Effects of Verapamil on Daunomycin Cellular Retention and Cytotoxicity in P388 Leukemic Cells

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ABSTRACT

We have utilized digitized video microscopy to investigate the influence of verapamil, a calcium channel blocker, on the in vitro effects of daunomycin in P388 sensitive and resistant sublines. In this study, verapamil enhanced the uptake of daunomycin and its cytotoxicity in both sublines, but the effect was more pronounced in the resistant cells. Flow cytometry showed a pronounced accumulation of resistant cells at G2-M when treated with daunomycin in the presence of verapamil, with no effects observed in the absence of verapamil. Analysis of individual subpopulations of cells using the digitized video fluorescence microscopy technique demonstrated that the influence of verapamil on daunomycin uptake affected all cells and was not restricted to certain subpopulations of cells.

MATERIALS AND METHODS

Cell Lines. P388 cells sensitive and resistant to daunomycin were maintained by serial i.p. passages in DBA/2 mice. Ascitic cells were collected on Days 5 to 7 after passage. Erythrocytes were removed by Ficoll-Hypaque density gradient sedimentation. The P388 cells were then washed and resuspended in Hank's balanced salt solution without phenol red (at room temperature). Cells were used immediately for in vitro experiments.

Drugs. Daunomycin (Sigma Chemical Co.) was used at concentrations of 0.2, 0.4, 1, and 1.5 μg/ml.

Incubation Conditions. Sensitive and resistant P388 cells (5 x 10⁶ cells) were allowed to settle on to the surface of a glass coverslip coated previously with poly-L-lysine (26). This coating permits the cells to attach to the coverslip without impairing the viability of the cells or interfering with fluorescence measurements under incident light stimulation. The coverslip is inverted and placed so as to form the top of a chamber with openings designed to allow the inflow and outflow of medium. The temperature was maintained at 37°C by placing the injection syringe in a heated block controlled by a Thermistemp (Yellow Springs Instruments). Flow of the medium was regulated by a motorized syringe pump at a rate of 0.50 ml/min. Since the chamber volume is 40 μl, the chamber fluid was exchanged at a rate of 12 times/min. For every experiment, between 30 and 40 cells were analyzed. Most cells (>90%) exclude trypan blue at the end of the experiment at the frequency of fluorescence stimulation used in these studies. Cells that take up trypan blue are excluded from the analysis.

Determination of Intracellular Drug Levels. Uptake and efflux of daunomycin were quantitated by measuring the fluorescence intensity emitted by drug associated with the cells using digitized video fluorescence microscopy. This system consists of a light microscope equipped with an HBO 50-watt mercury source; a vertical fluorescence illuminator with the appropriate filters is used for routine fluorescence studies. In this system, the most efficient excitation and emission filters for daunomycin are 540 and 580 nm, respectively. The vertical body tube of the microscope is coupled to a specially modified RCA TC-1040 intensified silicon intensifier target camera. The modification of the camera, system calibration, and analysis of fluorescence have been described in detail in a previous paper (25).

Cytotoxicity Studies. Suspension cultures of P388 sensitive or resistant cells were incubated with RPMI medium with 10% FCS. Cells were incubated with daunomycin, daunomycin-verapamil, verapamil, and without drugs for 2 hr at 37°C in a humidified 5% CO₂ atmosphere. The cells were subsequently diluted with RPMI medium, centrifuged, and resuspended in drug-free medium at a concentration of 1 x 10⁶ cells/ml. To determine the percentage of survival after drug exposure, cells were then plated (5 x 10⁶ cells/plate) in 35- x 10-mm Petri dishes with RPMI 1640 medium supplemented with 10% FCS, 10 μg/ml 2-mercaptoethanol, and 0.35% agar. After 10 days of incubation in a humidified 5% CO₂ atmosphere, colonies (>50 cells) in untreated and treated plates were enumerated using a colony-counting unit. Plating efficiencies of controls were 30 and 60% for sensitive and resistant cells, respectively. In all cases, the cloning efficiency of the untreated cells was normalized to 100%, and the cloning efficiency of the treated cells was expressed as

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2 The abbreviation used is: FCS, fetal calf serum.
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Cell Cycle Analysis. To determine the effects of daunorubicin, daunorubicin-verapamil or verapamil on the traverse of cells through the cell cycle, resistant cells treated previously were incubated for 24 or 48 hr in drug-free medium (RPMI with 5% FCS and 10 μM 2-mercaptoethanol). After this period, the cells were counted, and viability was determined. Dead cells were removed prior to cell cycle analysis by Ficoll-Hypaque gradient sedimentation. Portions of the cell suspension were washed and treated with an equal volume of propidium iodide-staining solution consisting of 3.8 mM sodium citrate, 0.1% Triton X-100, RNase B (7,000 units/ml), and 0.05 mg propidium iodide/ml for 30 min at room temperature. The stained cells were then analyzed with a Coulter Electronics TBS-IL at a laser setting of 35 milliwatts at 488-nm excitation.

