Calcium antagonists potentiate P-glycoprotein-independent anticancer drugs in chronic lymphocytic leukemia cells in vitro

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ABSTRACT

Background and Objectives. A major obstacle to the successful use of chemotherapy in the treatment of leukemia and other cancers is the emergence of drug resistance. One of the most studied resistance mechanisms is mediated by P-glycoprotein, which can be modulated by calcium channel blockers. Here we investigated whether the Ca\(^{2+}\) channel blockers verapamil and nifedipine are toxic alone and in combination with P-glycoprotein-independent anticancer drugs against chronic lymphocytic leukemia (CLL) cells in vitro.

Design and Methods. Verapamil cytotoxicity was investigated in peripheral blood samples of 35 patients with B-cell CLL and 10 healthy control subjects. Cytotoxicity was assessed in vitro 4-day cultures using \(^{14}\)C-leucine incorporation as an indicator of cell viability. Interactions were tested with Ca\(^{2+}\) channel blockers and cyclosporine or 7 anticancer drugs: (i) chlorambucil, (ii) 2-chlorodeoxyadenosine, (iii) cisplatin, (iv) fludarabine, (v) prednisolone, (vi) adriamycin, and (vii) vincristine. The mode of cell death was assessed by annexin binding and DNA ladder formation.

Results. Verapamil induced dose- and time-dependent death of CLL cells in vitro. A statistically significant effect (p = 0.0085) was noted with as little as 4 µM verapamil. The mode of cell death was apoptotic as determined by annexin positivity and condensation of verapamil-treated cells. Verapamil effectively potentiated the toxicity of cyclosporine and all the anticancer drugs mentioned above. Furthermore, nifedipine, a more specific L-type calcium channel antagonist, significantly potentiated the effect of chlorambucil against CLL cells.

Interpretation and Conclusions. Calcium channel blockers enhance the effect of P-glycoprotein-independent anticancer drugs remarkably. This indicates that the death signals initiated by calcium depletion and anticancer drugs together facilitate cell death. This novel finding opens a new avenue to modulate, by using calcium channel antagonists, the effect of traditional anticancer drugs having different mechanisms of P-glycoprotein-independent action.

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well known Ca^2+ channel blockers verapamil and nifedipine significantly potentiate the cell killing action of several drugs which are not known to be directly affected by P-gp.

Design and Methods

Patients and samples

Clinical specimens were obtained after informed consent from 35 consecutive CLL patients referred to the CLL out-patient clinic at Tampere University Hospital (Finland). The inclusion criterion was a blood leukocyte count of 30x10^9/L or higher. Diagnosis and staging were based on standard clinical, morphologic and immunophenotyping criteria. All patients had the B-CLL phenotype. The CLL scores, when available, ranged from three to five. The clinical hematologic details at the time of sampling are given in Table 1.

Peripheral blood mononuclear cells from CLL patients and from 10 healthy donors were isolated from heparinized (Noparin, Novo Nordik, Dagsvaerd, Denmark) blood samples by centrifugation over a Lymphoprep layer (Nycomed, Oslo, Norway) at a density of 1.077 g/mL. The cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and once with complete medium consisting of RPMI 1640 (20 mM Hepes, ICN Biochemicals, Costa Mesa, CA, USA), 2 mM L-glutamine (Gibco BRL) and antibiotics (Gibco BRL, penicillin 50 U/mL, streptomycin 50 µg/mL). Freshly isolated cells were used for immunophenotyping and cytotoxicity studies. Cell counting was performed by using Technicon H1, H2 or H3 analyzers (Bayer Diagnostica). Density gradient centrifugation yields of mononuclear cells – without other purification steps – were good; range 29-92%, median 58.0%, mean 61.1%, SD 16.0%, n=35. We regard this information as essential for interpretation of the results. The proportion of monocytes plus polyclonal T- and B-lymphocytes was 1-10%, indicating that 90-99% of the isolated cells represented the leukemic population.

