indicated by the arrows. B = control group, showing intact cytoplasm, nuclear pores, and preserved organelles indicated by the arrows. C = celecoxib group, indicating heterochromatization. Moreover, it was not possible to identify the organelles, as in B. D = heterochromatization (arrow) and loss of normal cell architecture. 254 × 190 mm (72 × 72 DPI)

the hepatic transformation of alanine are found to be diminished when W256 is present, characterizing the cachexia induced by the tumor. The animals treated with celecoxib showed a reestablishment of these pathways [26]. The maintenance of metabolic pathways may explain, at least partly, the gain of weight and high survival rate (100%) of the animals of the present study after 14 or 30 days of treatment, respectively, if cachexia is considered as the main death-inducing factor in this cancer model. A previous report using the antiinflammatory drugs indomethacin, ibuprofen, and aspirin in W256 cancer had already suggested that prostaglandin inhibitors may improve cachexia in cancer [27].

The therapeutic and accompanying effects of NSAIDs such as celecoxib are principally the consequence of COX inhibition [28]. In this study, no significant difference was found between groups, both in the expression of the COX-2 enzyme in the tumor and in its activity, measured through PGE_2 levels in the tumor tissue. A previous study failed to show significant quantities of PGE_2 in cultured W256 cells [29]. These data suggest that celecoxib probably acts in W256 cells through a COX-2-independent pathway, confirming a study in which the involvement of the lipoxygenase enzymes, but not the cyclooxygenases, was well established in the W256 cells [30]. The inhibition of cellular growth induced by celecoxib was reversed through the addition of exogenous PGE_2 in a low-invasive cell line (MDA-MB-468), but not in the high-invasive cell line (MDA-MB-231). This indicates that the method of action of celecoxib may be extremely complex and variable in the different tumor cell types [31].

Still in this sense, an in vitro study investigated the effect of celecoxib in three lines of nasopharynx carcinoma: HK-1, Hone-1, and CNE-2. Celecoxib inhibited the growth of all the three cell lines in a dose-dependent manner. This inhibition showed to be independent of the COX-2 levels expressed in the cells [32]. Sauter et al. [33] showed in women with a high risk of developing breast cancer that celecoxib was not able to reduce the PGE_2 levels in aspirated local fluids and in the plasma. The authors attributed these results to several hypotheses, such as the long intervening period between the final administration of celecoxib and the collection of the samples, considering the half-life of the drug (11.2 hours). In this study, the period between the final administration of the drug and the measurement of PGE_2 was greater than 24 hours, which may have influenced the effects or activity of the enzyme. Analyses with a shorter interval between the final treatment and the collection of the samples may confirm or exclude this hypothesis.

One hypothesis that explains the possible celecoxib antitumoral effect, independent of COX-2, is its antioxidant action [6], considering that ROS play an important role in the pathogeneses of different diseases, including cancer. However, the results of the assays with the free radical DPPH proved that celecoxib did not possess antioxidant activity per se, suggesting that it could have some effect on the activity of anti- or pro-oxidant enzymes. The cumulative production of ROS in many tumor cells is related to an impaired *redox* regulation of the signaling cascades. The current results show an increase in the SOD activity in the tumor tissue of the treated group (9.11 ± 0.18 U SOD

mg^{-1} ptn), in comparison to the control group (8.28 ± 0.16 U SOD mg^{-1} ptn), suggesting that an increase in the antioxidant activity of the tumor occurred as a response to the generation of the superoxide anion. It is probable that the generation of superoxide anion (O_2°) in animals bearing W256 occurs in the respiratory chain, because the activities of succinate oxidase and NADH oxidase were decreased in those animals. In fact, the energy metabolism of the tumor cell is modified in comparison with the tissue of origin. The most well-known alteration of the energy metabolism is the increase in the glycolytic flux, which is sometimes considered a response to the decrease of oxidative phosphorylation. In addition, it has been accepted that this mitochondrial dysfunction is associated with an increase in mitochondrial production of ROS [34, 35], but celecoxib was unable to revert this condition. The inhibition of NADH oxidase and succinate oxidase by celecoxib was not a surprise because several antiinflammatory drugs, such as mefenamic acid, aspirin, and diclofenac, can affect mitochondrial activities by inhibiting the oxygen consumption [36, 37].

