Antiproliferative Effect of Verapamil Alone on Brain Tumor Cells in Vitro

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ABSTRACT

Recent studies have shown that the calcium channel blockers, when combined with standard anticancer drugs, help overcome resistance that often develops to those drugs. Little is known about the effects of the calcium channel blockers themselves on tumor cells. We have studied the effects of one calcium channel blocker, verapamil, on human tumor cell lines in vitro. Our results show a reversible, antiproliferative action of verapamil on human medulloblastoma, pinealoblastoma, glioma, and neuroblastoma tumor lines established from pediatric patients. Growth rates are inhibited 10 to 100% by 10 to 100 μM verapamil with 50% inhibition occurring between 25 and 50 μM verapamil. No cell line proliferates in 100 μM verapamil, yet washing the cells after 72 h of incubation with 100 μM verapamil results in resumed cell growth. Growth inhibition is accompanied by dose-dependent decreases in DNA, RNA, and protein synthesis which occur within minutes after addition of verapamil. DNA flow cytometry on propidium iodide-stained nuclei shows that, after incubation for 48 h with 100 μM verapamil, the medulloblastoma and neuroblastoma tumor lines as well as normal, human foreskin and lung fibroblast cell lines are reversibly blocked throughout the cell cycle with slight increases in G1. Verapamil appears to have no effect on nucleic acid precursors or on calcium influx or efflux in human medulloblastoma cells.

INTRODUCTION

Several years ago, it was suggested that verapamil, the prototype calcium channel blocker, might have some role in anticancer therapy when it was shown that verapamil enhanced the cytotoxic effect of the Vinca alkaloids in P388 leukemia cells previously resistant to vincristine (1). Since then, many studies have confirmed that calcium channel blockers and calmodulin inhibitors (e.g., trifluoperazine), in 5 to 50 μM drug concentrations, augment the cytotoxicity of many different standard anticancer agents including vincristine, adriamycin, etoposide, and melphalan on a variety of tumor cell types (2–8). In all cases, the calcium channel blockers and calmodulin inhibitors are thought to act by blocking efflux of the standard anticancer drug from the cell (1, 7, 9). Although the precise mechanism of action is unknown, recent data indicate that both verapamil and trifluoperazine cause an increase in the phosphorylation of the M1, 170,000 to 180,000 glycoprotein which is specific to multidrug-resistant tumor cells (10).

Because the high lipid solubility of the calcium channel blockers increases the likelihood of their crossing the blood-brain barrier, we became interested in studying whether verapamil might increase the cytotoxicity of selected anticancer drugs in the treatment of human brain tumor cells. Since little information was available on the effects of the calcium channel blockers on brain tumor cells in vitro, we began with an investigation on the effects of verapamil on human tumor cell lines in the absence of cytotoxic drugs. Our results show that verapamil alone rapidly and reversibly inhibits cell proliferation in a dose-dependent manner that does not appear to be coupled to calcium movements across the cell membrane.

MATERIALS AND METHODS

Cell Culture and Drug Treatment. The human TE671 medulloblastoma and A172 glioma cell lines were obtained from the American Type Culture Collection (doubling times of 1.3 and 0.8 days, respectively). The normal human lung fibroblast cell line IMR-90 was generously provided by Dr. Clive Bunn (Department of Biology, University of South Carolina). This line was studied at the 25th of 55 generations and had a doubling time of 2 days (11). The neuroblastoma cell line Wade-1 was a generous gift of Dr. Lawrence Helson (Memorial Sloan Kettering) and had a doubling time of 2.3 days. The pinealoblastoma cell line was recently established in this laboratory and had a doubling time of 2 days. All cell lines were studied within 2 mo after thawing new stock cultures. Cultures were routinely monitored and found free of Mycoplasm infection using the Mycotrim assay (New England Nuclear, Boston, MA). Cells were maintained in RPMI medium (KC Biological, Lenexa, KA) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin, and streptomycin. The growth chamber was maintained at 37°C with 5% CO2.

A stock solution of verapamil (Sigma) was prepared in ethanol and stored at 4°C protected from light. Stock solutions were diluted with the final ethanol concentration in cell cultures always 0.1% or less. This concentration of ethanol did not affect any of the parameters studied. Verapamil concentration was assayed spectrophotometrically with ε 280 = 122 at 280 nm. All chemicals used were of the highest grade available.

Cell viability experiments were conducted in 35-mm Petri dishes that were plated on Day zero with 104 cells in 2 ml of medium. Four h later, after cell attachment, a constant volume of verapamil solution or ethanol was added. Cells from triplicate cultures under each condition were harvested at timed intervals by trypsinization and dispersed into single cell suspensions with RPMI plus fetal calf serum. Cell viability experiments were conducted in 35-mm Petri dishes that were plated on Day zero with 104 cells in 2 ml of medium. Four h later, after cell attachment, a constant volume of verapamil solution or ethanol was added. Cells from triplicate cultures under each condition were harvested at timed intervals by trypsinization and dispersed into single cell suspensions with RPMI plus fetal calf serum. Cell viability was determined by adding trypan blue (final concentration, 0.2%) and counting duplicate samples from each culture in a hemacytometer.

