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Malignant Lymphomas

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 *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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Key words: anticancer drugs, calcium channel blockers, chronic lymphocytic leukemia, cytotoxicity

Chemoresistance is a major problem in cancer chemotherapy. The mechanisms underlying the clinical phenomenon of *de novo* and acquired drug resistance have been studied using *in vitro* models. Drug resistance may arise from alterations at any step in the cell killing pathway. These include drug transport, drug metabolism, drug targets, cellular repair mechanisms, and the ability of cells to recognize a toxic insult and engage apoptosis.[1,2]

While P-glycoprotein (P-gp) may represent the best understood mechanism of multidrug-resistance (MDR), many other mechanisms have also been identified, as recently reviewed:[3] P-gp-mediated MDR has particularly been associated with vinca alkaloids, anthracyclines, epipodophyllotoxins, taxanes and some other drugs, while the resistance against platinum derivatives, antimetabolites, alkylating agents and bleomycin is evidently mediated by other mechanisms.

B-cell chronic lymphocytic leukemia (CLL) is the most common form of leukemia in Western countries. CLL cells commonly express a multidrug resistance phenotype. These cells frequently express P-gp at diagnosis and display functional drug efflux *in vitro*.[4] Verapamil, the most studied calcium channel blocker has been used as a *first-generation P-gp modulator* to reverse MDR *in vitro*.[3] This drug is toxic alone and potentiates the effect of vincristine and anthracyclines against chronic lymphocytic leukemia *in vitro*.[5-8] Interestingly, in addition to the *in vitro* effects, verapamil alone may also have *in vivo* cytoreductive potential: four CLL patients, treated with verapamil for cardiac problems, showed improvement in their hematologic malignancy (substantial reduction of lymphadenopathy in one patient, 3- and 5-year stabilization in two patients, and a dramatic decrease in lymphocyte count, lymphadenopathy and splenomegaly in one stage IV patient).[9]

It is generally assumed that the mechanism of action of verapamil is based on its ability to block the selective drug efflux pump.[2] Very little attention has been paid to another possibility that Ca^{2+} channel blockers interfere directly with the mechanisms of Ca^{2+} signaling as part of the cell death pathway. This kind of effect could be synergistic with death signals initiated by cytotoxic drugs. Here we show that the

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.[10-13] All patients had the B-CLL phenotype. The *CLL scores*, when available, ranged from three to five.[14] 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), 10% heat-inactivated fetal calf serum (Gibco BRL, Paisley, Scotland), 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.[15] 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.

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Table 1. Clinical and hematologic data on 35 CLL patients.

Patient no.	*Age/sex (years)*	*B-Lymph. ($x10^9$/L)*	*Binet class[a]*	*FAB type[b]*	*Histology[c]*	*Immunophenotype score[d]*	*Progression[e]*	*Verapamil toxicity[f]* ID_{50}	ID_{80}
1	54/m	63	A	CLL	M	5	S	12.5	47
2	64/m	46	A	CLL	I	5	T	71	265
3	72/m	84	B	CLL	D	4 (Igκ)	F	123	285
4	69/m	92	B	CLL	I	5	F	13.5	46
5	61/f	86	A	CLL	I	N.A. (CD22 missing, CD23 21%+)	S	68	220
6	50/f	130	A	CLL	N	4 (Igλ)	S	68	225
8	58/m	310	C	CLL/PL	D	4 (Igλ)	T	43	90
9	68/m	67	B	CLL	I	3(CD5, CD23)	S	58	188
10	68/m	51	A	CLL	M	5	I	57	135
11	68/f	88	C	CLL	D	5	T	21	230
12	64/m	132	C	CLL	D	5	T	72	235
13	68/m	114	C	CLL	M	3 (Igλ, CD5)	F	102	270
14	66/f	188	C	CLL/PL	D	4 (Igλ)	T	70	265
15	57/m	63	A	CLL	D	5	F	64	205
16	68/m	81	A	CLL	I	5	I	57	135
17	57/m	120	A	CLL	I	5	F	49	230
18	73/f	79	C	CLL/mix	M	3 (Igκ, CD23)	F/T	33	88
19	55/m	36	A	CLL/PL	M	4 (CD5)	F/T	180	340
20	53/m	69	B	CLL/PL	D	5	I	66	255
21	73/m	142	B	CLL/mix	D	4 (Igλ)	I	10.7	45
22	55/m	59	B	CLL/PL	I	4 (Igλ)	I	120	285
23	62/m	59	B	CLL	M	3 (CD23, Igλ)	S	180	350
24	79/f	97	B	CLL/mix	M	4 (CD23)	T	69	250
25	54/m	216	B	CLL/mix	D	4 (Igκ)	T	27	88
26	48/m	93	A	CLL	I	5	S	47	150
27	67/m	67	A	CLL	M	5	I	29	66
28	67/m	178	C	CLL	D	N.A. (FMC7 missing, λ strong)	F/T	31	62
29	78/m	224	B	CLL	M	4 (CD5)	F	39	86
30	69/f	69	A	CLL	M	4 (Igλ)	F	41	70
31	59/m	98	A	CLL	M	5	I	41	70
32	70/m	134	A	CLL	D	3 (Igκ, CD22)	F	34	74
33	57/m	68	A	CLL	M	4 (Igλ)	S	44	73
34	75/f	173	B	CLL	D	4 (Igκ)	I	33	65
35	76/f	206	C	CLL	M	5	F	20	58
36	78/f	155	A	CLL	I	4 (Igκ)	S	36	68