Drugs

The test compounds and their proposed mechanisms of action are listed in Table 2.
Cytotoxicity tests
The cytotoxic effects against human peripheral blood mononuclear cells from CLL patients and from healthy control subjects were assessed using 4-day cultures on microplates by adding the indicated concentrations of the test compounds to cultures in 96-well microplates; 200,000 cells per well in a volume of 100 µL, i.e. 2x10^6 cells per mL. The effects of the test compounds were monitored by assessing protein synthesis by using [14C]-leucine incorporation. The cells were first cultured for 3 days, but in some experiments for 0, 1 and 2 days. [U-14C]-leucine (specific activity 1.3 mCi/mmol, 0.5 µCi/mL) was then added for the final 24 h of culture. After incubation, the proteins were precipitated with 0.2 M perchloric acid and collected on glass fiber filters using a multiple cell harvester (LKB Wallac 1295-001, Turku, Finland). The radioactivity incorporated was measured in a liquid scintillation counter (Wallac 1410). Living cells were counted hemocytometrically using trypan blue dye exclusion. The ID50 (50% decrease in leucine incorporation) and ID80 (80% decrease in leucine incorporation) values were calculated from dose-response curves representing duplicate or triplicate cultures of 6 different drug concentrations.

Flow cytometry
Immunophenotyping was performed by flow cytometry (EPICS C, Coulter Electronics, Hialeah, CA, USA; FACScan and FACScalibur, Becton Dickinson, San José, CA, USA) using commercial mouse monoclonal antibodies and respective immunoglobulin isotype controls, as recommended by the manufacturers. An annexin-binding assay was used as an indicator of membrane changes seen particularly in apoptotic cells. We used FITC-conjugated annexin V (Annexin V-FITC Apoptosis Detection Kit, Genzyme Diagnostics, Cambridge, MA, USA) as instructed by the manufacturer.

DNA electrophoresis
The DNA of verapamil-exposed cells and unexposed control cells was purified using an Apoptotic DNA Ladder Kit (Boehringer Mannheim, Mannheim, Germany). DNA electrophoresis was performed on 1% agarose gel. DNA was stained with ethidium bromide and photographed. As positive control we used U937 cells treated with camptothecin (4 µg/mL) for 3 hours, resulting in about 30% apoptotic cells. A DNA ladder (Combined, New England Biolabs, Beverly, MA, USA) was used for molecular weight markers.

Results
Cytotoxicity of verapamil
Verapamil induced time- and dose-dependent death of leukemic CLL cells, were observed for all 35 CLL patients investigated. Individual ID50 and ID80 values, as determined from dose-inhibition curves from routine 4-day cultures, are given in Table 1. A representative example of cell loss kinetics is illustrated in Figure 1. There was very good correlation between the number of living cells and the incorporation of 14C-leucine per well (Figure 1). This made it possible to use the latter parameter instead of labor-intensive cell counting in the assessment of cytotoxicity.

As low a concentration of verapamil as 4 µmol/L induced significant toxicity when the results from 35 CLL patients were compared with non-treated cultures (Figure 2). Furthermore, at higher doses the
Calcium channel blockers

Polyclonal peripheral blood mononuclear cells from healthy donors were slightly less susceptible to verapamil than CLL cells (Figure 2).

Analysis of variance did not reveal clear interrelationships between the clinical-hematologic parameters and verapamil ID\textsubscript{80} cytotoxicity values. The tested parameters were sex, age, Binet class, FAB class, histology, immunophenotypic score, and progression velocity (Table 1).

Flow cytometry

Flow cytometric analysis of cells from several patients revealed that verapamil induced time- and dose-dependent changes in the light scattering properties of leukemic cells. Furthermore, the occurrence of annexin-positive events increased simultaneously. Usually three distinctive event populations emerged during verapamil treatment: (i) normal cells, (ii) condensed cells, and (iii) fragments smaller in size than in the two previous populations, evidently representing cell debris. The results of flow cytometric analy-

Table 3. Flow cytometric analysis of immunophenotype and annexin positivity of verapamil-treated versus control cells in four different SSC-FSC matrix gates.

