ABSTRACT

Verapamil and a number of other Ca\(^{2+}\) antagonists were found to potentiate DNA damage induced by 4'-demethyl epipodophyllotoxin-9-(4,6-O-ethylidene-\(\beta\)-d-glucopyranoside (VP-16) in L1210 cells in vitro. The potentiating effect of verapamil on DNA single-strand breaks in vitro was concentration dependent, relevant to clinically achieved levels of Ca\(^{2+}\) antagonists, and showed good correlation with enhanced cytotoxicity when VP-16 and Ca\(^{2+}\) antagonists were combined in soft agar colony-forming assays. Onset of verapamil activity was observed within 20 min of addition to cells whether VP-16 had been preincubated with cells or was added simultaneously with the Ca\(^{2+}\) blocker. The presence of the extracellular Ca\(^{2+}\) antagonist was required for potentiation as evidenced by the rapid reversal of increased DNA single-strand breaks when cells were washed free of verapamil. Neither ethylenglycoll bis(\(\beta\)-aminoethyl ether)-N,N',N\(^\prime\)-tetraacetic acid nor the Ca\(^{2+}\) ionophore A23187 altered verapamil potentiation of VP-16-induced DNA damage, suggesting that this Ca\(^{2+}\) antagonist acts by a mechanism other than by inhibition of Ca\(^{2+}\) influx. In isolated L1210 nuclei, verapamil did not enhance VP-16 or 4'-demethyl epipodophyllotoxin-9-(4,6-O-2-thienyldene-\(\beta\)-d-glucopyranoside (VM-26)-induced single-strand breaks suggesting a requirement for the intact cytoplasm. Even though VM-26 was 5- to 10-fold more potent than VP-16, verapamil potentiated the DNA damage caused by these two epipodophyllotoxins in L1210 cells to the same extent when these agents were used at equipotent doses. Potency differences between VM-26 and VP-16 were evident in isolated nuclei suggesting that nuclear binding or activation is a more important parameter than were previously reported membrane transport differences. The significance of Ca\(^{2+}\) antagonist potentiation of VP-16-induced DNA damage is discussed in terms of overcoming resistance to epipodophyllotoxins and characterizing more precisely the intracellular disposition, binding, and activation of VP-16.

INTRODUCTION

VP-16\(^4\) (etoposide), a semisynthetic epipodophyllotoxin, is active against a variety of leukemias and solid tumors (8, 18) and has become an important chemotherapeutic agent for the treatment of small cell carcinoma of the lung (11). VP-16 introduces damage at the level of DNA by causing strand scissions (13, 16, 21, 31) which correlate over a wide dose range with cytotoxicity (31). Removal of the drug results in rapid reversibility of DNA strand breakage (16). Therefore, intracellular concentration is probably an important factor for VP-16 activity. Accordingly, it is important to identify and characterize the factors which regulate the intracellular disposition of VP-16.

Previous reports have shown that Ca\(^{2+}\) antagonists, a class of drugs first synthesized in the 1960s (6), are able to overcome resistance to anthracyclines and Vinca alkaloids when used in combination with these agents in vivo (26, 27). The Ca\(^{2+}\) antagonists caused enhanced accumulation of Adriamycin, vincristine, and vinblastine in murine and human leukemia cells in vitro, especially in those cells which were resistant based on altered membrane transport for these chemotherapeutic agents (27, 28). Since cross-resistance between the epipodophyllotoxins, anthracyclines, and Vinca alkaloids in these cell lines has been reported and is associated with altered membrane transport (7, 12), VP-16 activity might be similarly enhanced in the presence of Ca\(^{2+}\) antagonists.

In this paper, we demonstrate that a variety of Ca\(^{2+}\) antagonists, when used in combination with VP-16, increased the frequency of SSBs found in DNA of intact L1210 cells when compared to cells treated with VP-16 alone. This enhancement of VP-16-induced DNA damage correlated with increased cytotoxicity. We have characterized a number of additional properties of the epipodophyllotoxin-Ca\(^{2+}\) antagonist interaction in order to more closely define the pharmacological effects of both VP-16 and the Ca\(^{2+}\) antagonists.

MATERIALS AND METHODS

Chemicals. [2-\(^{14}\)C]Thymidine (53 mCi/mmol) and [methyl-\(^3\)H]thymidine (20 Ci/mmol) were obtained from New England Nuclear (Boston, MA). VP-16 and VM-26 were provided by Bristol Laboratories (Syracuse, NY). Verapamil and D-600 were provided by Knoll Pharmaceutical ( Whippany, NJ). Diltiazem was a gift (Marian Laboratories, Kansas City, MO) as were bepridil (McNeil Pharmaceutical, Spring House, PA) and pirenzepine (Hoechst-Roussel Pharmaceuticals, Summit, NJ). All drugs were dissolved in dimethyl sulfoxide. Solvent concentration did not exceed 0.6% in the culture medium after drug treatment and was present in control flasks at equivalent levels. Cell culture medium and fetal calf serum were purchased from Grand Island Biological Co. (Grand Island, NY). Proteinase K was obtained from E. Merck (Darmstadt, Germany). Tetrabromophenol blue hydroxide was obtained from RSL Corp. (Ardsdale, NY). Unless otherwise noted, all remaining chemicals were purchased from Sigma (St. Louis, MO).