[a]Binet et al. (1981);[12] [b]Bennett et al. (1989);[10] [c]Rozman et al. (1981):[13] D, diffuse; I, interstitial; M, mixed; N, nodular; N.A, not available; [d]Matutes et al. (1994);[14] A score of five means CD22$^-$, CD23$^+$, CD5$^+$, FMC7$^-$, and undetectable or weak SmIgκ/λ. Markers causing a deviation from score 5 are indicated in parentheses; Igκ or λ means moderate to strong. CD5 means CD5 negativity. CD23 means CD23 negativity; [e]abbreviations: S (slow) = blood lymphocytes increased less than 20% within a year; F (fast) = lymphocyte doubling time 1 year or less; I (intermediate) between S and F; T (therapy) = chemotherapy given, natural disease progression non-evaluable; [f]Determined from dose-response curves examined as described in Design and Methods section.

Table 2. Drugs and their proposed mechanisms of action.[28]

Drug name	*Source*	*Mechanism(s) of action*	*P-glycoprotein-dependent efflux*
Chlorambucil	Sigma Chemical Co., St. Louis, MO, USA	Bifunctional alkylating agent; DNA base modification and intra/interstrand cross-link formation	No
2-chloro-2'-deoxyadenosine	Sigma	Inhibition of semiconservative and repair replication of DNA	No
Cyclosporine; Cyclosporin A	Sandoz Pharmaceutical Co., Basle, Switzerland	Binds to cyclophilin; the complex binds to calcineurin suppressing calcineurin-stimulated effects de-pendent on Ca^{2+}	No (Modulator)
Cisplatinum (II) diammine-dichloride; cisplatin	Sigma	Similar to that of alkylating agents (see chlorambucil above)	No
Doxorubicin hydrochloride; Adriamycin 2 mg/mL	Pharmacia, Uppsala, Sweden	Intercalation in DNA; stabilizes topoisomerase-2 complex leading to single- and double-strand DNA breaks	Yes
2-fluoroadenine-9-β-D-arabinofuranoside; fludarabine	Sigma	Inhibition of semiconservative and repair replication of DNA; also inhibits ribonucleotide reductase and incorporates into RNA	No
Nifedipine	Sigma	Ca^{2+} channel blocker, a dihydropyridine; specific to L-type channels[29,30]	Modulator
Prednisolone natrium succinate; Di-Adeson-F; prednisolone	N.V. Organon, Oss, the Netherlands	lymphocytotoxic; mechanisms not understood in detail	No
Verapamil; Verpamil 2.5 mg/mL	Orion Ltd, Espoo, Finland	Ca^{2+} channel blocker, a phenylalkylamine; less specific than dihydropyridines[29,30]	Modulator
Vincristine sulfate; Oncovin 1 mg/mL	Lilly France SA, Fegersheim, France	Interaction with tubulin; inhibits mitotic spindle formation	Yes

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 [^{14}C]-leucine incorporation. The cells were first cultured for 3 days, but in some experiments for 0, 1 and 2 days. [U-^{14}C]-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 ID_{50} (50% decrease in leucine incorporation) and ID_{80} (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.[16]

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 ID_{50} and ID_{80} 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 ^{14}C-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

