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Contribution of Nitric Oxide in Doxorubicin-Related Cytotoxicity in Murine Tumour Model

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ABSTRACT

Background: Doxorubicin (DOX) is an anthracycline antibiotic with broad spectrum antineoplastic activity against different types of experimental and human tumours. However, the optimal usefulness of this important anticancer drug has been limited due to the development of tumour cell drug resistance. Several anticancer drugs including cisplatin, hydroxyurea and DOX have been shown to stimulate nitric oxide (NO) production, which has been demonstrated to affect several aspects of tumour biology.

Purpose: This study has been initiated to determine if DOX stimulates NO production in EAC-cells and if so, whether NO interferes with p-glycoprotein (p-gp) function and to determine if DOX-stimulated NO production contributes to DOX's cytotoxicity in vivo against solid Ehrlich carcinoma (SEC).

Materials and methods: To achieve these goals, the in vitro and in vivo effects of DOX and the nitric oxide synthase (NOS) inhibitors, aminoguanidine (AG) and NG-nitro-L-arginine-methyl ester (L-NAME) on the growth of SEC, NO production, rhodamine 123 (RD 123) accumulation and endothelial nitric oxide synthase (eNOS) were studied in EAC-cells.

Results: A single dose of DOX (10 mg/kg i.p.) resulted in significant decrease in tumour volume and significant increase in serum NO level. Administration of AG (100 mg/kg i.p.), or L-NAME (100 mg/kg i.p.) one hour prior to DOX, significantly decreased DOX-induced NO production and attenuated doxorubicin's effect on tumour volume. Treatment with L-NAME or AG alone induced an insignificant change in NO production. Incubation of EAC-cells with AG or L-NAME resulted in significant decrease in NO production, endothelial nitric oxide synthase (eNOS) activity and RD 123 accumulation. The addition of AG or L-NAME to DOX-incubated cells resulted in significant decrease in NO production and RD 123 accumulation compared to DOX alone.

Conclusion: This study suggests that NO may contribute to DOX's antitumour activity. Increased NO production by DOX may decrease p-gp function with the consequent increase in DOX accumulation in EAC-cells. Further studies are needed to confirm these findings.

Key Words: *Nitric oxide - Doxorubicin - Aminoguanidine - L-NAME - p-glycoprotein - Drug-interaction.*

INTRODUCTION

The anthracycline antibiotic, DOX, has been widely and successfully employed for the treatment of various types of cancers [1]. The usefulness of DOX has been limited due to its dose-related myocardial toxicity [9] and the development of tumour drug resistance [10,23]. Several antineoplastic agents including cisplatin, hydroxyurea, DOX and relaxin, have been shown to stimulate NO production [2,19,26]. It has been demonstrated that tumour cells are susceptible to NO cytostasis [27].

Nitric oxide may influence several aspects of tumour biology including modulation of cell growth, apoptosis, differentiation, angiogenesis and metastatic capability [27]. NO exerts this action through several mechanism, including inhibition of DNA synthesis [20], mitochondrial respiration [28] and cytochrome P-450 activity [33] and an interference with iron-sulfur proteins [22]. NO also enhances cellular oxidative injury [17] and may affect DOX metabolizing enzymes or cellular drug efflux pump mechanisms including p-glycoprotein (p-gp) expression [13]. This latter protein is hypothesized to be an active transporter that uses the energy from ATP to pump DOX out of cells, reducing its intracellular concentrations and hence, toxicity and activity [5,15,16]. The effect of DOX-induced NO production on p-gp function is not studied yet. Therefore, this study has been initiated with the

following specific aims: (1) to determine if DOX stimulates NO production in EAC-cells and if so, whether or not NO interferes with p-gp function and (2) to determine whether or not DOX-stimulated NO production contributes to DOX's cytotoxicity in vivo against solid Ehrlich carcinoma (SEC). To achieve these goals, the in vivo and in vitro effects of DOX and the nitric oxide synthase (NOS) inhibitors, aminoguanidine (AG) and NG-nitro-L-arginine-methyl ester (L-NAME) on the growth of SEC, NO production, Rhodamine 123 (RD 123) accumulation and endothelial NOS (eNOS) were studied in EAC-cells.

MATERIALS AND METHODS

Animals and tumours:

Female Swiss albino mice, weighing 20-22g were obtained from the animal house of the National Cancer Institute (NCI), Cairo University. Animals were allowed free access to standard diet and water ad libitum. A line of Ehrlich ascites carcinoma (EAC) cells was supplied through the courtesy of Dr.C. Benckhuijsen, Amestrerdam, the Netherlands since 1982 and maintained in our laboratory by weekly I.P. transplantation of 2.5 X 10^6 cells/mouse.

