



Acute oxidant damage promoted on cancer cells by amitriptyline in comparison with some common chemotherapeutic drugs

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Oxidative therapy is a relatively new anticancer strategy based on the induction of high levels of oxidative stress, achieved by increasing intracellular reactive oxygen species (ROS) and/or by depleting the protective antioxidant machinery of tumor cells. We focused our investigations on the antitumoral potential of amitriptyline in three human tumor cell lines: H460 (lung cancer), HeLa (cervical cancer), and HepG2 (hepatoma); comparing the cytotoxic effect of amitriptyline with three commonly used chemotherapeutic drugs: camptothecin, doxorubicin, and methotrexate. We evaluated apoptosis, ROS production, mitochondrial mass and activity, and antioxidant defenses of tumor cells. Our results show that amitriptyline produces the highest cellular damage, inducing high levels of ROS followed by irreversible serious mitochondrial damage. Interestingly, an unexpected decrease in antioxidant machinery was observed only for amitriptyline. In

conclusion, based on the capacity of generating ROS and inhibiting antioxidants in tumor cells, amitriptyline emerges as a promising new drug to be tested for anticancer therapy. *Anti-Cancer Drugs* 00:000–000 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The vast majority of cancer chemotherapeutic drugs are potent substances that kill cells by interfering with their ability to divide, and thus block tumor growth. As malignant cells are more mitotically active than normal cells, they are more susceptible to be poisoned. However, normal tissues with a high turnover, such as hematopoietic tissue and gastrointestinal epithelia, exhibit high mitotic indices and thus are frequently damaged by cancer chemotherapeutic drugs. Several anticancer strategies are currently being developed with the expectation to kill cancer cells in a more effective manner with minimal adverse side effects to normal tissues. In this sense, as the mitochondria are the gateway of death for cells receiving external signals or internal cytotoxic agents, many efforts have been made recently to investigate the role of this organelle in mediating cytotoxicity of anticancer therapies [1–4].

Mitochondria are the major consumers of molecular oxygen in cells, representing an important source of reactive oxygen species (ROS), an internal cytotoxic agent [5]. Although a moderate increase of ROS may induce cell proliferation, excessive amounts of ROS can

cause oxidative damage to lipids, proteins, and DNA, provoking oncogenic transformation, increased metabolic activity, and mitochondrial dysfunction [6–9]. There is enough evidence to suggest that many types of cancer cells, compared with their normal counterparts, exhibit increased intrinsic ROS levels [10,11]. In contrast, the majority of tumor cells frequently possess very few antioxidative enzymes, such as catalase, superoxide dismutase, and glutathione peroxidase, which are known to play a protective role against ROS in normal cells [12–15]. The lack of proper antioxidant defenses makes tumor cells very vulnerable to oxidative stress. Therefore, manipulating ROS levels by redox modulation is a way to selectively kill cancer cells without causing significant toxicity to normal cells [16,17].

A promising new anticancer strategy named oxidation therapy has been developed by inducing cytotoxic oxystress for cancer treatment. This could be achieved by basically using two different methods: first, inducing the generation of high level of ROS to solid tumors, and second, inhibiting the antioxidant system of tumor cells [17,18]. It is well known that ROS, such as hydrogen peroxide (H₂O₂) and superoxide anion (O₂⁻), induce

apoptosis to a wide range of tumor cells through the activation of the caspase cascade [19–22]. It has been described that mitochondrial damage induced by the use of drugs provokes an increment of oxidative stress and cell death [2]. Major ROS-modulating agents are based on the capacity to induce high ROS generation or to reduce the antioxidant defense machinery of cancer cells. To our knowledge, only motexafin gadolinium has the capacity of producing both effects [17,23].

An interesting drug for oxidative cancer therapy is amitriptyline, a tricyclic antidepressant commonly prescribed for depression and therapeutic treatments of several neuropathic and inflammatory illnesses, such as fibromyalgia, chronic fatigue syndrome, migraine, irritable bowel syndrome, and atypical facial pain [24,25]. Clomipramine, another tricyclic antidepressant, has been already proposed as a novel anticancer agent targeted to mitochondria as it induced caspase-3-dependent apoptosis [1]. The administration of amitriptyline to cell cultures has been proven to induce several signs of toxicity. Furthermore, several reports showed that the toxicity of this drug is because of an increase in oxidative stress by generating high amounts of ROS [26–28]. In contrast, it has been proven in mouse 3T3 fibroblasts culture that provokes an increase in intracellular lipidic peroxidation, one of the important consequences of disproportionate free radicals [29].

It is also well known that mitochondrial damage and oxidative stress could induce apoptosis by the intrinsic pathway [30–32]. In fact, tricyclic antidepressants have been shown to cause apoptotic cell death in normal human lymphocytes [33], non-Hodgkin's lymphoma cells [34], and neurons [35]. We have recently shown that amitriptyline causes mitochondrial dysfunction and oxidative stress in human fibroblasts, producing a dose-dependent growth inhibition [36]. Moreover, we have also observed a decrease in antioxidant defenses when fibroblasts were treated with amitriptyline, showing that the ROS increment originated in the mitochondria [32]. Therefore, amitriptyline increases ROS levels and decreases antioxidant defenses, features not commonly found in anticancer drugs.

We proposed that amitriptyline could be an excellent candidate for oxidation therapy and cytotoxicity studies should be carried out to be tested in tumor cells with other chemotherapeutic drugs to establish a reasonable comparison. The objective of this work is to study the anticancer capacity of amitriptyline compared with other common anticancer drugs in different cancer cell lines. For the achievement of these goals, we have used three of the most representative antitumor drugs frequently used in cancer therapy: camptothecin (CPT), doxorubicin (Doxo), and methotrexate (Metho); and human cell lines H460 (non-small-cell lung cancer), HeLa (epithelial cervical cancer), and HepG2 (hepatoma).

Materials and methods

Reagents

Amitriptyline, Doxo, CPT, Metho, and trypsin were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). Anticytochrome *c* antibody (7H8.2C12) and Hoechst 3342 were purchased from Molecular Probes (Eugene, Oregon, USA). Anticytochrome *c* antibody was purchased from PharMingen (BD Bioscience, San Jose, California, USA). Antiactive caspase-3 antibody was purchased from Cell Signalling Technology (Beverly, Massachusetts, USA). Antigliyceraldehyde 3-phosphate dehydrogenase monoclonal antibody, clone 6C5, was purchased from Research Diagnostic, Inc. (Flanders, New Jersey, USA), and mouse anti-MnSOD (manganese superoxide dismutase) was purchased from Chemicon International (Temecula, California, USA).

Cell culture

H460 (non-small cell lung cancer), HeLa (epithelial cervical cancer), and HepG2 (hepatoma) cells were purchased from the American Type Culture Collection (Manassas, Virginia, USA) and cultured at 37°C in a 5% CO₂ atmosphere in RPMI-1640 medium (except HepG2, cultured in Dulbecco's modified Eagle's medium) supplemented with L-glutamine, an antibiotic/antimycotic solution (Sigma Chemical Co.), and 10% fetal bovine serum (FBS).

