Vol. 135, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1986 Pages 330-335

PREVENTION OF DOXORUBICIN-INDUCED KILLING OF MCF-7 HUMAN BREAST CANCER CELLS BY OXYGEN RADICAL SCAVENGERS AND IRON CHELATING AGENTS

James H. Doroshow

Department of Medical Oncology and Therapeutics Research, City of Hope National Medical Center, Duarte, California 91010

Received January 21, 1986

This study investigated the effect of oxygen radical scavengers and iron chelating agents on the toxicity of doxorubicin for MCF-7 human breast cancer cells. Superoxide dismutase and catalase, but not the heat-inactivated enzymes, the hydroxyl radical scavenger N-acetylcysteine, and the organo-selenium compound 2-phenyl-1-2-benzisoselenazol-3(2H)-one, which possesses glutathione peroxidase-like activity, significantly reduced or abolished tumor cell killing by doxorubicin. Similar protective activity was found only for those iron chelating agents capable of penetrating the tumor cell plasma membrane. These experiments suggest that an iron-dependent oxygen radical cascade contributes to the antineoplastic action of the anthracycline antibiotic doxorubicin.

The anthracycline antibiotics, including doxorubicin, daunorubicin, and related analogs, constitute one of the most important groups of drugs used in cancer chemotherapy because they possess significant therapeutic potential in the treatment of most hematologic malignancies as well as carcinomas of the lung, breast, and ovary (1). Although the cytotoxic effect of the anthracyclines for malignant cells has in the past been related to effects on DNA structure or synthesis as a consequence of intercalation or alkylation (2), current evidence suggests that tumor cell killing, at least for doxorubicin, may also be due to lethal events occurring at extranuclear sites (3). Furthermore, despite investigations demonstrating that cyclical reduction and oxidation of the quinone function of the anthracyclines produces a reactive oxygen cascade capable of damaging DNA in vitro (4),

Abbreviations used in the text: ·OH, hydroxyl radical; SOD, superoxide dismutase; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; TPEN, N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine; FCS, fetal calf serum; PZ 51, 2-phenyl-1-2-benzisoselenazol-3(2H)-one; MEM, minimal essential medium.

0006-291X/86 $1.50

a role for the quinone moiety in the antineoplastic action of these compounds for intact cells has not been definitively established.

Recent studies, however, have indicated that doxorubicin stimulates superoxide anion and hydrogen peroxide production by microsomal, mitochondrial, and nuclear preparations from murine carcinomas (5,6) as well as hydroxyl radical (·OH) formation after anthracycline treatment of intact tumor cells (7). Because these investigations strongly suggested that oxygen radical metabolism might play a role in the antineoplastic action of doxorubicin, we examined the effect of reactive oxygen scavengers on the toxicity of doxorubicin for human MCF-7 breast cancer cells. We found that agents which detoxify H_2O_2 or ·OH, including proteins that do not penetrate the cell surface, significantly reduced or abolished the cytotoxicity of doxorubicin. Thus, both extracellular and intracellular oxygen radical attack could be involved in the tumor cell toxicity produced by anthracycline antibiotics.

MATERIALS AND METHODS

Materials. Doxorubicin hydrochloride was purchased from Adria Laboratories, Inc., Wilmington, Del. Superoxide dismutase (SOD), diethylenetriamine-pentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), ethylene-glycol-bis-(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), and N-acetylcysteine were purchased from Sigma Chemical Co., St. Louis, Mo. 2,2-bipyridine was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J. Catalase of analytical grade (65,000 units/mg protein) was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and was devoid of SOD activity when assayed by the method of McCord and Fridovich (8). Deferoxamine mesylate was obtained from Ciba Pharmaceutical Co., Summit, N.J. N, N, N', N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) was purchased from Molecular Probes, Junction City, Oregon. The synthetic, seleno-organic compound 2-phenyl-1-2-benzisoselenazol-3(2H)-one (PZ 51) (9) was a gift of Dr. E. Graf, A. Nattermann and Cie GMb.H, Cologne, F.R.G. Eagle's minimal essential medium with 1 mM sodium pyruvate (MEM) and heat-inactivated fetal calf serum (FCS) were purchased from GIBCO (Grand Island, NY). The mean iron concentration of the MEM containing 10% FCS was 5 μM when examined by atomic absorption spectroscopy.

