The in-vitro antiproliferative effect of PRI-2191 and imatinib applied in combined treatment with cisplatin, idarubicin, or docetaxel on human leukemia cells

Marta Świtalska[a], Anna Nasulewicz-Goldeman[a], Aleksandra Opolska[a], Magdalena Maciejewska[a], Andrzej Kutner[b] and Joanna Wietrzyk[a]

Imatinib mesylate (Gleevec, STI571) is a specific inhibitor of the Bcr/Abl fusion tyrosine kinase that exhibits potent antileukemic effects in chronic myelogenous leukemia. Bcr/Abl-positive K562 and Bcr/Abl-negative HL-60 human leukemia cells were used to investigate the effect of PRI-2191, a calcitriol analog, on the biological effects of imatinib combined with other anticancer drugs.

The results show that PRI-2191 enhances the antiproliferative effect of imatinib on HL-60 cells. When these two agents together are applied with either docetaxel or cisplatin, but not with idarubicin, the antiproliferative effect could still be enhanced. Moreover, when the interaction between the chemotherapy agents was antagonistic or additive, PRI-2191 could even shift it to synergism. This effect correlated with an accumulation of HL-60 cells in the G0/G1 phase of the cell cycle and a decrease in the percentage of cells in the G2/M and S stage in the ternary combinations used. PRI-2191 did not influence apoptosis induced by imatinib alone or in ternary combinations with all the chemotherapy agents used. These results may suggest that the stronger antiproliferative effect of the combined treatment with PRI-2191 on HL-60 cells is related to cell cycle arrest rather than to the induction of apoptosis. ***Anti-Cancer Drugs*** **23:70–80 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2012, **23:**70–80

Keywords: cisplatin, combined antitumor therapy, docetaxel, idarubicin, imatinib mesylate, PRI-2191

[a]Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, R. Weigla, Wroclaw and [b]Pharmaceutical Research Institute, L. Rydygiera, Warsaw, Poland

Correspondence to Joanna Wietrzyk, PhD, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla St. 12; 53–114 Wrocław, Poland
Tel: +48 71 337 11 72; fax: +48 71 337 13 82;
e-mail: wietrzyk@iitd.pan.wroc.pl

Received 31 March 2011 **Revised form accepted** 30 July 2011

Introduction

Imatinib mesylate (STI571) selectively inhibits Bcr/Abl, platelet derived growth factor receptor (PDGFR), and c-Kit kinase activity. It has significant and rapid antileukemic activity against chronic myelogenous leukemia and Philadelphia-positive (Ph^+) acute lymphoblastic leukemia. However, refractoriness and early relapse have been reported frequently, particularly in patients with advanced disease [1]. Several mechanisms of refractoriness and relapse have been reported, including point mutations within the Abl kinase domain, *Bcr-Abl* gene amplification, *Bcr-Abl* mRNA overexpression, and increased drug efflux through the P-glycoprotein-mediated process, all of which lead to reactivation of the Bcr-Abl kinase [2–8]. These findings suggest that therapeutic agents that can augment the anti-Ph^+ leukemia activity of imatinib mesylate may be mandatory to overcome resistance and relapse. To improve response rates and prolong survival, a number of preclinical and clinical evaluations of combinations of imatinib mesylate and other commonly used antileukemic agents have been investigated [9–11].

Ras signaling pathways are frequently overactive in Ph^+ leukemia. As a result of Bcr-Abl or c-Kit activation, proteins of the Ras/Erk and phosphoinositide-3 kinase pathways are subsequently activated. Therefore, targeting one of the Bcr-Abl or c-Kit downstream signaling proteins essential for leukemogenesis, in addition to Bcr-Abl or c-Kit itself, was intriguing. Several lines of evidence have implicated Ras/mitogen-activated protein kinase (MAPK) signaling pathway as an important molecular target [12,13].

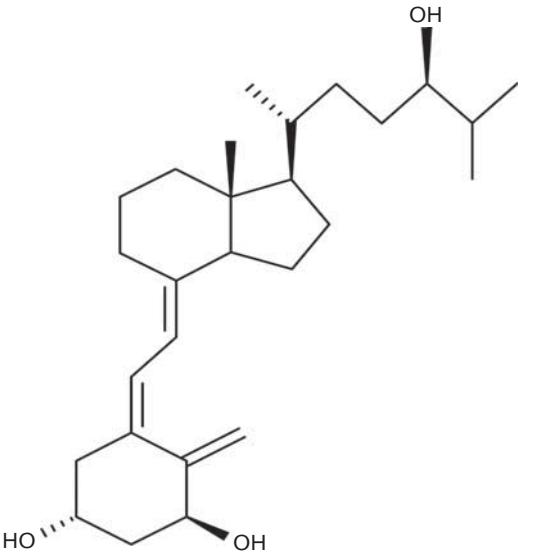
The active hormonal form of vitamin D_3 [calcitriol, 1,25-$(OH)_2D_3$] reveals antitumor activity both *in vitro* and *in vivo* [14–17]. As has been shown, calcitriol is involved in regulating the proliferation and differentiation of a variety of normal and cancer cells [14,15,18,19]. This effect is mediated mainly by the interaction of calcitriol with an intracellular receptor protein [vitamin D receptor (VDR)] [20]. Several mechanisms have been implicated in calcitriol's arrest of the cell cycle at the G0/G1 transition, including a reduction of *c-myc* expression or activation of the cyclin-dependent kinase inhibitor p21 [21,22]. Recent advances show the role of calcitriol, as well as deltanoids such as tacalcitol (PRI-2191) in cell differentiation. It is manifested in the principal MAPK pathways, their potential downstream target CCAAT/enhancer binding protein β; cell cycle-related proteins; and cyclin-dependent kinase 5 [23–27].

0959-4973 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32834b72de

Numerous vitamin D_3 analogs have been synthesized in recent years to obtain compounds with favorable biological and therapeutic profiles [28–35]. In our group, a series of vitamin D_2 analogs with a highly unsaturated side chain and a series of vitamin D_3 analogs with an additional one or two hydroxyl groups in the side chain, as well as geometric and diastereomeric analogs of calcipotriol, were examined for their antiproliferative activity *in vitro* against various human normal and cancer cell lines. On the basis of our studies, we selected a more potent and less toxic analog of vitamin D_3, coded PRI-2191 ((24*R*)-1,24-dihydroxyvitamin D_3, tacalcitol) [16,31,32,36–39].

In this study, we examined the cytotoxic effects of imatinib mesylate in combination with PRI-2191 and/or other anticancer agents [cisplatin (CIS), docetaxel (DC), or idarubicin (ID)] in human leukemia cell lines, which are VDR and c-kit (HL-60) or Bcr/Abl (K562) positive. Moreover, the effects of these combinations on apoptosis and cell-cycle distribution are presented.

Fig. 1



The structural formula of (24*R*)-1,24-dihydroxycholecalciferol (PRI-2191, tacalcitol).

