Potentiation of Cytotoxicity of Mitoxantrone Toward CHO-K1 Cells *in Vitro* by Dipyridamole

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Dipyridamole (DP), a clinically used vasodilator and an antiplatelet compound, augmented the activity of the anticancer drug mitoxantrone (MXN) toward chinese hamster ovary (CHO-K1) cells in culture. Clonogenic assays indicated that DP (1.0, 2.5, and 5.0 μ*M*) decreased the survival of cells treated with MXN (5 to 25 n*M*) in a dose-dependent manner. Further, DP (1 and 5 μ*M*) decreased the MXN concentration required for 50% inhibition of cell growth from 3.2 to 1.8 and from 3.0 to 0.5 n*M*, respectively, over a period of 3 days. DP (10 μ*M*) increased the accumulation of MXN by 1.8-fold in exponentially growing cells exposed to MXN. The enhanced levels of MXN in CHO-K1 cells in the presence of the chemosensitizer may account for the potentiation of MXN-cytotoxicity by DP.

KEY WORDS: mitoxantrone; cytotoxicity; chemosensitization; CHO-K1 cells; dipyridamole.

INTRODUCTION

Mitoxantrone (MXN) is an anthracenedione chemotherapeutic agent which was designed to reduce the dose-dependent cardiotoxicity of anthracyclines such as doxorubicin while retaining the clinically important antitumor activity (1). Although the comparative frequency of cardiotoxicity of MXN and doxorubicin is still under investigation, it appears that MXN is less cardiotoxic than doxorubicin (2,3). MXN has exhibited activity against leukemia, lymphoma, and breast cancer (3,4). This drug is reportedly more potent than doxorubicin (5). Moreover, studies have shown that tumor cells that develop resistance to anthracyclines may not be cross-resistant to MXN (6). These advantages of MXN over anthracyclines make it a clinically important antineoplastic drug.

Dipyridamole (DP), a clinically used vasodilator and an antiplatelet compound, appears to be a promising modulator of the activity of antineoplastic agents. It has a history of safe clinical use and has been shown to potentiate the cytotoxic action of various compounds. DP, a potent inhibitor of nucleoside transport into cells, has been employed to potentiate the cytotoxicity of antimetabolite antitumor agents. Examples of such anticancer drugs include methotrexate (7), 5-fluorouracil (8), acivicin (9), and *N*-phosphonacetyl-L-aspartate (10). Subsequently, studies have been undertaken to examine the clinical usefulness of DP for this purpose (11,12). Other studies have shown that DP also enhances the cytotoxicity of compounds which are mechanistically different from antimetabolites. DP has been shown to augment the activity of doxorubicin (13,14), vinblastine (14), and etoposide (14,15). In each of these studies it was observed that the presence of DP in the culture medium enhanced the intracellular levels of the anticancer drug.

We have now obtained evidence that DP can markedly potentiate the cytotoxicity of MXN toward CHO-K1 cells *in vitro*, possibly due to enhanced intracellular concentrations of MXN.

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MATERIALS AND METHODS

Drugs and Chemicals

Mitoxantrone hydrochloride was a gift from Dr. Narendra Desai, (American Cyanamid Company, Pearl River, NY). Dipyridamole was obtained from Sigma Chemical Company (St. Louis, MO).

Solutions of DP were prepared by dissolving the compound in a small quantity of dimethyl sulfoxide (DMSO) and then diluting it with complete culture medium. MXN solutions were prepared by dissolving the drug in complete culture medium to obtain appropriate stock solutions (usually 50×). All the solutions were sterilized by filtration through a 0.22-μm-pore size membrane. Drug-free controls contained the appropriate amount of DMSO. From our preliminary spectrophotometric analysis it appears that MXN is stable in alkaline incubation medium (pH 7.4) at 37°C at all periods of drug treatment employed in our experiments.

Cell Culture

Wild-type chinese hamster ovary (CHO-K1) cells were obtained from Dr. Naveen Mishra (University of South Carolina, Columbia). Cells were cultured in minimum essential medium (GIBCO Laboratories, Grand Island, NY), supplemented with 10% fetal bovine serum, glutamine (2 m*M*), penicillin (50 units/ml), and streptomycin (50 μg/ml) (KC Biologicals, Inc., Lenexa, KS). CHO-K1 cells had a doubling time of approximately 24 hr when grown as monolayer cultures. Cell cultures were routinely tested for mycoplasma contamination. Exponentially growing cells were employed in all our experiments.

