Cancer Chemother Pharmacol (1995) 37: 70–78

ORIGINAL ARTICLE

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In vitro effect of r-verapamil on acute myelogenous leukemia blast cells: studies of cytokine secretion and cytokine-dependent blast proliferation

Received: 3 November 1994/Accepted: 17 February 1995

Abstract The in vitro effect of the dextroisomer r-verapamil on blast cells derived from patients with acute myelogenous leukemia (AML) was studied. R-verapamil caused a dose-dependent inhibition of AML blast proliferation in the presence of stem-cell factor, leukemia inhibitory factor, interleukin 4, interleukin 6, and interleukin 10 when these cytokines were tested both alone and in different combinations. R-verapamil also inhibited the growth of clonogenic AML blast cells. The antiproliferative effect was not specific for AML blast cells, because r-verapamil also inhibited cytokine-dependent proliferation of blast cells derived from patients with acute lymphoblastic leukemia. The inhibitory effects of r-verapamil and anti-IL1 serum were additive, suggesting that the antiproliferative effect of r-verapamil does not depend solely on inhibition of IL1-mediated effects. Although r-verapamil inhibited spontaneous AML blast proliferation, for a majority of patients it caused only minimal, if any, inhibition of spontaneous cytokine secretion (IL1α, IL1β, TNFα, IL6) by AML blast cells. Thus, although inhibition of IL1 effects may contribute in certain patients to the antiproliferative effect of r-verapamil, mechanisms other than IL1 inhibition seem to be more important in mediating the effects of r-verapamil.

Key words: Acute myelogenous leukemia · R-verapamil

Abbreviations *ALL*, Acute lymphocytic leukemia · *AML* acute myelogenous leukemia · *cpm* counts per minute · *ELISA* enzyme-linked immunosorbent assay · *G-CSF* granulocyte colony-stimulating factor · *GM-CSF* granulocyte-macrophage colony-stimulating factor · *IL* interleukin · *IF* leukemia inhibitory factor · *PBMC* peripheral blood mononuclear cells · *RR* relative response · *SCF* stem cell factor · *TNF*α tumor necrosis factor α

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Introduction

The calcium antagonist verapamil has been used for decades in the treatment of cardiovascular diseases [15,21,32]. However, in vitro studies have demonstrated that verapamil also has effects on cell metabolism other than calcium antagonism. First, verapamil has antiproliferative effects on different cells, including T-lymphocytes [7], chronic myelogenous leukemia cells [4], and acute myelogenous leukemia (AML) blasts [4,6]; and for T-lymphocytes it has been suggested that this nontoxic antiproliferative effect is caused by alteration either of the utilization of essential nutrients or of transmembrane potassium transport [12,25]. Second, verapamil can inhibit the function of the p-glycoprotein, thereby increasing intracellular drug concentrations and rendering malignant cells more susceptible to cytotoxic drugs [22,29]. These additional effects have been clearly demonstrated only in vitro and often only at concentrations exceeding the therapeutic serum level [32]. However, r-verapamil has fewer cardiotoxic side effects than does racemic verapamil, and higher in vivo concentrations can therefore be attained [3,15,24]. The potential use of r-verapamil in cancer therapy is therefore being investigated [3,24], and both its antiproliferative effects and its inhibition of p-glycoprotein may become clinically important when higher in vivo concentrations are reached.

The two chemically unrelated drugs dipyridamole and verapamil have antiproliferative effects [4,6]. As both drugs also inhibit p-glycoprotein, it has been suggested that they should be used in combination with chemotherapy to enhance cytotoxic effects

[2,3,22,24,27,29]. For both drugs, the exposure sequence or interval between exposure to dipyridamol/verapamil and chemotherapy seems to be important for the achievement of maximal enhancement of cytotoxicity in vitro [2,22]. One possible explanation for this could be that depending on the exposure sequence, antiproliferative drug effects may alter the susceptibility of malignant cells to cell-cycle-specific cytotoxic drugs and thereby counteract the effect on the p-glycoprotein. The possible use of p-glycoprotein-inhibitory drugs in combination with chemotherapy in leukemia treatment is being investigated [22,27], and in this context we further characterized the antiproliferative effect of r-verapamil on acute leukemia blast cells.

Patients and methods

Patients

The clinical data of all patients are summarized in Table 1.

Reagents

The culture medium was RPMI 1640 with HEPES and glutamine (Gibco, UK), to which 10% inactivated fetal calf serum (HiClone, USA) and gentamicin (100 μg/ml) were added. Conditioned medium was prepared by culturing peripheral blood mononuclear cells (PBMC, 1×10^6/ml) from a healthy individual together with phytohemagglutinin (PHA HA 16, 1 μg/ml; Wellcome, UK) for 72 h. The culture supernatant was then collected and referred to as conditioned medium [5].