Cells, Media, and Incubation Techniques. Mouse leukemia L1210 cells were grown in suspension culture in Roswell Park Memorial Institute Tissue Culture Medium 1630 containing 20% fetal calf serum, penicillin, and streptomycin. Cells were labeled overnight with [2-\(^{14}\)C]thymidine (0.01 uCi/ml) or with [methyl-\(^3\)H]thymidine (0.1 uCi/ml). Unlabeled thymidine was used to adjust radiolabel specific activity and to allow for a final nucleoside concentration of 1 uM in the culture medium. Isolated nuclei were prepared by washing \(^3\)H-labeled whole cells in an
ice-cold buffer (pH 6.4) containing 1 mM KH₂PO₄, 5 mM MgCl₂, 150 mM NaCl, and 1 mM EGTA (3). The cells were resuspended in 1 ml of this buffer, and an additional 9 ml of the original buffer containing 0.3% Triton X-100 (E. Kodak Co., Rochester, NY) were added to lyse the cells. After sitting on ice for 30 min, 40 ml of buffer were added, and nuclei were separated by centrifugation at 1000 rpm for 10 min. Nuclei density was adjusted to 1 x 10⁶/ml in cold buffer. After warming at 37° for 15 min, the nuclei were treated for 1 hr at 37° with VP-16 or VM-26 in the presence or absence of the Ca²⁺ antagonist verapamil.

Epipodophyllotoxin-mediated DNA damage in isolated nuclei was assessed utilizing the alkaline elution technique for high-frequency DNA SSBs (15). Nuclei containing ³H-labeled DNA were 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 0.5 mg of proteinase K/ml. The DNA was eluted from the filter with tetrapropylammonium hydroxide, pH 12.1. The elution flow rate was 0.16 to 0.2 ml/min with a fractional interval of 5 min and a total elution time of 30 min.

In other experiments, intact L1210 cells at a density of 5 x 10⁵ cells/ml, labeled previously with [2-¹⁴C]thymidine, were treated with VP-16 or VM-26 for 1 hr in the presence or absence of various calcium channel antagonists. The DNA SSB frequency was then measured using the alkaline elution assay as described above, except that cells which contained ³H-DNA and had received 1500 rads of radiation were included as an internal standard. 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. All charts using relative retention as the ordinate value are replots of primary elution data of the type depicted in Chart 1. A calibration curve for relating the frequency of VP-16-induced DNA 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 (data not shown). 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.

Cytotoxicity was measured using a modification of the soft agar colony-forming assay of Chu and Fisher (2). Unless stated otherwise, data shown are representative of at least 3 similar experiments.

RESULTS

Potentiation of VP-16 SSB Frequency and Cytotoxicity by Verapamil and D-600. Previous studies have indicated that VP-16 causes SSBs in the DNA of L1210 cells. Enhancement of the VP-16 (5 μM)-induced SSB frequency by simultaneously added verapamil is demonstrated in Chart 1 to occur as a function of the extracellular concentration (2 to 20 μM) of this calcium antagonist. No DNA damage was present in cells treated with 20 μM verapamil alone. Chart 2 demonstrates that D-600 and verapamil reduce the relative retention of ¹⁴C-labeled DNA of L1210 cells when used over a wide concentration range (2 to 40 μM). Verapamil is more effective in potentiating VP-16-induced SSBs in whole cells than its methoxy derivative, D-600. Both agents maximally enhanced DNA damage in the range of 20 to 40 μM.

It was of interest to determine whether enhanced DNA damage mediated by Ca²⁺ antagonists correlated with an increase in cellular cytotoxicity under the same experimental conditions. Chart 3 illustrates the results of soft agar colony-forming assays using L1210 cells treated with 5 μM VP-16 for 1 hr. Cytotoxicity due to VP-16 was progressively increased with increasing con-
potentiation of VP-16-induced DNA damage. L1210 cells were incubated for 1 hr with 5 μM VP-16 in order to approach a steady-state SSB frequency. A portion of the cells was then removed and placed in an incubation flask which contained verapamil, so that the final Ca²⁺ antagonist concentration was 10 μM. Within 20 min after exposure of cells to verapamil in the presence of VP-16, there was an enhancement of the SSB frequency (Chart 5), which soon reached a new plateau.

