The effects of verapamil and a tiapamil analogue, DMDP, on adriamycin-induced cytotoxicity in P388 adriamycin-resistant and -sensitive leukemia in vitro and in vivo

S. Radel, I. Bankusli, E. Mayhew, and Y. M. Rustum

Summary. DMDP [N-(3,4-dimethoxyphenethyl)-N-methyl-2-(2-naphthyl-m-dithane-2-propylamine] a recently developed calcium antagonist analogue, caused a greatly increased intracellular retention of adriamycin and concomitant enhanced cytotoxicity in adriamycin-resistant P388 leukemia cells in vitro. These effects of DMDP were greater than those of another calcium channel blocker, verapamil, and occurred at one-half the dosage levels. Only slight enhancement in adriamycin toxicity was observed for either of these agents in the adriamycin-sensitive parental cell line. However, no significant therapeutic potentiation of adriamycin activity occurred with either verapamil or DMDP treatment in vivo. In vivo maximum DMDP tumor intracellular concentrations, as analyzed by HPLC, were the same in vitro tumor cell levels required to overcome adriamycin resistance. This inability to overcome drug resistance in vivo at acceptable levels of host toxicity is not only a function of maintaining necessary calcium antagonist concentrations in resistant tumor cells.

Materials and methods

Chemicals. Adriamycin (ADR) was obtained from ADRIA Laboratories, Columbus, Ohio. Verapamil (VRP) was purchased from SIGMA Chemicals Company, St. Louis, Mo and DMDP was kindly provided by Hoffman-LaRoche, Basel, Switzerland.

Tumor cells. P388/ADR and P388/O murine leukemia cells were supplied by the National Cancer Institute's Frederick Cancer Research Facility, Frederick, Md. These cells have been maintained in our laboratory for 2 years with routine drug resistance checks (once every 2 months). P388/ADR cells have remained 95-100 times more resistant to ADR than P388/O cells throughout this period.

Cell culture. Tumor cells were maintained in plastic 75-cm² (250 ml) T-flasks (Becton-Dickinson Labware, Oxnard, Calif) in Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal bovine serum [Grand Island Bottling Co. Labs (GIBCO), Chagrin Falls, Ohio], 1% penicillin and streptomycin (GIBCO, Grand Island, NY) and 10⁻⁵ M concentration of 2-mercaptoethanol (Fisher Scientific Co., Fair Lawn, NJ). Oxygen free radical scavengers may be involved in the cytotoxic effects of ADR (1). Some studies have reported that oxygen radical scavengers may decrease the cytotoxic effects of ADR (6). 2-ME can act as an oxygen radical scavenger and could alter the cytotoxic effects of ADR. However, no effects of 2-ME on ADR ED₅₀ were found, and so the culture studies employing 2-ME did not interfere with the interpretation of the results. In addition Tsuruo used 2-ME in his in vitro studies (27). Cells were grown in an 37°C humidified atmosphere of 5% CO₂, in air. Each cell line was resupplied with fresh medium every 24-28 h, maintaining a concentration of 4.0-6.0 x 10⁵ cells/ml. Under these conditions exponential growth, with a cell doubling time of approximately 24 h, occurred for both cell lines.

Drug treatment. Each cell line was washed in phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS w/out) (GIBCO, Grand Island, NY) and resuspended in 75 cm² T-flasks at a concentration of 5 x 10⁵ cells/ml. The flasks were then placed in a CO₂ incubator and the cells left to equilibrate for 1 h before experimentation. VRP or DMDP (at a final concentration of 6.1 μM or 3.1 μM, respectively) was added to the appropriate flasks and incubated for 5 min prior to addition of ADR (at 5 x 10⁻⁶ M). The flasks

Introduction

Resistance to chemotherapeutic agents is a common problem experienced in the treatment of cancer. A number of studies have shown that resistant cells accumulate lower drug concentrations than their sensitive counterparts [8, 12]. One mechanism of drug-resistance has been postulated to be due to an enhanced cellular efflux of drug [22]. Overcoming this protective efflux mechanism in drug-resistant cells should lead to enhanced tumor cytotoxicity. Verapamil, a calcium channel blocker, has been shown to increase cellular adriamycin retention and cytotoxicity in drug-resistant tumor cells [9], and this effect has also been reported for a number of other calcium antagonists and calmodulin inhibitors [26].

DMDP [N-(3,4-dimethoxyphenethyl)-N-methyl-2-(2-naphthyl-m-dithane-2-propylamine], an analogue of the calcium antagonist tiapamil, has been reported to be more potent than verapamil, 'VRP' [15]. The aim of our investigation was to determine the effects of DMDP on ADR retention and toxicity compared to VRP in P388 adriamycin-resistant (P388/ADR) and -sensitive (P388/O) tumor cells in vitro and in vivo.

