Nicotine Decreases the Cytotoxicity of Doxorubicin towards MCF-7 and KB-3.1 Human Cancer Cells in Culture

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The harmful effects of tobacco use and nicotine are well known. There is strong epidemiological evidence for smoking as a risk factor for cancer of the lung and oral cavity. The evidence for second-hand smoke as a risk factor for breast cancer is rapidly accumulating. The anthracycline doxorubicin is used in the treatment of many types of malignancies, including breast cancer. The effect of nicotine on doxorubicin toxicity was evaluated in MCF-7 and KB-3.1 cancer cell cultures. Nicotine partially inhibited doxorubicin toxicity towards MCF-7 and KB-3.1cells, as judged by clonogenicity and flow cytometry assays. Flow cytometric analysis showed that <10% of cells treated with doxorubicin underwent apoptosis, while necrosis was the major mode of cell death. Inhibition of apoptosis and necrosis in cancer cells by nicotine can diminish the effectiveness of doxorubicin in cancer therapy.

Key words: nicotine ■ doxorubicin ■ cancer ■ chemotherapy

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INTRODUCTION

The deleterious effects of smoking and tobacco use have been well documented.[1-9] About 30% of cancer deaths in the United States are associated with the use of tobacco products.[2] Tobacco use is well recognized as the major cause of lung, and head and neck cancers.[2,3] Evidence for breast cancer risk from active and passive smoking is accumulating.[4-7] There is increasing evidence that the use of tobacco can decrease the efficacy of cancer treatment and increase the risk of recurrence.[8-10] Recent studies have shown that nicotine inhibits apoptosis of cancer cells treated with a variety of chemotherapeutic drugs. Blunting of anticancer efficacy of chemotherapeutic drugs has been associated with inhibition of drug-induced apoptosis of cancer cells by nicotine.[10] However, it is important to recognize that apoptosis is not a major mode of cell death in solid tumors.[11,12] Necrosis rather than apoptosis is the major mode of cell death in some solid tumors. Clonogenicity assays are preferable to apoptosis assays for evaluating therapeutic potential of drugs and ionizing radiation against solid tumors.[11,12] Doxorubicin is a versatile anticancer drug that is used for treating a variety of malignancies, including breast cancer. This provides a strong rationale for investigating the influence of nicotine, a tobacco alkaloid, on the cytotoxicity of doxorubicin towards human breast cancer cells. Clonogenicity and apoptosis assays showed that nicotine partially protected MCF-7 human breast cancer cells against cytotoxicity of doxorubicin. The mechanisms by which nicotine may inhibit the cytotoxic effects of doxorubicin are discussed briefly.

MATERIALS AND METHODS

Reagents

Chemicals were of the highest available grade from commercial sources. (-)-Nicotine, doxorubicin, ethidium bromide, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and methylene blue were purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture reagents such as glutamine, sodium pyruvate, trypsin and fetal bovine serum were obtained from GIBCO, Grand Island, NY.

Cell Culture

MCF-7 human breast cancer cells and KB-3.1 human nasopharyngeal cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, glutamine (2 mM), sodium pyruvate (1 mM), 100 U penicillin/ml and 100 µg streptomycin/ml. Cells were subcultured twice weekly and maintained as exponentially growing monolayers in a humidified 5% carbon dioxide air atmosphere at 37°C.

Clonogenicity Assay

Cells were seeded at densities of 300 and 1,500 cells per 100-mm diameter tissue culture dish. The cells were allowed to attach overnight. The cultures were treated with nicotine (10 or 20 µM) for two hours. Control cultures were treated with same volume of medium without nicotine. After the two-hour exposure to nicotine, the cultures were treated with different concentrations of doxorubicin for 90 minutes. Appropriate controls without doxorubicin were also included. The medium was removed from each dish and the cells were washed with Dulbecco's phosphate buffered saline (PBS), and fresh drug-free culture medium (10 ml) was added; the cultures returned to the incubator for colony formation to progress for 10 days. Any colony containing >50 cells was considered to represent a viable clonogenic cell. The colonies in the dishes were counted after staining with 0.1% methylene blue in 50% ethanol.[13] Cell survival was calculated relative to a 100% value for untreated controls. The experiment was performed four times for each treatment.