For radioisotope studies, 35-mm Petri dishes were initially plated with 1 to 2 × 105 cells/dish. These were incubated until a cell density of approximately 2 to 106 cells/dish (subconfluent) was obtained. Except when the time course of verapamil's inhibitor effect was investigated, verapamil (or ethanol) was added 1 h prior to the start of the experiment.

Cell Cycle Phase Distribution. Nuclei were isolated as described by Thornthwaite (12) and stained with 50 μg/ml of propidium iodide, and cell cycle analysis was performed on a Coulter Electronics Epics V flow cytometer (Coulter Electronics, Hialeah, FL). The instrument was adjusted to achieve coefficients of variation for the nuclei of usually 3 to 5%. The proportion of 10,000 nuclei in G1, S, and G2-M was calculated using the Para-1 data analysis program of the flow cytometer. Each analysis was performed in triplicate, and experiments were performed at least 2 times.

Radioisotope Incorporation. Actively growing cells were always used when assessing isotope incorporation. Radiolabeled precursors were purchased from New England Nuclear (Boston, MA). L-[3,4,5-3H]-leucine, [5-3H]uridine, and [methyl-3H]thymidine were added to cell cultures at a final concentration of 1 to 5 μCi/ml. At timed intervals, cell samples were removed by trypsinization and added to an equal volume of cold 10% trichloroacetic acid. Precipitates were allowed to form for 30 min on ice before filtering through Whatman GF/C glass filters mounted in a vacuum manifold. After washing 3 times with 5%
Influx Studies. $^{46}$Ca (Amersham, Arlington Heights, IL) was added at a final concentration of 2 µCi/ml to cells grown in 35-mm Petri dishes as described above. At timed intervals, medium was aspirated, and the cells were washed 3 times with iced “transport buffer” which contained (mm): NaCl (107); KCl (5.3); NaHCO$_3$ (26.2); CaCl$_2$ (1.9); MgCl$_2$ (1.0); glucose (10); and Tris (10) (pH 7.40 at 37°C). To lyse the cells without disrupting the nuclei, 1 ml of 0.6% Nonidet P-40 non-ionic detergent (Sigma Chemical Co., St. Louis, MO) was added, and the mixture was incubated on ice for 5 to 10 min before transferring to microfuge tubes. The mixture was then centrifuged for 1 min in an Eppendorf centrifuge to remove the nuclei, and an aliquot of the supernatant fluid was counted in the scintillation spectrophotometer.

Efflux Studies. Cells were incubated with $^{46}$Ca for a minimum of 2 h (equilibrium) and subsequently immersed into 2 liters of transport buffer at 37°C for varied periods of time. Upon removal from the incubation, cells were washed rapidly with iced transport buffer, 0.6% Nonidet was added, and the nuclei-free portion was counted for radioactivity.

RESULTS

Effects of Verapamil on Cell Proliferation. Verapamil inhibits cell proliferation in a dose-dependent fashion, as illustrated by Fig. 1. Viability of the TE671 human medulloblastoma cells was not affected by as much as 100 µM verapamil, but cells ceased to grow at that drug concentration. The verapamil concentration required to inhibit cell growth by 50% was approximately 45 µM under these conditions. The inhibitory effect of verapamil on cell proliferation was not unique to TE671 cells as shown by Fig. 2. Inhibition of cell proliferation was similar for A172 glioma, USC-P pinealoblastoma, Wade-I neuroblastoma, and IMR90 normal lung fibroblast human cell lines.

The reversibility of the antiproliferative effects of verapamil is illustrated by Fig. 3. After incubation for 3 days with 100 µM verapamil, cells were washed free of the drug and then reincubated in fresh medium lacking verapamil. Cells resumed growth immediately. Moreover, when 100 µM verapamil was maintained in the culture medium, viable tumor cells persisted even after 8 days of incubation, indicating again that verapamil is antiproliferative and not cytotoxic.