Cytokines were used at the following concentrations [1,11,14,16,18,23,28,30]: GM-CSF, 100 ng/ml (Sandoz, Switzerland); G-CSF, 100 ng/ml (Roche, Switzerland); TNFα, 100 ng/ml (kind gift from Knoll AG, Germany); IL3, 50 ng/ml (R&D Systems, UK); IL4, 50 ng/ml (R&D Systems); IL6, 50 ng/ml (PeproTech, UK); IL7, 50 ng/ml (R&D Systems); (LIF), 50 ng/ml (PeproTech); Leukemia inhibitory factor (SCF), and stem-cell factor 50 ng/ml (PeproTech). Cytokine combinations were also tested for SCF (SCF + IL3, SCF + GM-CSF), IL4 (IL4 + IL3, IL4 + GM-CSF), LIF (LIF + IL3, LIF + IL6, LIF + GM-CSF), and IL7 (IL7 + IL2, IL7 + IL3), and all cytokines were used in the same concentrations when tested in combinations as when tested separately.

Normal goat serum and anti-IL1α and anti-IL1β polyclonal goat antiserum were purchased from R&D Systems (UK).

Cell preparation

PBMC were isolated by density-gradient separation (Ficoll-Hypaque, NyCoMed, Norway; specific density, 1.077). To reach a high percentage of AML blast cells among the PBMC (> 95%), only patients with a high leukocyte count in peripheral blood ($> 30 \times 10^9$/l; at least 90% blast cells) were included in the study [6,8]. The high percentage of blast cells was confirmed by light

Table 1 Clinical characteristics of acute leukemia patients (*CHOP* Cyclophosphamide/Doxorubicin/Vincristine/Prednisone, *CML* chronic myelocytic leukemia, *FAB* French-American-British, *NT* Not tested)

Patient number	Sex (M/F)	Age (years)	Previous hematological disease	FAB classification	Membrane molecule expression[a]							
					CD3	CD13	CD14	CD15	CD19	CD20	CD33	CD34
1	F	64		AML-M2	−	+	+	NT	−	−	−	−
2	F	56	Primary myelodysplastic syndrome	AML-M2	−	+	−	−	−	−	+	−
3	M	47		AML-M4	−	+	+	NT	−	−	−	−
4	M	67	Primary myelodysplastic syndrome	AML-M2	−	+	−	NT	−	−	+	−
5	F	81	Primary myelodysplastic syndrome	AML-M2	−	+	−	NT	−	−	+	−
6	M	73	Chronic myelofibrosis	AML-M2	−	+	−	NT	−	−	+	NT
7	F	67		AML-M1	−	+	−	NT	−	−	−	−
8	M	80		AML-M4	−	−	+	NT	−	−	−	−
9	F	72	Multiple myeloma	AML-M2	−	−	+	NT	−	−	+	−
10	F	67		AML-M4	−	+	−	NT	−	−	+	−
11	M	30		AML-M2	−	−	−	NT	−	−	+	−
12	M	75	Non-Hodgkin's lymphoma, CHOP therapy	AML-M2	−	+	−	NT	−	−	+	NT
13	M	64		AML-M2	−	+	−	+	−	−	+	+
14	F	69		AML-M2	−	+	−	+	−	−	+	+
15	M	33	CML	AML-M4	−	+	−	+	−	−	+	+
16	F	42		AML-M5	−	+	−	+	−	−	+	−
17	M	82		AML-M2	−	+	−	+	−	−	+	+
18	M	84		ALL	−	−	−	−	+	NT	−	−
19	F	16		ALL	−	−	−	−	+	+	−	−
20	F	58		ALL	−	−	−	−	+	+	−	−
21	F	29		ALL	+	−	−	−	−	−	−	−
22	M	78		ALL	−	−	−	−	+	NT	−	+

[a]Patients were regarded as positive when more than 20% of blast cells stained positive as judged from flow-cytometric analysis

microscopy of PBMC. Cells were stored frozen in liquid nitrogen; the methods used for freezing and thawing of cells have been described elsewhere [4].

Proliferation assay

As previously described [4,6], leukemia blast cells (5×10^4/well) were cultured in 200 µl culture medium in flat-bottomed microtiter plates (Costar, USA). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. [^{3}H]-thymidine was added at 37 kBq/well (TRA 310, 1 mCi/mmol; Amersham, UK) after 3 days for acute lymphoblastic leukemia (ALL) cells and after 6 days for AML cells, and the nuclear incorporation of [^{3}H]-thymidine was determined after 18 h by liquid scintillation counting. All cultures were performed in triplicate, and median responses were used for calculations and statistical analysis. Proliferation was regarded as significant when it (a) exceeded the negative control value by at least 1,000 cpm and (b) exceeded the mean cpm obtained for the negative control by at least 3 SD.

Colony-forming assay

AML blast cells (2.5×10^4/well) were cultured in 24-well tissue-culture plates (Costar, USA); each well contained 250 µl culture medium with 0.36% agarose (SeaPlaque; FMC Bio Products, USA). After 12 days the number of colonies exceeding 20 cells were counted.

Cytokine secretion

Cytokine secretion was investigated as described previously [8, 20]. In each well of 24-well tissue-culture plates (Costar), AML blast cells (1×10^6/well) were cultured in 1 ml culture medium. Cultures were incubated for 48 h and supernatants were then collected and stored frozen until analyzed. Cytokine secretion was measured as the concentrations of free cytokines (IL1α, IL1β, IL6, and TNFα) in culture supernatants, concentrations being determined by cytokine-specific ELISA analysis (Quantikine ELISA kits; R&D Systems, UK). All ELISA analyses were performed strictly according to the manufacturer's instructions as described elsewhere [8].