MTT Assay for Cell Viability

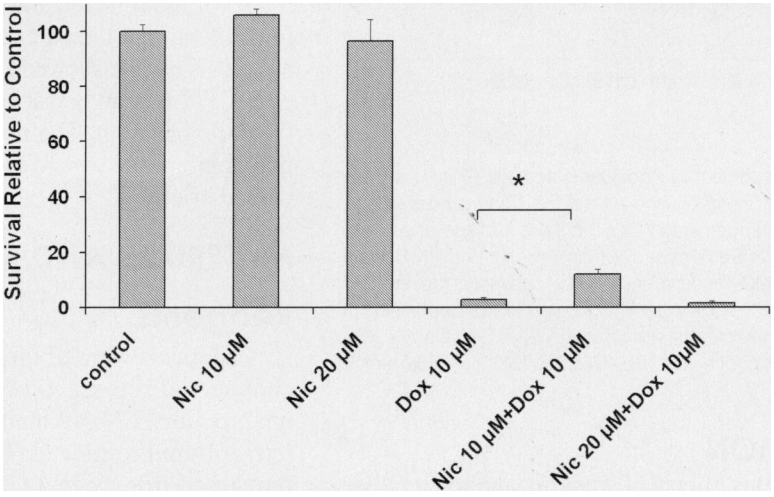
Exponentially growing cells were harvested and plated in 96 well flat-bottom plates at 10,000 cells per well containing 200 µl of complete culture medium, and the cells were allowed to attach overnight. The cells were then pretreated with nicotine (1–10 µM) for two hours, followed by exposure to doxorubicin (0.1–1.0 µM) with or without nicotine (1–10 µM) for 72 hours at 37°C. Mitochondrial dehydrogenase levels, which are correlated to cell viability, were determined by the enzyme-mediated cleaving of the tetrazolium salt ring of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide to yield purple formazan crystals. These crystals were dissolved in isopropanol, and the absorption at 560 nm was determined spectrophotometrically. Appropriate control experiments were run to determine spec-

Table 1.Effect of nicotine in combination with doxorubicin on MCF-7 cell proliferation

	Control	Dox 0.1 mM	Dox 0.5 mM	Dox 1.0 mM
Nic 0 µM	100 ± 5 *	91 ± 5*	26 ± 1*	21 ± 2*
Nic 1 µM	87 ± 8	97 ± 5	30 ± 1	20 ± 3
Nic 5 µM	105 ± 7	117 ± 3*	38 ± 4*	25 ± 2
Nic 10 µM	126 ± 5 *	123 ± 6*	40 ± 3*	28 ± 3*

Cell viability was determined using the MTT assay for cell proliferation as described under methods. Data are presented as mean ± SD; * p<0.05 (Student's t test)

Figure 1. Effect of nicotine on doxorubicin cytotoxicity towards MCF-7 cells determined using clonogenicity assays



For combination treatment, cells were pretreated with nicotine (10 or 20 µM) for two hours followed by a 90-minute treatment with dox (10 µM) as described under methods. Data represent the mean ± S.D of four experiments; * p<0.05 (Student's t test)

trophotometric background and absorption due to reagent blanks.

Apoptosis Assays

The levels of apoptosis after treatment of MCF-7 cells with nicotine (10 and 20 μM) or doxorubicin alone and in combination with each other were measured using Annexin V-EGFP (enhanced green fluorescent protein) apoptosis detection kit (MBL, Nagoya, Japan). For these experiments, a two-hour pretreatment with nicotine was followed by 90 or 120 minutes of treatment with doxorubicin (10 μM). After treatment with or without drug(s), the cells were harvested by trypsinization followed by centrifugation. The cell pellet was resuspended to obtain $5x10^5$ cells in 500 μl of binding buffer and followed by addition of 5 μl of Annexin V-EGFP and 5 μl of propidium iodide solution. The resulting mixture was incubated at room temperature for 5 minutes in the dark. Annexin binding and propidium iodide staining were analyzed using a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA).

Apoptosis was also estimated in the case of MCF-7 cells, by measuring caspase-3 and caspase-8 activities using a caspase-3/CPP32 fluorometric protease assay kit and a caspase-8/Flice fluorometric protease assay kit (MBL, Nagoya, Japan).[14,15] About $2x10^6$ cells were seeded per 75-cm^2 tissue culture flask and allowed to grow overnight and then cultures were subjected to different treatments. After treatment with or without drug(s), the cells were harvested by trypsinization followed by centrifugation to obtain cell pellets. The pellet was resuspended in 50 μl of chilled cell lysis buffer and kept on ice for 10 minutes. This was followed by addition of 50 μl of 2x reaction buffer containing 10 μM DTT and 5 μl of 1 mM DEVD-AFC substrate for caspase-3 (50 μM final concentration) and the mixture incubated at 37°C for 1–2 hours. Fluorescence intensity was measured in a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter. Caspase-3 activity was normalized to the protein content of the samples, and the protein concentration was determined using Biorad protein assay kit. Similarly, caspase-8 activity was estimated using 5 μl of 1 mM IETD-AFC substrate for caspase-8.