Cell Line, Drug Treatment, and Clonogenic Cell Survival Determinations. The human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection, Bethesda, Md. Cells were grown in monolayer culture in MEM with 10% FCS in 75 cm^2 plastic flasks (Corning #25110, Corning, NY) at 37°C in a humidified atmosphere containing 5% CO_2 in air. Stock cultures of MCF-7 cells in exponential growth phase were used for experiments 4 days after plating 0.5×10^6 cells per 25 ml of MEM:FCS. Paired groups of tumor cells in 75 cm^2 flasks were treated with either MEM:FCS (10 ml) or an equal volume of medium with the protective agent under investigation for 1 hr; cells were then exposed to either 36 μl of medium or to an equal volume of doxorubicin for 1 hr. At the completion of drug exposure, the culture

medium was removed and cells were washed in phosphate-buffered physiologic saline; cells were then harvested by treatment with 4 ml of a mixture containing 0.05% (w/v) crystalline trypsin and 680 μM EDTA (GIBCO) for 1 min at 37°C. The cell suspension was removed from the tissue culture flasks, passed through a 5 ml pipette, and resuspended in 5 ml of MEM:FCS; it was then centrifuged at 200 x g for 5 min at room temperature. The resulting pellet was resuspended in 3 ml of a 1:1 (v/v) mixture of MEM:FCS and 0.02% EDTA, 0.8% NaCl, 0.035% $NaHCO_3$, 0.04% KCl, 0.1% dextrose. Cells were passed through a 2 ml pipette and then counted in a hemocytometer; cell viability (in all cases > 90%) was measured by exclusion of 0.1% trypan blue dye. Tumor cell clonogenicity was determined by modification of a recently described technique (10); dilutions of 1000 and 2500 cells were prepared in 3 ml of MEM:FCS and were plated in triplicate in 60 x 15 mm tissue culture dishes (Corning #25010). The MCF-7 cells were incubated for 12 days at 37°C in a humidified 5% CO_2 atmosphere; tumor cell colonies consisting of ≥ 40 cells were fixed and stained with 0.005% brilliant cresyl blue in 70% ethanol and counted using an inverted microscope. The cloning efficiency of the MCF-7 cells in this system consistently ranged from 20-40%; the percent clonogenic cell survival has been expressed as the number of colonies produced by drug-treated cells divided by the number of colonies produced by control cells X 100. Detailed control experiments revealed that in the concentrations used for these studies none of the free radical scavenging agents tested produced any significant effect on the plating efficiency of the tumor cells. Data for these studies were analyzed with the two-tailed t test for independent means; differences were tested at the $p \leq 0.05$ level (11).

RESULTS

As shown in Table 1, we found that SOD and catalase, scavengers of the superoxide anion and H_2O_2, respectively, significantly inhibited the killing

Table 1
Effect of Reactive Oxygen Scavengers on the Cytotoxicity of Doxorubicin for MCF-7 Human Breast Cancer Cells

Reactive Oxygen Scavenger	Doxorubicin (μM)		
	0.4	0.5	0.75
	(percent of control colonies)		
None	60 ± 1[a]	49 ± 5	42 ± 1
SOD (50 μg/ml)		93 ± 1[b]	
Heat-inactivated SOD (50 μg/ml)[c]		31 ± 5	
Catalase (3000 Units/ml)	91 ± 1[b]	87 ± 2[b]	69 ± 1[b]
Heat-inactivated Catalase (3000 Units/ml)[c]		41 ± 1	
N-acetylcysteine (100 mM)		100 ± 4[b]	
PZ 51 (5 μM)[d]		74 ± 2[b]	

[a] These studies were performed as described in "Materials and Methods" and the data represent the mean ± S.E. of 3 experiments.