Materials and methods

Cells

The in-vitro-cultured human promyelocytic leukemia HL-60 and erythroleukemia K562 cell lines were purchased from American Type Culture Collection (ATCC, USA). Cells were maintained in RPMI-1640 GLUTAMAX (Gibco, Paisley, UK) medium containing 1.0 mmol/l of sodium pyruvate, 4.5 g/l of glucose (both from Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and 100 U/ml of penicillin and 100 µg/ml of streptomycin (both from Polfa Tarchomin S.A., Warsaw, Poland), supplemented with 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Cells were maintained under standard cell culture conditions (humidified atmosphere and 5% CO_2 at 37°C).

Compounds

The calcitriol side-chain-modified analog PRI-2191 (tacalcitol, Fig. 1) was a certified synthetic material provided by the manufacturer (Pharmaceutical Research Institute, Warsaw, Poland). Samples of the compounds were stored in amber ampoules in argon at −20°C. Before use, the compounds were dissolved in absolute ethanol to a concentration of 0.1 mmol/l and subsequently diluted in the culture medium to reach the required concentrations. The anticancer drugs were as follows: CIS, obtained from KZF Polfa, Krakow, Poland; DC from Sigma, Milan, Italy; and imanitib mesylate (GV) and ID, both from the Pharmaceutical Research Institute, Warsaw, Poland.

In-vitro antiproliferative assay

Cells were placed in 96-well flat-bottom plates (Sarstedt, Inc., Newton, North Carolina, USA) at a density of 5×10^3 cells per well, 24 h before addition of the tested compounds. Cells were exposed for 96 h to various concentrations (1, 10, and 100 nmol/l for K562; and 1 and 10 nmol/l for HL-60 cells) of PRI-2191 and simultaneously to various concentrations of GV (10, 100, 1000, and 10 000 ng/ml) and other agents (DC: 0.01, 0.1, and 1 ng/ml; ID: 0.001, 0.1, 1, and 10 ng/ml; and CIS: 1, 10, 100, and 1000 ng/ml). The MTT assay for evaluating the antiproliferative effect was performed as described previously [32].

Results were calculated as IC_{50} values (inhibitory concentration 50%) – that is, the dose of the tested compound that inhibits the proliferation of cancer cells by 50%. IC_{50} values were calculated for each experiment separately and the mean values ± standard deviation are presented in Tables 1–4. Each compound at a given concentration was tested in triplicate in a given experiment; each experiment was repeated 3–5 times. Ethanol, which was used as a solvent (in the dilution corresponding to its highest concentration applied to the tested compounds), did not exert any inhibitory effect on cell proliferation ($P > 0.05$).

Evaluation of combination effects

The cytotoxic effects obtained from the different combinations of vitamin D analogs with the chemotherapeutic agents were analyzed according to the method of Chou and Talalay [40,41]. The interaction between two agents (mutually nonexclusive) was assessed by means of the combination index (CI) calculated for the IC_{50} values from in-vitro experiments:

$$CI_{A+B} = (D_{A/A+B} / D_A) + (D_{B/A+B} / D_B) + (D_{A/A+B} \times D_{B/A+B}) / D_A D_B$$

Table 1 The antiproliferative effect of PRI-2191 combined with GV alone or applied with either DC, CIS, or ID on human HL-60 cells

PRI-2191 imatinib	10 (nmol/l) IC_{50} (μg/ml)	10 (nmol/l) CI[a]	1 (nmol/l) IC_{50} (μg/ml)	1 (nmol/l) CI[a]	0 IC_{50} (μg/ml)	0 CI[a]
Combined treatment with (μg/ml):						
–	3.7 ± 2.0	0.774	2.5 ± 1.7	0.517	4.8 ± 3.0	–
DC						
0.001	1.3 ± 0.2	0.669	4.0 ± 0.1	1.407	3.1 ± 0.3	1.169
0.0001	2.5 ± 1.5	0.566	5.9 ± 1.5	1.285	4.8 ± 1.3	1.054
0.00001	2.0 ± 0.2	0.414	4.5 ± 0.8	0.944	4.1 ± 0.5	0.849
CIS						
0.1	1.4 ± 0.3	0.824	2.6 ± 1.4	1.182	3.8 ± 0.5	1.528
0.01	3.9 ± 2.1	0.874	2.5 ± 1.5	0.589	5.0 ± 1.8	1.111
0.001	4.1 ± 3.6	0.856	3.0 ± 1.0	0.622	3.8 ± 0.3	0.784
ID						
0.001	2.3 ± 1.6	1.389	3.4 ± 1.9	1.762	3.3 ± 0.7	1.732
0.0001	5.5 ± 1.7	1.263	4.4 ± 2.2	1.030	5.5 ± 1.5	1.281
0.00001	2.6 ± 1.1	0.539	4.5 ± 1.1	0.937	4.8 ± 1.4	0.998

IC_{50} value for PRI-2191 alone = 6.7 ± 1.1 nmol/l.
CI, combination index; CIS, cisplatin; DC, docetaxel; GV, imanitib mesylate; ID, idarubicin.
[a]Calculated for GV combined with the given anticancer agent with or without PRI-2191.

Table 2 The antiproliferative effect of DC, CIS, or ID combined with PRI-2191 against human HL-60 cells

PRI-2191	10 (nmol/l) IC_{50}	10 (nmol/l) CI[a]	1 (nmol/l) IC_{50}	1 (nmol/l) CI[a]	0 IC_{50}
DC (ng/ml)	2.0 ± 0.8	0.625	3.0 ± 0.2	0.955	3.1 ± 0.1
CIS (μg/ml)	0.16 ± 0.05	0.667	0.26 ± 0.05	1.083	0.24 ± 0.04
ID (ng/ml)	0.20 ± 0.50	0.120	0.28 ± 0.19	0.169	1.66 ± 0.54

IC_{50} value for PRI-2191 alone = 6.7 ± 1.1 nmol/l.
CI, combination index; CIS, cisplatin; DC, docetaxel; ID, idarubicin.
[a]Calculated for the anticancer agent combined with the given concentration of PRI-2191.