Apoptosis was also detected on the basis of DNA fragmentation analysis after gel electrophoresis (1.5% agarose gel) and ethidium bromide staining.[16,17]

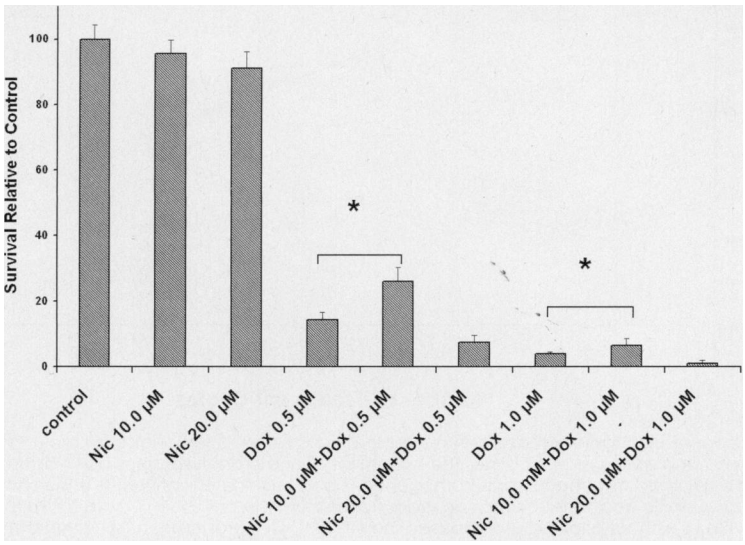
RESULTS

Partial Inhibition of Doxorubicin Toxicity towards Cells Pretreated with Nicotine

Exposure of MCF-7 monolayers to doxorubicin decreased cell survival as determined on the basis of clonogenicity assays. A 90-minute treatment of MCF-7 cells with doxorubicin (10 μM) alone killed >97% cells. However, pretreatment with 10 μM but not 20 μM of nicotine afforded significant protection to MCF-7 cells treated with doxorubicin (10 μM) (Figure 1). Similar results were obtained with KB-3.1 nasopharyngeal carcinoma cells pretreated with nicotine (10 μM and 20 μM), followed by exposure to doxorubicin (0.5 and 1.0 μM) (Figure 2). Significant protection against doxorubicin cytotoxicity was observed in cells pretreated with nicotine (10 μM but not 20 μM).

The effect of nicotine on doxorubicin cytotoxicity towards MCF-7 cells was also examined using MTT assay

Figure 2. Influence of nicotine on clonogenic potential of KB-3.1 cells treated with doxorubicin



For combination treatment, cells were pretreated with nicotine (10 or 20 μM) for two hours followed by a 90-minute treatment with dox (0.5 and 1.0 μM) as described under methods. Data represent the mean ± S.D of four experiments; * $p<0.05$ (Student's t test)

for cell viability. A dose-dependent protection by nicotine was seen after 72 hours exposure to graded doses of doxorubicin (Table 1). Interestingly, nicotine appeared to increase the number of viable cells over a 72-hour period and consistently protected against doxorubicin toxicity.

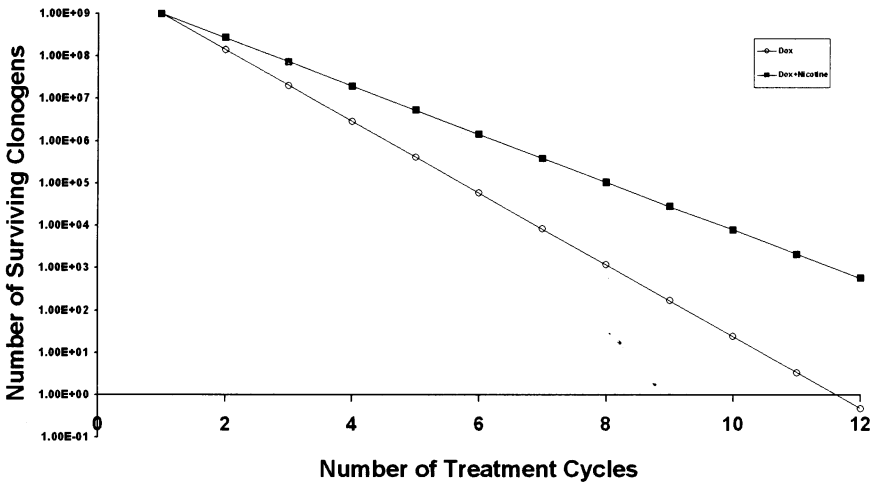
In clonogenicity experiments with KB-3.1 cultures, the survival was 14.19% after a 90-minute treatment with 0.5 μM doxorubicin alone, but the survival increased to 27.16 (average of four experiments, range 22.22–34.57%) when a two-hour treatment with 10-μM nicotine preceded doxorubicin (0.5 μM) treatment. Such inhibition of doxorubicin cytotoxicity by nicotine will have a profound effect on tumor control. This is illustrated by simulating the effect of multiple treatments with doxorubicin alone and in the presence of nicotine. The graph (Figure 3) depicts the number of clonogens remaining after multiple courses of treatment of a hypothetical 1-g tumor containing 10^9 clonogenic cells at the start of therapy. It is assumed that the tumor is homogeneous and that the effectiveness of treatment is the same for each cycle of therapy. Effects of tumor cell proliferation between