* Supported by NIH grants CA 28494 and CA 18420

Offprint requests to: E. Mayhew
were then placed in a CO₂ incubator on a rocker platform, and at 1, 6, 24 and 48 h thereafter cell samples were removed and assayed for ADR and protein content. Samples were also removed at each time interval to determine cell viability by visual scoring for cellular exclusion of 0.1% trypan blue stain.

A number of methods have been used to assess cell viability, including cell doubling time, thymidine labeling index, chromium release, in vitro colony formation and dye exclusion. Although in vitro colony formation may be a reliable index of cell viability [17], dye exclusion methods are simple, quick and reliable for comparative studies such as that described in the present report.

The short-term ADR/long-term DMDP in vitro P388/ADR experiments were conducted in the same manner as the aforementioned drug treatment experiments, except that after an initial 2-h treatment with ADR (at 5 × 10⁻⁸ M) the cells were washed twice in 37°C PBS w/ out and then resuspended in the presence or absence of DMDP (3.1 µM). Net intracellular ADR accumulation and cell viability were then determined for different times during a 48-h treatment period.

High-pressure liquid chromatography Assay. HPLC analysis of DMDP associated with plasma and cells (in vitro and in vivo) was determined using a µBondapak phenyl C-18 reversed phase column with acetonitrile/H₂O/ammonium hydrochloride (45:54.5:0.5% w/v) buffered to pH5 with formic acid as the mobile phase. Cells were washed twice with PBS w/out, extracted with methanol (0.4 ml/10⁶ cells) and centrifuged at 3000 × G for 15 min. The flow rate was 2 ml/min and the DMDP peak assayed at 229 nm had a retention time of 13 min.

Adriamycin equivalent assay and protein assay. For the assays duplicate cell samples (2.5 × 10⁶ cells) were removed, placed in 15 ml disposable centrifuge tubes (Falcon, Oxnard, Calif) and spun down on an IEC CRU-5000 centrifuge at 450 g for 5 min at 4°C. The samples were washed twice in 5 ml cold PBS w/out extracted with methanol (0.4 ml/10⁶ cells) and centrifuged at 3000 × G for 15 min. The flow rate was 2 ml/min and the DMDP peak assayed at 229 nm had a retention time of 13 min.

Animal studies. DBA/2J female mice (Jackson Laboratory, Bar Harbor, Maine) were inoculated i.p. with 10⁵ tumor cells on day 0, with treatment beginning on day 1. VRP therapy consisted of i.p. injections of VRP (at 50 or 75 mg/kg) and/or ADR (at 1 mg/kg) for 10 consecutive days as described by Tsuruo et al. [26]. DMDP therapy was administered according to two schedules: (1) DMDP was injected i. p. once an hour for 4 h with ADR (10 mg/kg) injected i. p. between the 3rd and 4th DMDP injection; (2) DMDP was administered by continuous i.v. infusion, using a Harvard pump (Harvard Apparatus, South Natick, Mass), for 3 days (at 750 mg/kg per day) with ADR (at 1.0 or 3.0 mg/kg per day) injected i.p.

Results

Toxicity of VRP and DMDP

The in vitro toxicity of VRP and DMDP was determined for the P388/ADR and P388/O cell lines (Fig. 1). The ED₅₀ (effective dose resulting in 50% cell viability) for P388/ADR and P388/O cells exposed for 48 h to VRP was 82 µM±1.7 and 94 µM±2.1, respectively, whereas for DMDP treated cells it was 12 µM±0.7 and 16µM±1.0, respectively. At final concentrations of 6.1 µM for VRP and 3.1 µM for DMDP, which were used in all the experiments, no effect on either cell viability or growth was observed for either P388/ADR or P388/O cells.

Potentiation of ADR cytotoxicity by VRP and DMDP

The cytotoxicity of ADR to P388/ADR cells was enhanced greatly after treatment with VRP or DMDP (Fig. 2). There were 3- and 11-fold increases in cytotoxicity to P388/ADR cells exposed for 24 and 48 h, respectively, to VRP + ADR, whereas DMDP + ADR treatment resulted in 4- and 19-fold enhancements, respectively, compared with ADR only treatment (Fig. 2A). Neither DMDP nor VRP treatment enhanced ADR cytotoxicity against P388/O cells (Fig. 2B).

Enhanced intracellular accumulation of ADR by VRP and DMDP

The intracellular accumulation of ADR in P388/O and P388/ADR cells is shown in Fig. 3. ADR retention in P388/ADR cells increased markedly on exposure to VRP or DMDP. Treatment of P388/ADR cells with VRP + ADR (Fig. 3A) resulted in 1.8-, 3.7- and 4.4-fold enhancements of drug accumulation in comparison with ADR only treated cells at 1, 6 and 24 h, respectively (P < 0.05). There were 2.2-, 4.5- and 5.5-fold increases in intracellular drug retention for DMDP + ADR-treated