Chart 6 illustrates the rapid reversibility of enhanced DNA SSB after cells have been washed free of extracellular verapamil. Cells were incubated for 60 min in the presence of 5 μM VP-16 alone or together with 10 μM verapamil. After being washed free of all extracellular drugs, cells which were exposed to VP-16 alone were again resuspended in 5 μM VP-16. Cells which had been incubated with VP-16 and verapamil were resuspended in 5 μM VP-16 in the absence or in the presence of 10 μM verapamil. One hr after the removal of verapamil, DNA SSB frequency had returned to a level close to that found with VP-16 alone.

To test the possibility that verapamil is exerting its effects by inhibiting DNA repair processes, cells incubated for 1 hr with 5 μM VP-16 and verapamil were washed free of VP-16 in the continued presence of verapamil. During the first 15 min after
VP-16 removal, there was a delay in strand break rejoining. Over the next 100 min, the rapidity and extent of repair in verapamil-treated cells were unaltered compared to cells not exposed to verapamil (not shown).

Effects of EGTA and A23187 on VP-16-induced DNA Damage and Verapamil Potentiation. L1210 cells were incubated in the presence or absence of 2 mM EGTA for a period of 1 hr. This concentration of EGTA is 5-fold higher than the medium Ca\textsuperscript{2+} concentration and assures that extracellular Ca\textsuperscript{2+} is chelated. Cells were subsequently treated with VP-16 (5 \mu M) or VM-26 in the presence of verapamil (10 \mu M) for 1 hr. EGTA had no effect on either VP-16-induced SSB or verapamil-induced potentiation (not shown). Similarly, the Ca\textsuperscript{2+} ionophore A23187 (10 \mu M) did not perturb either VP-16-induced DNA damage or verapamil-mediated potentiation.

Effects of Verapamil on VP-16- and VM-26-induced SSBs in L1210 Nuclei. Epipodophyllotoxin effects on DNA strand scission were analyzed in isolated nuclei from L1210 cells. It was of interest to note that VM-26 was at least 5-fold more potent than VP-16 (Chart 7). When verapamil was added along with the epipodophyllotoxins, there was no potentiation of SSB frequency as demonstrated above (Chart 2) in the whole cell. Thus, whatever the mechanism by which the Ca\textsuperscript{2+} antagonists potentiate VP-16-induced DNA damage, these data suggest that there is a requirement for the intact cell.

Comparison of Ca\textsuperscript{2+} Antagonist Effects on Epipodophyllotoxin-induced DNA Damage in L1210 Cells. VP-16 and VM-26 concentrations were adjusted so that the DNA SSB frequencies produced in whole cells were comparable (VM-26, 0.5 \mu M; VP-16, 5 \mu M). Under these conditions, in 7 experiments run on separate days, there was no significant difference in the degree of potentiation of SSB damage by 10 \mu M verapamil (not shown).

As illustrated in Table 1, verapamil is the most potent of the agents tested for ability to potentiate VP-16-induced DNA damage. Diltiazem, on the other hand, did not enhance SSB frequency when used at 10 \mu M. Diltiazem at 80 \mu M did potentiate to the same level as 10 \mu M bepridil. In all cases, the Ca\textsuperscript{2+} antagonists alone did not cause DNA damage.

**DISCUSSION**

Results presented previously have indicated that the epipodophyllotoxin, VP-16, damages DNA (13, 16, 17, 21, 31) by inducing DNA-protein cross-links and single- and double-strand breaks. Recent observations (30) have led to the hypothesis that VP-16 is activated via an enzyme, such as a dehydrogenase, whereby the pendant ring phenol group of VP-16 undergoes an oxidation-reduction reaction. In other recent experiments, it has been demonstrated that VP-16 stimulates DNA cleavage induced by purified calf thymus type II topoisomerase.\textsuperscript{5} The present study demonstrates a new element of the pharmacology of the epipodophyllotoxins, namely that Ca\textsuperscript{2+} antagonists, which are known to block Ca\textsuperscript{2+} influx in a variety of tissues (4, 5, 14, 25), potentiate the DNA SSB frequency caused by VP-16 in intact L1210 cells in vitro.

Ca\textsuperscript{2+} antagonists have been used recently to overcome resistance to the anthracycline antibiotics, Adriamycin and daunorubicin, and to vincristine (24, 26–28). Drug resistance in these systems is characterized by altered membrane transport (9, 10, 22, 23, 29). The Ca\textsuperscript{2+} antagonist verapamil has been shown to enhance the cellular level of vincristine 2-fold in sensitive P388 cells and 10-fold in vincristine-resistant P388 cells (27). Since verapamil did not alter vincristine binding to microtubules, the suggestion was made that verapamil inhibits the active unidirectional efflux of vincristine, thereby resulting in elevated intracellular drug levels (27).