Table 2. Combined effect of nicotine and doxorubicin on apoptosis and necrosis

	Apoptosis (Early Stage) (Lower Right Quadrant)	Necrosis and Late-Stage Apoptosis (Upper Left and Right Quadrant)	Mixture of Late-Stage Apoptosis and Necrosis (Upper Right Quadrant)
MCF-7 Cells			
Control	1.18	10.61	5.51
Nic 10 μM	2.38	11.36	7.12
Dox 10 μM 90 min	1.96	63.26	6.68
Nic 10 μM + dox 10 μM 90 min	1.71	47.60	6.84
KB-3.1 Cells			
Control	2.48	2.91	2.11
Nic 10 μM	3.28	4.69	3.24
Dox 1.0 μM 90 min	1.13	63.42	5.38
Nic 10 μM + dox 1.0 μM 90 min	1.82	49.27	5.77
Control for 120-min treatment	2.77	2.03	1.33
Nic 10 μM	4.80	8.79	5.98
Dox 1.0 μM 120 min	0.79	68.46	4.30
Nic 10 μM + dox 1.0 μM 120 min	1.64	60.14	7.48

Nic: nicotine; Dox: doxorubicin; Data are percentages of apoptosis and necrosis, as determined by counting cells in each of the four quadrants of the flow cytometer dot plots obtained using the annexin V-EGFP apoptosis kit (Figure 4).

Figure 3. Simulation of the effect of multiple cycles of doxorubicin treatment with and without nicotine



The graph shows the number of clonogens remaining in a hypothetical one gram tumor after multiple cycles of treatment with doxorubicin in the presence and absence of nicotine. The hypothetical one gram tumor has been assumed to contain 10^9 clonogens at the start of therapy. It is assumed that the tumor is homogeneous and that the effectiveness is the same for each cycle of therapy. The level of cytotoxicity for a single treatment cycle is arbitrarily taken from the cell survival data for 90 minute treatment of KB-3.1 cells to doxorubicin (0.5 μM) with or without prior exposure to nicotine (10 μM). Cell proliferation occurring between cycles has been ignored and the duration of action of each dose of doxorubicin has been assumed not exceed 90 minutes. Open circles represent data for doxorubicin alone and closed squares represent doxorubicin treatment following nicotine pretreatment. It is clear that less than one clonogen is expected to survive after 12 cycles of doxorubicin alone, 555 clonogens would survive after 12 cycles of doxorubicin following nicotine pretreatment.

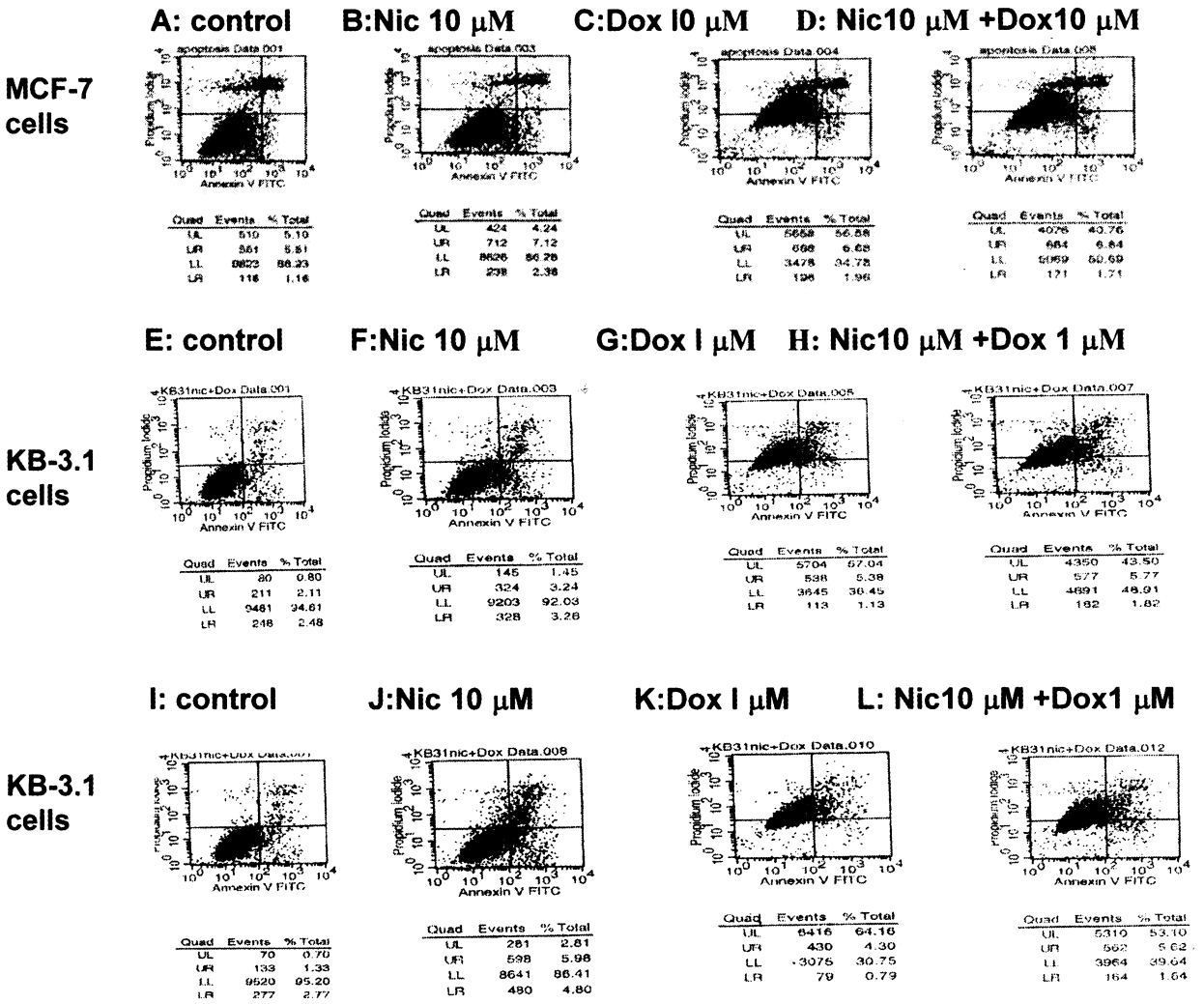
treatment cycles have been ignored in this simulation based on the equation for (N), the number of surviving clonogens that remain at the end of n cycles of therapy:

$N=I\,(SF)^n$, where (I) is the number of clonogens in the tumor at the start of (n) cycles of therapy and SF is the surviving fraction after one cycle of treatment.[18,19]

Pretreatment of Cells with Nicotine Decreases Doxorubicin Induced Necrosis and Apoptosis

Two-color flow cytometry with annexin V and propidium iodide labeling showed necrosis to be the predominant mode of cell death in MCF-7 cells treated with doxorubicin (10 μM) for 90 minutes, while early apoptosis accounted for <5% cell death. Pretreatment with nicotine (10 μM) decreased necrosis and late apoptosis due to doxorubicin (10 μM) treatment of MCF-7

Figure 4. Flow cytometric measurement of apoptosis in MCF-7 and KB-3.1 cells treated with nicotine in combination with doxorubicin



Data are presented as dot plots in which the vertical axis represents fluorescence due to PI staining and the horizontal axis represents the fluorescence associated with annexin V. The upper left quadrant contains necrotic (PI positive) cells, the upper right region contains late stage apoptotic and necrotic (mixture of PI and annexin V positive) cells. The lower left region contains viable non-apoptotic (PI and annexin V negative) cells, and the lower right region contains early apoptotic (PI negative and annexin V positive) cells.

A,B, C and D are the results for MCF-7 cells pretreated with nicotine followed by 90 minutes of doxorubicin (10 μM) treatment. A: Control; B: Nicotine (10 μM); C: Doxorubicin (10 μM); D: Doxorubicin (10 μM) + Nicotine (10 μM).

E, F, G and H are the results for KB-3.1 cells pretreated with nicotine followed by 90 minutes of doxorubicin (1μM) treatment. E: Control; F: Nicotine (10 μM); G: Doxorubicin (1 μM); H: Doxorubicin (1 μM) + Nicotine (10 μM).

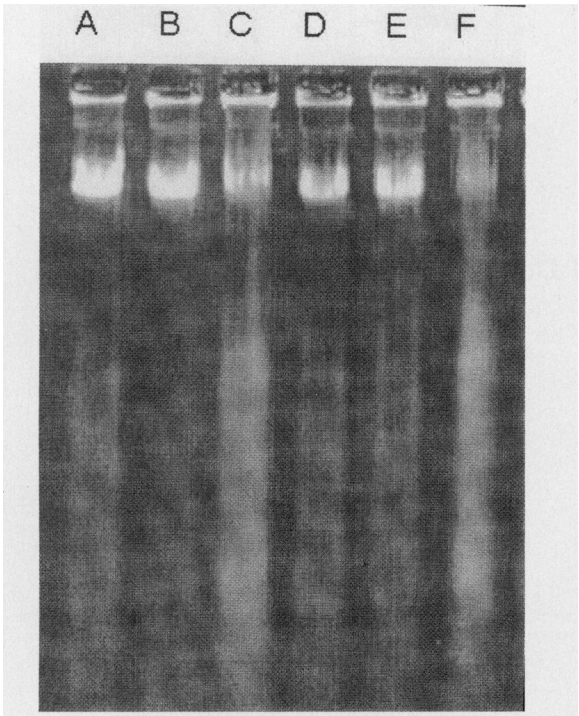
I, J, K and L are the results for KB-3.1 cells pretreated with nicotine followed by 120 minutes of doxorubicin (1μM) treatment. I: Control; J: Nicotine (10 μM); K: Doxorubicin (1 μM); L: Doxorubicin (1 μM) + Nicotine (10 μM).

cells (Figure 4 and Table 2). Similar results were obtained using KB-3.1 human nasopharyngeal cells treated with nicotine and doxorubicin (0.5 and 1.0 μM), (Figure 4 and Table 2).