Statistical analysis

The relative response (RR) was defined as the proliferation (cpm) in drug-containing cultures relative to that in drug-free controls. Wilcoxon's test for paired samples was used for statistical analysis, and differences were regarded as statistically significant when $P < 0.05$.

Results

Effect of r-verapamil on cytokine-dependent proliferation of blast cells derived from AML patients

The effects of r-verapamil on AML blast proliferation ([^{3}H]-thymidine incorporation) in the presence of SCF, IL4, IL6 and LIF were investigated for patients 1–8. Consistent with the results described in previous studies [4,6], both spontaneous and cytokine-dependent proliferation showed a wide variation range for the patients studied, and the number of patients showing significant proliferation and the variation range are therefore given for each cytokine or cytokine combination investigated. In the presence of SCF alone, significant proliferation was seen for five patients ($n = 5$; range, 1,466–44,634 cpm), and r-verapamil showed a dose-dependent inhibitory effect on blast proliferation for all these patients (Fig. 1). A similar antiproliferative effect was also seen when AML blast cells were cultured in medium alone and in the presence of SCF + IL3 ($n = 6$; range, 1,286–26,334 cpm; $P = 0.016$ for r-verapamil at 2.5 and 25 µg/ml) and SCF + GM-CSF ($n = 6$; range, 2,130–68,656 cpm; $P = 0.016$ for r-verapamil at 2.5 and 25 µg/ml).

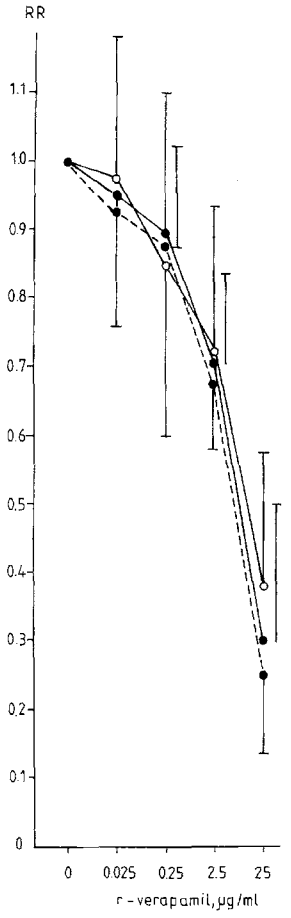

Fig 1 Effect of r-verapamil (25, 2.5, 0.25 and 0.025 µg/ml) on AML blast proliferation assayed as [^{3}H]-thymidine incorporation in suspension cultures. AML blast cells were cultured in the presence of SCF ($n = 5$, ●—●), IL3 + IL4 ($n = 6$, ○—○), and IL3 + LIF ($n = 6$, ●---●). The results are expressed as mean RR ± SD obtained for the AML patients tested

Significant AML blast proliferation was seen for two patients in the presence of IL4 and for six patients in the presence of IL4 + IL3 (range, 1,168–11,692 cpm) and IL4 + GM-CSF (range, 1,286–31,792 cpm). For all patients showing significant proliferation, r-verapamil caused a dose-dependent antiproliferative effect when

cells were cultured with IL4, IL4 + GM − CSF ($n = 6$, $P = 0.016$ for r-verapamil at 2.5 and 25 μg/ml), and IL4 + IL3 (Fig. 1; $n = 6$, $P = 0.016$ for r-verapamil at 2.5 and 25 μg/ml).

Because AML blast cells from only two patients showed significant proliferation in the presence of IL6, statistical analysis could not be performed. However, for these two patients, r-verapamil also caused a dose-dependent inhibition in the presence of IL6 (data not shown).

AML blast cells from two patients showed significant proliferation in the presence of LIF alone, and r-verapamil caused a dose-dependent inhibitory effect for both patients (data not shown). Significant proliferation was seen when AML blasts were cultured with LIF + IL3 ($n = 6$; range, 1,140–22,132 cpm; $P = 0.016$ for r-verapamil at 2.5 and 25 μg/ml), LIF + IL6 ($n = 3$; range, 3,294–10,736 cpm), and LIF + GM − CSF ($n = 4$; range, 1,166–16,238 cpm), and r-verapamil caused a dose-dependent antiproliferative effect for all three cytokine combinations for all patients studied. The results obtained for LIF + IL3 are presented in Fig. 1.

When AML blasts were cultured with IL10 at 3 ng/ml, significant proliferation was seen only for six (patients 5–10) of ten patients tested. Although both increased and decreased AML blast proliferation could be seen in the presence of IL10, r-verapamil inhibited AML blast proliferation for all five patients in the presence of IL10. The results obtained for R-verapamil at 2.5 μg/ml ($n = 6$, $P = 0.016$) are presented in Fig. 2.