Active efflux of vincristine, daunorubicin, and Adriamycin has been demonstrated (10, 23, 27); alteration of this exit pump is accompanied by cross-resistance to these agents in a number of cell types (7, 9, 12, 23). Since several of the cell lines which have been shown to be cross-resistant to anthracyclines and vinca alkaloids are also resistant to VM-26 and VP-16 (7, 12), it is possible that the epipodophyllotoxins share similar transport parameters (with the other classes of anticancer agents) and may be affected by Ca\textsuperscript{2+} antagonists in a similar manner.

In our work, DNA SSBs are observed in 20 min of exposure of cells to 5 \mu M VP-16 (Chart 5). In fact, DNA SSBs rapidly reach a plateau which probably relates to a complex interplay between VP-16 membrane transport (influx and efflux), the rate of DNA strand scission, and the rate of DNA strand rejoining. Addition of verapamil results in a rapid enhancement of SSB frequency, but again, a steady state is quickly achieved. These observations are consistent with verapamil allowing for an elevated intracellular level of VP-16 and consequently more DNA damage than seen in the presence of VP-16 alone. Since vera-

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5\textsuperscript{W. Ross, T. Rowe, J. Yalowich, B. Glisson, and L. Liu. Epipodophyllotoxin-induced DNA cleavage is mediated by topoisomerase II, submitted for publication.}
pamil does not enhance DNA damage in isolated nuclei (Chart 7), it would appear that an intact plasma membrane is required and suggests Ca2+ antagonist effects on drug transport. However, our data do not allow firm conclusions regarding the mechanism by which verapamil modifies the effects of VP-16.

Although there was a delay in strand rejoicing in the presence of verapamil during the first 15 min after washout of VP-16, the rapidity and extent of strand rejoicing were subsequently unaltered as compared to cells not exposed to verapamil. These data suggest, but do not prove, that verapamil does not exert its effect by inhibiting strand rejoicing. Thus, based on our studies to date and data cited previously in which Ca2+ antagonists were combined with Vinca alkaloids and anthracycline (26-28), we believe that the most probable mechanism for the effects of verapamil is by increasing available intracellular VP-16 by either altering transport or perturbing intracellular binding. Preliminary studies of VP-16 cellular uptake in this laboratory suggest that the former possibility is more likely. Thus, it is probable that the initial delay of strand rejoicing in the presence of verapamil is due to a retardation of the cellular loss of VP-16.

The next obvious question is, how do verapamil and other so-called "Ca2+ antagonists" alter uptake of various anticancer drugs? Our studies have shown that neither EGTA nor A23187 perturbs VP-16-induced DNA damage or verapamil potentiation. These results suggest that alteration of Ca2+ influx does not play a significant role in VP-16 transport or Ca2+ antagonist activity in the L1210 cells and that other mechanisms must be sought. Similarly, Ramu et al. (20) have reported recently that EGTA and A23187 do not affect Ca2+ antagonist-mediated potentiation of Adriamycin- or vinblastine-treated resistant P388 cells.

Potency differences between VP-16 and VM-26 have been noted previously both in vitro (1, 17) and in vivo (18, 19). Also, although VM-26 has been found previously to cause more DNA SSBS on a molar basis than VP-16 (17), our results in isolated nuclei (Chart 7) suggest that VM-26 is more potent than VP-16 due to more extensive binding (1) and/or greater activation in the nucleus and not because of unidirectional influx differences in L1210 cells which had been reported previously (1). When VM-26 and VP-16 concentrations were adjusted in L1210 cells so that SSBS frequency was comparable, Ca2+ antagonist-mediated potentiation was the same for both agents, suggesting that the 2 may share common transport mechanisms. These results tend to confirm the earlier study of Allen (1) of efflux parameters for VM-26 and VP-16 from the L1210 cell, whereby no difference in the rate constant for exit from the cell was noted for these 2 agents.

The possible clinical importance of Ca2+ antagonists used in combination with anthracyclines and Vinca alkaloids has been suggested (24, 26, 28). Our report similarly suggests that epipodophyllotoxins and Ca2+ antagonists should be tested together in more clinically relevant models. It would be most helpful, however, to first determine the sensitivity of normal hematopoietic cells to VP-16 and the ability of the Ca2+ antagonists to potentiate VP-16 effects. Such studies are underway in our laboratory (32). Whether or not the epipodophyllotoxin-Ca2+ antagonist combination is useful clinically, it is clear that, as a biochemical probe, Ca2+ antagonists will be extremely useful to facilitate the study of the intracellular disposition and mechanism of not only VP-16 but other classes of chemotherapeutic drugs as well.

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in Vitro

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