Verapamil-induced Augmentation of Etoposide Accumulation in L1210 Cells in Vitro

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ABSTRACT

The effects of the calcium antagonist verapamil on the intracellular disposition of 4'-demethylepipodophyllotoxin-9-(4,6-O-ethylidene-D-glucopyranoside) (etoposide) (VP-16) as well as on subsequent DNA damage and cytotoxicity were studied in L1210 cells in vitro. At extracellular VP-16 concentrations of 1 to 5 µM, verapamil (10 µM) addition resulted in an increase of DNA single-strand break frequency comparable to that found when VP-16 was present alone at a 3-fold higher concentration. In addition, the elevation of cellular VP-16 levels in the presence of verapamil was linearly correlated with the enhancement of DNA damage and increased cell kill. Verapamil-mediated increase in net VP-16 transport was rapid (1 to 2 min), and allowed for the same elevation of steady-state VP-16 concentration, whether verapamil was added simultaneously with VP-16 or was added after a steady-state level of VP-16 was achieved. Verapamil-mediated elevation of VP-16 levels was not seen at reduced temperature (0 °C). Studies of bidirectional VP-16 transport revealed that verapamil (40 µM) did not alter influx of VP-16 (15 µM), but lowered the unidirectional rate constant for efflux by 93%, resulting in the observed increase of steady-state level of the epipodophyllotoxin. Removal of verapamil resulted in a rapid return of VP-16 to levels comparable to that seen with VP-16 alone. When VP-16 was allowed to flow out of the cell in the presence of verapamil, less than 5% of cellular epipodophyllotoxin was retained, suggesting that the calcium antagonist is not acting by enhancing intracellular binding of VP-16. These results indicate that verapamil potentiates VP-16 activity by elevation of intracellular exchangeable epipodophyllotoxin; an activity which seems to be due to inhibition of the efflux mechanism for VP-16. The low intracellular retention of this epipodophyllotoxin and the good correlation between intracellular VP-16 and subsequent DNA damage and cytotoxicity suggest that the epipodophyllotoxin class of anticancer agents may be more useful for probing calcium antagonist effects on drug transport in sensitive cells and in cells exhibiting pleiotropic drug resistance than the vinca alkaloids and anthracyclines which have large tight binding intracellular components.

INTRODUCTION

We have previously reported that a number of calcium antagonists, when used in combination with the semisynthetic epipodophyllotoxin VP-163 (etoposide), increased both the frequency of DNA strand breaks and cellular toxicity in L1210 cells compared to cells treated with VP-16 alone (33). Based on the rapid reversibility of the calcium antagonist effect, and the absence of potentiation of VP-16 effects in isolated L1210 nuclei, we have suggested that these agents act by perturbing VP-16 membrane transport processes. The few reports of membrane transport of VP-16 suggest that the drug enters cells by passive diffusion but is actively extruded (1, 22). This so called "leak and pump" mechanism for transport has also been suggested for Vinca alkaloids and anthracyclines (13-15, 24, 25). Alteration of active efflux resulting in lower steady-state accumulation of drug has been associated with acquired resistance to Vinca alkaloids and anthracyclines, and cross-resistance to epipodophyllotoxins (7, 13, 16, 24, 25). Inhibition of accelerated drug efflux has been proposed as one mechanism by which calcium-modifying agents overcome resistance to Vinca alkaloids and anthracyclines (7, 21, 29, 30). Therefore, it is not unreasonable to predict that calcium antagonists may operate similarly to block efflux of VP-16.

In this report, studies were undertaken to more clearly define the mechanisms by which calcium antagonists potentiate VP-16 activity. Using [3H]VP-16, we have been able to demonstrate a correlation between intracellular VP-16 levels and the consequential damaging effects on DNA and cell clonogenicity in the presence or absence of the calcium antagonist verapamil. In addition, we have observed that verapamil inhibits the unidirectional efflux of VP-16.