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Parameters</th>
<th>Gate 1</th>
<th>Gate 2</th>
<th>Gate 3</th>
<th>Gate 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>% events in the gate</td>
<td>1.1-1.5</td>
<td>93.5-97.1</td>
<td>2.0-2.5</td>
<td>0.1-0.3</td>
</tr>
<tr>
<td>0</td>
<td>None</td>
<td>% T cells in the gate</td>
<td>2.4-7.8</td>
<td>2.3-2.9</td>
<td>1.6-6.1</td>
<td>0.1-1.8</td>
</tr>
<tr>
<td>0</td>
<td>None</td>
<td>% annexin + in the gate</td>
<td>0.5-7.7</td>
<td>0.3-0.6</td>
<td>18.3-30.3</td>
<td>15.5-73.3</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>% events in the gate</td>
<td>0.4-0.6</td>
<td>84.3-87.7</td>
<td>4.6-16.5</td>
<td>0.3-0.5</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>% T cells in the gate</td>
<td>1.7-9.6</td>
<td>1.6-2.1</td>
<td>2.8-4.9</td>
<td>2.0-5.9</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>% annexin + in the gate</td>
<td>9.1-18.4</td>
<td>2.7-3.4</td>
<td>82.3-92.9</td>
<td>67.4-94.2</td>
</tr>
<tr>
<td>3</td>
<td>Verapamil</td>
<td>% events in the gate</td>
<td>2.5-3.1</td>
<td>4.7-4.7</td>
<td>43.7-46.1</td>
<td>33.4-4.7</td>
</tr>
<tr>
<td>3</td>
<td>Verapamil</td>
<td>% T cells in the gate</td>
<td>1.4-5.0</td>
<td>1.9-3.3</td>
<td>2.9-3.9</td>
<td>2.7-4.7</td>
</tr>
<tr>
<td>3</td>
<td>Verapamil</td>
<td>% annexin + in the gate</td>
<td>87.6-100</td>
<td>88.1-91.7</td>
<td>94.9-97.3</td>
<td>85.1-100</td>
</tr>
</tbody>
</table>

*95% confidence interval (assuming Poisson distribution of events).31

Figure 2. Effect of different concentrations of verapamil on cell viability as determined by \textsuperscript{14}C-leucine incorporation into peripheral blood mononuclear cells among 35 CLL patients (black columns) and 10 healthy control subjects (open columns). Each column represents the mean. The bars represent 1 SD.
sis of an index patient are illustrated in Figure 3 and Table 3. In this case the normal cell population consisted of two overlapping subpopulations (R1 and R2 in the Figure). Three separate event populations (marked R1 through R3) were observed at the beginning of 4-day culture, but a fourth population (R4) started to appear after the first day of culture. Detailed analysis of these event populations was performed at the beginning of culture and on day 3, as illustrated in Table 3. Gates R2 and R3 always contained more than 90% of all events. Two interesting changes were observed in verapamil-treated versus untreated cells. Firstly, a remarkable increase of cells in gate R3 was observed. This was accompanied by a comparable decrease of events in gate R2. Secondly, the proportion of annexin-positive cells in gates R2+R3, analyzed from verapamil-treated cultures, was 84.2% whereas it was only 12.0% in control cultures. This difference was highly significant (p < 0.0001; Chi squared test). This indicates effective induction of apoptosis-like membrane changes in verapamil-treated cultures. The proportion of T-cells remained approximately the same in the gates containing the majority of cells. This indicates that the susceptibilities of polyclonal T-cells and leukemic B-cells to verapamil were very similar.

DNA fragmentation

Remarkable apoptotic ladder formation was seen in the DNA of U937 cells treated with camptothecin. In contrast, despite several cases investigated, a highly toxic dose of verapamil did not induce visible ladder formation in CLL cells (Figure 5).

Potentiation by verapamil and nifedipine of the action of cyclosporine and seven different chemotherapeutic agents

The synergistic action of verapamil plus chlorambucil was constantly demonstrable within a broad range of concentrations. Representative examples are illustrated in Figures 4 and 6. Figure 4 also shows that 14C-leucine incorporation is in excellent accordance with living cell count and obviates the use of labor-intensive cell counting.