[b] Significantly different from doxorubicin-treated cells not pretreated with a reactive oxygen scavenger, $p < 0.05$.

[c] Enzymes were heat-inactivated by autoclaving for 60 min.

[d] PZ 51 was initially dissolved in concentrated form in 100% (v/v) dimethyl sulfoxide; in these experiments the final concentration of dimethyl sulfoxide when used with PZ 51 was 2.5 mM which had no effect on the cytotoxicity of doxorubicin for MCF-7 cells.

of MCF-7 human breast cancer cells; since the heat-denatured proteins were not protective, it is unlikely that these results were simply due to a nonspecific protein effect or to alterations in drug uptake by the anti-oxidant enzymes. It is of interest that the doxorubicin concentration found to inhibit clonogenic cell survival by 50% (Table 1) approximates quite closely the peak plasma concentration of doxorubicin after intravenous administration in man (1). We also found that the thiol-containing hydroxyl radical scavenger N-acetylcysteine abolished the cytotoxicity of doxorubicin at the concentrations tested in this study; N-acetylcysteine has recently been demonstrated in our laboratory to ameliorate damage to the Ca^{2+} pump of cardiac sarcoplasmic reticulum produced when the doxorubicin quinone is reduced by exogenous NADH dehydrogenase (12). Finally, we determined that the lipophilic organo-selenium compound PZ 51, which possesses glutathione peroxidase-like activity for intact cells and which can effectively detoxify H_2O_2 in the presence of glutathione (9), significantly reduced the cytotoxicity of doxorubicin for MCF-7 cells (Table 1).

In addition to scavengers of $\cdot OH$, H_2O_2, and the superoxide anion, we examined whether iron chelating agents could affect tumor cell killing by doxorubicin (Table 2). All of the agents tested, except EDTA, complex iron

Table 2
Effect of Iron Chelating Agents on the Cytotoxicity of Doxorubicin for MCF-7 Human Breast Cancer Cells

Iron Chelator	Doxorubicin (μM)		
	0.5	1.25	2.5
	(percent of control colonies)		
None	49 ± 5[a]	33 ± 1	9 ± 1
2,2-bipyridine (150 μM)	98 ± 2[b]	45 ± 2[b]	20 ± 1[b]
TPEN (150 μM)	81 ± 2[b]		
Deferoxamine (150 μM)	52 ± 1		
DTPA (150 μM)	54 ± 6		
EDTA (150 μM)	49 ± 1		
EGTA (150 μM)	52 ± 5		

[a]The data represent the mean ± S.E. of 3 experiments.

[b]Significantly different from doxorubicin-treated cells not pretreated with an iron chelating agent.

in a form that is unavailable for participation in reactions generating ·OH (13); however, DTPA and EDTA are incapable of penetrating the cell surface (14), deferoxamine enters only certain mammalian cells and not others (15), and bipyridine and TPEN are freely permeable across the plasma membrane (14,16). We found that both bipyridine (over a five-fold concentration range) and TPEN significantly reduced the toxicity of doxorubicin for MCF-7 cells, whereas the chelators which do not enter the cell produced no effect on cytotoxicity (Table 2).

DISCUSSION

In these experiments we have demonstrated that the killing of MCF-7 human breast cancer cells by doxorubicin can be reduced or abolished by chemicals and antioxidant proteins that detoxify H_2O_2, ·OH, or the superoxide anion. Previous studies with doxorubicin have focused on the possibility that oxidation-reduction cycling of the anthracycline quinone could produce DNA strand scission (4). However, our finding that the antioxidant proteins catalase and SOD diminished the cytotoxicity of doxorubicin is of particular interest because enzymes of this size are unlikely to traverse the tumor cell surface (17). Since H_2O_2 is freely permeable across cell membranes, the protective effect of exogenous catalase could result from a decrease in the intracellular peroxide concentration by the extracellular enzyme. However, superoxide anion itself does not easily cross the tumor cell outer membrane (18); and yet, SOD effectively reduced the cytotoxicity of doxorubicin (Table 1). Thus, superoxide anion produced by redox cycling at or near the cell surface could contribute to the killing of MCF-7 cells by doxorubicin.