Clonogenic Assays

Cells were harvested with trypsin, centrifuged free of trypsin, and then resuspended in complete medium to obtain a suspension of single cells. The cell density (number of cells/ml of suspension) was determined using a hemocytometer. Cells were plated in triplicate onto 60 × 15-mm plastic tissue culture plates (Corning Glass Works, Corning, NY) at a density of 200 cells/plate in 5 ml culture medium. After an incubation period of 24 hr, graded doses of MXN (5 to 25 n*M*) were added to the culture in the presence and the absence of DP (1.0, 2.5, or 5.0 μ*M*). Following a drug treatment period of 6 hr, the drug-containing medium was discarded

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and the cells were washed and replenished with fresh medium. Cells were then incubated for 10 days and stained with methylene blue, and colonies containing 50 or more cells were counted. The control dishes usually contained 160–185 colonies.

Inhibition of Cell Growth

Cells were plated onto 60 × 15-mm plastic dishes as described earlier, at a density of 1×10^5 cells/dish in 5 ml culture medium. The cell cultures were maintained in the incubator for 24 hr. MXN was added to cell cultures in the presence of graded concentrations of DP (1.0, 2.5, or 5.0 μ*M*). Cells were trypsinized and harvested at intervals of 24, 48, or 72 hr following the drug treatment and cell counts were determined using a hemocytometer.

MXN Accumulation and Retention Studies

Cells were harvested and seeded into 75-cm^2 flasks (2×10^6 cells/flask) in 10 ml of culture medium. Twenty-four hours after seeding, MXN (10 μ*M*) was added in the presence and the absence of DP (10 μ*M*). Cells were harvested with trypsin at different time intervals (1, 2, 5, 8, and 12 hr) after treatment with MXN and centrifuged. The cell pellet was washed thrice with ice-cold (0°C) sodium phosphate buffer solution (0.1 *M*, pH 7.4). The supernatant was aspirated and the cell pellet was dissolved in NaOH (1 *N*, 1.5 ml) to obtain a clear solution. MXN is an intensely blue-colored compound with a strong absorbance at λ_{max} = 608 nm, which facilitates the use of a spectrophotometer to measure the intracellular drug concentration without the need to use radiolabeled compound. The alkaline solutions obtained were assayed at λ = 608 nm for MXN content using a Beckman Model DU-6 spectrophotometer. Control cells, processed similarly but without the drug treatment, served as blanks. Standard curves were obtained by spiking blanks with different amounts of MXN.

The uptake of MXN by the cells was also studied as a function of DP concentration. Cells were plated and maintained as described above. MXN (10 μ*M*) was added to the cell cultures with or without DP (1.0, 2.5, 5.0, 7.5, 10, 15, and 20 μ*M*). After a drug exposure period of 5 hr cells were harvested by trypsinization, treated as described above, and assayed for MXN content.

RESULTS

Enhancement of MXN Activity by DP Evaluated Using Colony Formation Assays

Clonogenic assays indicated that several doses of DP potentiated the cytotoxicity of MXN. Cells were treated with MXN (5 to 25 n*M*) in the presence and the absence of various doses of DP for a period of 6 hr. DP enhanced the cytotoxicity of MXN at all concentrations used in a dose-dependent fashion (Fig. 1). Under the influence of MXN (25 n*M*) alone, the survival of CHO-K1 cells relative to controls was 23.5%. At the same MXN concentration, the presence of DP (1.0, 2.5, and 5.0 μ*M*) decreased the cell survival to 7.5, 1.5, and 0.29%, respectively.

Potentiation of cytotoxicity of MXN by DP was observed only when the cells were exposed to MXN and DP simultaneously. Incubation of cells with various concentrations of DP for 6, 4, 2, 1, and 0.5 hr prior to the initiation of MXN treatment, with DP not present during the MXN treatment, did not potentiate the cytotoxicity of MXN. Similarly the addition of DP 0.5, 1, 2, 4, and 6 hr after the termination of MXN treatment did not alter the activity of MXN.