RESULTS

Effects of Verapamil on Daunorubicin Drug Retention. Chart 1, A and B, illustrates the marked reduction of daunorubicin (0.2 μg/ml) uptake in P388 resistant as compared to sensitive cells. The degree of fluorescence found in sensitive cells was 6-fold higher than in resistant cells at 30 min. The addition of verapamil (3 μg/ml) to the medium resulted in enhanced uptake of daunorubicin in both cell lines, but the effect was more pronounced in the resistant cells. In the latter cells, the level of fluorescence increased 5-fold in comparison to a 67% increase in sensitive cells.

To evaluate the effects of verapamil on retention of daunorubicin, the chamber containing resistant cells was perfused with 1 μg of daunorubicin/ml with or without verapamil. After 30 min, the chamber was perfused with daunorubicin-free medium with or without verapamil, and efflux was determined (Chart 2). In cells exposed to verapamil during the uptake and efflux interval, efflux after 60 min was not pronounced as cells retained 69% of the daunorubicin (Chart 2, Curve a). In contrast, if verapamil was excluded from the efflux perfusion, after a short delay, efflux was rapid, and the cells lost 80% of their fluorescence (Chart 2, Curve b). In cells initially exposed only to daunorubicin, efflux was quite rapid and complete (8% retention) in the absence of verapamil (Chart 2, Curve d); in the presence of verapamil in the efflux medium, efflux was slower (Chart 2, Curve c), and cells retained 25% of the daunorubicin.

Previously, we demonstrated drug uptake heterogeneity in individual P388 sensitive and resistant cells (26). As the effect of verapamil on daunorubicin uptake was greater in resistant cells, we investigated whether this enhancement was restricted to certain subpopulations of cells or involved all cells. Chambers containing resistant cells were perfused with daunorubicin (0.2 μg/ml) for 60 min (control cells) or initially for 30 min followed by perfusion with daunorubicin plus verapamil for another 30 min (Chart 3A). Recordings of intracellular fluorescence were obtained at 30 and 60 min. A distinct difference in daunorubicin uptake is observed at the end of the 60-min incubation when verapamil is included in the perfusion medium. When individual cells are grouped according to their fluorescence accumulation, it is clear that verapamil enhances daunorubicin uptake in virtually all cells (Chart 3B).
Growth of P388 Sensitive and Resistant Colony-forming Units on Agar. Prior to plating of cells on agar, viability as established by trypan blue was determined and found to be 95% in every case. Table 1 illustrates the effects of verapamil on the cytotoxicity of daunomycin in sensitive and resistant tumor cell populations. No change in the number of colonies (as compared to the control population) was observed when resistant cells were incubated with 1 µg of daunomycin/ml for 2 hr, while incubation with 1.5 µg/ml induced only a slight decrease in the number of colonies. When verapamil (at nontoxic concentrations) was added to the incubation medium containing 0.2 µg of daunomycin/ml, no colony formation was detected in resistant cells. In the case of sensitive cells, increased cytotoxicity could be induced by verapamil when the daunomycin concentration was lowered from 0.2 to 0.05 µg/ml.

Cell Cycle Analysis of P388 Resistant Cells. The effects of daunomycin or daunomycin plus verapamil on the cell cycle distribution of resistant cells were determined by flow cytometry.

Table 1

<table>
<thead>
<tr>
<th>Daunomycin concentration (µg/ml)</th>
<th>Verapamil concentration (µg/ml)</th>
<th>Survival (% of control)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Sensitive</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>0.05</td>
<td>0</td>
<td>48 ± 4</td>
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<tr>
<td>0.2</td>
<td>0</td>
<td>100 ± 7</td>
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<tr>
<td>0.4</td>
<td>0</td>
<td>100 ± 4</td>
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<tr>
<td>1</td>
<td>ND</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>1.5</td>
<td>ND</td>
<td>87 ± 6</td>
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<tr>
<td>0.05</td>
<td>3</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>0.2</td>
<td>3</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>0.4</td>
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</tbody>
</table>

*P388 sensitive and resistant cells were treated with various concentrations of daunomycin in the presence or absence of verapamil and plated in soft agar.

Survival is based on colony counts expressed as percentage of the untreated control.

Mean ± S.E. from triplicate experiments.