Effect of Nicotine on DNA Fragmentation and Caspase-3 and Caspase-8 Levels in MCF-7 Cells Treated with Doxorubicin

The results of DNA fragmentation assays (Figure 5) showed that treatment of MCF-7 cells with doxorubicin alone (lane F) induced most DNA fragments compared to untreated control (lane E) and other treatment groups, including treatment with nicotine alone (lanes A, B) or in combination with doxorubicin (lanes C, D). Nicotine (10 and 20 μM) pretreatment for 120 minutes followed by doxorubicin treatment for 90 minutes caused less DNA fragmentation (lanes C, D) compared to treatment with doxorubicin alone (lane F) (Figure 5). Nicotine at 20 μM was more effective than nicotine (10 μM) in preventing doxorubicin-mediated DNA fragmentation. Treatment with nicotine alone did not have any appreciable effect (lanes A, B). This was further examined on the basis of caspase-3 and caspase-8 assays for apoptosis (Figure 6).

Figure 5. Effect of nicotine on DNA fragmentation induced by doxorubicin in MCF-7 cells



The agarose gel patterns for DNA fragmentation are shown for the different treatment groups. The different treatment groups in lanes A–F are: Lane A: nicotine 10 μM; Lane B: nicotine 20 μM; Lane C: nicotine 10 μM + doxorubicin 10 μM; Lane D: nicotine 20 μM + doxorubicin 10 μM; Lane E: control; Lane F: doxorubicin 10 μM.

Treatment of MCF-7 cells with 10 μM of doxorubicin alone for 90 minutes increased caspase-3 activity to 142% and caspase-8 activity to 126% relative to 100% activity for each enzyme in untreated control cultures. However, a two-hour pretreatment with 20 μM of nicotine followed by doxorubicin treatment for 90 minutes resulted in caspase-3 activity of 114% and caspase-8 activity of 101% relative to control (Figure 6). This corresponds to a 20% inhibition of doxorubicin-induced apoptosis by 20 μM of nicotine in these cells. A two-hour pretreatment with 10 μM of nicotine did not have a significant effect on doxorubicin induced caspase-3 and caspase-8 activities.

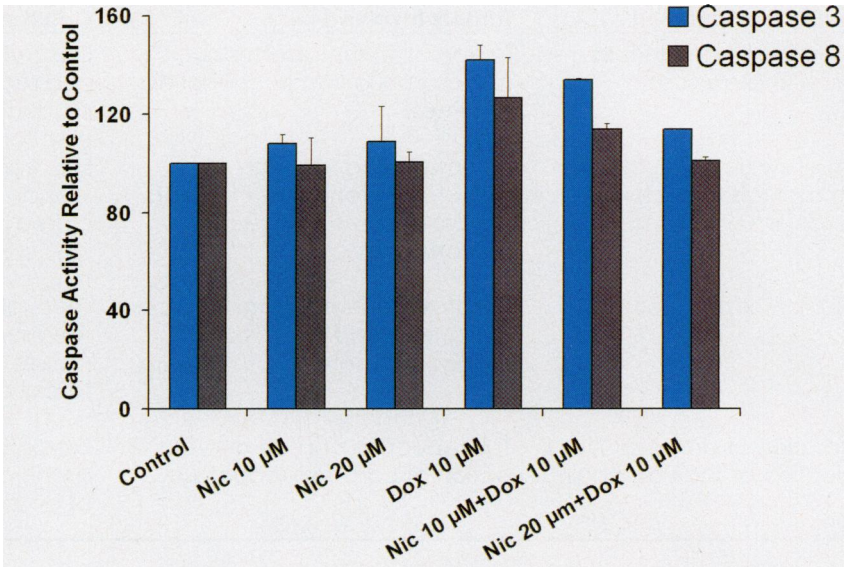
DISCUSSION

Nicotine at 10 μM but not 20 μM protected against doxorubicin mediated loss of clonogenic potential of MCF-7 and KB-3.1 cells. This is probably due to differences in the effect of nicotine on cell signaling at these two concentrations.[20] The aim of cancer therapy is to eliminate clonogenic cells in a tumor since tumor recurrence depends on continued growth of surviving clonogens. Programmed cell death or apoptosis occurs in both normal tissues and tumors. There is concern that apoptosis assays may be inadequate for predicting the response of tumors to drug and radiation treatment.[11,12] The treatment outcome depends on several decades of cell killing (multilog cell kill), which is related to clonogenic cell survival. Loss of colony formation from surviving clonogens is the main event in treated tumor cells, and apoptosis assays do not reflect the clonogenic potential of tumor cells. Moreover, studies comparing apoptosis and cell survival responses in tumor cells usually fail to demonstrate a causal effect.[11,21,22] Inhibition of apoptosis of damaged normal cells may be important in carcinogenesis, while inhibition of apoptotic response may be associated with tumor resistance to therapy. It is known that apoptosis is not the main mechanism for death of cells in nonhematogenous solid tumors.[11,12] This leads to the conclusion that other modes of cell death are also important in the response to therapy. Doxorubicin is a cell cycle-independent cytotoxic agent that is quite effective in decreasing the clonogenic potential of cells. This drug owes its cytotoxic properties to several mechanisms including, but not restricted to, the formation of an array of free radicals through diverse mechanisms. (Table 3 and Figure 7). Free radicals formed during doxorubicin metabolism can initiate DNA damage that can lead to cell death via apoptosis or necrosis depending on the cellular levels of ATP (Figure 6).[34]