Table 3 The antiproliferative effect of PRI-2191 combined with GV alone or applied with either DC, CIS, or ID on K562 cells

PRI-2191 Imatinib	100 (nmol/l) IC_{50} (μg/ml)	100 (nmol/l) CI[a]	10 (nmol/l) IC_{50} (μg/ml)	10 (nmol/l) CI[a]	0 IC_{50} (μg/ml)	0 CI[a]
Combined treatment with (μg/ml):						
–	0.31 ± 0.05	–	0.40 ± 0.05	–	0.36 ± 0.11	–
DC						
0.001	0.19 ± 0.14	2.31	0.20 ± 0.09	2.38	0.31 ± 0.04	3.15
0.0001	0.36 ± 0.04	1.51	0.31 ± 0.10	1.31	0.30 ± 0.08	1.27
CIS						
1.0	0.15 ± 0.05	1.32	0.12 ± 0.09	1.18	0.08 ± 0.06	0.99
0.1	0.37 ± 0.15	1.22	0.29 ± 0.14	0.97	0.24 ± 0.11	0.81
0.01	0.43 ± 0.06	1.28	0.33 ± 0.12	0.98	0.27 ± 0.09	0.81
ID						
0.01	0.12 ± 0.01	1.16	0.03 ± 0.02	0.76	0.05 ± 0.04	0.85
0.001	0.28 ± 0.06	0.89	0.32 ± 0.05	1.0	0.30 ± 0.11	0.95
0.0001	0.31 ± 0.08	0.87	0.30 ± 0.16	0.85	0.35 ± 0.05	0.98

IC_{50} value for PRI-2191 alone – above 1000 nmol/l.
CI, combination index; CIS,cisplatin; DC, docetaxel; GV, imanitib mesylate; ID, idarubicin.
[a]Calculated for GV combined with the given anticancer agent with or without PRI-2191.

where $\mathbf{CI_{A+B}}$ is the CI for the experimentally achieved effect F (IC_{50}) for the combination of compound A (the given chemotherapy agents) and compound B (calcitriol analog); $\mathbf{D_{A/A+B}}$ is the concentration of compound A in the combination A+B giving the effect F; $\mathbf{D_{B/A+B}}$ is the concentration of compound B in the combination A+B giving the effect F; $\mathbf{D_A}$ is the concentration of compound A alone giving the effect F; $\mathbf{D_B}$ is the concentration of compound B alone giving the effect F.

Table 4 The antiproliferative effect of DC, CIS, or ID combined with PRI-2191 against human K562 cells

PRI-2191	100 (nmol/l) IC_{50}	100 (nmol/l) AW[a]	10 (nmol/l) IC_{50}	10 (nmol/l) AW[a]	0 IC_{50}
DC (μg/ml)	0.0019 ± 0.0009	0.5	0.0012 ± 0.0007	0.9	0.001 ± 0.0009
CIS (μg/ml)	2.1 ± 0.9	0.8	1.6 ± 0.3	1.0	1.6 ± 0.5
ID (μg/ml)	0.0068 ± 0.0052	2.4	0.0052 ± 0.0017	3.1	0.016 ± 0.013

IC_{50} value for PRI-2191 – above 1000 nmol/l; CIS, cisplatin; DC, docetaxel; ID, idarubicin.
[a]AW, IC_{50} for anticancer agent alone/IC_{50} for the anticancer agent with PRI-2191 calculated for the anticancer agent combined with the given dose of PRI-2191.

A CI less than 0.8 indicates synergism, CI more than 1.2 indicates antagonism, and CI = 0.8 – 1.2 indicates an additive effect.

Cell-cycle analysis

The cultured HL-60 cells were seeded at a density of 1×10^5 cells/ml in the culture medium in 24-well plates (Sarstedt, Germany) at a final volume of 2 ml. After 24 h of incubation, the cells were exposed to PRI-2191 at 10 nmol/l, GV 1 μg/ml, DC 0.01 ng/ml, CIS 0.05 μg/ml, or ID 0.1 ng/ml, either alone or in combinations, for 24, 48, 72, or 96 h. Ethanol itself (the solvent for the compounds tested), at a dilution corresponding to its highest concentration used for the compounds, produced no toxicity to any of the cell types studied. After 24, 48, 72, and 96 h of incubation, the cells were collected, washed in phosphate-buffered saline (PBS), and counted in a hemacytometer. The cells (1×10^6) were washed twice in cold PBS and fixed for 24 h in 70% ethanol at –20°C. Thereafter, the cells were washed twice in PBS and incubated with RNAse (8 μg/ml, Fermentas, Germany) at 37°C for 1 h. Afterwards, they were stained for 30 min. with propidium iodide (PI) (50 μg/ml, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) at 4°C, and cellular DNA content was determined using the Cell Quest program (Becton Dickinson, San Jose, California, USA).

Apoptosis determination by annexin V staining

Cultured HL-60 cells were seeded at a density of 5×10^4 cells/ml in culture medium on 24-well plates (Sarstedt, Germany) at a final volume of 2 ml. After 24 h of incubation, the cells were exposed to PRI-2191 at 10 nmol/l, GV 4 μg/ml, CIS 0.5 μg/ml, ID 2 ng/ml, or DC 2 ng/ml, either alone or in combinations, for 48 or 96 h. Ethanol itself (the solvent for the compounds tested), at a dilution corresponding to its highest concentration used for the compounds, produced no toxicity to any of the cell types studied. After 48 and 96 h of incubation, the cells

were collected, washed in PBS, and counted in a hemacytometer. The cells (1×10^6) were washed twice with PBS. FITC-annexin V (Alexis Biochemicals, San Diego, California, USA) was diluted at a concentration of 1 mg/ml in binding buffer (Hepes buffer: 10 mmol/l HEPES/NaOH, pH 7.4, 150 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgCl_2$, 1.8 mmol/l $CaCl_2$), and the cells were suspended in 1 ml of this solution (prepared freshly each time). After 10 min of incubation in the dark at room temperature, 0.1 ml of PI solution (10 mg/ml) was added before analysis to yield a final concentration of 1 mg/ml. Data analysis was carried out by flow cytometry using the CellQuest program for data acquisition. The data were displayed as a two-color dot plot with FITC-annexin V (FL1-H, Y-axis) versus PI (FL3-H, X-axis). Double-negative cells were live cells, PI^+/annexin $V^{+/-}$ necrotic cells, PI weak/annexin V^+ apoptotic cells, and PI^-/annexin V^+ early apoptotic cells.

Statistical evaluation

Statistical analysis was carried out using STATISTICA version 7.1 (StatSoft Inc., USA). The Mann–Whitney *U* test and one-way ANOVA, followed by the Fisher least significant difference test, were applied. A *P* value less than 0.05 was considered significant.

Results

The influence of PRI-2191 combined with imatinib and chemotherapy agents on HL-60 and K562 cell proliferation

Tables 1 and 2 present the IC_{50} values (the dose of tested agent that results in 50% inhibition of proliferation of cancer cells) and the CI for imatinib (GV) used alone or in combination with tacalcitol (PRI-2191) and/or DC, CIS, or ID. The cells were exposed to PRI-2191 (in doses of 1, 10, or 100 nmol/l) and simultaneously to different doses of imatinib and/or chemotherapy drugs.