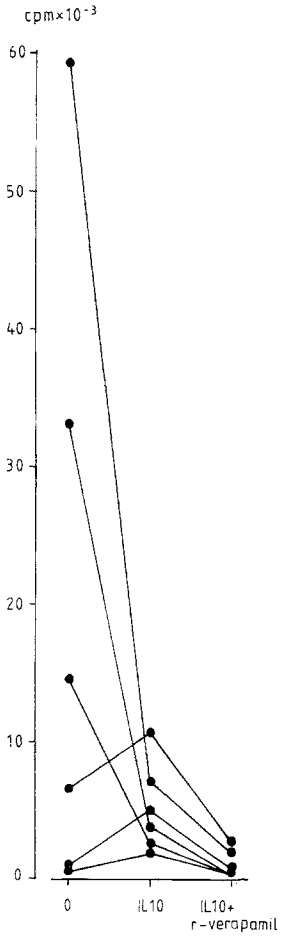

Fig 2 Proliferation of AML blast cells derived from 6 patients when cells were cultured in medium alone (*0*), medium + IL10 3 ng/ml (*IL10*); and medium + IL10 3 ng/ml + r-verapamil 2.5 μg/ml (*IL10 + r-verapamil*). For each patient the results are presented as mean cpm obtained for triplicate cultures, the SD corresponding to < 10% of the mean

Effect of r-verapamil on AML blast colony formation.

AML blast cells from five patients were studied using the colony-forming assay. For each patient, blast cells were cultured with the cytokine causing the strongest proliferation as judged from the [^{3}H]-thymidine incorporation assay. R-verapamil inhibited proliferation of clonogenic AML blast cells for all patients investigated (Table 2).

R-verapamil shows an additive inhibitory effect with IL1-specific antibodies on AML blast proliferation

AML blast cells from patients 6–10 showed high in vitro spontaneous proliferation and were used to investigate whether r-verapamil had an additive antiproliferative effect together with neutralizing anti-IL1 antibodies. Anti-IL1α and anti-IL1β antiserum caused low inhibition when tested separately over a wide concentration range (50–0.05 μg/ml). However, when anti-IL1α + anti-IL1β were added together in equal concentrations an inhibition was seen with a plateau being observed at concentrations of 10 μg/ml or higher. R-verapamil at 2.5 μg/ml added together with anti-IL1α + anti-IL1β caused an additive inhibitory effect for all patients ($n = 5$; $P = 0.036$); the results obtained for two patients are presented in Fig. 3.

Effects of different calcium antagonists on AML blast proliferation

The effects on AML blast proliferation of the calcium antagonists r-verapamil, amlodipin, diltiazem, felodipin, nifedipin, and nimodipin were studied for six patients (patients 3,4, and 6–9). All these patients showed significant proliferation in tests of both spontaneous and cytokine-dependent (G-CSF, GM-CSF) proliferation. The drugs were dissolved in ethanol to give a final concentration of 0.5% ethanol for the highest drug concentration tested, and further dilutions were made in culture medium. Ethanol caused no significant alteration of spontaneous or cytokine-dependent AML blast proliferation (data not shown). When

Table 2 Effect of r-verapamil on AML blast-cell colony formation

Patient number	Cytokine	Colony formation[a]	
		Cultures without r-verapamil	Cultures with r-verapamil (25 μg/ml)
2	IL3	8,6	0,1
3	G-CSF	8,7	0,0
8	G-CSF	19,15	0,2
16	IL3	10,7	0,2
17	G-CSF	31,24	3,1

[a]Results are expressed as the number of colonies per well; all tests were performed in duplicate and the numbers of colonies for both duplicates are given

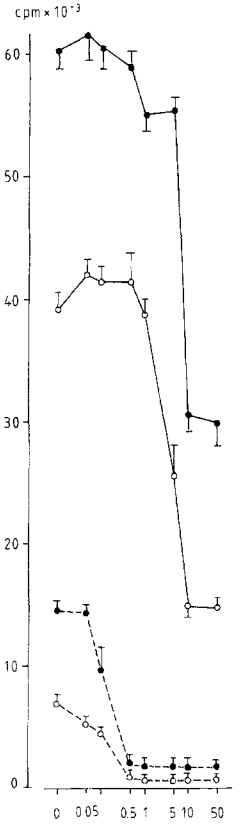

Fig 3 Effect of r-verapamil (2.5 μg/ml) on AML blast proliferation in the presence of different concentrations of anti-IL1α + anti − IL1β antiserum. The results obtained for patients 9 (——) and 10 (– –) when cells were cultured with anti-IL1 alone (●) and with anti-IL1 + r-verapamil (○) are shown. The results are presented as mean cpm ± SD obtained for triplicate cultures