Apoptosis assessment

Apoptosis was analyzed by observing nuclei fragmentation by Hoechst staining (0.05 µg/ml), cytochrome *c* release, and caspase-3 activation by immunofluorescence microscopy. Ten random fields and more than 500 cells were counted. For cytochrome *c* and active caspase-3 immunofluorescence microscopy, the cells were grown on 1-mm² glass coverslips (Goldseal No. 1) in a culture medium containing 10% FBS. After drugs treatment (50 µmol/l) for 24 h, the cells were rinsed once with PBS, fixed in 3.8% paraformaldehyde for 5 min at room temperature, and permeabilized in 0.1% saponin for 5 min. For immunostaining, the glass coverslips were incubated with anticytochrome *c* antibodies (6H2.B4) and antiactive caspase-3 (17kDa) diluted 1:100 in PBS for 1–2 h at 37°C in a humidified chamber. Excess antibody binding was removed by washing the coverslips with PBS (three times, 5 min). The secondary antibodies, a fluorescent fluorescein isothiocyanate-labeled goat antimouse and a tetramethylrhodamine goat antirabbit immunoglobulin G (Molecular Probe), diluted 1:100 in PBS, were added and incubated for 1 h at 37°C. The coverslips were then rinsed with PBS for 3 min, incubated for 1 min with PBS containing Hoechst 33342 (1 µg/ml), and washed with PBS (three times, 5 min). Finally, the coverslips were mounted onto microscope slides using a Vectashield Mounting Medium (Vector Laboratories, Burlingame, California, USA) and were analyzed using a fluorescence microscope.

Measurement of intracellular generation of reactive oxygen species

Flow cytometric analysis of the intracellular generation of ROS was carried out using dihydrorhodamine 123. The cells were cultured in 6-well plates (35 mm diameter well) and, at confluence were treated with drugs (50 $\mu\text{mol/l}$). After the indicated incubation times, dihydrorhodamine 123 (1 $\mu\text{mol/l}$) was added and the incubation was prolonged for 30 min. Once the incubation was completed, the cells were harvested, washed, centrifuged (500g), resuspended in the RPMI medium, and analyzed by flow cytometry (excitation at 488 nm and fluorescence detection at 530 nm). Fluorescence was analyzed in viable cells characterized by forward scatter versus side scatter.

Antioxidant status

Biochemical determination of coenzyme Q levels

Lipid extraction from cell samples was performed as described earlier [37]. Coenzyme Q9 (CoQ9) was used as an internal standard. The cell samples were lysed with 1% SDS and vortexed for 1 min. A mixture of ethanol:isopropanol (95:5) was added and the samples were vortexed for 1 min. To recover CoQ, 5 ml of hexane was added and the samples were centrifuged at 1000g for 5 min at 4°C. The upper phases from three extractions were recovered and dried on a rotatory evaporator. The lipid extract was suspended in 1 ml of ethanol, dried in a speed vac and kept at -20°C. The samples were suspended in the suitable volume of ethanol before high-performance liquid chromatography (HPLC) injection. The lipid components were separated by a Beckmann 166-126 HPLC system (Beckman Coulter, Brea, California, USA) equipped with a 15-cm Kromasil C-18 column (Deltatechprod; Des Plaines, Illinois, USA) in a column oven set to 40°C, with a flow rate of 1 ml/min and a mobile phase containing 65:35 methanol/n-propanol and 1.42 mmol/l of lithium perchlorate. CoQ levels were analyzed using an ultraviolet (System Gold 168) and electrochemical (Coulchem III ESA, Esainc, Chelmsford, Massachusetts, USA) detector.

Western blotting for manganese superoxide dismutase

Whole cellular lysates were prepared in a buffer consisting of 0.9% NaCl, 20 mmol/l Tris-HCl, pH 7.6, 0.1% triton X-100, 1 mmol/l phenylmethylsulfonylfluoride, and 0.01% leupeptine by gentle shaking. Electrophoresis was carried out in a 10–15% acrylamide SDS/PAGE. Proteins were transferred to Immobilon membranes (Amersham Pharmacia, Biotech; Madrid, Spain). Mouse anti-MnSOD (Chemicon International) and mouse antiglyceraldehyde 3-phosphate dehydrogenase antibodies were used to detect proteins by western blotting. The proteins were electrophoresed, transferred to nitrocellulose membranes, and, after blocking overnight at 4°C, incubated with the respective antibody solution at 1:1000 dilutions. Then, the membranes were probed with their respective

secondary antibody labeled with fluorescence (1:2500). Immunolabeled proteins were detected by using a chemiluminescence method (Bio-Rad Laboratories Inc., Hercules, California, USA). The protein concentration was determined by the Bradford method [38].

Catalase determination

A spectrophotometric method described by Beers and Sizer [39] was used for measuring the breakdown of H₂O₂ by catalase. In brief, catalase activity was assayed by adding 0.1 ml of cell extracts to 1.4 ml of freshly prepared 13.2 mmol/l H₂O₂ in 0.05 mol/l K₂HPO₄ (pH 7.0; 0.15 ml of 30% H₂O₂ per 100 ml). The solution was mixed, and a loss of absorbance was determined at 240 nm by using a Gilford 2000 recording spectrophotometer for 1–3 min.

Mitochondrial status

Cytochrome *c* determination

Cytochrome *c* fluorescence immunostaining was performed to analyze the mitochondrial density in cells treated with 50 $\mu\text{mol/l}$ of amitriptyline, CPT, Doxo, and Metho. The cells were grown on 1-mm² glass coverslips (Goldseal No. 1) for 24 h in a culture medium containing 10% FBS. After treatment for 24 h, the cells were rinsed once with PBS, fixed in 3.8% paraformaldehyde for 5 min at room temperature, and permeabilized in 0.1% saponin for 5 min. For immunostaining, the glass coverslips were incubated with anticytochrome *c*. The primary antibodies were diluted 1:100 in PBS and were incubated for 1–2 h at 37°C in a humidified chamber. Excess antibody binding was removed by washing the coverslips with PBS (three times, 5 min). The secondary antibody, a fluorescein isothiocyanate-labeled goat antimouse antibody (Molecular Probes), diluted 1:100 in PBS, was added and incubated for 1 h at 37°C. The coverslips were then rinsed with PBS for 3 min, incubated for 1 min with PBS containing Hoechst 33342 (1 $\mu\text{g/ml}$), and washed with PBS (three times, 5 min). Finally, the coverslips were mounted onto microscope slides using a Vectashield Mounting Medium (Vector Laboratories) and analyzed using a fluorescent microscope.

For western blotting, whole cellular lysates were prepared in a buffer consisting of 0.9% NaCl, 20 mmol/l Tris-HCl, pH 7.6, 0.1% triton X-100, 1 mmol/l phenylmethylsulfonylfluoride, and 0.01% leupeptine by gentle shaking. Electrophoresis was carried out in a 10–15% acrylamide SDS/PAGE. The proteins were transferred to Immobilon membranes (Amersham Pharmacia). A mouse anticytochrome *c* antibody was used to detect proteins by western blotting. The proteins were electrophoresed, transferred to nitrocellulose membranes, and after blocking overnight at 4°C, were incubated with the antibody solution at 1:1000 dilutions. Then, the membranes were probed with the secondary antibody (1:2500). Immunolabeled proteins were detected by using a chemiluminescence

method (Bio-Rad). The protein was determined by the Bradford method [38].

Mitochondrial membrane potential ($\Delta\Psi_m$)

Cells were cultured in 6-well plates (35 mm diameter well) and, at confluence, were treated with drugs (50 $\mu\text{mol/l}$). After 24 h, Mitotracker (Mitotracker Red CMXRos packaging; Molecular Probes) of 100 mmol/l was added and the incubation was prolonged for 30 min. Once the incubation was completed, the cells were harvested, incubated with fresh medium, washed, centrifuged (500g), resuspended in the RPMI medium and analyzed by flow cytometry (excitation: 579 nm, emission: 599 nm).

Citrate synthase

Biochemical determination of mitochondrial mass was achieved by measuring the citrate synthase activity in whole extracts from cells. The activity was measured spectrophotometrically at 412 nm at 30°C. Cell and liver homogenates were added to the buffer containing 0.1 mmol/l of 5,5-dithio-bis-(2-nitrobenzoic) acid, 0.5 mmol/l of oxaloacetate, 50 $\mu\text{mol/l}$ of EDTA, 0.31 mmol/l of acetyl coenzyme A, 5 mmol/l of triethanolamine hydrochloride, and 0.1 mol/l of Tris-HCl, pH 8.1. Citrate synthase data were expressed as enzyme activity.