MATERIALS AND METHODS

Chemicals. [2-14C]Thymidine (53 mCi/mmole) and [methyl-3H]thymidine (20 Ci/mmole) were obtained from New England Nuclear (Boston, MA). VP-16 was provided by Bristol Laboratories (Syracuse, NY). Verapamil was provided by Knoll Pharmaceuticals (Whippany, NJ). [3H]VP-16 was obtained from Moravek Biochemicals (Brea, CA). The radiochemical purity of the [3H]VP-16 was determined to be >92%, as assessed by a high-performance liquid chromatography technique (23). All drugs were dissolved in either dimethyl sulfoxide or 50% ethanol. Solvent concentrations did not exceed 0.8% in the culture media after drug treatment and was present in control flasks at equivalent levels. Cell culture media and fetal calf serum were purchased from Grand Island Biological Co. (Grand Island, NY). Proteinase K was obtained from E. Merck (Darmstadt, West Germany). Tetrapropylammonium hydroxide was obtained from RSA Corp. (Ardsdale, NY). Unless otherwise noted, all remaining chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells, Media, and Incubation Techniques. Mouse leukemia L1210 cells were grown in suspension culture in RPMI 1630 containing 20% fetal calf serum and penicillin, and streptomycin. Cells were labeled overnight with [2-14C]thymidine (0.01 µCi/ml) or with [methyl-3H]thymidine (0.1 µCi/ml). Unlabeled thymidine was used to adjust radiolabel specific activity, and to allow for a final nucleoside concentration of 1 µM in the culture medium. VP-16-mediated DNA damage was assessed using the alkaline elution technique for high-frequency single-strand breaks (18). Intact L1210 cells at a density of 5 x 10⁶ cell/ml previously labeled with [2-14C]thymidine, were treated with VP-16 for 1 h in the presence or absence of verapamil.
Cells containing [3H]DNA which had received 1500 rads irradiation were added as an internal standard to drug-treated cells containing [14C]-labeled DNA. Cells were then layered onto a polyvinyl chloride filter (pore size, 2 μm; Millipore Corp., Bedford, MA) and lysed with a solution of 2% sodium dodecyl sulfate, 10 mM disodium EDTA, and proteinase K (0.5 mg/ml). The DNA was eluted from the filter with tetrapropylammonium hydroxide, pH 12.1. The elution flow rate was 0.16 to 0.20 ml/min, with a fractional interval of 5 min, and a total elution time of 30 min. Cells containing [3H] DNA were irradiated on ice with a 137Cs source (Mark I irradiator, J. I. Sheppard and Associates, Glendale, CA) using an exposure rate of 2250 rads/min. Relative retention is defined as the fraction of 14C-labeled DNA remaining on the filter when 75% of the [3H]-labeled internal standard DNA remains. A calibration curve for relating the frequency of VP-16-induced SSBs to an equivalent effect of radiation was obtained by plotting rads versus [14C]DNA retention at 75% retention of the [3H]DNA internal standard. The relative retention of [14C]DNA was found to be proportional to X-ray dose on a first-order plot in the range of 1 to 6 kilorads.

Uptake Studies. L1210 cells were suspended in RPMI 1630 in the presence of 20% fetal calf serum at a final concentration of 10^7 cell/ml. Cells were stirred in specially designed flasks by revolving Teflon paddles in a 37 °C water bath, as described previously (10).

For determination of cellular [3H]VP-16 uptake, 1-ml portions of cell suspension containing [3H]epipodophyllotoxin were periodically injected into 10 volumes of 0.85% NaCl solution at 0 °C. Cells fractions were then separated by centrifugation (500 x g for 2 min) and washed twice with 0.85% NaCl solution at 0 °C. The washed pellet was drawn up into a plastic pipet tip, extruded onto a polyethylene tare, and dried overnight at 70 °C. The dried pellets were weighed, placed in a scintillation vial, and dissolved in 0.25 ml of 1 N NaOH for 2 h at 70 °C. The digest was neutralized with 0.25 ml of 1 N HCI, 4.5 ml of Safety-Solv (Research Products International Corp., Mt. Prospect, IL) were added, and radioactivity was determined on a liquid scintillation spectrometer. Counting efficiencies were determined using [3H]toluene standards.

RESULTS

Correlation of Intracellular VP-16, DNA Single-Strand Breaks, and Cytotoxicity in the Presence and Absence of Verapamil. We have previously demonstrated that calcium antagonists such as verapamil enhance both the DNA strand break frequency and cytotoxicity of VP-16 (33). These effects were found to increase with increasing concentrations of calcium antagonist, suggesting dose-dependent enhancement either of VP-16 intracellular levels or activation intracellularly. Chart 1 shows that DNA strand break frequency is linearly correlated with extracellular VP-16 concentration. Addition of 10 μM verapamil allows for a potentiation of VP-16-induced single-strand breaks, which again shows a linear correlation with VP-16 dose. These data indicate that 10 μM verapamil used over a VP-16 range from 1 to 5 μM potentiates VP-16 activity, so that relative retention values are equivalent to that found for VP-16 used at approximately 3-fold higher levels. In other words, verapamil has modified the activity of VP-16 so that DNA damage reflects that of a 3-fold greater extracellular VP-16 concentration.