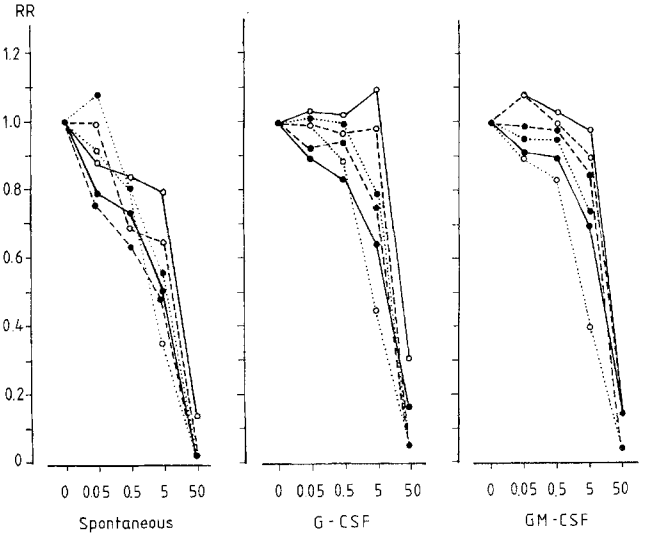

Fig 4 Effect of calcium antagonists on in vitro proliferation of AML blast cells in medium (*Spontaneous*) and in the presence of G-CSF and GM-CSF. The results are presented as mean RR obtained for 6 AML patients when the drugs were tested at equimolar concentrations (μ*M*). The following calcium antagonists were tested: r-verapamil (●—●), amlodipine (●– –●), diltiazem (●. .●), felodipine (○__○), nifedipine (○—○), and nimodipine (○ . . . ○). Cultures containing only ethanol did not differ significantly from ethanol-free controls

tested at equimolar concentrations, all calcium antagonists caused a dose-dependent inhibition of both spontaneous proliferation and G-CSF- and GM-CSF-dependent AML blast proliferation (Fig. 4). The inhibition was statistically significant for spontaneous proliferation ($n = 6$) and for G-CSF-($n = 6$) and GM-CSF-dependent ($n = 6$) proliferation when drug concentrations of both 50 μ*M* ($P = 0.016$ for all drugs) and 5 μM ($P = 0.016$ for r-verapamil, diltiazem, and nemodipin; $P = 0.031$ for felodipin and nifodipin) were tested.

For all calcium blockers, cultures were microscoped regularly for evaluation of the frequency of necrotic (dense, swollen, granular) cells as compared with drug-free controls. Drug-containing cultures showed no increased frequency of necrotic cells (data not shown).

Effect of r-verapamil on in vitro proliferation of ALL blast cells

Blast cells derived from five ALL patients showed no proliferation when cultured in medium alone. However, significant proliferation could be detected when cells were cultured with 10% conditioned medium + IL2 (10 ng/ml), and r-verapamil then caused a dose-dependent inhibition of ALL blast proliferation

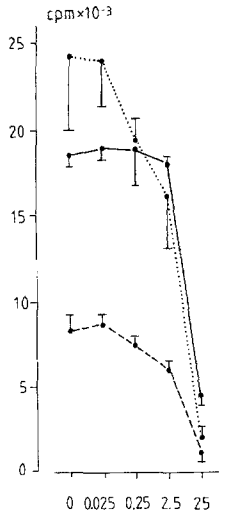

Fig 5 Effect of r-verapamil on cytokine-dependent in vitro proliferation ([^{3}H]-thymidine incorporation assay) of ALL blast cells from patients 18 (——), 19 (· · · · ·) and 20 (– –). The results are presented as mean cpm ± SD obtained for triplicate cultures when proliferation was assayed after 5 days

for all patients tested (Fig. 5; $n = 5$, $P = 0.036$ for r-verapamil at 25 and 2.5 μg/ml).

ALL blast cells were also incubated in culture medium containing recombinant IL2, IL3, IL7, IL2 + IL7, IL3 + IL7, and TNFα (see patients and methods). For three patients, low but significant proliferation (< 2,000 cpm; negative controls, < 300 cpm) could also be detected when ALL blasts were cultured with IL2 (patient 21), IL2 + IL7 (patient 21), IL3 (patients 18 and 19), or IL3 + IL7 (patient 19), and r-verapamil caused a dose-dependent inhibition of all these responses (data not shown).

Effect of r-verapamil on cytokine secretion from AML blasts

The effect of r-verapamil at 25 μg/ml on cytokine secretion was investigated for nine AML patients (patients 6,7, and 9–15). The secretion of cytokines differs between individual AML patients [8], and for these experiments we selected patients showing detectable secretion of several cytokines. R-verapamil caused a strong inhibitory effect of AML blast proliferation when tested in parallel with cytokine secretion after 48 h of culture (mean RR, 0.21; $n = 9$, $P = 0.002$). The results obtained for those patients showing detectable cytokine secretion are presented in Fig. 6. In contrast to the effect on AML blast proliferation, r-verapamil caused no significant alteration of spontaneous AML blast secretion of IL1α ($n = 8$), IL1β ($n = 9$), TNFα ($n = 9$), or IL6 ($n = 7$).

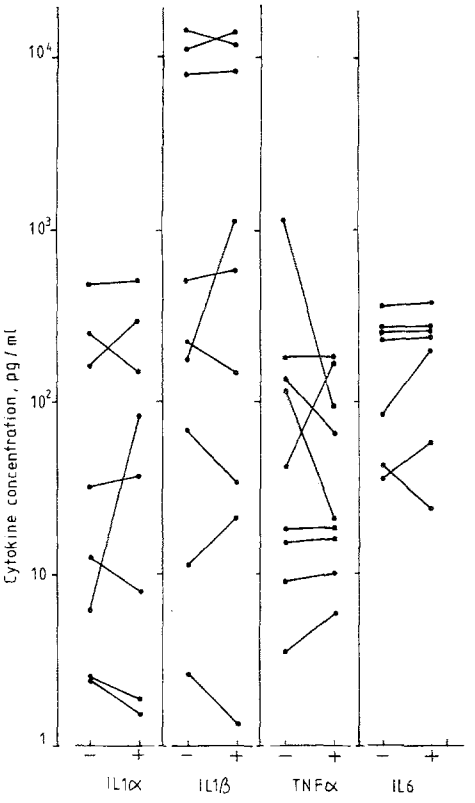

Fig 6 Effect of r-verapamil (25 μg/ml) on in vitro cytokine secretion from AML blast cells. Cytokine secretion was measured as concentrations of IL1α, IL1β, TNFα and IL6 (pg/ml) in supernatants from cultures of AML blast cells, and the figure includes only the results obtained for those patients showing detectable cytokine levels. Cultures were prepared in culture medium with (+) and without (−) r-verapamil 25 μg/ml.