Nicotinamide adenine dinucleotide cytochrome c reductase (complexes I and III) activity

Activity of nicotinamide adenine dinucleotide: cytochrome c reductase (complexes I and III) was determined in cell extracts using spectrophotometric methods described [earlier 40]. The results are expressed as units per milligrams of protein (mean \pm SD). The proteins of tumor cells homogenates were analyzed by the Lowry procedure [41].

Electron microscopy

The cells were incubated with drugs (50 $\mu\text{mol/l}$) for 24 h, and centrifuged at 1300g for 5 min. The supernatant was removed and the cell pellet obtained was then fixed overnight in 3% buffered glutaraldehyde in 0.1 mol/l of sodium cacodylate buffer (pH 7.4) at 4°C. Tissue samples were postfixed in 2% osmium tetroxide, stained with uranyl acetate in 50% ethanol, dehydrated through an ethanol series, and embedded in Spurr. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a Philips CM-10 electron microscope.

Statistical analysis

All results are expressed as mean \pm SD. The measurements were statistically analyzed using Student's *t*-test for comparing two groups and analysis of variance for more than two groups. The level of significance was set at *P* value of less than 0.05.

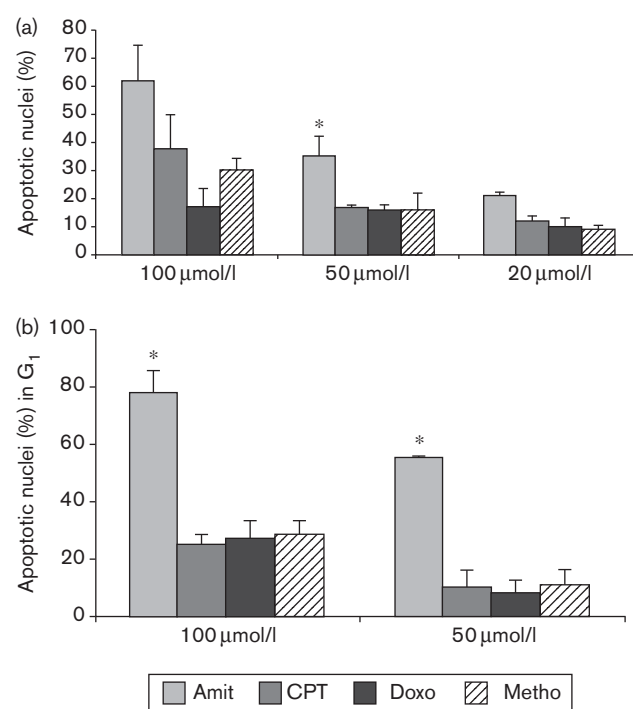
Results

Apoptosis assessment

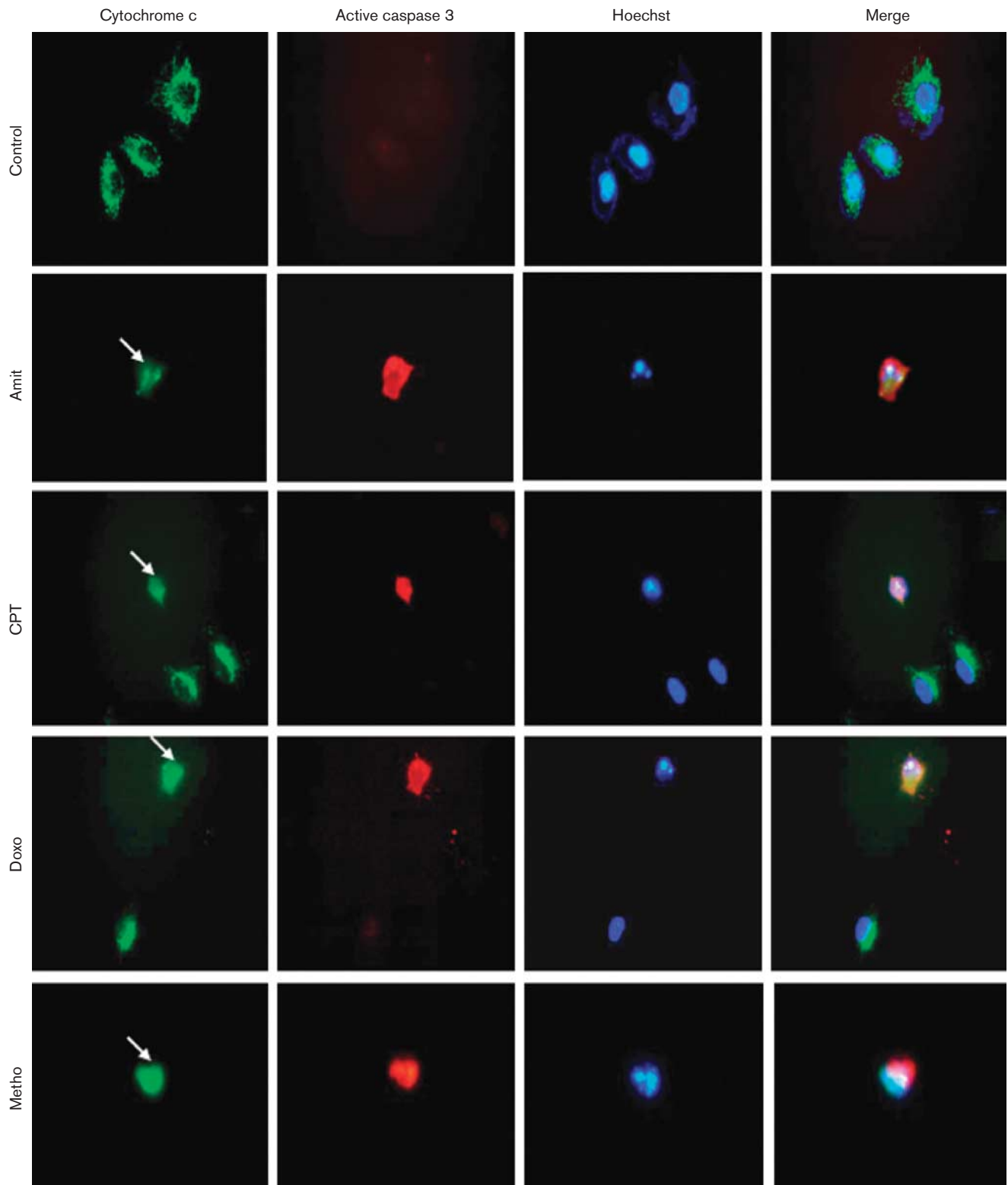
Apoptotic cell death in H460 cell cultures was determined by the quantification of apoptotic nuclei and caspase-3 activation by immunofluorescence microscopy. After 24 h of culturing, the average number of dead cells was higher in the presence of amitriptyline at all concentrations tested in comparison with the chemotherapeutic drugs, especially significant at 50 $\mu\text{mol/l}$ and 100 $\mu\text{mol/l}$. Figure 1a shows percentages of apoptotic cells when treated with different drugs and concentrations, evidencing the dose-dependent effect of amitriptyline.