The linear relationship between extracellular VP-16 and the steady-state level of the intracellular epipodophyllotoxin is illustrated in Chart 2. In the presence of verapamil (10 μM), the steady-state level of VP-16 is increased; and again, a linear relationship is obtained.

Chart 3 demonstrates the rapid onset of verapamil enhancement of VP-16 levels, when verapamil was added after steady-state epipodophyllotoxin levels were achieved. No difference in time of onset or magnitude of potentiation was observed when verapamil was added simultaneously with [3H]VP-16 (not shown). Verapamil activity is observed within 1 to 2 min, and a new steady-state level of intracellular VP-16 is rapidly achieved. As verapamil concentration was increased to 20 and 40 μM, the enhancement of steady-state VP-16 levels was increased to 2.9 ± 0.2 (SE) and 3.5 ± 0.2-fold over control levels of the epipodophyllotoxin, respectively. No elevation of VP-16 levels could be seen if verapamil was added to cells at 0 °C.

In addition to the data presented in Charts 1 to 3 which suggest that verapamil enhances VP-16-induced DNA damage by elevation of intracellular epipodophyllotoxin, Chart 4 indicates the correlation between intracellular VP-16 levels and DNA damage when cells were incubated with various extracellular concentrations of VP-16 alone, or together with verapamil. These results indicate that verapamil is not altering the relationship between intracellular epipodophyllotoxin and resultant DNA damage. Chart 5 illustrates a similar analysis of cellular VP-16 compared...
ENHANCED VP-16 ACCUMULATION BY VERAPAMIL

Chart 3. Effect of verapamil on net uptake of VP-16 in L1210 cells. Cells were brought to steady state with 5 mM VP-16. Arrow, a portion of the cell suspension was transferred to another flask containing verapamil to achieve a final concentration of 10 mM.

Chart 4. Relationship between steady-state cellular VP-16 levels and DNA damage in the presence or absence of verapamil. Cells were incubated with [3H] VP-16 (1 to 15 mM) alone (○), or together with verapamil (●, 5 to 40 mM) for 20 to 40 min, during which time 2 to 4 measurements of steady-state cellular VP-16 were made. Not all concentrations of VP-16 were used in combination with verapamil. The steady-state levels of cellular VP-16 were unaltered if incubation times were extended through 1 h. In separate experiments, cells were incubated for 1 h with VP-16 alone, or together with verapamil at extracellular concentrations corresponding to those used in the previously mentioned uptake studies. Verapamil used alone as high as 40 μM had no effect on colony formation. Points, mean for each parameter from 3 to 18 experiments run on separate days; bars, SE.

Effects of Verapamil on Initial Uptake of [3H]VP-16. In order to examine the effects of verapamil on VP-16 uptake, the bidirectional fluxes of [3H]VP-16 were studied. Verapamil (40 μM) was preincubated with cells for a short period (less than 1 min), after which [3H]VP-16 (15 mM) was added, and cellular accumulation was quantitated over 15 to 105 s. Chart 6 indicates that over the first 30 s there is little if any effect of verapamil on unidirectional influx of [3H]VP-16. In addition, uptake over this interval approximates initial influx, since uptake points can be extrapolated through zero time with little or no positive y-intercept. Similar results are observed if verapamil is incubated with cells for 20 min prior to addition of [3H]VP-16 (not shown).

Beyond the first 30 s of incubation, there is a definite and progressive enhancement of intracellular [3H]VP-16 in the presence of verapamil. These data again suggest that verapamil has not altered the basic linear relationship between intracellular drug and its lethal consequence.