Potentiation of the antiproliferative effect of GV by PRI-2191, analyzed by the Chou and Talalay method [41], was observed in the HL-60 cell line. Synergy was shown in almost all the combinations tested in the HL-60 cell line when PRI-2191 at a concentration of 10 nmol/l was used (Table 1). The interactions between GV and DC were additive, but when a third compound, namely PRI-2191, at a dose of 10 nmol/l, was added, these interactions shifted to synergism (Table 1). A similar effect was shown in combination with CIS; the interactions of GV with CIS at doses of 0.1 and 0.01 μg/ml were antagonistic and additive, respectively. This changed after incubation with 1 or 10 nmol/l of PRI-2191 and shifted to an additive or even synergistic interaction. In the case of GV in combined treatment with CIS at a dose of 0.001 μg/ml, PRI-2191 did not affect the interactions between these two compounds. GV combined with ID interacted additively (0.00001 μg/ml of ID) or antagonistically (at two other doses). PRI-2191 at a dose of 10 nmol/l significantly affected only the additive interaction observed in the combination of GV with 0.00001 μg/ml of ID; the observed effect of the triple combination was synergistic (Table 1). The interactions of PRI-2191 with the chemotherapy agents used on the HL-60 cell line were synergistic or additive, depending on the dose of PRI-2191 (Table 2).

In the K562 cell line, no influence on the antiproliferative effect of GV by PRI-2191 was observed when analyzed by the Chou and Talalay method (Table 3). The interactions between GV and DC were either antagonistic or additive, and when a third compound, namely PRI-2191, was added these interactions did not change (Table 3). The interactions of GV with CIS were additive. This changed after incubation with 10 or 100 nmol/l of PRI-2191 and even became antagonistic (Table 3). GV combined with ID interacted in an additive manner. PRI-2191 in both doses did not influence these interactions significantly (Table 3). No interactions of PRI-2191 with CIS or DC on the K562 cell line were observed. In the case of ID, potentiation of the antiproliferative effect of this anticancer agent by PRI-2191 was observed. This interaction was estimated by a simple comparison of IC_{50} values for the chemotherapy agent alone and for the agent used in combination with PRI-2191 (Table 4). Chou and Talalay's method could not be used because of the low antiproliferative activity of PRI-2191 alone (inhibition of proliferation lower than 50%).

For the following investigations, the concentrations of agents in various combinations were chosen on the basis of the IC_{50} value of each agent (Tables 1 and 2) and on the basis of proliferation curves (not shown in the manuscript). In the case of cell-cycle analysis to show the influence of tested agents on cell-cycle progression we chose the doses that do not induce cell death (10% or lower percentage of cell death). In contrast, to show the influence of tested combinations of agents on apoptosis, the doses similar to IC_{50} were used.

The effect of PRI-2191 and/or GV on the chemotherapy agent-induced cell-cycle distribution of HL-60 cells

The results of DNA analysis in FACS (WinMDI 2.8 program) are summarized in Fig. 2. The data are for 24, 48, 72, and 96 h of incubation with the compounds tested. Figure 2a presents an analysis of the cell-cycle distribution when a combination with DC was applied. After 24 h of incubation, statistically significant decreases in the percentage of HL-60 cells in G0/G1 stage, in the majority of protocols used, were observed. When the incubation time was prolonged to 72 or 96 h, all the cells in groups incubated with PRI-2191 accumulated in the G0/G1 phase of the cell cycle. When exposed to PRI-2191 alone or with almost all combinations, a parallel progressive decrease was observed in the percentages of HL-60 cells in the S phase. In contrast, in almost all protocols without PRI-2191, a tendency to increase the

percentage of cells in this stage was observed. PRI-2191 or GV used alone significantly decreased the percentage of cells in the G2/M phase starting from 48 h of observation. An analogous effect was observed when all types of combinations with these two compounds were used. DC alone showed a tendency to increase the proportion of cells in the G2/M stage after 72 h of incubation, and DC+GV had no effect on the percentage of cells in the G2/M phase over the time of examination when compared with the control group (Fig. 2a).

Figure 2b shows an analysis of the cell-cycle distribution for the combinations with CIS. After 24 h of incubation, statistically significant decreases in the percentage of HL-60 cells in G0/G1 stage, in all the protocols used, were observed. Parallel increases in the accumulation of HL-60 cells in G2/M stage were observed when CIS was used alone or combined with GV or in a triple combination with PRI-2191. A similar effect, but not statistically significant, as in the G2/M stage was observed in stage S after this period of incubation. When the incubation time was prolonged to 48 h, the percentage of all cells in the G2/M stage decreased in the groups incubated with PRI-2191 and/or GV. GV alone increased the percentage of cells in the S phase; however, in combination with CIS (as for CIS alone), a decrease in the percentage of cells in the G0/G1 phase was observed. After 72 and 96 h of exposure to PRI-2191 alone or in all combinations with this agent, cells accumulated in the G0/G1 phase of the cell cycle and decreased in S stage. In all protocols without PRI-2191, a tendency to decrease the percentage of cells in G0/G1 stage and increase in S stage was observed. Changes in the G2/M stage were also observed after 72 or 96 h of incubation. PRI-2191 or GV, used alone or in combination, decreased the percentage of cells in the G2/M phase. CIS alone or in combination with GV increased the percentage of cells in G2/M stage after 72 h of incubation. This effect was diminished after 96 h of incubation. After this period, exposure to all combinations with PRI-2191 resulted in a decrease in G2/M stage (Fig. 2b).

Figure 2c presents an analysis of the cell-cycle distribution when combinations with ID were applied. The dose of ID (0.1 ng/ml) applied in this study was chosen on the basis of preliminary data. The concentration higher than 0.1 ng/ml strongly induced apoptosis, whereas lower concentration did not influence the cell cycle. However, it acted synergistically on cell proliferation with GV and PRI-2191 in triple combination. After 24 h of incubation, statistically significant decreases in the percentages of HL-60 cells in G0/G1 stage in all the protocols used were observed. A parallel tendency to increase the accumulation of HL-60 cells in G2/M stage was observed when ID was combined with GV. When the incubation time was prolonged to 48 h, the percentage of all cells in the groups incubated with PRI-2191 and/or GV decreased in G2/M stage. GV alone increased the percentage of cells in the S phase. Significant decreases in G2/M stage were also observed in combinations of GV with ID or in ternary combination. After 72 and 96 h of exposure to PRI-2191 alone or in all combinations, cells accumulated in the G0/G1 phase of the cell cycle but decreased in the S stage. In all the protocols without PRI-2191, a tendency to decrease the percentage of cells in G0/G1 stage and increase in S stage was observed. The changes in G2/M stage were also observed after 72 or 96 h of incubation. GV and PRI-2191, used alone or in combination, decreased the percentage of cells in the G2/M phase (Fig. 2).

The effect of GV and/or PRI-2191 on chemotherapy agent-induced apoptosis of HL-60 cells

The results of apoptosis analysis after 48 and 96 h of incubation are presented in Fig. 3. After the analysis of cells by flow cytometry with the use of annexin V and PI staining, we selected three domains, that is, viable cells, necrosis, and apoptosis. The maximal percentage of necrotic cells did not exceed 20%, and the changes were parallel to those of apoptotic cells (data not shown). PRI-2191 or GV were used in doses that did not influence apoptosis. The chemotherapy agents induced apoptosis in approximately 15 and 50% of HL-60 cells after 48 and 96 h of incubation, respectively. All combinations with CIS were similar to CIS alone after 48 h of incubation. After 96 h of exposure to the tested compounds, an increase in the percentage of apoptotic cells was observed when CIS was combined with PRI-2191. However, the combination of GV with CIS or the triple combination was less potent in inducing apoptosis in HL-60 cells (Fig. 3a).