In addition to chlorambucil, the potentiation by verapamil of the action of seven other drugs was demonstrable with leukemic cells from all six patients investigated. Examples are given in Figure 6A and 6B.
The results concerning peripheral blood mononuclear cells from a healthy study subject are illustrated in Figure 6C. The results were essentially similar to those obtained with CLL cells. The only clear exception was a remarkable relative resistance of normal mononuclear cells to vincristine.

Nifedipine, a specific L-type Ca$^{2+}$ channel blocker, also appeared to be toxic to CLL cells and enhanced the toxicity of chlorambucil against CLL cells remarkably, as illustrated in Figure 7.

Discussion

In the present work we examined cytotoxic interactions of calcium channel blockers with 7 anticancer drugs and with cyclosporine using CLL cells and normal peripheral blood mononuclear cells as targets. We confirmed previous results showing that verapamil alone is toxic to CLL cells. We extended the data to cover a considerable number of patients. This enabled us to make calculations concerning the variability of verapamil responses among individual patients and different patient groups. Relative small overall variability was observed on the basis of the narrow range of ID$_{50}$ values (10.7-180 µM) and ID$_{80}$ values (40-330 µM). In contrast to this 8.25-fold variation in the ID$_{80}$ values for verapamil, 35-fold differences for vincristine$^{17}$ and a 100-fold and even a greater range for purine analogs$^{18}$ have been demonstrated under identical conditions. This, together with similar susceptibility of normal polyclonal blood mononuclear cells to verapamil, indicates good predictability of verapamil’s cytotoxicity. Furthermore, the relatively constant effect of verapamil was substantiated by similar sensitivity of various clinical and hematologic forms of CLL. Although the present data imply poor selectivity of verapamil against malignant versus normal lymphocytes, the situation in vivo may be different. This has already been substantiated by preliminary observations according to which verapamil may cause remarkable cytoreduction among CLL cells in vivo, even when used at ordinary doses.$^{9}$ All this warrants further clinical testing of verapamil as an anti-CLL agent.

Verapamil induced remarkable annexin positivity of CLL cell plasma membranes. This and cell condensation are compatible with an apoptotic form of cell death. Interestingly, apart from vincristine-induced cell death (unpublished), no DNA ladder formation was

![Figure 5. DNA electrophoresis of CLL cells (patient # 24) exposed for 24 h to verapamil. Lane A, molecular weight markers. Lane B, cells used to start the cultures. Lane C, control CLL cells after 24 h incubation. Lane D, CLL cells exposed to 100 µM verapamil for 24 h. Lane E, U937 cells exposed for 3 h to 4 µg camptothecin/ ml showing a clear nucleosome-sized DNA fragmentation ladder.](haematologica-085-08-27613-f5)

![Figure 6. Synergism of verapamil and various anticancer drugs against CLL cells in 4-day cultures. Cell survival was indicated as $^{14}$C-leucine incorporation during the final 24 h of culture. For technical details, see Design and Methods section. The cells were incubated without additives (columns with stripes, n = 12; error bar = 1 SD), with 20 µM verapamil (next column, n = 12 ± SD), with drug alone (open columns, n = 6), and with the same drug plus 20 µM verapamil (adjacent column, black, n = 6). Panel A represents patient #36. Panel B represents patient #15. In panels A and B the differences (drug alone versus drug + verapamil) were statistically significant at the level of p < 0.0001 in all cases (Student’s t test). Panel C represents a healthy control person.](haematologica-085-08-27613-f6)
Moreover, the more specific L-type Ca\(^{2+}\) channel antagonist verapamil potentiated the effect of P-gp-independent compounds. Cancer drugs, we observed that verapamil also potentiated the cytotoxicity of vincristine against CLL cells. This clearly illustrates the remarkable selectivity of verapamil plus vincristine against normal cells was demonstrated in the present study. The viability of cells was investigated by 14C-leucine incorporation as described in Design and Methods section. The experiment provided strong evidence (p < 0.001) that nifedipine potentiated chlorambucil-induced cytotoxicity as assessed on the basis of differences between cultures with and without chlorambucil.