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Beyond the first 30 s of incubation, there is a definite and progressive enhancement of intracellular [3H]VP-16 in the presence or absence of verapamil. Cells were incubated with [3H]VP-16 (1 to 15 mM) alone (○) or together with verapamil (●, 5 to 40 mM) for 20 to 40 min, during which time 2 to 4 measurements of steady-state cellular VP-16 were made. Not all concentrations of VP-16 were used in combination with verapamil. The steady-state levels of cellular VP-16 were unaltered if incubation times were extended through 1 h. In separate experiments, cells were incubated for 1 h with VP-16 alone, or together with verapamil at extracellular concentrations corresponding to those used in the previously mentioned uptake studies. Cytotoxicity was assessed by quantitating colony-forming ability according to a modification of the assay of Chu and Fisher (6). Verapamil used alone as high as 40 μM had no effect on colony formation. Points, mean for each parameter from 3 to 18 experiments run on separate days; bars, SE.
ence of verapamil. However, even by 105 s, the elevation of VP-16 levels is relatively small, compared to that seen after steady-state levels have been achieved (see Chart 2). Chart 6 demonstrates that even at an elevated verapamil concentration (40 μM), influx of VP-16 is initially unaltered, suggesting that there is an inhibitory effect on VP-16 efflux from the cell under these conditions, to account for the marked elevation in VP-16 levels at steady state.

Effects of Verapamil on VP-16 Efflux. Chart 7 illustrates an experiment in which cells were loaded with VP-16 (15 μM) in the presence or absence of 40 μM verapamil. Cells were then washed free of drugs and resuspended into VP-16-free media again, with or without 40 μM verapamil, respectively. Dimethyl sulfoxide was present at the same concentration under both conditions. Averaging the results of 4 experiments, steady-state VP-16 levels were elevated by 2.4 ± 0.2-fold. Upon efflux of VP-16 in the presence of verapamil, there was a small increase in the nonexchangeable component, although less than 5% of radioactivity was retained. In Chart 7 (inset), the log of the fraction of exchangeable intracellular VP-16 is plotted as a function of time after resuspension into VP-16-free buffer in control and verapamil-treated cells. In 4 experiments, there was a 93 ± 2% decline in the unidirectional rate constant for efflux of exchangeable VP-16 in the presence of verapamil. In separate experiments, cells loaded to the same level of VP-16 in the presence of 40 μM verapamil were resuspended into VP-16-free media in the presence or absence of verapamil. When verapamil was removed, VP-16 efflux returned to the more rapid control rate depicted in Chart 7, while in the continued presence of verapamil, the efflux rate constant was decreased by more than 90%. These results again indicate verapamil inhibition of efflux, and suggest rapid reversibility of verapamil effects.

DISCUSSION

We have previously reported that VP-16-induced DNA damage and cytotoxicity is enhanced in the presence of a number of calcium-modifying agents (33). Although the mechanism for potentiation of epipodophyllotoxin effects is unknown, we have suggested that these so called “calcium antagonists” act by perturbing membrane transport processes, most probably by inhibition of efflux. Previous reports have suggested that calcium antagonists such as verapamil, or calmodulin inhibitors such as trifluoperazine slow the energy-dependent efflux of Vinca alkaloids and anthracyclines, thus allowing for circumvention of resistance to these classes of chemotherapeutic agent (26-31).

Seeber et al. (22) have demonstrated that VP-16 resistance in Ehrlich ascites tumor cells is associated with reduced VP-16 accumulation. Metabolic blockade by sodium azide increased net VP-16 accumulation. Addition of glucose allowed for extrapolation of [3H]VP-16 from those metabolically blocked cells, suggesting an “active pump” system in these cells. We have confirmed that, in L1210 cells incubated in glucose-free buffer, sodium azide (10 mM) allows for enhanced drug levels which are brought back to control levels by addition of glucose (not shown). Sodium azide (10 mM) had no effect on steady-state VP-16 levels when cells were incubated in the presence of glucose, in agreement with results previously obtained by Allen (1), in which L1210 cells were incubated in media which contains glucose. Thus, there is ample documentation that the efflux of VP-16, like that of the Vinca alkaloids and anthracyclines, is linked to cellular energy metabolism. Since cross-resistance between the epipodophyllotoxins, anthracyclines, and Vinca alkaloids has been reported to be associated with altered membrane transport (11, 16), it seemed reasonable to postulate that VP-16 activity might be enhanced by calcium antagonists by inhibition of drug efflux.