We also found that two iron chelating agents capable of entering tumor cells, bipyridine and TPEN, significantly reduced the cytotoxicity of doxorubicin. These experiments suggest that intracellular iron or iron-proteins contribute to the antineoplastic activity of the anthracycline antibiotics. Although the precise source and biochemical form of the iron

involved in drug-related tumor cell killing remain to be determined, our studies support the possibility that treatment of human breast cancer cells with doxorubicin initiates a free radical cascade culminating in the formation of a highly reactive species with the chemical characteristics of .OH. Whether .OH production occurs by the iron-catalyzed Haber-Weiss reaction (13), by direct interaction of the doxorubicin semiquinone with H_2O_2 in presence of transition metals (4), or after formation of a stable doxorubicin-iron complex (19) is unknown. On the other hand, the oxidizing power of the hydroxyl radical is such that potentially lethal events, including alterations in calcium homeostasis (12), energy production (20), membrane integrity (3), or reproductive potential (16), are likely consequences of a doxorubicin-induced oxygen radical cascade in MCF-7 cells.

ACKNOWLEDGMENT

I wish to thank Sunny Aure for her excellent secretarial help, and Linda Matsumoto for superb technical assistance. This study was supported by grant CA 31788 from the NCI and by the Leukemia Society of America.

REFERENCES

1. Young, R.C., Ozols, R.F., and Myers, C.E. (1981) N. Engl. J. Med. 305, 139-153.
2. DiMarco, A. (1982) in Cancer Medicine (Holland, J.F. and Frei, E., eds) pp. 872-906, Lea and Febiger, Philadelphia.
3. Tritton, T.R., and Yee, G. (1982) Science 217, 248-250.
4. Bates, D.A., and Winterbourn, C.C. (1982) FEBS Lett. 145, 137-142.
5. Doroshow, J.H. (1981) Proc. Amer. Assoc. Cancer Res. 22, 203.
6. Doroshow, J.H. (1982) Clin. Res. 30, 69a.
7. Doroshow, J.H. (1985) Proc. Amer. Assoc. Cancer Res. 26, 225.
8. McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.
9. Wendel, A., Fausel, M., Safayhi, H., Tiegs, G., and Otter, R. (1984) Biochem. Pharm. 33, 3241-3245.
10. Goldenberg, G., and Froese, E.K. (1982) Cancer Res. 42, 5147-5151.
11. Armitage, P. (1971) Statistical Methods in Medical Research, pp. 104-126, Blackwell Scientific Pub., Oxford.
12. Harris, R., and Doroshow, J.H. (1985) Biochem. Biophys. Res. Commun. 130, 739-745.
13. Rosen, H., and Klebanoff, S.J. (1981) Arch. Biochem. Biophys. 208, 512-519.
14. Arslan, P., DiVirgilio, F., Beltrame, M., Tsien, R., and Pozzan, T. (1985) J. Biol. Chem. 260, 2719-2727.
15. Hershko, C. (1978) Blood 51, 415-423.
16. Mello-Filho, A.C., Hoffman, E., and Meneghini, R. (1984) Biochem. J. 218, 273-275.
17. Michelson, A.M., and Puget, K. (1980) Acta Physiol. Scand. Suppl. 492, 67-80.
18. Powis, G., Svingen, B.A., and Appel, P. (1981) Mol. Pharmacol. 20, 387-394.
19. Eliot, H., Gianni, L., and Myers, C. (1984) Biochemistry 23, 928-936.
20. Tyler, D. (1975) Biochim. Biophys. Acta 396, 335-346.