When the cell cycle of synchronized cultures was stopped at the G_0/G_1 phase by depriving cells from serum, the difference of apoptosis percentage among amitriptyline and the remaining drugs was significantly higher than in normal cultures (Fig. 1b). These results suggest that the effect of amitriptyline does not depend on cell cycle stage, whereas CPT, Doxo, and Metho are more painful in

Fig. 1



Comparative study of amitriptyline and different chemotherapeutic drugs for the assessment of apoptosis. Control cells showed an apoptotic rate of about 6–8% (data not shown). (a) Percentages of apoptotic cells in H460 cell cultures 24 h after administration of 20, 50, and 100 $\mu\text{mol/l}$ of the different drugs. Amitriptyline shows the higher cell death, in a dose-dependent manner. $*P < 0.05$. (b) Percentages of apoptotic cells in synchronized cultures stopped at the G_0/G_1 phase. $*P < 0.001$. Data represent the mean \pm SD of three separate experiments. As expected, CPT-treated tumor cells presented less percentage of apoptosis when administered to nondividing cells [compare with (a)]. On the contrary, amitriptyline toxicity is independent of the cell cycle phase. Amit, amitriptyline; CPT, camptothecin; Doxo, doxorubicin; Metho, methotrexate.

Fig. 2

Apoptosis induction by different drugs in H460 cells. Cytochrome c release and caspase-3 activation by immunofluorescence microscopy was examined as described in Materials and methods. Arrows indicate cytochrome c release in apoptotic cells. Amit, amitriptyline; CPT, camptothecin; Doxo, doxorubicin; Metho, methotrexate.

dividing cells, as most chemotherapeutic drugs. The treatment of amitriptyline and the chemotherapeutic drugs used in this study killed the cells by apoptosis through a mechanism dependent on cytochrome *c* release and caspase-3 activation (Fig. 2).

Oxidative stress analysis

Intracellular generation of ROS was determined by flow cytometry. The level of ROS in H460 tumor cells treated with 50 $\mu\text{mol/l}$ of amitriptyline during 24 h was significantly increased (1.9 ± 0.14 , arbitrary units) with respect to CPT (1.25 ± 0.07), Doxo (1.05 ± 0.07), or Metho (1.45 ± 0.07). So, amitriptyline induced an increment of 100% of ROS with respect to control (0.95 ± 0.07), whereas CPT, Doxo, and Metho produced an increment of 31, 10, and 52%, respectively.

Antioxidant defenses

Several antioxidants were analyzed to study the defense machinery of H460 cells against ROS production. As observed by flow HPLC, amitriptyline induced a 40%

decrement of CoQ10 with respect to control (184.83 ± 19.46 and 454.05 ± 7.18 pmol/mg protein, respectively). All chemotherapeutic drugs increased the levels of CoQ10 slightly (CPT: 529.81 ± 53.3 , Doxo: 517.38 ± 6.71 , Metho: 518.34 ± 72.17 ; Fig. 3a).

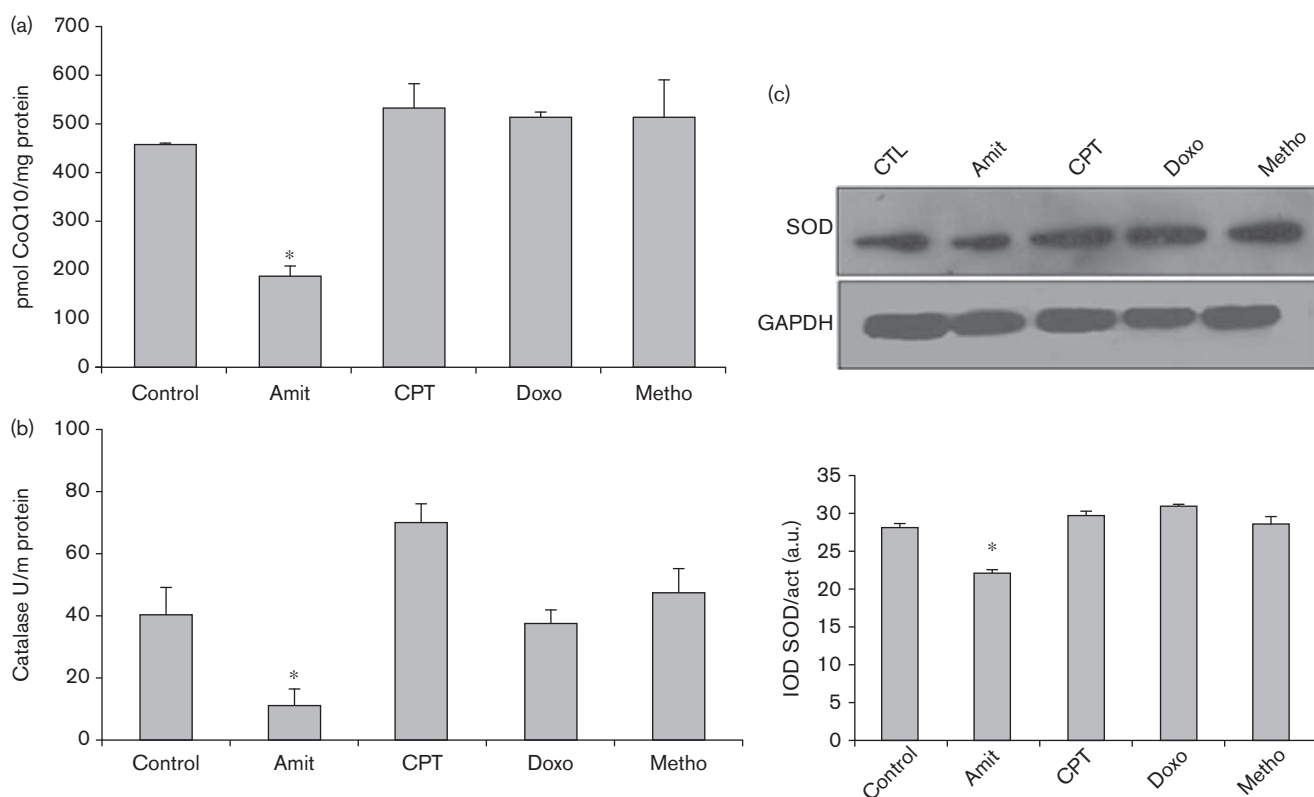
Both catalase and MnSOD showed a significant decrease in amitriptyline-treated cells, in contrast to CPT, Doxo, and Metho. Spectrophotometric determination of catalase yielded the following data (unit per milligrams of protein): 39.73 ± 9.36 for control, 11.27 ± 5.32 for amitriptyline, 70.09 ± 6.60 for CPT, 37.79 ± 4.11 for Doxo, and 47.34 ± 8.37 for Metho (Fig. 3b). The protein levels of MnSOD were analyzed by western blotting, resulting in a significant drop in this antioxidant when cells were treated with amitriptyline (Fig. 3c).

Mitochondrial damage

Mitochondrial dysfunction

As CoQ10 is an essential component of the mitochondrial respiratory chain, transporting electrons from complexes I