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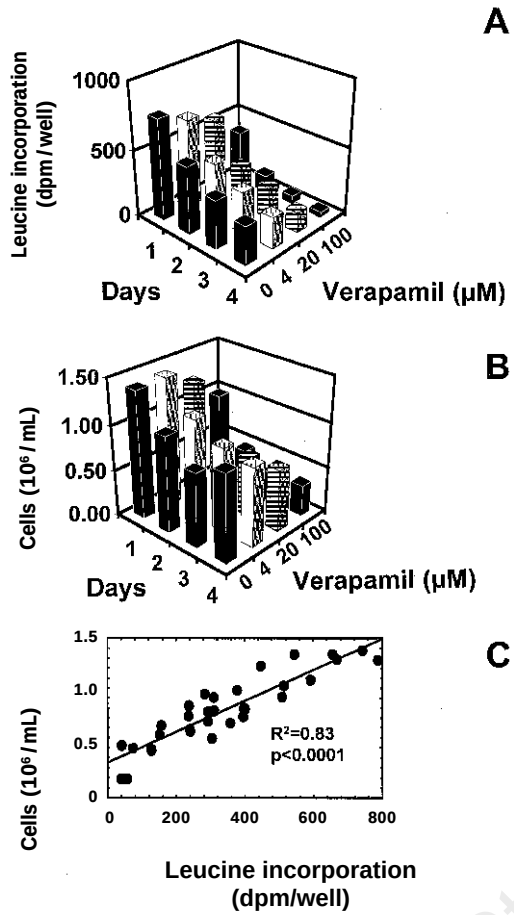

Figure 1. Kinetics of ^{14}C-leucine incorporation (A) and concordant changes in living cell counts (B) *in vitro*. Leukemic cells from an index CLL patient (#15) were cultured and exposed to verapamil as indicated in *Design and Methods*. Radioactive leucine was present for the final 24 h of culture. Very good correlation was observed between the numbers of living cells and the rate of leucine incorporation (C).

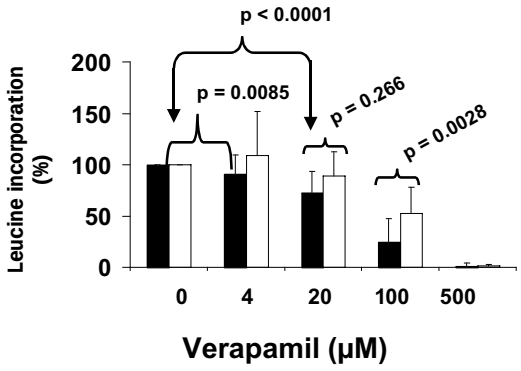

Figure 2. Effect of different concentrations of verapamil on cell viability as determined by ^{14}C-leucine incorporation into peripheral blood mononuclear cells among 35 CLL patients (black columns) and 10 healthy control subjects (open columns). Each column represents the mean. The bars represent 1 SD.

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_{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.

Day	*Treatment*	*Parameters*	*Events**			
			Gate 1	*Gate 2*	*Gate 3*	*Gate 4*
0	None	% events in the gate	1.1-1.5	93.5-97.1	2.00-2.5	0.1-0.3
0	None	% T cells in the gate	2.4-7.8	2.3-2.9	0.6-3.1	0-1.8
0	None	% annexin + in the gate	0-5.7	0.3-0.6	18.3-30.3	15.5-73.3
3	None	% events in the gate	0.4-0.6	84.3-87.7	4.6-16.5	0-3.5
3	None	% T cells in the gate	1.7-9.6	1.6-2.1	2.8-4.9	2.0-5.9
3	None	% annexin + in the gate	9.1-18.4	2.7-3.4	82.3-92.9	67.4-94.2
3	Verapamil	% events in the gate	2.5-3.1	44.7-47.1	43.7-46.1	3.3-4.7
3	Verapamil	% T cells in the gate	1.4-5.0	1.9-3.3	2.9-3.9	2.7-4.7
3	Verapamil	% annexin + in the gate	87.6-100	88.1-91.7	94.9-97.3	85.1-100