Verapamil is the most studied P-gp modulator, but the mechanisms of action of these agents have not been elucidated. The conclusion from these previous studies is that in vitro verapamil potentiates the effect of the P-gp-dependent drugs vincristine and anthracyclines, as also observed in the present study. Interestingly, we found that verapamil also strongly potentiates the effect of doxorubicin against normal blood mononuclear cells, which indicates poor selectivity. This is in sharp contrast to the selectivity of vincristine. In accordance with previous studies, normal mononuclear cells were approximately 200 times more resistant to vincristine than the leukemic cells were. Furthermore, only marginal, although statistically significant, enhancement by verapamil of vincristine cytotoxicity in normal cells was demonstrated in the present work. This clearly illustrates the remarkable selectivity of verapamil plus vincristine against CLL cells.

In addition to the effect of P-gp-dependent anticancer drugs, we observed that verapamil also potentiated the effect of P-gp-independent compounds. Moreover, the more specific L-type Ca\(^{2+}\) channel blocker, nifedipine, significantly potentiated the cytotoxicity of chlorambucil, which is a known P-gp-independent drug. These results are novel and unexpected on the basis of traditional thinking about verapamil as a chemomodulator of P-gp.

Recent work in other laboratories has demonstrated that Ca\(^{2+}\) channel blockers may have several kinds of interactions, the mechanisms of which are not yet understood. In addition to the cytoprotective properties of Ca\(^{2+}\) channel blockers, a number of publications imply that they are toxic alone and in combination with other chemical or physical factors. However, we are aware of only two cases in which the obvious mechanism has been P-gp-independent. These are: (i) the verapamil-enhanced growth inhibitory effect of hyperthermia on human colon carcinoma cells in vitro; (ii) the verapamil-enhanced growth inhibitory effect of 5-fluorouracil and 5-fluorouracil plus hyperthermia on human adenocarcinoma cells in vitro. Verapamil also reversed 5-fluorouracil resistance in a human hepatocellular carcinoma cell line.

A prolonged period of Ca\(^{2+}\) signaling is an important growth signal for many cells. Calcium is also involved in the proliferation of B- and T-lymphocytes. During the antigen response of immune cells, release of Ca\(^{2+}\) from internal stores takes place. Once these stores are empty, entry of external Ca\(^{2+}\) is activated through so-called store-operated Ca\(^{2+}\) channels in the plasma membrane. The present work clearly demonstrated that Ca\(^{2+}\) channel blockers, alone and together with different types of anticancer drugs, kill CLL cells in vitro. It is likely that in addition to proliferating lymphocytes, a continuous Ca\(^{2+}\) supply is also vital for resting lymphocytes such as CLL cells. If the Ca\(^{2+}\) store within the endoplasmic reticulum were depleted, the mitochondria would become overloaded and there would be two main consequences. First, the decline of levels of Ca\(^{2+}\) in the endoplasmic reticulum would lead to activation of stress signals which switch on the genes associated with cell death. Some of these genes also specify proteins that bind Ca\(^{2+}\) in the endoplasmic reticulum, and this may further disturb the correct Ca\(^{2+}\) balance between the endoplasmic reticulum and the mitochondria. This part of the death-signaling network may be the area where Ca\(^{2+}\) blocking and anticancer drug-induced signals converge. Second, the build-up of mitochondrial Ca\(^{2+}\) initiates a program of events that leads to cell death. The molecular details of these mechanisms are slowly emerging. Our present study clearly demonstrated that Ca\(^{2+}\) channel blockers enhance the toxicity of several non-P-gp-dependent anticancer drugs. The most likely mechanism is synergistic induction of two different or converging cell death pathways by these two types of drug. The mechanistic details and clinical significance of these observations remain to be investigated.

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JV: conception and design; part of the practical work, analysis and interpretation of data, drafting the article, final proval of the version to be published TK: analysis and interpretation of data, drafting the article, final proval of the version to be published LV: conception and design; most of the practical work, analysis and interpretation of data, final proval of the version to be published. Cyclosporin A was a generous gift from the Sandz Pharmaceutical Co., Basle, Switzerland. We thank...
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Potential implications for clinical practice
Understanding the mechanisms of drug resistance will allow the development of strategies to overcome it.

References