Fig. 3

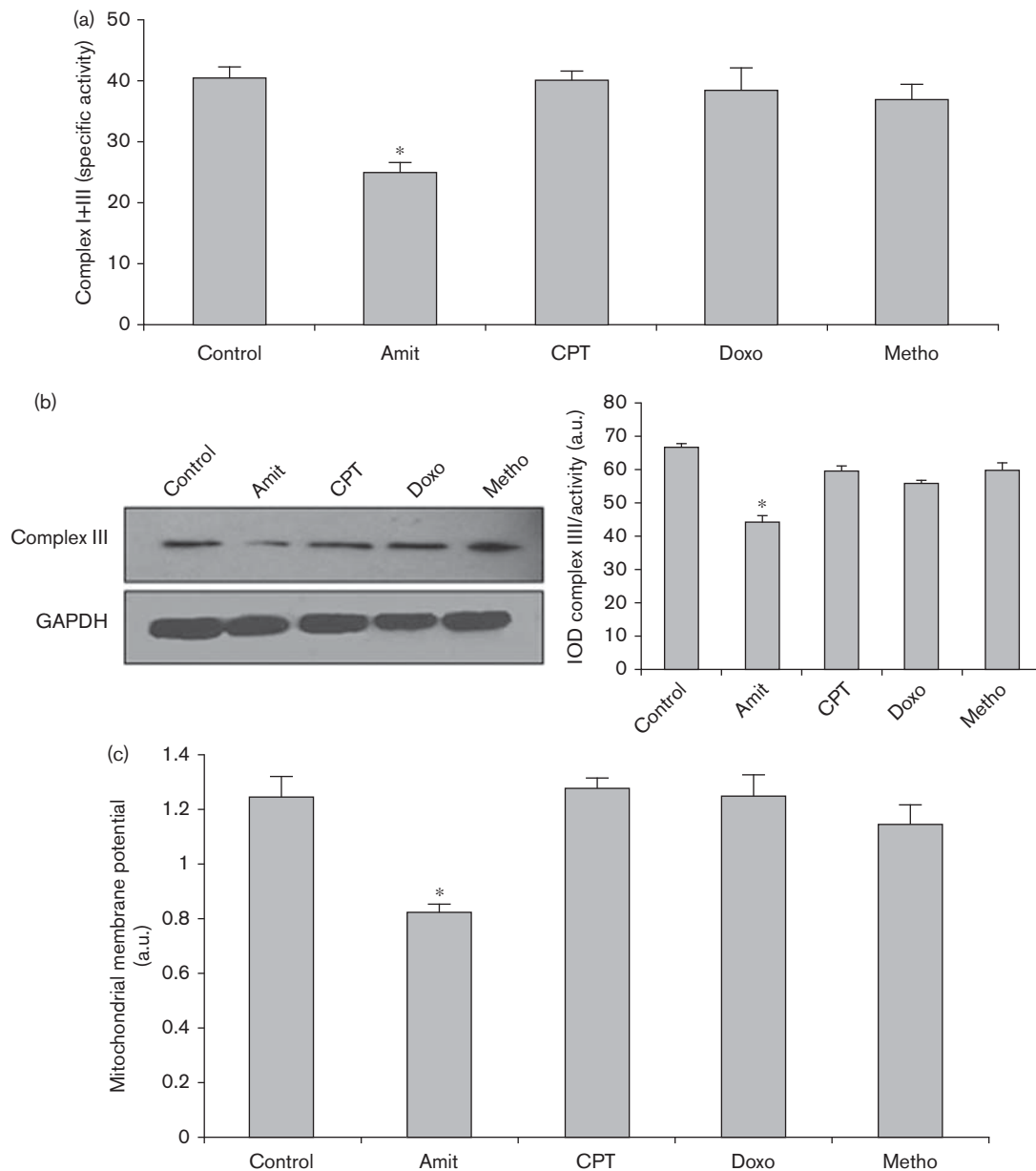


Analysis of the antioxidants status of H460 cells treated with 50 $\mu\text{mol/l}$ of the different drugs. A significant decrease is observed in all antioxidants analyzed in the cells treated with amitriptyline. (a) Coenzyme Q10 (CoQ10) determination by high-performance liquid chromatography. Results are expressed as picomole of CoQ10 per milligrams of protein. * $P < 0.005$ vs. control cells. (b) Spectrophotometric determination of catalase, expressed as unit per milligrams protein. * $P < 0.02$ vs. control cells. Data represent the mean \pm SD of three separate experiments. (c) Western blotting and fluorescence quantification of cellular extracts. Membrane blot was incubated with antibodies against superoxide dismutase. * $P < 0.05$ vs. control cells. Amit, amitriptyline; a.u., arbitrary unit; CPT, camptothecin; Doxo, doxorubicin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IOD, integrated optical density; Metho, methotrexate.

and II to complex III, reduced levels of CoQ10 in amitriptyline-treated tumor cells should induce an alteration in the normal mitochondrial electron flow. To determine this putative mitochondrial dysfunction, we measured the respiratory enzymatic activities in control and H460 cells treated with amitriptyline, CPT, Doxo, and Metho. As expected, we found that the amitriptyline treatment induced a significant de-

crease in complex I and III enzymatic activity compared with control (Fig. 4a). Moreover, a significant decrease in the protein expression of complex III was observed in cells treated with amitriptyline, corroborating the damage provoked by this drug in the respiratory chain (Fig. 4b). The mitochondrial damage generated by the other drugs is less harmful than that of amitriptyline.

Fig. 4



Mitochondrial dysfunction promoted by amitriptyline, (CPT), Doxo, and Metho in H460 cells. (a) Amitriptyline treatment induced a significant decrease in the respiratory enzymatic activities of the nicotinamide adenine dinucleotide:cytochrome c reductase (complex I and complex III). Data represent the mean \pm SD of three separate experiments. * $P < 0.02$ vs. control cells. (b) Western blot and densitometric analysis were carried out to determine complex III protein levels. A significant decrease in the complex III protein expression was observed in amitriptyline-treated cells. * $P < 0.05$, vs. control cells. (c) Mitochondrial membrane potential of the H460 cells treated with 50 $\mu\text{mol/l}$ of the different drugs. Mitochondrial membrane potential decreased significantly in amitriptyline-treated tumor cells. * $P < 0.02$ vs. control cells. Amit, amitriptyline; CPT, camptothecin; CTL, control; Doxo, doxorubicin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IOD, integrated optical density; Metho, methotrexate.

In contrast, changes in mitochondrial membrane potential were observed by a significant decrease in fluorescence after incubation with amitriptyline. Flow cytometry quantification data were 1.25 ± 0.07 for control, 0.82 ± 0.03 for amitriptyline, 1.27 ± 0.03 for CPT, 1.25 ± 0.07 for Doxo, and 1.15 ± 0.07 for Metho. It can be observed that after 24 h of 50 $\mu\text{mol/l}$ of amitriptyline incubation, the mitochondrial membrane potential was 34% less of control value, indicating a lower capacity to produce ATP in amitriptyline-treated tumor cells (Fig. 4c).