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

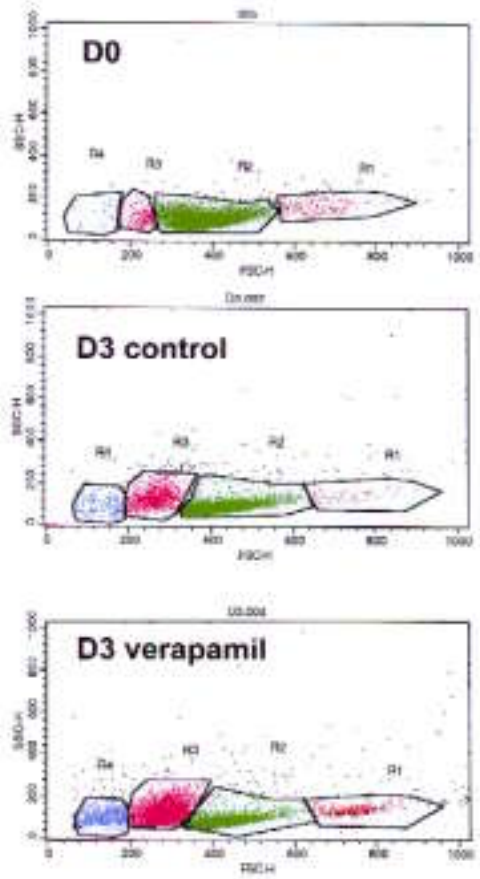

Figure 3. Flow cytometric analysis of cells from an index patient (# 15, as in Figure 1) with chronic lymphocytic leukemia at the beginning (upper panel) and at the end of 3-day culture (middle and lower panels). Spontaneous changes are illustrated in the middle panel. In the lower panel, the cells were exposed to 100 µM verapamil. The events were gated in four populations. R1 and R2 represent overlapping populations of normal cells. Condensed cells are located inside R3. Subcellular events (apoptotic bodies) are gated in R4. For detailed numerical description, see Table 3.

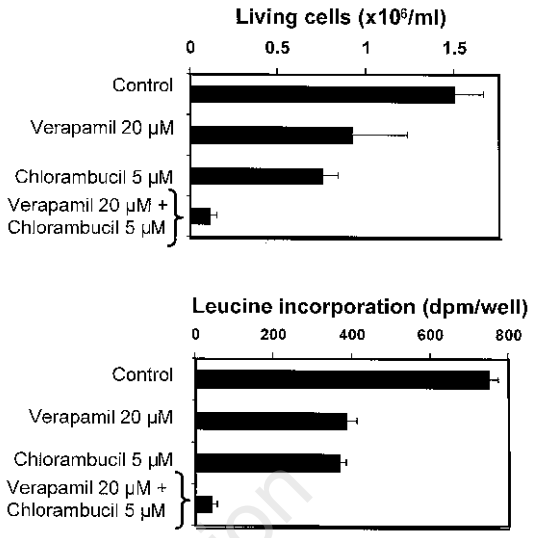

Figure 4. Interaction of chlorambucil and verapamil as demonstrated in 4-day cultures of cells from an index CLL patient (#36). Remarkable enhancement of cytotoxicity was observed as assessed by leucine incorporation and by counting the living cells on the basis of trypan blue dye exclusion. Each column represents the mean (±1 SD) of six cultures. There was very good correlation between the means of ^{14}C-leucine incorporation results and those of living cell counts per well (r = 1.0, $p < 0.0001$, Pearson's correlation test).

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 ^{14}C-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.

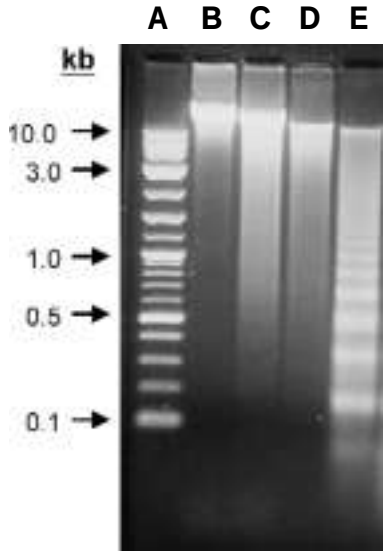

Figure 5. DNA electrophoresis of CLL cells (patient # 24) exposed for 24 h to verapamil. Lane A, molecular weight markers. Lane B, cells used to start the cultures. Lane C, control CLL cells after 24 h incubation. Lane D, CLL cells exposed to 100 µM verapamil for 24 h. Lane E, U937 cells exposed for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

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. Relatively 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