Intracellular accumulation of [3H]VP-16 was found to linearly correlate with DNA damage in a dose-dependent manner in the presence and absence of 10 μM verapamil (Charts 1 and 2). These results indicate that verapamil does not change the basic relationship between intracellular VP-16 levels and subsequent lethal damage which presumably occurs at the level of DNA. Similar correlations between intracellular VP-16, DNA damage, and cytotoxicity were demonstrated over a wide range of verapamil and VP-16 concentrations (Charts 4 and 5).

In our previous report (33), we were unable to exclude the possibility that verapamil was potentiating VP-16 activity by inhibiting DNA strand rejoining. If verapamil is inhibiting DNA repair, then at any given intracellular level of VP-16 there would be a correspondingly greater level of DNA damage in the presence than in the absence of verapamil. In this report, we have demonstrated that the correlation between cellular VP-16 levels and resultant DNA damage is unaltered in the presence of verapamil (Chart 4). Therefore, these results suggest that the
calcium antagonist verapamil is not inhibiting DNA strand rejoining. In contrast, it is interesting to note that calmodulin inhibitors have been shown to inhibit DNA repair (5).

Overall, our data indicate that verapamil is most probably not perturbing drug activation or repair processes, but rather allows for enhanced intracellular VP-16 levels. Addition of verapamil to cells which have accumulated VP-16 to a steady state results in a rapid (within 1 to 2 min) enhancement of epipodophyllotoxin levels and establishment of a new steady-state level (Chart 3). If verapamil is added together with VP-16, no change in the time for onset of stimulatory effects, or in the magnitude of enhanced VP-16 levels, was observed (not shown). This rapid onset of action has been observed previously in terms of rapid enhancement of VP-16-induced DNA damage (33), and again suggests that verapamil effects are mediated by increasing cellular VP-16 levels, which allows for elevated drug-induced perturbation of DNA.

Initial uptake of [3H]VP-16 in the presence and absence of verapamil was unaltered, whether or not verapamil had been preincubated with cells prior to [3H]VP-16 addition. These results suggest that over the short period of our experiments, calcium antagonists do not perturb membrane structure and/or function, so that enhanced influx of VP-16 would occur. Fluorescence polarization measurements using diphenyl hexatriene as a probe of membrane fluidity indicate no changes when whole cells were incubated with 20 to 100 μM verapamil. These studies are in contrast to those of Lazo et al. (19), which have demonstrated a slight decrease in membrane fluidity in the presence of 100 μM verapamil.

The inhibitory effects of verapamil on [3H]VP-16 efflux (Chart 7) demonstrates either that the calcium antagonist is in fact inhibiting a membrane carrier which operates to extrude VP-16, or is allowing for enhanced intracellular VP-16 binding to a heretofore unrecognized site. Interestingly, Kessel and Wilberding (17) have recently reported that in P388 cells, verapamil is a substrate for the outward transport system utilized by anthracyclines; competition for efflux sites results in elevated levels of anthracycline. Thus, it is possible that in our system verapamil is similarly slowing VP-16 efflux by competing for transport sites.

The efflux of exchangeable VP-16 was characterized by a single exponential decay process, but a small fraction of drug was observed to be nonexchangeable. Verapamil increased this nonexchangeable component only slightly. We interpret these data to mean that the primary effect of verapamil is to increase total drug by inhibition of efflux. While we cannot exclude the possibility that the bound component is important for VP-16 mechanism of action, it does not appear that verapamil significantly alters the relationship between total intracellular drug and pharmacological effect (e.g., DNA damage and cytotoxicity).

Thus, it is unlikely that verapamil alters the relationship between free drug and drug bound at the site of action.

The apparent lack of intracellular VP-16 binding is in contrast to the well-documented tight-binding loci for both Vinca alkaloids and anthracyclines (4, 8, 12, 20, 32, 35). In fact, the absence of VP-16 cellular binding facilitates the analysis of studies of cellular drug loss in the presence of calcium modifiers. Since considerable controversy exists as to the mechanism by which calcium antagonists overcome pleiotropic drug resistance, i.e., whether calcium modifiers alter affinity of anticancer agents for intracellular binding sites (2, 3, 9, 30, 34) or are perturbing drug transport, it is apparent that the lack of intracellular VP-16 binding makes the epipodophyllotoxins a desirable class of agent for the study of drug resistance.

REFERENCES
Verapamil-induced Augmentation of Etoposide Accumulation in L1210 Cells *in Vitro*

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