Discussion

In the present study we further characterized the effect of r-verapamil on in vitro functions of AML blast cells. R-verapamil exerted a strong inhibitory effect on AML blast proliferation for all patients and all in vitro models studied. In contrast, the effect of r-verapamil on AML-blast cytokine secretion varied between patients, and decreased secretion was seen for only a minority of patients.

Combination treatment with cytotoxic drugs and p-glycoprotein inhibitors is being investigated in leukemia therapy [27]. The drugs verapamil and dipyridamole inhibit p-glycoprotein in malignant cells and may thereby increase intracellular concentrations of cytotoxic drugs [2,29]. Recent in vitro studies have also shown that combination therapy with r-verapamil/dipyridamole and cytotoxic drugs will increase cytotoxic effects on malignant cells, but the increased cytotoxicity depends on the interval between treatments or the sequence of drug administration [2]. Both dipyridamole and verapamil have antiproliferative effects on malignant cells [6]. Thus,

dipyridamole and r-verapamil have common pharmacological effects, although they are chemically unrelated. For both drugs, the importance of the treatment sequence in increasing cytotoxicity can be explained by a similar antiproliferative effect rendering malignant cells less sensitive to cell-cycle-specific cytotoxic drugs. This possible importance of the antiproliferative effect should be considered for the future design of clinical studies.

When racemic verapamil is used the cardiotoxic effects are dose-limiting [15,32]. R-verapamil is less cardiotoxic than racemic verapamil [15], and antiproliferative effects of r-verapamil are seen at concentrations corresponding to levels reached in vivo by r-verapamil and its active metabolite norverapamil (1–4 μg/ml or 1–7 μ*M* [3, 24].

We used leukemia blast cells isolated by density-gradient separation from peripheral blood of patients with a high number of blasts [6,8]. By this approach a highly enriched blast population could be prepared without extensive cell-separation procedures causing in vitro induction of cytokine expression [26]. However, the number of blast cells in peripheral blood seems to be a prognostic factor for AML patients and may thus reflect intrinsic properties of the leukemia cells [17]. Because of this patient selection, our results may thus be representative only for the subset of AML patients with high blast counts in peripheral blood and not for AML patients in general.

In our study we investigated the effect of r-verapamil in suspension cultures of AML blasts, proliferation being assayed as [^{3}H]-thymidine incorporation after 7 days of culture. During this interval the exponential growth of clonogenic leukemia cells can be maintained without reculture of the cells, this in vitro culture then leading to an enrichment of clonogenic AML cells [19]. AML blast proliferation is then assayed after 7 days, when proliferation has reached a plateau, and proliferation is then determined by cells that can be maintained in culture [6,8].

Proliferation of acute leukemia blast cells is influenced by neighboring cells in the in vivo microenvironment through the cytokine network. SCF, IL3, IL6, G-CSF, and GM-CSF function as hematopoietic growth factors that can also modulate the proliferation of AML blast cells [1,6,9,10,16,23,28]. However, several other factors can also modulate AML blast functions: (a) both IL4 and IL10 can interact with other hematopoietic growth factors and thereby modulate blast proliferation, and both cytokines can also decrease the blast secretion of several cytokines ([8,18]; Bruserud, manuscript in preparation); (b) IL7 can function as a growth factor for ALL blasts [(14]; and (c) LIF can also be a growth-enhancing factor for AML blast cells [28].

In the present study we used cytokine concentrations that had previously been shown to be within the optimal range for acute leukemia blasts cultured in vitro [1,14,16,23,28,30], the only exception being SCF, which was used at a concentration known to be optimal for normal hematopoietic stem cells [11]. Thus, we used concentrations known to be optimal for in vitro AML blast culture rather than concentrations corresponding to cytokine serum levels. However, serum cytokine concentrations may not reflect cytokine concentrations in the in vivo microenvironment, as local cytokine concentrations at the site of an inflammatory reaction can exceed the serum level [31].

AML blast cells are functionally heterogeneous with regard to responses to and secretion of cytokines [1,6,8,12,16,18], and to investigate the effects of r-verapamil on various cellular functions we had to select different AML patients, depending on the cytokine or cytokine combination investigated. For certain cytokines (IL4, IL6, LIF, LIF + IL6), significant proliferation could be detected only for very few patients, and a statistical analysis could not be performed. However, an antiproliferative effect of r-verapamil could also be detected for individual patients when these cytokines were tested. The antiproliferative effect of r-verapamil also affected the clonogenic AML blast subset. Thus, in our in vitro model the antiproliferative effect of r-verapamil could be demonstrated in the presence of all hematopoietic growth factors or growth factor combinations investigated (Figs. 1,2; [6]).