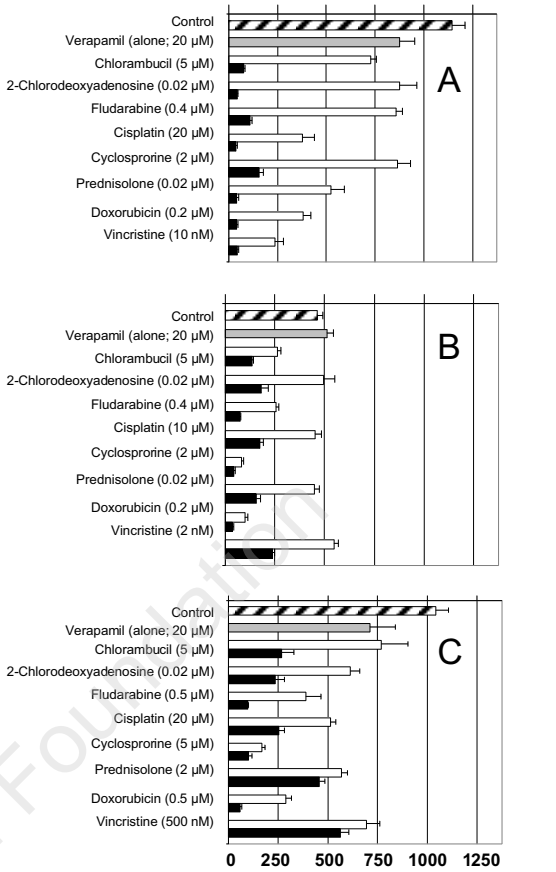

Figure 6. Synergism of verapamil and various anticancer drugs against CLL cells in 4-day cultures. Cell survival was indicated as ^{14}C-leucine incorporation during the final 24 h of culture. For technical details, see *Design and Methods* section.The cells were incubated without additives (columns 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.

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

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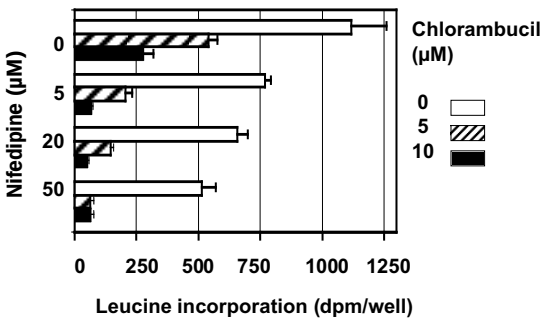

Figure 7. Synergistic cytotoxicity of nifedipine and chlorambucil in 4-day cultures (patient #2). Each column represents the mean (±SD) of six cultures. The viability of cells was investigated by 14C-leucine incorporation as described in *Design and Methods* section. The experiment provided strong evidence ($p < 0.001$) that nifedipine potentiated chlorambucil-induced cytotoxicity as assessed on the basis of differences between cultures with and without chlorambucil.

induced by verapamil. This is consistent with a need for Ca^{2+} to complete the apoptotic pathway of cell death, and nucleosome-sized DNA fragmentation.

Verapamil is the most studied P-gp modulator, but very few studies have been carried out in CLL.[5-8] 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 our previous studies,[17] 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-pg-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, depending on the target cells and experimental conditions, have either cytoprotective or toxic effects. Examples of known cytoprotective situations are: (i) verapamil partially prevented cadmium-induced apoptosis in human T-cells;[19] (ii) verapamil and nifedipine attenuated 2-methoxyacetic acid-induced apoptosis in human spermatocytes;[20] (iii) verapamil and nifedipine inhibited apoptosis induced by 25-hydroxycholesterol in human aortic smooth muscle cells;[21] (iv) verapamil prevented renal reperfusion injury;[22] (v) verapamil prevented nerve growth factor-induced cell death in a hypoglycemic environment.[23] These examples mean that Ca^{2+} channel blockers may have several kinds of effects and 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 vivo*;[24] (ii) the verapamil-enhanced growth inhibitory effect of 5-fluorouracil and 5-fluorouracil plus hyperthermia on human adenocarcinoma cells *in vitro*.[25] Verapamil also reversed 5-fluorouracil resistance in a human hepatocellular carcinoma cell line.[26]

A prolonged period of Ca^{2+} signaling is an important growth signal for many cells.[27] 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.[27] 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+} stored within the endoplasmic reticulum were depleted, the mitochondria would become overloaded and there would be two main consequences.[27] 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.[27] 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 Sandoz 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.

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