In contrast, when a combination with ID was applied, a tendency to increase apoptosis was observed in the ternary combination after 48 h of incubation. However, this tendency reversed after 96 h (Fig. 3c). In contrast, the combined protocol using DC indicated no differences in the percentage of apoptotic cells in all combinations with these chemotherapy agents (Fig. 3a).

In the above-mentioned experiments, GV was used in doses that inhibit proliferation but do not induce apoptosis in the schedules and methods we used. Independent experiments were conducted to demonstrate the influence of PRI-2191 on GV-induced apoptosis. GV used alone induced apoptosis in HL-60 cells after 48 h of exposure at a dose of 40 μg/ml. The lower dose, i.e. 20 μg/ml, induced apoptosis of HL-60 cells only after 96 h of incubation. Apoptosis induced by 20 or 40 μg/ml of GV did not change after simultaneous incubation with PRI-2191, nor was a tendency to increase the percentage of apoptotic cells observed (Fig. 4).

Discussion

There are many results of preclinical and clinical studies indicating that inhibition of the molecules targeted by imatinib might improve the efficacy of chemotherapy. An example is the inhibition of PDGFR, which should

improve therapeutic effect by lowering interstitial tumor pressure and allowing increased tumor penetration by chemotherapy agents. However, results of the phase II clinical trials, in which GV was added to DC in the treatment of patients with metastatic breast cancer or with nonsmall-cell lung cancer, showed that such treatment was tolerated relatively poorly and produced a low objective response rate [42,43]. Studies by Skorta *et al.* [44] showed that GV sensitizes Bcr-Abl$^+$ cells to CIS by simultaneous inhibition of p53 transactivation, induction of p53 accumulation predominantly in the cytoplasm, and reduction of Bcl-x_L. Moreover, the combination of GV and CIS was reasonably well tolerated and may provide stabilization in locally advanced and metastatic adenoid cystic carcinoma of the salivary glands [45]. GV is also synergistic with ID in chronic myelogenous leukemia in the blast phase and with DC in human K562 chronic myeloid leukemia cells [46,47]. As most patients with leukemia will not be cured with currently available chemotherapy regimens, there is an ongoing effort to develop new agents to treat this disease. The example is the phase I study in patients with relapsed/refractory acute leukemia, in which treatment with CIS combined with temozolomide was effective and well tolerated [48].

Our results show that the calcitriol analog PRI-2191 strengthens the antiproliferative activity of GV used alone or in ternary combination with DC or CIS on the HL-60 cell line. Moreover, PRI-2191 could switch the additive or

Fig. 2

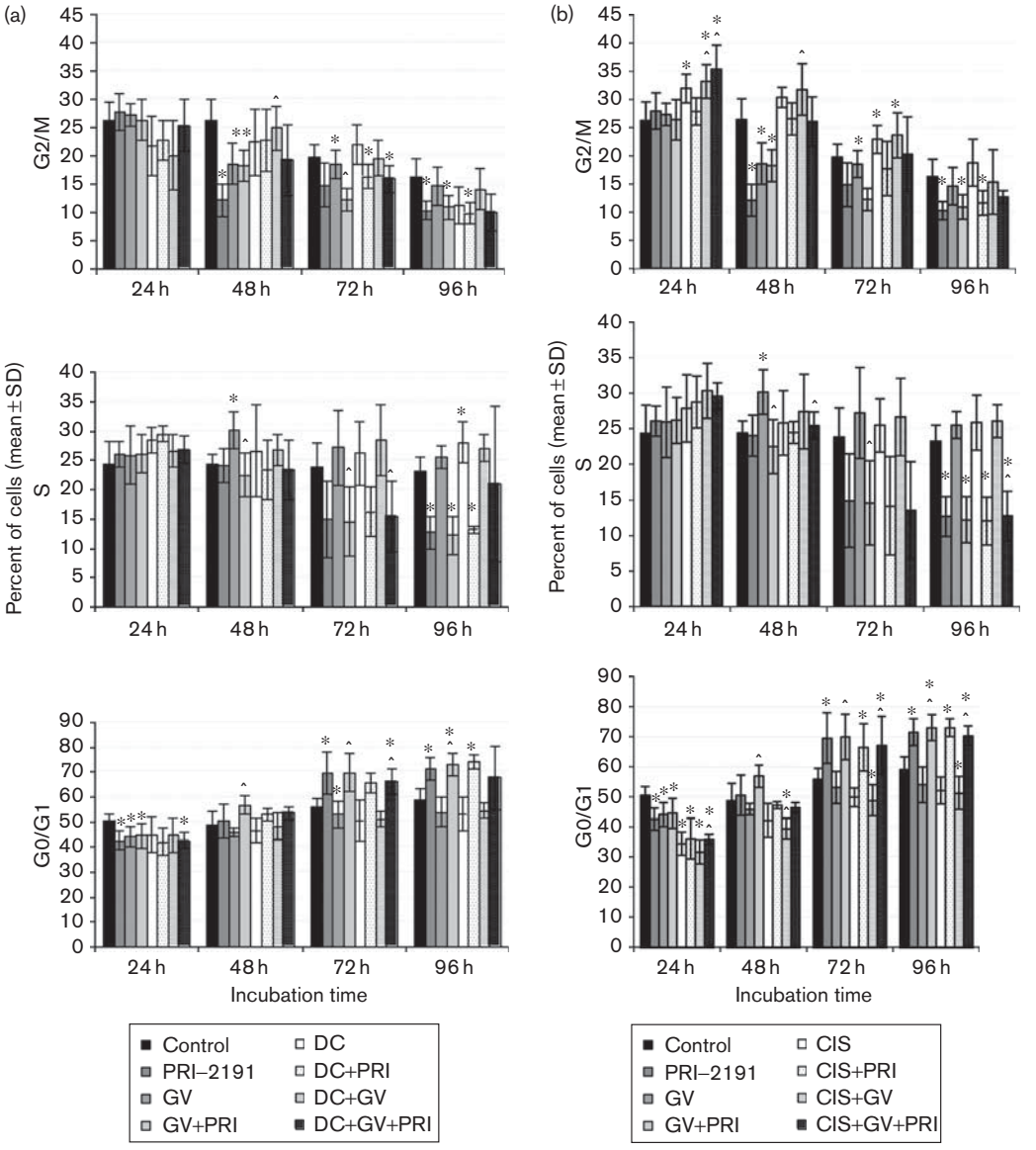
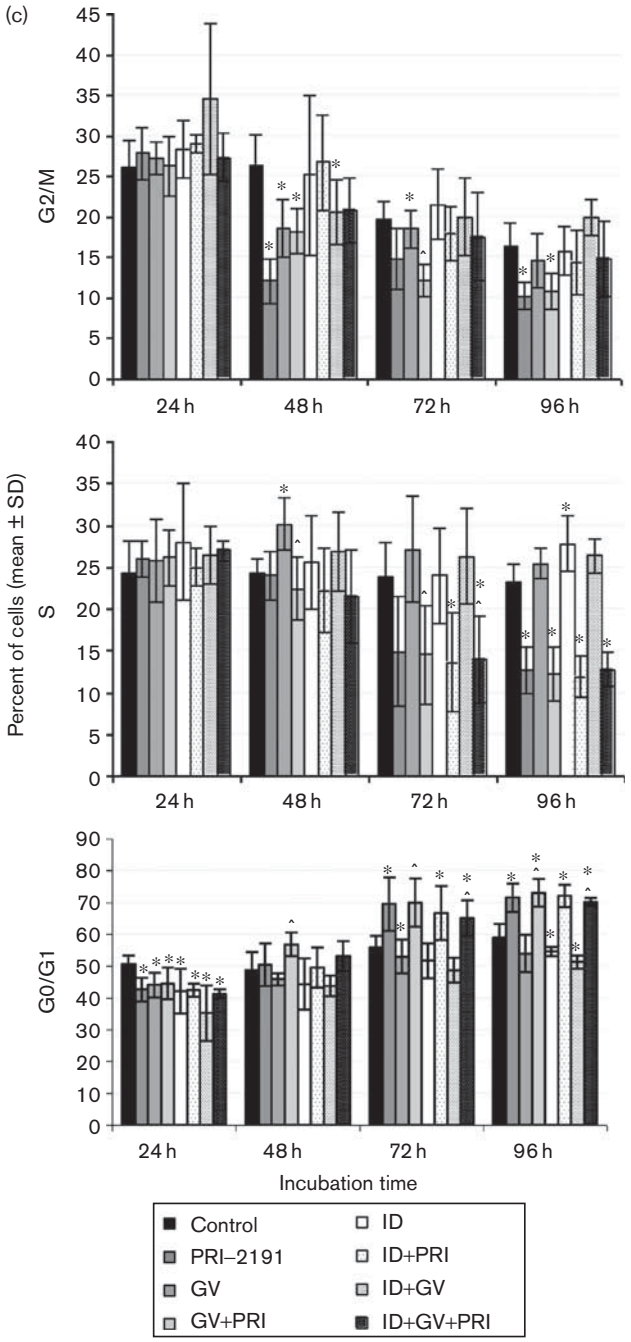