All calcium blockers showed a similar dose-dependent inhibitory effect on both spontaneous and cytokine-dependent AML blast proliferation. Thus, despite their pharmacological differences [21], the antiproliferative effect on AML blasts seems to be common for several calcium blockers. This antiproliferative effect may be caused by an alteration in the use of essential nutrients, as has previously been suggested for the antiproliferative effects of different calcium antagonists during T-cell activation [25].

Conditioned medium contains several cytokines. However, IL2 concentrations are low [5], and ALL blast cells were therefore cultured with conditioned medium + IL2. ALL blasts were also cultured with recombinant cytokines or cytokine combinations stimulating in vitro ALL blast proliferation [14,30]. R-verapamil inhibited ALL blast proliferation in the presence of both conditioned medium + IL2 and recombinant cytokines. These results show that the antiproliferative effect of r-verapamil is not specific for myelogenous leukemia cells.

IL1α and IL1β function as autocrine growth factors for AML blast cells [13], and AML cells from a majority of patients are capable of IL1 secretion [8]. However, for a majority of patients the growth-enhancing effects of IL1α and IL1β are modulated by blast secretion of IL6 and TNFα [23,26]. Because these cytokines interact as growth factors for AML blast cells, we investigated the effect of r-verapamil on spontaneous blast secretion of all these cytokines. R-verapamil

caused no significant alteration in the secretion of these cytokines. A small decrease in cytokine secretion was seen only for a minority of patients, even when a high r-verapamil concentration (25 μg/ml), was tested, whereas this concentration caused a strong antiproliferative effect. We conclude that for a majority of patients the antiproliferative effect of r-verapamil is caused by mechanisms other than inhibition of AML blast cytokine secretion.

AML blasts were also cultured with neutralizing anti-IL1 antibodies. IL1α and IL1β bind to common receptors, and to neutralize the IL1 effects we used a mixture of anti-IL1α and anti-IL1β antibodies. R-verapamil showed an additive inhibitory effect with anti − IL1α + anti − IL1β antibodies on AML blast proliferation, and these results support the conclusion that the antiproliferative effect of r-verapamil is caused by mechanisms other than inhibition of IL1-dependent cellular functions.

From the present study we conclude that r-verapamil exerts an antiproliferative effect on AML blast cells at concentrations corresponding to the serum level attained in vivo. This antiproliferative effect may become clinically important when r-verapamil is combined with cell-cycle-specific cytotoxic drugs and should therefore be considered in the design of future clinical investigations.

Acknowledgements This study received financial support from Knoll AG, Germany; MEDA, Norway; DFG SFB 120; and the Norwegian Cancer Society. The technical assistance of Laila Menzoni is gratefully acknowledged.