ND, not determined.
Viability of the cells prior to analysis was 90% for control, verapamil-, or daunomycin-treated resistant cells after 24 or 48 hr. For the population of cells treated with 0.2 µg of daunomycin/ml plus 3 µg of verapamil/ml for 2 hr, the viability after 24 and 48 hr of incubation in drug-free medium was 81 ± 5% (S.E.) and 27 ± 4% (S.E.), respectively. When daunomycin was used at a concentration of 0.4 µg/ml in the presence of verapamil (3 µg/ml) for 2 hr, the viability of the cells decreased to 69 ± 4% (S.E.) and 19 ± 3% (S.E.) after 24 and 48 hr of incubation in drug-free medium. Exposure of tumor cells to concentrations of daunomycin as high as 1 µg/ml for 2 hr failed to alter the cell cycle distribution as compared to the untreated cells (Chart 4A; Table 2). When daunomycin was used at a concentration of 0.2 or 0.4 µg/ml for 2 hr in the presence of nontoxic concentrations of verapamil, a daunomycin dose-dependent accumulation of cells in G2-M phase was observed (Chart 4B, C and D) when the cells were isolated 24 hr later. This block in the viable cells (Table 2) was only partially evident 48 hr later, when the daunomycin concentration was 0.4 µg/ml. It is probable that part of this reversal is an in vitro artifact due to cell selection, since cells blocked on G2-M are the first to die, but it is also possible that some cells may be capable of cell division under liquid medium conditions, but not when they are plated on agar.

No significant differences (data not included) in the cell cycle distribution were observed when sensitive cells were incubated with daunomycin (0.05 µg/ml) in the presence or absence of verapamil (3 µg/ml).

**DISCUSSION**

Verapamil, a calcium channel blocker without antitumor activity, potentiates the effects of cytotoxic agents, such as Vinca alkaloids and anthracyclines (17, 19–23). The present study confirms previous findings that verapamil increases intracellular levels of daunomycin. Although the intracellular fluorescence levels in P388 resistant cells exposed to daunomycin and verapamil for 30 min were found to be lower than in similarly treated sensitive cells, the overall enhancement in daunomycin uptake by verapamil was much greater in resistant cells. The increase in the intracellular accumulation of daunomycin is partially irreversible, as removal of verapamil from the perfusion medium increases efflux of daunomycin. Previously, it has been suggested (19, 21–23) that verapamil inhibits the energy-dependent efflux of daunomycin. Although these data do not clarify the basic mechanism(s) by which verapamil increases the intracellular level of daunomycin, these studies are consistent with the alternative possibility that verapamil increases the level of a slowly exchanging (i.e., bound) drug component. This is consistent with previous studies from this laboratory (26), which suggest increased intracellular daunomycin binding in P388 sensitive as compared to resistant cells, and in sensitive and resistant cells exposed to metabolic inhibitors.

Uptake of daunomycin in P388 sensitive and resistant sublines has been shown to be heterogeneous (26). Hence, in a resistant cell population, there is a small percentage of cells with near normal daunomycin uptake; similarly, sensitive populations include a small percentage of cells with very low drug uptake. This finding is consistent with the notion of subpopulations of tumor cells which differ in growth rate, karyotype, metastatic behavior, and response to chemotherapeutic agents (24, 7). In the present study, perfusion of resistant cells with verapamil-daunomycin induced a significant increase in the fluorescent level of the cells compared to cells treated with daunomycin alone. Furthermore, there was a shift in the predominant subpopulation of cells with low fluorescence (observed in the daunomycin-treated cells) to one with a much higher drug accumulation. These data indicate that enhanced daunomycin uptake induced by verapamil is not restricted to an effect on individual cell subpopulations but affects the majority of tumor cells, suggesting that this agent converts a heterogeneous resistant subpopulation to a more homogeneous and sensitive group of cells.

Levels of intracellular daunomycin as determined by fluorescence techniques provide only a relative value due to a well-described fluorescence-quenching phenomenon which occurs when anthracyclines intercalate with DNA (5, 6, 12). In these studies, fluorescence levels were correlated with the biological activity of the drug. Using a soft-agar colony assay, findings of increased uptake and retention of daunomycin correlated with an increase in cytotoxicity, which was demonstrated for both resistant and sensitive sublines. In the former cells, the addition of verapamil to concentrations of daunomycin as low as 0.2 µg/ml completely circumvented drug resistance.

Studies on synchronized cultured cells have indicated that the cytotoxic effect of daunomycin is at a maximum when cells are exposed during the S phase (13). In proliferating mammalian cells, daunomycin effectively prevents cells from reaching mitosis, without greatly affecting the G1-S transition (18). In the present study, a 2-hr exposure to daunomycin at a concentration of 1 µg/ml had no effect on the cellular kinetics of the P388 resistant cells as the cell cycle phase distribution was virtually identical to the untreated control population. When the resistant cells were incubated with 0.2 or 0.4 µg of daunomycin/ml in the presence of verapamil, there was a significant accumulation of cells in G2-M, a block which partially persisted even after 48 hr of incubation in drug-free medium.

It is not clear that calcium channel blockers will play a role in the application of anthracyclines to the treatment of human cancers. It is possible that verapamil might increase the toxicity of daunomycin, limiting the clinical usefulness of this drug combination. One interesting possibility is the use of these agents to synchronize malignant cells, as verapamil-daunomycin can induce a G2-M proliferation arrest even in the presence of low...
concentrations of daunomycin. This arrest may cause a partial synchronization, and therefore with proper drug scheduling or the use of other cell cycle-specific agents, it may result in increased therapeutic synergy and cell kill.

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