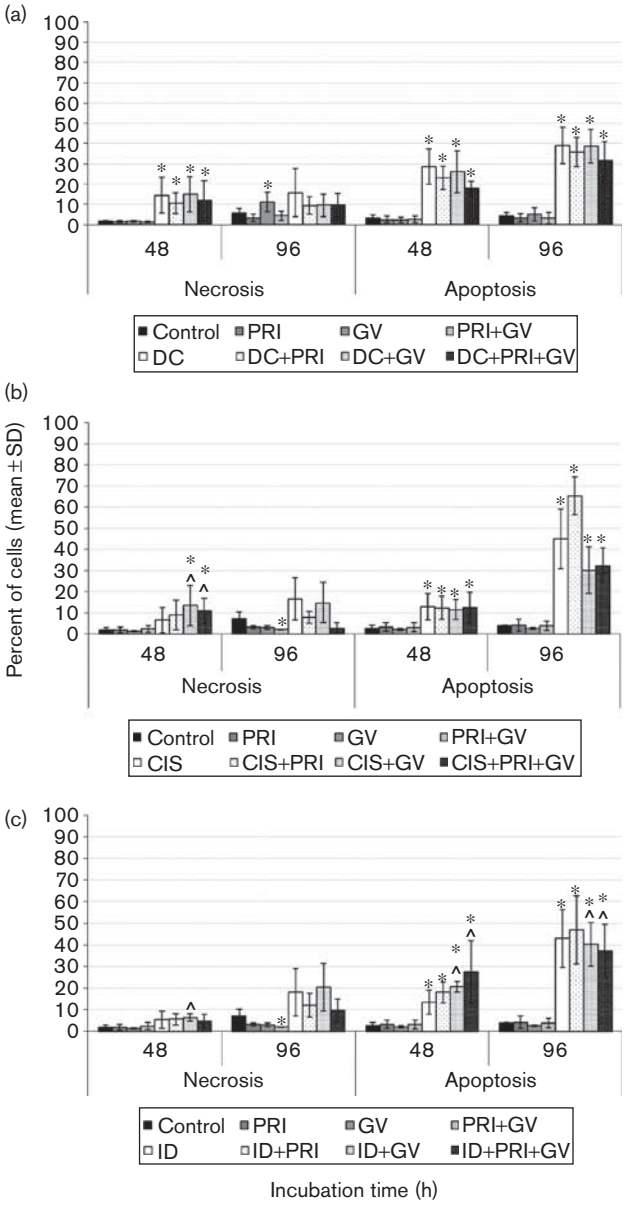
Fig. 2



The effect of exposure of HL-60 cells to PRI-2191 applied alone or with imanitib mesylate (GV) and/or an anticancer agent on cell-cycle distribution. (a) Ternary combination of PRI-2191+GV+docetaxel (DC); (b) ternary combination of PRI-2191+GV+cisplatin (CIS); (c) ternary combination of PRI-2191+GV+ID. * indicates statistically significant [$P \leq 0.05$; ANOVA, the Fisher least significant difference (LSD) test] differences versus control (ethanol). ^ indicates statistically significant ($P \leq 0.05$. ANOVA, Fisher LSD test) differences versus group treated with GV. The results are presented as a mean percentage of the cell population qualified to one of the four groups: M1 – cells in the phase G0/G1, M2 – cells in phase the S, M3 – cells in the phase G2/M, and M4 – apoptotic cells.