References

1. Assano Y, Okamura S, Shibuya T, Harada M, Niho Y (1988) Growth of clonogenic myeloblastic leukemia cells in the presence of human recombinant erythropoietin in addition to various human recombinant hematopoietic growth factors. Blood 72: 1682
2. Barberi-Heyob M, Griffon G, Merlin JL, Weber B (1993) Sequence-dependent growth-inhibitory effects of the in vitro combination of fluorouracil, cisplatin and dipyridamole. Cancer Chemother Pharmacol 28: 163
3. Bisset D, Kerr DJ, Cassidy J, Meredith P, Traugott U, Kaye SB (1991) Phase I and pharmacokinetic study of D-verapamil and doxorubicin. Br J Cancer 64: 1168
4. Bruserud Ø (1992) Effect of dipyridamole, theophyllamine and verapamil on spontaneous in vitro proliferation of myelogenous leukemia cells. Acta Oncol 31: 53
5. Bruserud Ø, Moen T (1983) Production of interleukin 2 containing growth medium for use in cloning of human T lymphocytes. J Immunol Methods 71: 175
6. Bruserud Ø, Pawelec G (1993) Effects of dipyridamole and R-verapamil on in vitro proliferation of blast cells from patients with acute myelogenous leukemia. Leuk Res 17: 507
7. Bruserud Ø, Hamann W, Patel S, Pawelec G (1992) $CD4^+$ TCRα β^+T cell clones derived shortly after allogeneic bone marrow transplantation: theophyllamine and verapamil inhibit proliferation of functionally heterogeneous T cells. Int J Immunopharmacol 14: 783
8. Bruserud Ø, Nesthus I, Bühring H-J, Pawelec G (1995) Cytokine modulation of interleukin 1 and tumor necrosis factor α secretion by acute myelogenous leukemia blast cells in vitro. Leukemia Res 19:15
9. Bühring H-J, Ullrich A, Schaudt K, Müller CA, Busch FW (1991) The product of the proto-oncogen c-kit (P145 c-kit) is a human bone marrow surface antigen of hematopoietic precursor cells which is expressed on a subset of acute nonlymphoblastic leukemic cells. Leukemia 5: 854
10. Butturini A, Santucci MA, Gale RP, Perocco P, Tura S (1990) GM-CSF incubation prior to treatment with cytarabine or doxorubicine enhances drug activity against AML cells in vitro: a model for leukemia chemotherapy. Leuk Res 14: 743
11. Carow CE, Hangoc G, Cooper SH, Williams DE, Broxmeyer H (1991) Mast cell growth factor (c-kit ligand) supports the growth of human multipotential progenitor cells with a high replating potential. Blood 78: 2216
12. Chandy KG, DeCoursey TE, Cahalan MD, McLaughlin C, Gupta S (1984) Voltage-gated potassium channels are required for human T lymphocyte activation. J Exp Med 160: 369
13. Cozzolino F, Rubartelli A, Aldinucci D, Sitia R, Torcia M, Shaw A, diGuglielmo R (1989) Interleukin 1 as an autocrine growth factor for acute myeloid leukaemia cells. Proc Natl Acad Sci USA 86: 2369
14. Digel W, Schmid M, Heil G, Conrad P, Gillis S, Porzsolt F (1991) Human interleukin 7 induces proliferation of neoplastic cells from chronic lymphocytic leukemia and acute leukemias. Blood 78: 753
15. Echizen H, Brecht T, Niedergan S, Vogelgesang B, Eichelbaum M (1985) The effect of dextro-levo and racemic verapamil on AV-conduction in humans. Am Heart J 109:210
16. Lemoli RM, Gulati SC, Strife A, Lambek C, Perez A, Clarkson BD (1991) Proliferative response of human acute myeloid leukemia cells and normal marrow enriched progenitor cells to human recombinant growth factors IL3, GM-CSF and G-CSF alone and in combination. Leukemia 5: 386
17. McGlave PB, Haake RJ, Bostrom BC, Brunning R, Hurd DD, Kim TH, Nesbit TE, Vercelotti GM, Woods WG, Ramsay NKC, Kersey JH (1988) Allogeneic bone marrow transplantation for acute nonlymphoblastic leukemia in first remission. Blood 72: 1512
18. Miyauchi J, Clarc SC, Tsunematsu Y, Shimizu K, Park J-W, Ogawa T, Toyama K (1991) Interleukin 4 as a growth regulator of clonogenic cells in acute myelogenous leukemia in suspension culture. Leukemia 5: 108
19. Nara N, McCulloch EA (1985) The proliferation in suspension of the progenitors of the blast cells in acute myeloblastic leukemia. Blood 65: 1484
20. Rambaldi A, Torcia M, Bettoni S, Vannier E, Barbui T, Shaw AR, Dinarelli CA, Cozzolino F (1991) Modulation of cell proliferation and cytokine production by interleukin 1 receptor antagonist and lack of its expression by leukemic cells. Blood 78: 3248
21. Reicher-Reiss H, Barasch E (1991) Calcium antagonists in patients with heart failure. Drugs 42:343
22. Roller E, Klumpp B, Krause J, Eichelbaum M, Schumacher K (1993) Influence of sequential exposure to R-verapamil or B8509-035 on rhodamine 123 accumulation in human lymphoblastoid cell lines. Cancer Chemother Pharmacol 32: 151
23. Salem M, Delwel R, Touw I, Mahmoud LA, Elbasousy EE, Löwenberg B (1990) Modulation of colony stimulating factor-dependent growth of acute myeloid leukemia cells by tumor necrosis factor. Leukemia 4: 37
24. Scheithauer W, Schenk T, Czejka M (1993) Pharmacokinetic interaction between epirubicin and the multidrug resistance reverting agent D-verapamil. Br J Cancer 68: 8
25. Schnell SR, Nelson DJ, Fozzard HA, Fitch FW (1987) The effects of K-channel-blocking agents on T lymphocyte proliferation and cytokine secretion are nonspecific. J Immunol 139: 3224

26. Sissolak G, Hoffbrand AV, Metha AB, Ganeshaguru K (1992) Effects of interferon-alpha (IFN) on the expression of Interleukin 1β (IL1), interleukin 6, granulocyte-macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor-α in acute myeloid leukemia (AML) blasts. Leukemia 6: 1155
27. Sotomatsu M, Yugami S-I, Shitara T, Kuroume T (1993) Dipyridamole enhancement of drug sensitivity in acute lymphoblastic leukemia cells. Am J Hematol 43: 251
28. Takanashi M, Motoji T, Masuda M, Oshimi K, Mizoguchi H (1993) The effect of leukemia inhibitory factor and interleukin 6 on the growth of acute myeloid leukemia cells. Leuk Res 17: 217
29. Tidefelt U, Sundman-Engberg B, Paul C (1988) Effects of verapamil on uptake and in vitro toxicity of anthracyclines in human leukemic cells. Eur J Haematol 40: 385
30. Uckun FM, Gesner TG, Song CW, Meyer DE, Mufson A (1989) Leukemic B cell precursors express functional receptors for human interleukin 3. Blood 73: 533
31. Waage A, Halstensen A, Shalaby R, Brandzæg P, Kierulf P, Espevik T (1989) Local production of tumor necrosis factor alpha, interleukin 1 and interleukin 6 in meningococcal disease. J Exp Med 170: 1859
32. Weiner DA, McCabe CH, Cutler SS, Creager MA, Ryan TJ, Klein MD (1983) Efficacy and safety of verapamil in patients with angina pectoris after one year of continuous high-dose therapy. Am J Cardiol 51: 1251