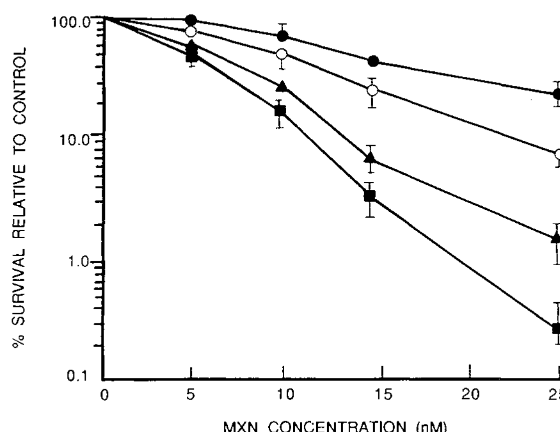

Fig. 1. Potentiation of MXN cytotoxicity by DP. Twenty-four hours after plating, cells were exposed to the indicated concentrations of MXN, with or without various doses of DP, for 6 hr. Colonies were counted at the end of 10 days of incubation in complete medium. (●) MXN alone; (○) MXN + DP (1.0 μ*M*); (▲) MXN + DP (2.5 μ*M*); (■) MXN + DP (5.0 μ*M*). Points, means of triplicate plates; vertical bars, range (shown when larger than symbol size).

DP Augments the Inhibition of Cell Growth by MXN

DP potentiated the inhibition of cell growth by MXN in a dose- and time-dependent manner. Non-growth-inhibitory doses of DP (1.0, 2.5, and 5.0 μ*M*) enhanced inhibition of cell growth by MXN (10 n*M*). The number of cells per milliliter

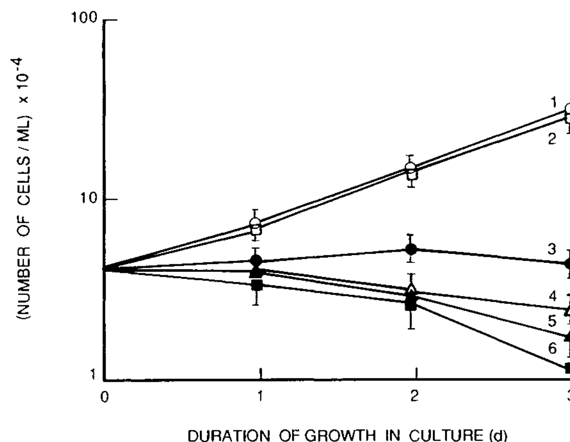

Fig. 2. Augmentation of cell growth inhibition of MXN by DP. Following an incubation period of 24 hr, cells were treated with MXN in the presence and the absence of DP on day 0. The number of cells per milliliter of culture medium was determined 24, 48, and 72 hr later. (1) Control (○); (2) 5 μ*M* DP (□); (3) 10 n*M* MXN (●); (4) 10 n*M* MXN + 1.0 μ*M* DP (△); (5) 10 n*M* MXN + 2.5 μ*M* DP (▲); (6) 10 n*M* MXN + 5.0 μ*M* DP (■). Points, means of duplicate plates; vertical bars, range (shown when larger than symbol size).