The inhibition of doxorubicin cytotoxicity by nicotine is in accord with accumulating evidence that nicotine can affect the cytotoxic effects of anticancer drugs.[8-10] The exact mechanisms by which nicotine affects cell viability is a matter of speculation, and several mechanisms have been proposed. Different possibilities are discussed below. There is convincing evidence that nicotine upregu-

lates antiapoptotic proteins such as XIAP and survivin to give a survival advantage to certain lung cancer cells.[10] Nicotine was shown to induce multisite phosphorylation of proapoptotic proteins Bad and Bax in lung cancer cells and suppress drug-induced apoptosis.[20,24] Inactivation of proapoptotic function of Bad and Bax via phosphorylation also involves nicotine-mediated activation of PI3K/AKT, which acts as a kinase for Bax and Bad phosphorylation. It is noteworthy that treatment of A549 human lung cancer cells for 60 minutes with nicotine (10 μM but not 20 μM) induced phosphorylation of AKT.[20] Therefore, nicotine (10 μM but not 20 μM) activates AKT, leading to suppression of apoptosis because of Bad and

Figure 6. Caspase assays for apoptosis in MCF-7 cells



Inhibition of doxorubicin-mediated apoptosis of MCF-7 cells was also demonstrated on the basis of caspase-3 (clear bars) and caspase-8 (stippled bars) assays as described under methods. Data represent the mean ± S.D of three experiments.

Figure 7. Scheme illustrating the pathways leading to apoptosis and necrosis in cells subjected to free radicals derived from doxorubicin[29]