Cell Cycle Phase Distribution. The growth of normal, nonneoplastic cells can be arrested reversibly in the G$_0$-G$_1$ cell cycle phase if medium conditions are unfavorable (negative pleiotypic response) (13). The ready reversibility of the antiproliferative effects of verapamil in tumor cells prompted us to examine whether any particular phase of the cell cycle is blocked by the drug. Twenty-four h after seeding, cells were incubated with 100 µM verapamil for 48 h. A fraction of the verapamil-treated cells was washed and then incubated in normal medium lacking verapamil. After 24 h, the cells were harvested, and cell cycle analysis was performed. Fig. 4 shows representative DNA histograms of human TE671 medulloblastoma and IMR-90 normal lung fibroblast lines. Incubation with verapamil for 48 h causes slight increases in the G$_1$-phase fraction with concomitant decreases in the S- and G$_2$-M-phase populations (Table 1). However, considerable numbers of cells remain in the S- and G$_2$-M-phase fractions after 48-h incubation with verapamil, indicating that cells are blocked in all phases of the cell cycle. Interestingly, even the normal nonfibroblast cell line IMR90 retained considerable numbers of cells in the S and G$_2$-M phases after treatment with verapamil, suggesting that the antiproliferative effect of verapamil does not provoke a negative pleiotropic response. Similar results were observed with the 172 glioma cell line and with another normal fibroblast cell line derived from foreskin (data not shown). When the verapamil-treated cells were washed after 48 h, then incubated another 24 h in verapamil-free medium, the resulting histograms show a striking increase in the G$_2$-M fraction. Parallel cell viability experiments using trypan blue exclusion techniques showed that more than 97% of the cells remained viable for up to 72 h after verapamil was removed from the medium. The doubling of the number of cells in the G$_2$-M fraction only 24 h after release from the drug, coupled with excellent survival, suggests that removal of the drug might be a strong stimulus for DNA synthesis and replication.

Macromolecule Synthesis. To help elucidate the kinetics and the mechanism of verapamil’s antiproliferative action, we studied the effects of verapamil on DNA, RNA, and protein synthesis. Cells were incubated with varying concentrations of verapamil for 1 h, radioactive precursors were added, and the cells were incubated for an additional 2 h. Macromolecule synthesis was inhibited by verapamil in a dose-dependent fashion. DNA synthesis, as assessed by $[^3]$H]thymidine incorporation, was inhibited by 50% with 25 µM verapamil. Similarly, protein synthesis, assessed by $[^3]$H]leucine incorporation, and RNA synthesis, assessed by $[^3]$H]uridine incorporation, showed...
dose-dependent decreases after 1-h incubation of cells with verapamil (data not shown). To examine the time required for verapamil to inhibit macromolecule synthesis, cells were incubated with drug for varying periods of time, and measurements of [3H]thymidine, [3H]leucine, and [3H]uridine incorporation were made after only 10-min exposure to the isotope. Fig. 5 shows that inhibition of macromolecule synthesis is rapid, with nearly 90% of the full effect seen after only 5-min incubation with verapamil.

The rapid onset of inhibition of macromolecule synthesis, combined with the ready reversal of inhibition of cell growth (Fig. 3), suggests that verapamil might be acting at the level of the cell membrane. To test for an effect of verapamil on transport of nucleic acid precursors, [3H]thymidine was added to the cells immediately after verapamil, and the amount of tracer taken up into the cells was measured at 30 s and then at 2-min intervals. In these experiments, nuclei were removed before counting radioactivity. [3H]Thymidine uptake into the cell peaked after 4 to 6 min. The initial slopes of these curves, representing an estimate of the flux of [3H]thymidine across the cell membrane, were similar in the presence and absence of 100 µM verapamil, suggesting that the drug does not interfere with nucleic acid precursor transport across the cell membrane (data not shown).

Calcium Transport. Calcium ions are an important element in the signal pathways leading to cell proliferation (14, 15), and verapamil is a known blocker of calcium influx in cardiac and smooth muscle cells (16). We investigated whether the blocking of calcium transport by verapamil could explain verapamil’s antiproliferative effect in TE671 human medulloblastoma cells. Cells were preincubated with 100 µM verapamil for 1 h before addition of isotope, and the amount of 45Ca in cells devoid of nuclei was measured at 10-min intervals as shown by Fig. 6. No difference in calcium flux across the cell membrane was observed between controls and verapamil-treated cells. Similarly, verapamil had no effect on the efflux of calcium from cells preloaded with 45Ca (data not shown). Thus, the rapid and reversible inhibition of cell proliferation by verapamil occurs through mechanisms other than the transmembrane fluxes of calcium.
Table 1 Effect of verapamil (100 μM) on cell cycle phase distribution in malignant and normal human cells in culture