Materials:

Doxorubicin (Farmitalia, Carlo Erba, Italy) was a generous gift from NCI drug store. Aminoguanidine (AG) and NG-nitro-L-arginine-Methyl ester (L-NAME) were Sigma products. All other chemicals used were of the highest analytical grade.

Effects of DOX, AG, L-NAME and their combination on the growth of SEC:

The effects of AG and L-NAME on the antitumour activity of DOX were evaluated in mice-bearing SEC. In brief, a total of 60 female mice were used and divided at random into 6 groups, 10 animals each. 2x10^6 EAC-cells were transplanted subcutaneously in the right thigh of each mouse. One hour later, mice received one of the following treatments according to the protocol reported by Lind et al. [13]. The first group received normal saline (0.5 ml/200 g, i.p.), the second group received single dose of DOX (10 mg/kg, i.p.), the third and fourth groups received single dose of AG (100 mg/kg, i.p.) or L-NAME (100 mg/kg, i.p.), respectively and the fifth and sixth groups received single dose of AG (100 mg/kg, i.p.) or L-NAME (100 mg/kg) one hour prior to a single dose of DOX (10 mg/kg, i.p.), respectively. Two weeks later. the tumour volume was measured using a Vernir caliper according to the formula of Osman et al. [18].

Tumour volume (mm^3) = 0.52 AB^2

Where A and B are the major and the minor axes, respectively.

Mice were anesthetized with ether and blood samples were obtained by heart puncture for determination of NO levels in serum.

Determination of nitric oxide:

Nitric oxide levels were determined in serum and culture supernatant according to the method of lgnarro et al. [8] which measures accumulation of stable NO end product, nitrite. This assay is based on the diazotisation of sulfanilic acid with nitric oxide at acidic pH and subsequent coupling with N-(10 naphthyl)-ethylene diamine to yield an intensely coloured product that is measured spectrophotometrically.

Detection of rhodamine 123 accumulation in EAC-cells:

The accumulation of RD 123 which monitors the function of p-gp in EAC-cells was determined according to Ludescher et al. [14]. This method depends on the fluorescent properties of RD 123 dye which is transported by the membrane efflux pump, p-gp. In brief, EAC-cells were withdrawn from tumour-bearing mice, washed, counted and 2 x 10^6 EAC-cells were then resuspended in 2 ml RPMI medium and incubated with one of the following treatments: (1) DOX (1000 uM), (2) AG (1000 uM), (3) L-NAME (1000 uM), (4) DOX plus AG, (5) DOX plus L-NAME and (6) Saline. Two hours later, NO was measured in the supernatant and RD 123 accumulation was measured in the cell pellet. 200 ng/ml RD 123 was added to each tube and incubated at 37°C for 1 hour. At the end of the incubation time, cells were washed twice with 2 ml cold PBS. For accumulation of RD 123, EAC-cells were resuspended in 2 ml cold PBS and the fluorescence intensity was quantitated spectrofluorometrically (SFM 25 Kontron, Italy) using excitation and emission wave lengths 470 and 590 nm, respectively.

Determination of endothelial NOS activity in EAC cells:

In brief, EAC-cells were withdrawn from tumour-bearing mice, washed, counted and 2 x 10^6 EAC-cells were then resuspended in 2 ml RPMI medium and incubated with one of the following treatments: (1) DOX (1000 uM), (2) AG (1000 uM), (3) L-NAME (1000 uM), (4) DOX plus AG, (5) DOX plus L-NAME and (6) Saline. Two hours later, EAC-cells were mixed with cell lysis buffer, then the level of eNOS was measured in the supernatant using ELISA technique according to the method of Salter et al. **[24]**.

Statistical analysis:

Differences between obtained values (mean ± SEM, n = 6) were tested for significance using the student *t*-test. In all data analyses, *p* values of 0.05 or less were considered significant.

RESULTS

Fig. (1) demonstrates that mice receiving DOX had tumor volume 61.5% smaller than in control mice. Tumor volume in mice receiving AG and DOX were nearly 1.5 fold the tumor size in mice receiving DOX alone. Administration of L-NAME before DOX decreased the tumor volume significantly by 54% of the control value. On the other hand, treatment with AG or L-NAME alone had no significant effect on tumor volume.