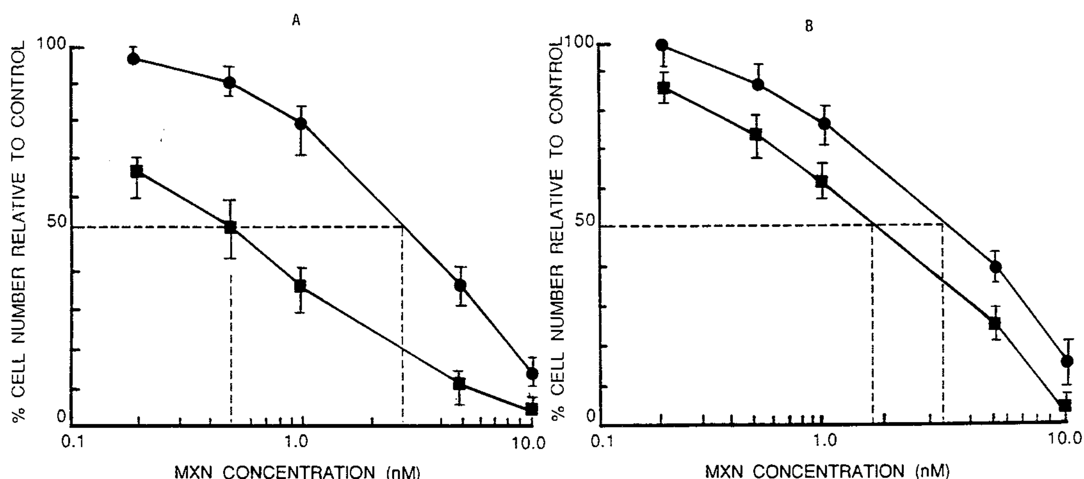

Fig. 3. Effect of DP on the concentration of MXN required for 50% inhibition of cell growth. Twenty-four hours after plating, cells were treated with the indicated concentrations of MXN in the presence and the absence of DP. The number of cells per plate was determined 72 hr later. (A) (●) MXN alone; (■) MXN + DP (5 μ*M*). (B) (●) MXN alone; (■) MXN + DP (1 μ*M*). Points, means of triplicate plates; vertical bars, range (shown when larger than symbol size).

decreased from 4.6×10^4 to 4.36×10^4 (14% of control value) between 24 and 72 hr after the addition of the drug in the culture medium, in the absence of DP. In the same duration, the number of cells per milliliter decreased from 3.46 $\times 10^4$ to 1.12×10^4 (3.5% of control value) in the presence of DP (5 μ*M*) (Fig. 2). We also examined the effect of DP on the concentration of MXN required for 50% inhibition of cell growth (IC_{50}). In the presence of DP (5 μ*M*), the IC_{50} of MXN decreased from 3.0 to 0.5 n*M* (Fig. 3A). In a separate experiment the presence of DP (1 μ*M*) decreased the IC_{50} of MXN from 3.2 to 1.8 n*M* (Fig. 3B).

Effect of DP on the Cellular Accumulation and Retention of MXN

The possibility that the effect of DP on the cytotoxicity of MXN was accompanied by alterations in the cellular accumulation and retention of MXN was evaluated. Cells treated with MXN (10 μ*M*) and DP (10 μ*M*) accumulated more drug compared to cells exposed to MXN alone (Fig. 4). The presence of DP increased the intracellular concentration of MXN approximately 1.8-fold at all time points examined. The observed augmentation of the intracellular concentration of MXN was dependent on the dose of DP employed (Fig. 5). Drug concentrations in cells exposed to MXN (10 μ*M*) in the absence of DP was 1.16 μg/10^6 cells, which increased to 1.36, 2.15, and 2.36 μg/10^6 cells in the presence of 1, 10, and 20 μ*M* DP, respectively. The accumulation and retention of MXN increased markedly, with an increase in the concentration of DP from 1 to 10 μ*M*. However, further increase in the concentration of DP had only a marginal influence.

DISCUSSION

The concentrations of DP employed in our studies are therapeutically achievable *in vivo*. The plasma levels of dipyridamole after p.o. administration for antiplatelet effects reportedly ranged from approximately 1 to 4.5 μ*M* (16,17). However, higher levels of DP in plasma can be achieved as indicated in clinical trials of DP in combination with anticancer drugs. In a phase I trial of DP and acivicin, continuous i.v. infusion at the maximum tolerated dose of DP (23.1 mg/kg/72 hr) produced a mean steady-state total plasma concentration of 11.9 μ*M* (11). In another phase I trial of 5-fluorouracil, folinic acid, and DP, p.o. administration of DP at a dose of 175 mg/m²/6 hr provided a peak total plasma DP concentration of 16.32 μ*M*. Dose-dependent toxicities such