The W256 tumor in nontreated animals was also capable of increasing the levels of SOD in the hypothalamus, cerebellum, and hippocampus [38]. Following our results, this increase may also occur in the hepatic tissue, because the mean value found for hepatic SOD in tumor-bearing animals (4.377 ± 0.1136 U SOD mg ptn^{-1}) is 2.2-fold the value of healthy animals (1.992 ± 0.1600 U SOD mg ptn^{-1}). In tumor-bearing animals, celecoxib treatment decreased the activity of this enzyme in liver (3.946 ± 0.09 U SOD mg ptn^{-1}), probably indicating a lower substrate generation for the enzyme.

It is evident from the current data that animals treated with celecoxib had increased hepatic levels of catalase (292 ± 31.03 μmol min^{-1} mg ptn^{-1}) compared to the control group (82.48 ± 22.90 μmol min^{-1} mg ptn^{-1}), which suggests a systemic oxidative state unleashed by the tumor. According to Valko et al. [39], the lessened capacity of various tumors to detoxify hydrogen peroxide is related to lower catalase levels. The specific activity of catalase in this model is quite greater in the hepatic versus tumor tissue, confirming the potential of the liver as an antioxidant organ in a systemic pathological condition such as the malignity of W256.

GST is another enzyme that participates in the normal cellular response against a wide variety of endogenous and ambient carcinogens through the conjugation reaction between glutathione and reactive and oxidative electrophiles [40]. In this study, there was no significant difference between the activities of hepatic GST in the animals receiving celecoxib treatment (610.7 ± 108.5 pmol min^{-1} mg ptn^{-1}) and those in the control group (588.8 ± 93.07 ρmol min^{-1} mg ptn^{-1}) and the basal values (607.5 ± 29.52 ρmol min^{-1} mg ptn^{-1}). The GST does not appear to be the target of celecoxib activity in the oxidative stress processes. Another aspect to be considered in oxidative stress is the injurious nature of lipoperoxidation (LPO), especially on membranes. Nevertheless, the present data indicate no significant difference in the LPO levels among the evaluated groups, both in the liver and in the tumor samples, which supports the findings of Kirkova et al. [6] in cells obtained from gastric and colonic mucosa.

Another explanation provided for the antitumor effects of NSAIDs, such as celecoxib, is based on studies in which these drugs inhibit tumor growth and induce apoptosis through COX-independent mechanisms [41]. Grösch et al. [2] cite specific studies involving celecoxib and intrinsic apoptotic pathways, such as the reduced expression of the antiapoptotic proteins *Bcl-2*, *Bcl-xL*, *Mcl-1*, and survivin, in addition to an increase in the expression of *Bad*, a proapoptotic protein. The data in this study corroborate that statement, because the expression of *Bcl-xL* in W256 tumor was significantly lower in the celecoxib-treated animals. Also, typical features of programmed cell death such as DNA fragments and apoptotic bodies had been observed by electronic microscopy. These data are in agreement with our findings since nuclear chromatin condensation, DNA fragmentation, and nuclear membrane alterations are signs of cellular apoptosis [30]. Those results agree with the ones found in our study, such as apoptose-suggesting alterations in the tumor cells observed through electronic microscopy, and decrease in the expression of the antiapoptotic protein *Bcl-xL* in the W256 tumor of the animals that received treatment with celecoxib.

In conclusion, the current results confirm the antitumoral potential of celecoxib, using the W256 tumor model, and its role in the restoration of treated animals, proved by the survival rate and tumor suppression, in association with weight gain, during the period of treatment. In addition, the antitumor effect in the W256 model appears to occur in a manner independent of modulation of the COX-2 enzyme activity, but dependent on the suppression of antiapoptotic routes and regulation of redox homeostasis. This may also regulate the activity of primary antioxidant enzymes, such as SOD and catalase.

Acknowledgment This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ, process 402414/2005-5). The authors thank the scientific support of Prof. Luiz Claudio Fernandas (UFPR).

References

1. Gasparini G, Longo R, Sarmiento R, Morabito A (2003) Inhibitors of cyclo-oxygenase 2: a new class of anticancer agents? Lancet Oncol 4:605–615