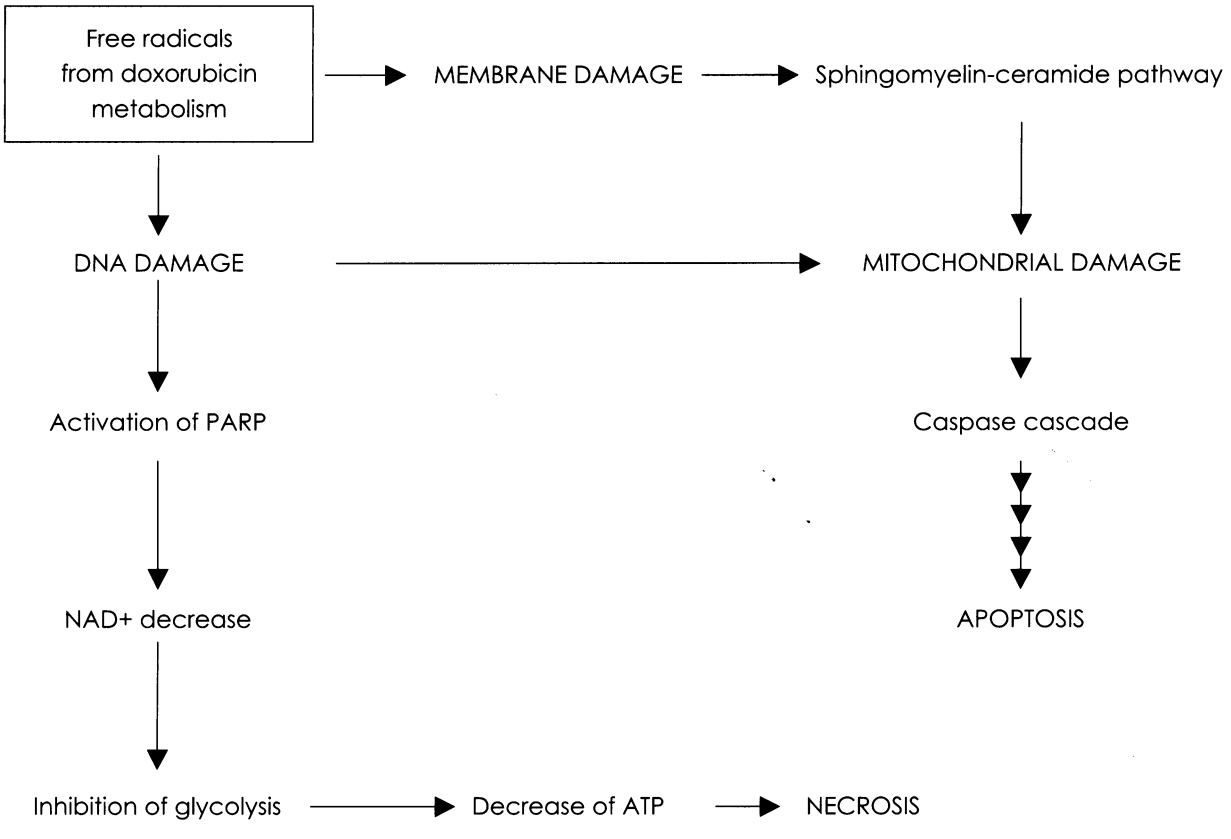


Table 3. Diverse mechanisms involved in the pharmacological action of doxorubicin

Mechanism Involved	Reactive Intermediates and Targets Involved	Comments
Formation of deleterious free-radical metabolites	Doxorubicin metabolism by microsomal and mitochondrial enzymes	Doxorubicin semiquinone radicals and reactive oxygen species are produced as documented by electron spin resonance studies.[22-26]
Non enzymic production of free radicals from doxorubicin interaction with iron	Iron-mediated reaction with a-hydroxyketone side chain of doxorubicin is a source of hydroxyl radicals.	Spin trapping with 5,5-dimethylpyrroline 1-oxide (DMPO) proved the formation of free radicals.[27-30]
Inhibition of topoisomerase	Cytotoxic effects of doxorubicin are attributed to inhibition of topoisomerase.	Cleavable complex of DNA, topoisomerase and doxorubicin causes DNA strand breaks leading to cell death.[31]
Formation of doxorubicinol by reduction of the side chain	This reduction can be due to the action of carbonyl reductase.	Doxorubicinol has been implicated as the cardiotoxic metabolite of doxorubicin.[32,33]

Bax phosphorylation.[20] In this context, it is interesting that nicotine (10 μM rather than 20 μM) protected against cell killing by doxorubicin in our experiments.

Nicotine itself has been reported to possess some antioxidant as well as prooxidant properties. As an antioxidant, nicotine can decrease cell killing mediated by reactive oxygen species.[36] Nicotine can affect mitochondria and decrease cellular ATP production[37,38] and modulate doxorubicin-mediated apoptosis and necrosis. Nicotine inhibits glycolysis and enhances oxygen uptake in isolated perfused rat liver.[39] Programmed cell death (apoptosis) requires an adequate supply of ATP.[34] Perhaps decreased levels of ATP in nicotine-treated cells may explain the slight inhibition of doxorubicin-mediated apoptosis by nicotine pretreatment.

Nicotine inhibits iron uptake and iron transport in certain biological systems without actually affecting the levels of transferrin. Nicotine at a high nonphysiological concentration of 15 mM inhibits iron uptake by about 60%.[40] Nicotine is a weak base, which inhibits release of iron from transferrin. Such perturbation of iron uptake by nicotine may affect the availability of iron for interaction with doxorubicin. Nicotine may interfere with free-radical generation resulting from the interaction of doxorubicin with iron. This may alter free-radical yield and decrease the cytotoxic effect of doxorubicin.

Nicotine may affect membrane permeability[41] and consequently affect the cytotoxicity of doxorubicin. This effect due to lysosomotropic effect of nicotine, if any, would be modest.

Nicotine induces oxidative stress and initiates cell signaling pathways involving transcription factor NF κB.[42-44] Interventions that decrease free-radical flux would decrease apoptosis. The influence of nicotine on apoptosis has been described in terms of its effect on the antiapoptotic protein Bcl2 and proapoptotic proteins Bad and Bax.[20,45] There are conflicting reports on the influence of nicotine on apoptosis. There are several references to proapoptotic[42,44,46,47] as well as antiapoptotic [10,20,35,45,48-50] effects of nicotine.

The concentration of nicotine used in our experiments is slightly higher than the physiological concentration of 1 μg per gram (about 6 μM) found in the tissue of smokers.[50] The plasma half-life of nicotine is approximately 2 hours.[51] Exposure to nicotine in our experiments was for 2 hours, whereas smokers will likely be subjected to more prolonged exposure.

CONCLUSION

Nicotine decreases the cytotoxicity of doxorubicin as indicated by assays for apoptosis, necrosis and clonogenicity. Necrosis, but not apoptosis, is the major cause of cell death in MCF-7 cells and KB-3.1 cells treated with doxorubicin under the conditions of our experiments. MCF-7 cells and KB-3.1 cells showed only a weak apoptotic response to doxorubicin treatment. Inhibition of apoptosis and reproductive cell death can affect tumor control. This statement must further be tempered by the fact that tumor response to therapy depends on tumor cell killing, cell proliferation and cell loss factor. The use of a transdermal nicotine patch during doxorubicin therapy may decrease overall efficacy.

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