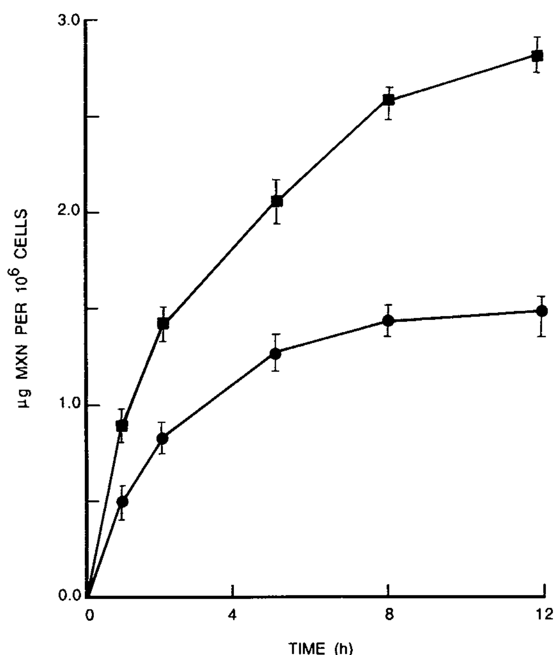

Fig. 4. Accumulation of MXN by cells in the presence and the absence of DP. Cells exposed to MXN (10 μ*M*), with or without DP (10 μ*M*), were harvested at the indicated time intervals and analyzed for the drug content as described under Materials and Methods. (●) MXN alone; (■) MXN + DP. Points, means of triplicate cultures; vertical bars, range.

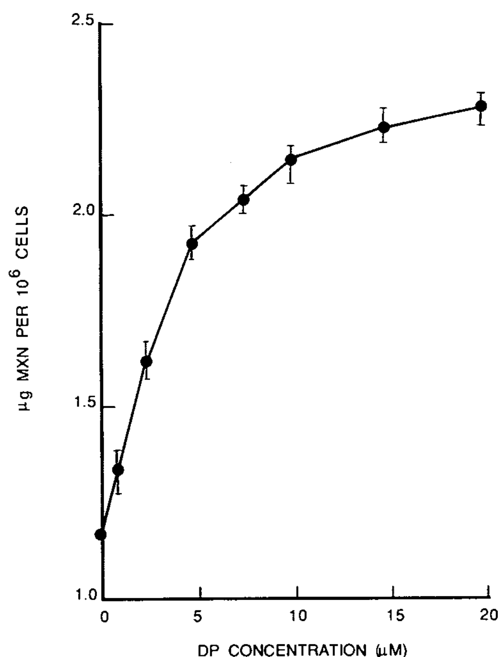

Fig. 5. Effects of varying the concentration of DP in the culture medium on the accumulation of MXN by cells. Cell cultures were treated with MXN (10 μM) in the presence and the absence of the indicated concentrations of DP. Cells were harvested 5 hr later and analyzed for the drug content as described under Materials and Methods. Points, means of triplicate cultures; vertical bars, range.

as neutropenia, mucositis, and nausea were observed at 200 mg/m^2 doses of DP (12). Thus it appears that DP may be one of the safer modulators with potential for clinical use.

The choice of CHO-K1 cells for our studies was based on the following considerations. CHO-K1 cells have been used extensively in several investigations involving studies on the cytotoxicity, uptake, and disposition of anticancer drugs by mammalian cells. These cells are relatively easy to grow and have a short doubling time and a high plating efficiency. This makes CHO-K1 cells useful for preliminary assessment of the cytotoxic effects of antitumor compounds. Furthermore, these cells have also been employed for investigating mechanisms of drug resistance. In this regard, a variety of mutants of wild type CHO-K1 cells, selected for resistance to anticancer drugs, has been isolated (18–20). Such drug-sensitive and drug-resistant cells are useful in studying the effects of modulators of drug action in a quantitative as well as a qualitative manner.

MXN is a potent inhibitor of DNA-topoisomerase II (21) and therefore, an increase in the intracellular concentration of MXN in the presence of DP can be expected to enhance the sensitivity of cells to MXN. The mechanism by which DP augments the accumulation and/or retention of MXN by the cells is not clear. This may involve, among other possibilities, DP-mediated inhibition of MXN efflux, an increase in the influx of MXN, or an alteration in the intracellular retention of MXN.

We plan to extend our studies to examine the DP-mediated modulation of the activity of MXN and other anticancer drugs in tumor cell lines with varying degrees of drug sensitivity *in vitro* and *in vivo*.

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