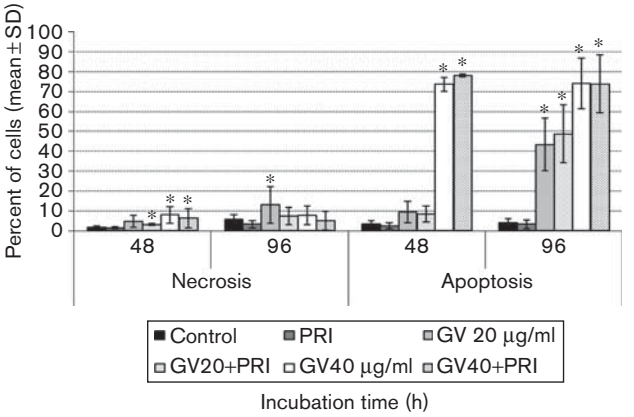
antagonistic interactions to synergistic. Interestingly, the effect of PRI-2191 on the antiproliferative activity of GV alone or used in a ternary combination with a chemotherapy agent against Bcr-Abl$^+$ K562 cells was not observed. We hypothesize that this phenomenon is related to the low activity of PRI-2191 on the proliferation of the K562 cell line (IC_{50} higher than 1000 nmol/l) (Table 3), even though both cell lines express the VDR [38,49]. The biological activity of vitamin D analogs is influenced by the genetic background of the cell lines

Fig. 3



The effect of exposure of HL-60 cells to PRI-2191 applied alone or with imanitib mesylate (GV) and/or anticancer drugs on apoptosis. * indicates statistically significant ($P \leq 0.05$) differences versus control (ethanol). ^ indicates statistically significant ($P \leq 0.05$) differences versus group treated with GV. After 24 h of incubation, HL-60 cells were exposed to PRI-2191 at 10 nmol/l, GV 4 μg/ml, and to the following doses: (a) Docetaxel (DC) (2 ng/ml), (b) cisplatin (CIS) (0.5 μg/ml), or (c) Idarubicin (ID) (2 ng/ml) for 48 or 96 h. The data are displayed as two-color dot plots with FITC-annexin V (FL1-H, *y*-axis) versus propidium iodide (PI) (FL3-H, *x*-axis). Double-negative cells were live cells (data not shown), PI^+/annexin $V^{+/-}$ necrotic cells, PI weak/annexin V^+ apoptotic cells, and PI^-/annexin V^+ early apoptotic cells (data not shown).

Fig. 4



The effect of exposure of HL-60 cells to PRI-2191 applied alone or with imanitib mesylate (GV) on apoptosis. * indicates statistically significant ($P \leq 0.05$) differences versus control (ethanol). After 24 h of incubation, HL-60 cells were exposed to PRI-2191 (PRI) at a dose of 10 nmol/l and GV 20 μg/ml (GV20) or 40 μg/ml (GV40) for 48 or 96 h. The data are displayed as two-color dot plots with FITC-annexin V (FL1-H, *y*-axis) versus PI (FL3-H, *x*-axis). Double-negative cells were live cells (data not shown), PI^+/annexin $V^{+/-}$ necrotic cells, PI weak/annexin V^+ apoptotic cells, and PI^-/annexin V^+ early apoptotic cells (data not shown).

used, suggesting the existence of cell-type-specific factors that can modulate activities. Such factors might include, for example, the cocktail of transcription factors present and the concentration of vitamin D metabolizing enzymes in cells, such as CYP24, CYP27A1, and CYP27B1. Human prostate cancer cell line PC3 is an example where weak sensitivity to the antiproliferative action of calcitriol is reported as being due to high expression of calcitriol catabolizing enzyme CYP24 [50]. It is also shown that K562 cells are less sensitive to proapoptotic action of calcitriol by a mechanism related to the lack of p53 and presence of Bcr-Abl. Moreover, as authors suggest 'it is also possible that Bax and Bcl-2 play nontraditional roles in apoptosis regulation in these cells' [51]. However, further studies are needed to explore the mechanisms of resistance of the K562 cell line to the antiproliferative effect of calcitriol and its analogs.

The observed effect on cell proliferation correlated with an accumulation of HL-60 cells in the G0/G1 cell-cycle phase and with a decrease in the percentage of cells in the G2/M and S stages in the triple combinations used. PRI-2191 did not influence apoptosis induced by GV alone or in ternary combinations with all the agents used. These results may suggest that the stronger antiproliferative effect of the combined treatment with calcitriol is related to cell cycle arrest rather than to induction of apoptosis. As demonstrated, calcitriol induces a significant G0/G1 arrest and increases the expression of p21 and p27, cyclin-dependent kinase inhibitors [21,22,24,52,53]. It has also been shown that, in the case of Bcr-Abl-positive cells, simultaneous interruption of two signal transduction pathways (using the kinase inhibitor GV and pharmacological antagonists of the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)/MAPK) may represent an effective antileukemic strategy [12]. Calcitriol and its

analogs are potentially good candidates for such combined treatment with GV in the light of findings that calcitriol signaling included perturbations in the MAPK pathways, especially the Raf/MEK/ERK cascade and the c-Jun-N-terminal kinase (JNK) cascades [24], which are also activated downstream after c-Kit or Bcr-Abl kinases activation [12,54]. However, the lack of dose relation in antiproliferative activity, especially observed when PRI-2191 was used in combination with GV, caught our attention (Table 1). Reiterer and Yen [55] using PDGFR inhibitor AG1296 along with vitamin D_3 have shown that inhibiting PDGFR enhances various features of vitamin D_3-induced monocytic differentiation, such as inducible oxidative metabolism. However, other typical properties of this induced differentiation process were diminished, including G_0 cell cycle arrest. It is possible that a balance between two different PDGFR inhibitors is necessary to effectively influence cell proliferation by inducing its differentiation.

In contrast, it was shown that GV potentiates the pharmacological activity of retinoic acid (RA) in acute promyelocytic leukemia cells. The authors observed that GV enhances the differentiating, antiproliferative, and proapoptotic activity of RA, which was associated with a decreased degradation of RARα induced by RA [56]. RA and calcitriol bind to specific transcription factors, RARs and VDR. RARs and VDR are members of the steroid/thyroid hormone nuclear receptor superfamily that, together with retinoid X receptors, form a subgroup of structurally homologous proteins with overlapping functions because of their ability to form functional heterodimers [20,57]. It seems possible that GV can also decrease the degradation of receptors other than RARα from this superfamily, thus contributing to the increase in the activity of PRI-2191.

Although potentiation of the antiproliferative activity of GV by PRI-2191 was demonstrated (Table 1), we could not exclude the role of interactions between PRI-2191 and the chemotherapy agents used in the observed synergistic effect of the ternary combinations. It has been demonstrated that the combination of calcitriol with CIS in various cancer cell lines acts synergistically to inhibit cell growth *in vitro* and *in vivo* [36–39,58–63]. In contrast, it has been shown that tumor cells in the G1 phase of the cell cycle, which is the case for cells treated with calcitriol or its analogs (Figs 2–4), are the most sensitive to the cytotoxic effect of CIS [59]. It has been revealed that sensitivity to taxol increases as proliferating cells progress from early G1 to late G2 phase [59]. Other authors have proposed that the increased cytotoxicity of combinations of calcitriol with CIS results from CIS's enhancement of calcitriol-induced apoptotic signaling through MEKK-1 [61,64]. Moreover, CIS and doxorubicin activate JNK. The increase in JNK activity was necessary for CIS-mediated apoptosis, but it was not needed for doxorubicin-induced cell death [65]. The activation of JNK1 pathways induced by calcitriol or its analogs may contribute to the stimulation of CIS activity in this way [24,66].