Data are from the same experiment depicted by Fig. 4.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>100 μM Verapamil (g)</th>
<th>% of cells in G1 phase</th>
<th>% of cells in S phase</th>
<th>% of cells in G2-M phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A172</td>
<td>-</td>
<td>67.2 ± 2.5</td>
<td>22.1 ± 3.1</td>
<td>10.7 ± 1.6</td>
</tr>
<tr>
<td>Glioma</td>
<td>+</td>
<td>81.4 ± 1.2</td>
<td>11.4 ± 1.3</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>TE671</td>
<td>-</td>
<td>40.5 ± 2.2</td>
<td>37.0 ± 1.7</td>
<td>21.7 ± 0.9</td>
</tr>
<tr>
<td>Medullo-</td>
<td>+</td>
<td>49.4 ± 2.6</td>
<td>33.1 ± 2.7</td>
<td>17.5 ± 0.1</td>
</tr>
<tr>
<td>Blastoma</td>
<td>±</td>
<td>32.4 ± 2.1</td>
<td>18.1 ± 3.1</td>
<td>49.5 ± 2.9</td>
</tr>
<tr>
<td>IMR-90</td>
<td>-</td>
<td>68.0 ± 1.4</td>
<td>24.5 ± 0.8</td>
<td>7.5 ± 0.7</td>
</tr>
<tr>
<td>Lung</td>
<td>±</td>
<td>72.6 ± 1.0</td>
<td>21.3 ± 1.4</td>
<td>6.1 ± 0.7</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>±</td>
<td>40.2 ± 1.3</td>
<td>28.9 ± 2.6</td>
<td>30.9 ± 1.6</td>
</tr>
</tbody>
</table>

* Mean ± SEM of 30,000 nuclei.
* Experiment where verapamil was washed from the cells after 48 h.

DISCUSSION

Although the calcium channel blockers are well known for their ability to enhance cytotoxicity when used in combination with the Vinca alkaloids (1–3, 8), anthracyclines (1–4), epipodophyllotoxins (5, 6), and melphalan (7), little is known about their ability to enhance cytotoxicity when used in combination with the Vinca alkaloids (1–3, 8), anthracyclines (1–4), epipodophyllotoxins (5, 6), and melphalan (7). This is in agreement with an earlier report that verapamil inhibits the proliferative response in rabbit vascular smooth muscle cells that is typically initiated by a change in medium (17) and by the recent report that 100 μM verapamil inhibits proliferation and promotes differentiation in human promyelocytic HL–60 cells (18).

The inhibitory effect of 100 μM verapamil does not seem to be cell cycle phase specific in the cell lines tested, but rather affects the cells in all phases of the cell cycle. There was only a slight increase in the number of cells in the G1 phase before addition of isotope. Two samples were collected from each of 2 Petri dishes at the times indicated on the abscissa. Control (○) and verapamil-treated cells (•) were prepared as described in "Materials and Methods." Bars, SEM.

The ready reversibility of verapamil’s inhibitory effect and the short preincubation time required to inhibit [3H]thymidine incorporation suggest that verapamil may be interacting with some component of the cell membrane that controls proliferation. Although the transport of nucleic acid precursors into the cell would appear a logical control point, it is clear from our study that the transport of [3H]thymidine into the cytoplasm of human medulloblastoma cells is not affected significantly by verapamil.

A key variable in cell proliferation in nonneoplastic cells is the cytoplasmic free calcium ion concentration (14, 15). Cytoplasmic calcium is determined, in part, by the movements of calcium across the cell membrane. Our studies show that, despite its designation as a calcium channel blocker, 100 μM verapamil has no effect on either calcium influx or efflux in TE671 human medulloblastoma cells. Thus verapamil, when inhibiting the growth of brain tumor cell lines, is working by a mechanism other than its known action in cardiac and smooth muscle cells. Recently, it has been shown that an increase in intracellular calcium mobilization accompanies the activation of cell proliferation (20, 21). In human promyelocytic leukemia HL–60 cells incubated in the absence of external calcium, 100 μM verapamil completely inhibits an ionomycin-induced increase in intracellular calcium, suggesting that verapamil inhibits calcium release from intracellular stores (18). This is in agreement with our findings of a lack of effect of verapamil
on the calcium fluxes across the cell membrane.

Whatever the mechanism of verapamil's antiproliferative effect, its action is readily reversible. Within 24 h of being washed free of verapamil the number of cells in G2-M has increased by over 100% (Table 1), and within 48 h, cell number increased (Fig. 3). These observations might eventually be important in designing chemotherapy regimens. For example, the Vinca alkaloids and epipodophyllotoxins are most effective against cells undergoing mitosis (22). Timing the administration of such cell cycle-specific drugs might provide for increased cell kill and decreased cell drug resistance.

The verapamil concentrations used in this study to fully inhibit cell proliferation (100 μM) are an order of magnitude higher than those used previously to enhance the cytotoxicity of standard anticancer agents (4–9). Such high concentrations of verapamil are toxic in vivo, totally blocking excitation-contraction, thereby causing heart block (23). However, as seen above, our studies suggest that the antiproliferative property of verapamil may not be related to its action as a calcium influx blocker. The usefulness of these and further studies will be in elucidating the pharmacology of verapamil's antiproliferative effect and aiding development of related drugs lacking verapamil's calcium influx-inhibiting and dose-limiting toxic effects.

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