Mitochondrial mass

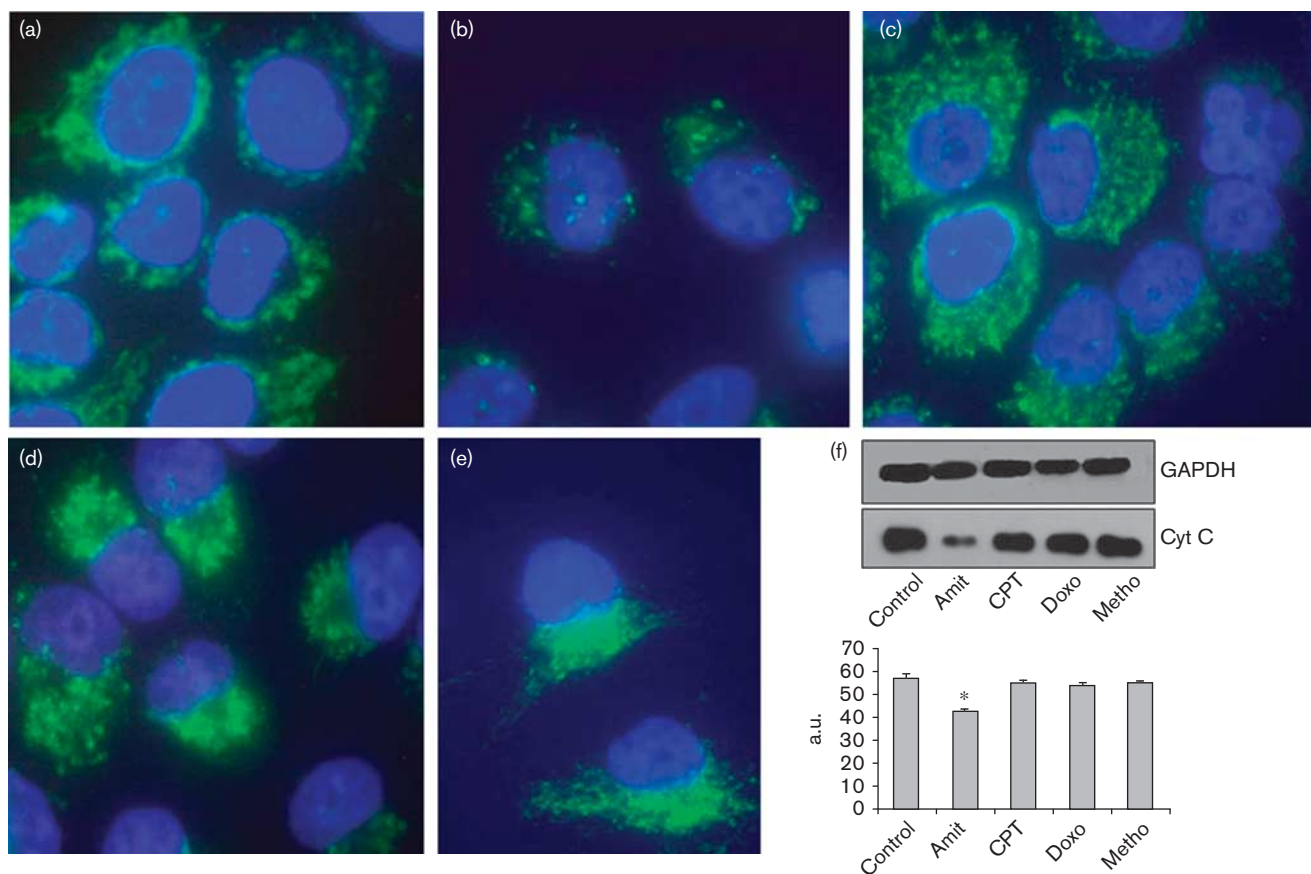
The number of mitochondria was analyzed, first, by cytochrome *c* fluorescence immunostaining. After treating the cells with 50 $\mu\text{mol/l}$ of amitriptyline, immunofluorescence decreased significantly with respect to controls. In contrast to the results for amitriptyline, the number of mitochondria did not vary much between the three

common chemotherapeutic drugs and the control. Quantification of fluorescence intensity showed values of 56.69 ± 1.90 for control, 42.33 ± 1.55 for amitriptyline, 55.15 ± 0.87 for CPT, 54.31 ± 1.15 for Doxo, and 54.62 ± 1.32 for Metho (Fig. 5a–e). Western blot for cytochrome *c* yielded the same results as immunostaining, confirming the significant decrease of mitochondria in cells treated with amitriptyline (Fig. 5f).

Mitochondrial mass was also determined by enzymatic activity of citrate synthase, resulting in a significant loss of mitochondria in amitriptyline-treated cells. Enzymatic activity data were 211.05 ± 33.73 for control, 139.00 ± 6.36 for amitriptyline, 251.05 ± 9.69 for CPT, 243.65 ± 31.04 for Doxo, and 270.15 ± 14.64 for Metho.

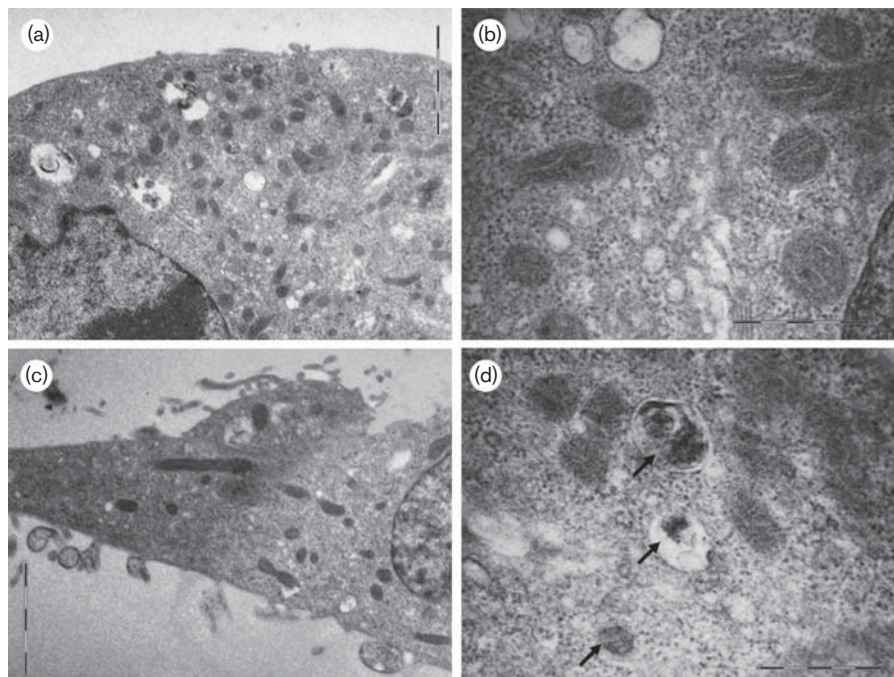
At the ultrastructural level, electron microscopy showed degenerating mitochondria and a decreased number of these organelles in amitriptyline-treated tumor cells compared with control cells (Fig. 6).

Fig. 5



Mitochondrial loss analyzed by cytochrome *c* (cyt *c*) fluorescence immunostaining and western blotting in H460 cells. Cyt *c* fluorescence immunostaining after treatment with 50 $\mu\text{mol/l}$ of the different drugs: control (a), amitriptyline (b), Metho (c), Doxo (d), CPT (e). (f) Mitochondrial protein expression levels in control and drug-treated cells. The different drugs (50 $\mu\text{mol/l}$) were administered for 24 h. The proteins from control and treated tumor cells extracts were immunostained with antibodies against cyt *c*. The protein levels were determined by densitometric analysis of three different western blots and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) signal. * $P < 0.05$ vs. control cells. Amit, Amitriptyline; CPT, camptothecin; Doxo, doxorubicin; Metho, methotrexate.

Fig. 6



Transmission electron microscopy showed damage and less number of mitochondria in H460 cells treated with 50 $\mu\text{mol/l}$ of amitriptyline. Degenerating mitochondria (arrows) are observed in treated tumor cells. (a) and (b) Nontreated tumor cells; bars: 2 μm , 500 nm, respectively. (c) and (d) Amitriptyline-treated tumor cells; bars: 2 μm , 500 nm, respectively.

Amitriptyline effects are common to several cancer cell lines

Along with non-small lung cancer cells (H460), amitriptyline toxicity has also been assayed in two other human cancer cell lines: HeLa (epithelial cervical cancer) and HepG2 (hepatoma). In these tumor cells we have analyzed apoptosis, ROS and CoQ levels, and changes in the mitochondrial membrane potential after the administration of 50 $\mu\text{mol/l}$ of amitriptyline, CPT, Doxo, and Metho for 24 h. As expected, apoptosis and ROS generation increased significantly in amitriptyline-treated cells, much more than in cells treated with the other drugs. In contrast, CoQ levels and mitochondrial membrane potential showed a significant decrease in HeLa and HepG2 cells treated with amitriptyline. Interestingly, this effect was not observed in the other cell lines treated with alternative chemotherapeutic drugs. Results are shown in Fig. 7 (HeLa cells) and Fig. 8 (HepG2 cells).