Fig. (2) shows that serum nitrite level was significantly increased by 67% of the control value after DOX treatment. Pretreatment with AG or L-NAME significantly decreased the nitric oxide production by 32% and 44% compared to DOX group, respectively. Treatment with L-NAME or AG alone induced an insignificant decrease in serum nitrite level.

Tables (1 & 2) show the relationship between NO production and RD123 accumulation in EAC-cells 2 hours after incubation with DOX, AG, L-NAME. Incubation of EAC-cells with either AG or L-NAME resulted in significant 62% and 88% decrease in NO production, respectively (Table 1). Similarly, the accumulation of RD 123 in EAC-cells is decreased by 44% and 30% after AG and L-NAME, respectively (Table 1). The addition of AG or L-NAME to DOX-incubated cells resulted in 27% and 50% decrease in NO production and significant decrease (34% and 46%) in RD 123 accumulation compared to DOX alone (Table 2).

Fig. (3) shows the effect of DOX, AG, L-NAME and their combination on eNOS level in EAC-cells. Incubation of EAC-cells with DOX resulted in significant increase (36%) in eNOS compared to the control level. Both AG and L-NAME significantly decreased the effect of DOX on eNOS by 53%.

Table (1): Effect of AG and L-NAME on nitrite production and RD 123 accumulation in EAC-cells.

Animals groups	Nitrite (µM)	Mean fluorescence ng RD 123 accumulation / $2X10^6$ cells/60 min
Control	0.8±0.06	11.6±1.01
AG	0.3±0.01*	6.5±1.03*
L-NAME	0.1±0.01*	8.1±0.08*

Data are presented as mean ± SEM (n=6).
* Indicates significant change from control group at $p < 0.05$.
AG = aminoguanidine.
L-NAME = L-arginine-methyl ester.

Table (2): Effect of DOX, DOX plus AG and DOX plus L-NAME on nitrite production and RD 123 accumulation in EAC-cells.

Animals groups	Nitrite (µM)	Mean fluorescence ng RD 123 accumulation / $2X10^6$ cells/60 min
DOX	11.96±0.51	95.00±4.05
AG + DOX	8.79±0.50#	63.00±3.23#
	73.50%	66.32%
L-NAME + DOX	5.94±0.25#	52.00±4.05#
	49.67%	54.74%

Data are presented as mean ± SEM (n=6).
# Indicates significant change from DOX group at $p < 0.05$.
DOX = Doxorubicin.
AG = Aminoguanidine.
L-NAME = L-arginine-methyl ester.

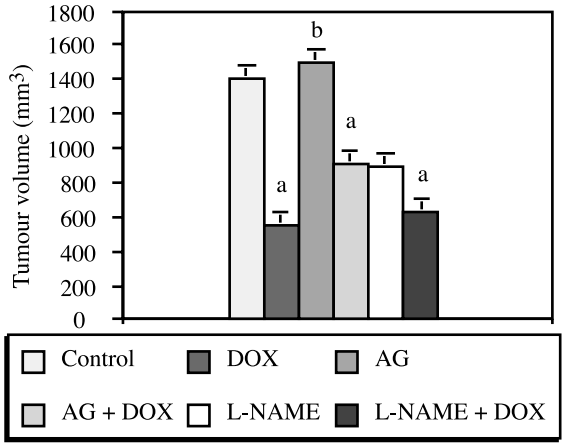

Fig. (1): Effect of DOX, AG, L-NAME and their combination on the growth of solid Ehrlich carcinoma in mice. The dose of DOX, AG and L-NAME were 10 mg/kg, 100 mg/kg and 100 mg/kg, respectively. Data are presented as mean±SEM (n=6).
[a] indicates significant change from control group, [b] indicates significant change from DOX group and [ab] indicates difference from [a] and [b] at $p < 0.05$.

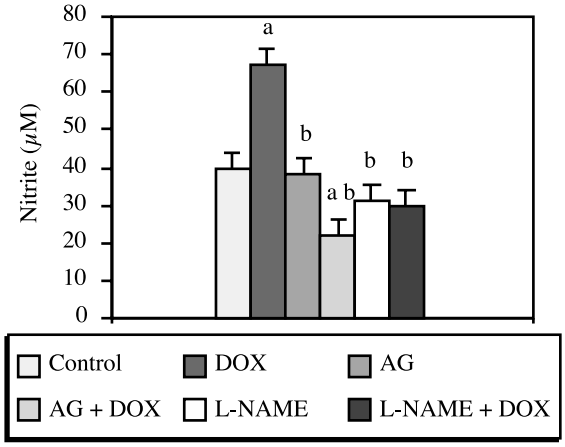

Fig. (2): Effect of DOX, AG, L-NAME and their combination on serum nitrite accumulation in mice. Data are presented as mean ± SEM (n=6).
[a] indicates significant change from control group, [b] indicates significant change from DOX group and [ab] indicates difference from [a] and [b] at $p < 0.05$.