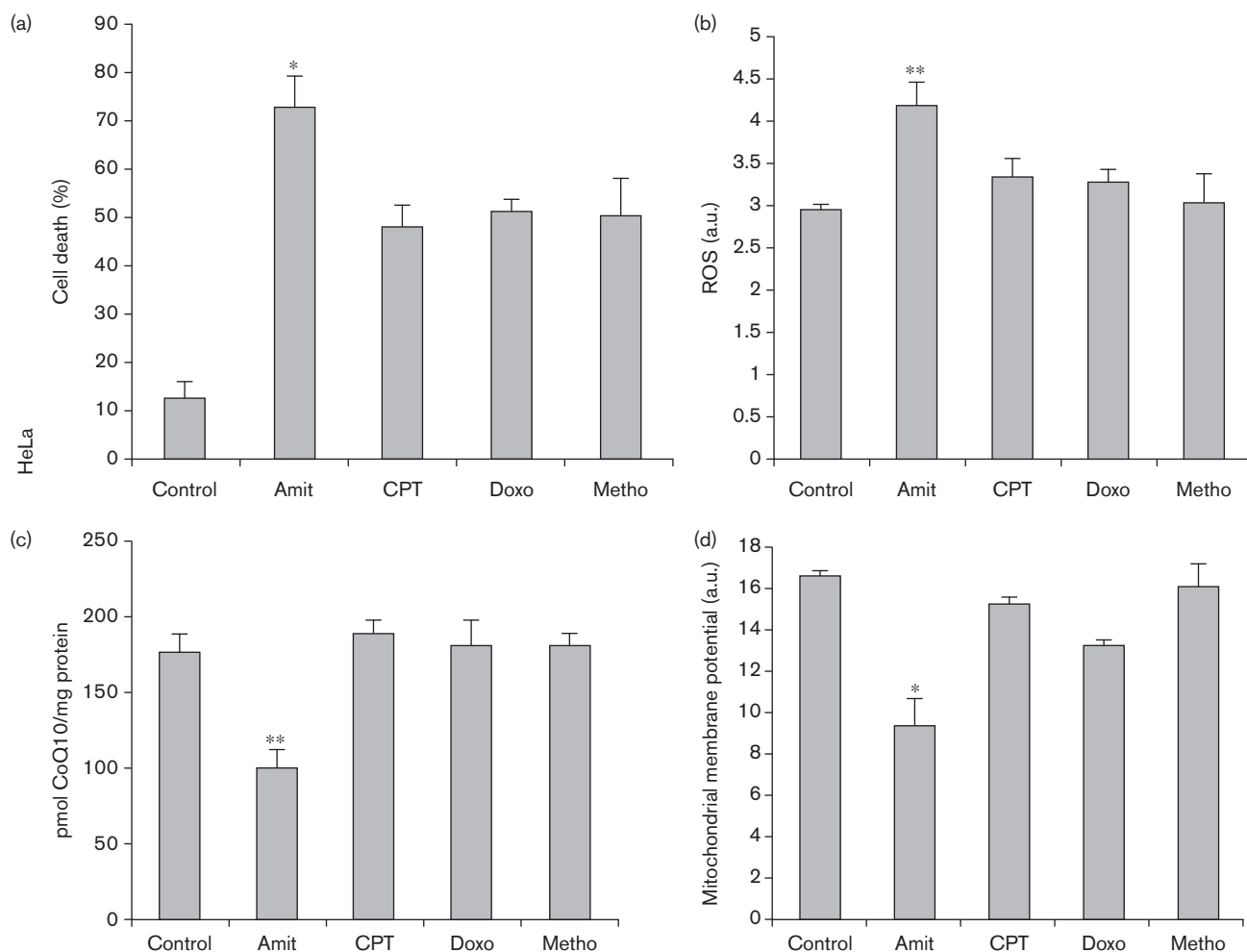
Discussion

In this study we have observed that amitriptyline induces important mitochondrial damage in tumor cell lines, generating high amounts of ROS and provoking apoptotic cell death. Moreover, amitriptyline effects have been compared with three antitumoral drugs frequently used in cancer therapy: CPT, Doxo, and Metho. Interestingly,

amitriptyline induces significantly higher ROS generation in comparison with the other drugs. Our results indicate that amitriptyline produces a dose-dependent increase of apoptosis in human cancer cells more efficiently than the other common chemotherapeutic drugs used, through a mechanism dependent on caspase-3 activation.

It has been shown that tricyclic antidepressants, in particular clomipramine, initiate the intrinsic pathway of caspase-3-dependent apoptosis through the inhibition of complex III of the mitochondrial respiratory chain [1]. By blocking the mitochondrial electron transport chain, the tricyclics initiate an increase in superoxide production and hence, H_2O_2 production, and a decrease in membrane potential. Consequently, cytochrome *c* is released and activation of procaspase 9, caspase 3, and endonuclease G results in DNA degradation and apoptotic death [4]. As we have described earlier [32], amitriptyline-treated fibroblasts showed reduced expression level of proteins of complex I (39 kDa subunit), complex III (core 1 subunit), cytochrome *c*, and reduced CoQ10 levels. These observations suggest that amitriptyline treatment affects the activity, organization, and assembly of mitochondrial complexes. Deficient mitochondrial protein expression levels and reduced levels of CoQ may impair normal mitochondrial electron flow and proton pumping, inducing a drop in mitochondrial membrane potential. Amitriptyline-treated fibroblasts possessed

Fig. 7



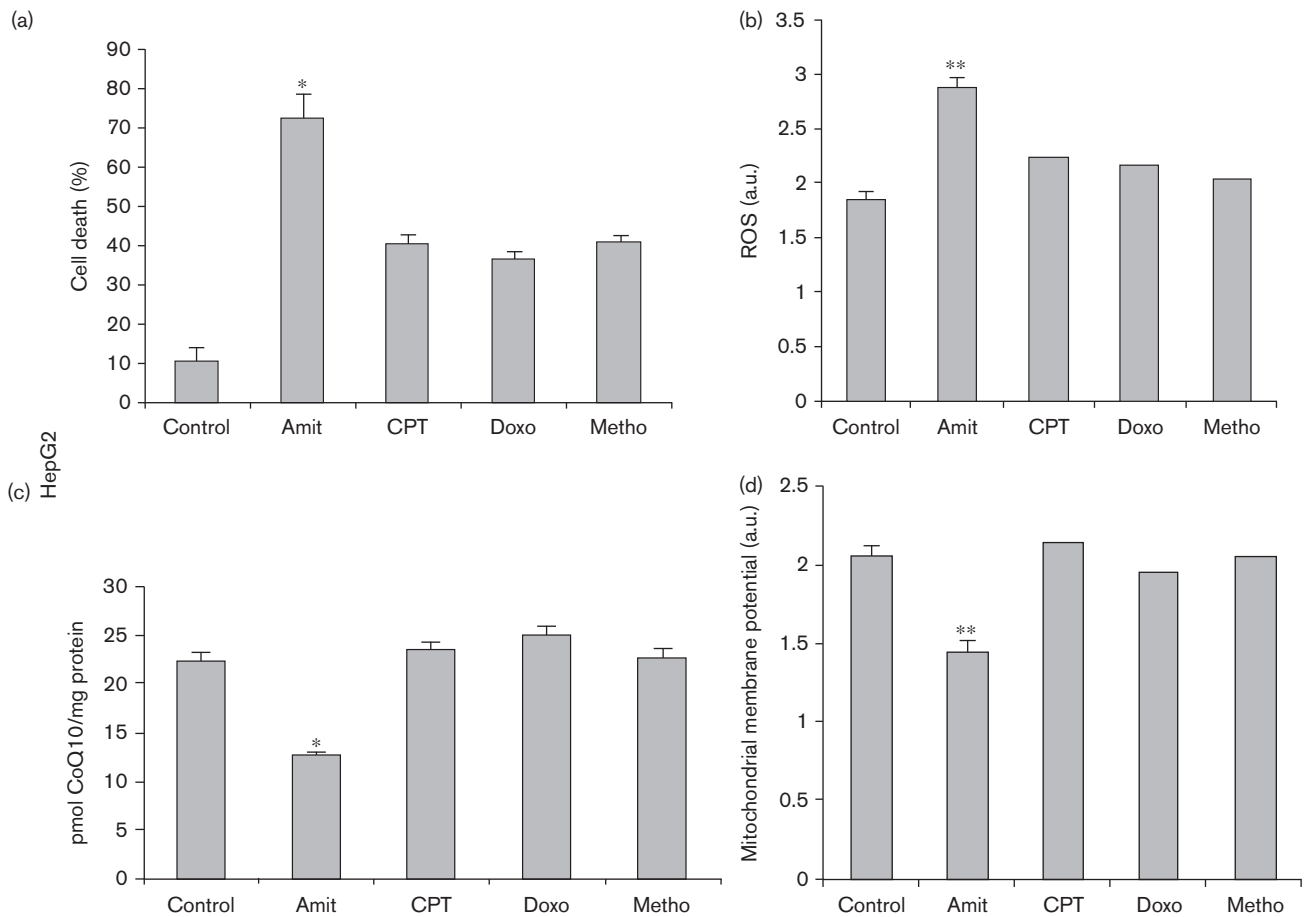
Effect of amiriptryline in HeLa cells. Apoptosis, reactive oxygen species (ROS), and coenzyme Q (CoQ) levels and changes in the mitochondrial membrane potential were determined, after the administration of 50 $\mu\text{mol/l}$ of amiriptryline, camptothecin (CPT), doxorubicin (Doxo), and methotrexate (Metho) for 24 h. * $P < 0.005$, ** $P < 0.05$. a.u., arbitrary unit.