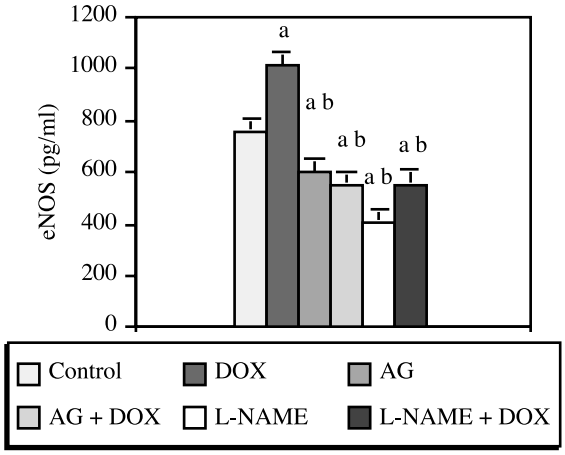

Fig. (3): Effect of DOX, AG, L-NAME and their combination on endothelial nitric oxide synthase (eNOS) in EAC cells. Data are presented as mean ± SEM (n=6).
[a] indicates significant change from control group, [b] indicates significant change from DOX group and [ab] indicates difference from [a] and [b] at $p < 0.05$.

DISCUSSION

Doxorubicin is the single most effective chemotherapeutic agent in the treatment of different types of cancer [7]. DOX cytotoxicity may involve several mechanisms including the generation of reactive oxygen intermediates, DNA fragmentation and programmed cell death [21]. Evidence suggested that nitric oxide (NO) may play diverse roles in DOX's antitumour activity as well as its cardiotoxicity [13,29].

Data from this study demonstrated that DOX increased NO production and decreased the growth of solid Ehrlich carcinoma. These results are in good agreement with previous studies which reported increased NO accumulation in a murine breast cancer cell line and during development of DOX related cardiomyopathy [9,13,25,32]. These results suggested that NO may contribute to DOX's antitumour activity. This hypothesis is confirmed by the data presented in this study which demonstrated that the NOS inhibitors, AG and L-NAME, attenuated DOX's effect on the growth of solid Ehrlich carcinoma. NO is a multifunctional messenger molecule derived from the amino acid L-arginine in a reaction catalyzed by NOS [11].

DOX may stimulate nitrite accumulation either directly or indirectly. The direct effect is through stimulation of NOS activity. Vasquez-Vivar et al. [30] reported that DOX undergoes redox-cycling at the reductase domains of three forms of NOS to produce NO and superoxide anion. Haywood et al. [6] and Vejlstrup et al. [31] observed high levels of NO production via inducible NOS. Increased NO production was due to increase of eNOS transcription and protein expression in bovine aortic endothelial cells [9]. Our results showed that DOX increased eNOS in EAC cells. The increase in nitrite accumulation may also be indirectly through calcium release and increase hydrogen peroxide formation induced by DOX [9]. Expo-

sure of endothelial cells to hydrogen peroxide [3] or calcium-mobilizing agents activates eNOS by dissociating the membrane-bound eNOS from caveolin [4]. Kalivendi et al. [9] reported that DOX induced Ca^{2+} release and H_2O_2 generation which are responsible for increase of eNOS and NO production. DOX may also stimulate nitrite accumulation indirectly through the production of cytokines. However, Lind et al. [13] reported that DOX stimulates NO production through a mechanism that did not involve cytokines. NO may affect cell proliferation via non-apoptotic mechanisms such as the inactivation of iron/sulfur-containing enzymes responsible for mitochondrial respiration [27], or the inhibition of ribonucleotide reductase [12]. NO also enhances cellular oxidative injury [17] and may affect DOX metabolizing enzymes [13].

In this study, the effect of DOX-induced NO production on p-glycoprotein function has been performed by monitoring the accumulation of RD 123 in EAC-cells. The observed decrease in NO production (Table 2) and NOS activity (Fig. 3) by NOS inhibitors, AG and L-NAME, was parallel with the decrease in the accumulation of RD 123 (Table 2) in EAC-cells. These results suggest that NO may interfere with the function and/or expression of p-glycoprotein. In conclusion, this study suggests that NO may contribute to DOX's antitumour activity. Increased NO production by DOX may decrease p-gp function with the consequent increase in DOX accumulation in EAC-cells.

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