reduced mitochondrial potential that could contribute to impaired mitochondrial protein import and aggravate mitochondrial dysfunction, ROS production, and oxidative damage. All these toxic effects were attenuated when supplemented with CoQ and α -tocopherol, two well-known lipophilic antioxidants, suggesting that the toxic side effects of amiriptryline could be provoked by increasing the oxidative stress. In this study, after treating cancer cells with amiriptryline, we have found increased ROS and several signs of mitochondrial damage, as attenuated complex I and III activity, decreased protein levels of complex III, decreased membrane potential, and a significant reduction of the number of this organelle, shown by cytochrome *c* and citrate synthase determination, and electron microscopy. As expected by our earlier investigations, this drug provokes oxidative stress in cancer cells, being mitochondria the target of amiriptryline toxicity. None of the chemotherapeutic drugs tested

in this study seemed to damage mitochondria seriously. However, these chemotherapeutic drugs induced apoptosis and increased ROS production in tumor cells, although not with the intensity of amiriptryline.

As already commented, evidences suggest that many types of cancer cells exhibit increased intrinsic ROS levels associated with carcinogenesis transformation [6–11]. The escalated ROS generation in cancer cells serves as an endogenous source of DNA-damaging agents that promote genetic instability and development of drug resistance. Malfunction of mitochondria also alters cellular apoptotic response to anticancer agents [15]. In contrast, the majority of tumor cells frequently possess very little antioxidative enzymes, such as catalase, SOD, and glutathione peroxidase, which are known to play a protective role against ROS in normal cells. Lack of a proper antioxidant defense makes tumor cells very

Fig. 8



Effect of amitriptyline in HepG2 cells. Apoptosis, reactive oxygen species (ROS), and coenzyme Q (CoQ) levels and changes in the mitochondrial membrane potential were determined, after the administration of 50 $\mu\text{mol/l}$ of amitriptyline, camptothecin (CPT), doxorubicin (Doxo), and methotrexate (Metho) for 24 h. * $P < 0.005$, ** $P < 0.05$. a.u., arbitrary unit.

vulnerable to oxidative stresses [12–14,18]. Among these enzymes, catalase plays a critical role in the antioxidant defense of many organisms, as it converts H_2O_2 to H_2O [42]. It has been reported that most tumors show a significantly reduced catalase activity, because of a marked suppression of catalase gene expression at the level of transcription [13]. In contrast, SOD, especially MnSOD, is crucial for aerobic organisms to develop cellular resistance to oxidative stress; moreover, it is an antioxidant with neuroprotective properties, preserving neurons from premature cell death [43,44].

In an earlier report, when amitriptyline was added to healthy fibroblasts, we found a decrease in antioxidant enzymes (catalase and MnSOD) 16 h after the treatment, followed by restored levels after 24 h, as a mechanism of antioxidant defense [36]. In this study, the same concentration of amitriptyline provoked an unrestorable decrease of catalase in cancer cells. The difference of the antioxidant status observed in cancer cells, in comparison

with healthy fibroblasts, may be because of the fact that cancer cells usually decrease the antioxidant defenses. Moreover, many tumor cells have a lower number of mitochondria than normal cells [45,46], being more vulnerable to mitochondrial damage.

Besides the decrease of catalase and MnSOD, we have found a significant decrease of CoQ expression when tumor cells were treated with amitriptyline. CoQ plays a critical protective role by either acting as antioxidant or by the noncompetitive inhibition of the neutral sphingomyelinase of plasma membrane, preventing ceramide production [13,42]. The chemotherapeutic drugs used in this study did not provoke any decrement of antioxidants. Instead, they induced a slight increase of antioxidants, only significant with CPT when catalase was analyzed. These results are in agreement with those reported earlier [37], in which we showed that chemotherapeutic drugs increment CoQ levels in cancer cell lines, probably as a protecting mechanism against ROS

generation, leading to lower cell death. Interestingly, amitriptyline provoked a significant decrease in antioxidants, catalase, and CoQ, dramatically affecting antioxidant cell defenses. Moreover, the toxicity of amitriptyline is independent of the cell cycle phase, as shown by the synchronized culture. CPT, on the contrary, showed dependence on cell cycle phase, resulting in lower toxicity on nondividing cells. These data are of special interest for cancer treatment during the nongrowing phases of certain tumors.

In this study, the effects of this drug have been shown in three different human cancer cell lines: non-small cell lung cancer (H460), epithelial cervical cancer (HeLa), and hepatoma (HepG2). To our knowledge, an antioxidant status study with tricyclic antidepressants has not been published yet. The fact that amitriptyline down-regulates both catalase and CoQ activity is an unprecedented and an attractive characteristic, because it destroys the already decreased antioxidant defenses present in cancer cells, being the oxidative stress produced by the amitriptyline-induced ROS generation in a more effective manner.

According to our results, amitriptyline promotes an enhanced oxidative damage to cancer cells as this drug attacks cells by two different mechanisms: by the production of high amount of ROS, provoking apoptosis; and by a significant decrease in antioxidant levels, seriously limiting cell reaction to oxidative stress. Therefore, amitriptyline could be used for anticancer oxidant therapy against tumors that present high levels of oxidative stress and/or low antioxidant defenses. For anticancer therapeutics on those tumors with a similar redox status than normal cells, a drug delivery vehicle could be used. Moreover, the doses of amitriptyline used in this study are in accordance with those acceptable in humans when used as an antidepressant.

In conclusion, our results suggest that amitriptyline induces high ROS generation as a result of mitochondrial dysfunction, provoking a higher level of apoptosis of tumor cells than common chemotherapeutic drugs. Moreover, it inhibits catalase, MnSOD, and CoQ, the three important antioxidants of the cell-defense machinery, dramatically limiting tumor cell response to the provoked oxidative stress. In this study, the effectiveness of amitriptyline has been widely shown to fit properly in the relatively new anticancer strategy named oxidation therapy, based on the capacity of this drug for generating ROS and inhibiting antioxidants in tumor cells. Although additional studies are necessary, amitriptyline could be considered as a putative new anticancer drug.

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