In a previous study, the protective effect of pretreatment with calcitriol or its side-chain-modified analogs on the apoptosis of HL-60 cells induced by CIS, doxorubicin, taxol, and genistein has been described [67]. It seems that HL-60 cells differentiated by exposure to calcitriol or its analogs become more resistant to apoptosis [67–70]. In contrast, our previous results also show that the differentiation of HL-60 cells induced by preexposure to calcitriol or its analogs does not decrease their sensitivity to the antiproliferative effects of doxorubicin, CIS, and genistein [37,39]. In addition, simultaneous incubation of ID or DC with PRI-2191 did not decrease the sensitivity of HL-60 or K562 cells to the antiproliferative activity of these agents (Tables 2 and 4). Our results on SCC cell lines are also in accordance with these observations. Moreover, we have shown on these cell lines a synergistic effect in the interaction of calcitriol or PRI-2191 with GV. However, a decrease in GV-induced apoptosis was observed after exposure of SCC-25 and FaDu cells to calcitriol or PRI-2191 [64,71].

Further studies are needed to explain the mechanism of interaction, particularly the synergistic antiproliferative effects, of GV combined with PRI-2191.

Acknowledgements

We thank Professor Czeslaw Radzikowski (IIET, PAS, Wroclaw) for critically reviewing the manuscript. Some of the results presented in this study were a part of the thesis of J. Wietrzyk DSc.

This research was supported by the Foundation for the Development of Pharmaceutical Sciences, Poland (grant no. 9/FB/2004) (2004–2007).

Conflicts of interest

There are no conflicts of interest.

References

1 Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, *et al*. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med* 2001; **344**:1038–1042.

2 Pfeifer H, Wassmann B, Pavlova A, Wunderle L, Oldenburg J, Binckebanck A, *et al*. Kinase domain mutations of BCR-ABL frequently precede imatinib-based therapy and give rise to relapse in patients with de novo Philadelphia-positive acute lymphoblastic leukemia (Ph+ ALL). *Blood* 2007; **110**: 727–734.

3 Ferrari G, Pastorelli R, Buchi F, Spinelli E, Gozzini A, Bosi A, *et al*. Comparative proteomic analysis of chronic myelogenous leukemia cells: inside the mechanism of imatinib resistance. *J Proteome Res* 2007; **6**:367–375.

4 Litzow MR. Imatinib resistance: obstacles and opportunities. *Arch Pathol Lab Med* 2006; **130**:669–679.

5 Wendel HG, De SE, Cepero E, Ray S, Emig M, Fridman JS, *et al*. Loss of p53 impedes the antileukemic response to BCR-ABL inhibition. *Proc Natl Acad Sci USA* 2006; **103**:7444–7449.

6 Nicolini FE, Corm S, Le QH, Sorel N, Hayette S, Bories D, *et al*. Mutation status and clinical outcome of 89 imatinib mesylate-resistant chronic myelogenous leukemia patients: a retrospective analysis from the French intergroup of CML (Fi(phi)-LMC GROUP). *Leukemia* 2006; **20**:1061–1066.
7 Deininger M. Resistance to imatinib: mechanisms and management. *J Natl Compr Canc Netw* 2005; **3**:757–768.
8 Sakai K, Ishikawa Y, Mori Y, Kobayashi M, Iriyama C, Ozawa Y, *et al*. A novel insertion mutation of K294RGG within BCR-ABL kinase domain confers imatinib resistance: sequential analysis of the clonal evolution in a patient with chronic myeloid leukemia in blast crisis. *Int J Hematol* 2011; **93**: 237–242.
9 Gorre ME, Ellwood-Yen K, Chiosis G, Rosen N, Sawyers CL. BCR-ABL point mutants isolated from patients with imatinib mesylate-resistant chronic myeloid leukemia remain sensitive to inhibitors of the BCR-ABL chaperone heat shock protein 90. *Blood* 2002; **100**:3041–3044.
10 Kano Y, Akutsu M, Tsunoda S, Mano H, Sato Y, Honma Y, *et al*. In vitro cytotoxic effects of a tyrosine kinase inhibitor STI571 in combination with commonly used antileukemic agents. *Blood* 2001; **97**:1999–2007.
11 La Rosee P, O'Dwyer ME, Druker BJ. Insights from pre-clinical studies for new combination treatment regimens with the Bcr-Abl kinase inhibitor imatinib mesylate (Gleevec/Glivec) in chronic myelogenous leukemia: a translational perspective. *Leukemia* 2002; **16**:1213–1219.
12 Yu C, Krystal G, Varticovksi L, McKinstry R, Rahmani M, Dent P, *et al*. Pharmacologic mitogen-activated protein/extracellular signal-regulated kinase kinase/mitogen-activated protein kinase inhibitors interact synergistically with STI571 to induce apoptosis in Bcr/Abl-expressing human leukemia cells. *Cancer Res* 2002; **62**:188–199.
13 Turhan AG, Solary E, Vainchenker W, Dusanter-Fourt I. Molecular pathophysiology of chronic myelogenous leukemia. *Hematol Cell Ther* 1998; **40**:217–221.
14 Asou H, Koike M, Elstner E, Cambell M, Le J, Uskokovic MR, *et al*. 19-nor vitamin-D analogs: a new class of potent inhibitors of proliferation and inducers of differentiation of human myeloid leukemia cell lines. *Blood* 1998; **92**:2441–2449.
15 Li J, Finch RA, Sartorelli AC. Role of vitamin D3 receptor in the synergistic differentiation of WEHI-3B leukemia cells by vitamin D3 and retinoic acid. *Exp Cell Res* 1999; **249**:279–290.
16 Opolski A, Wietrzyk J, Chrobak A, Marcinkowska E, Wojdat E, Kutner A, *et al*. Antiproliferative activity in vitro of side-chain analogues of calcitriol against various human normal and cancer cell lines. *Anticancer Res* 1999; **19**:5217–5222.
17 Blanke CD, Beer TM, Todd K, Mori M, Stone M, Lopez C. Phase II study of calcitriol-enhanced docetaxel in patients with previously untreated metastatic or locally advanced pancreatic cancer. *Invest New Drugs* 2009; **27**:374–378.
18 Bikle DD. 1,25(OH)2D3-regulated human keratinocyte proliferation and differentiation: basic studies and their clinical application. *J Nutr* 1995; **125**:1709S–1714S.
19 Zhou JY, Norman AW, Akashi M, Chen DL, Uskokovic MR, Aurrecoechea JM, *et al*. Development of a novel 1,25(OH)2-vitamin D3 analog with potent ability to induce HL-60 cell differentiation without modulating calcium metabolism. *Blood* 1991; **78**:75–82.
20 Baniahmad A, Tsai MJ. Mechanisms of transcriptional activation by steroid hormone receptors. *J Cell Biochem* 1993; **51**:151–156.
21 Hershberger PA, Modzelewski RA, Shurin ZR, Rueger RM, Trump DL, Johnson CS. 1,25-Dihydroxycholecalciferol (1,25-D3) inhibits the growth of squamous cell carcinoma and down-modulates p21(Waf1/Cip1) in vitro and in vivo. *Cancer Res* 1999; **59**:2644–2649.
22 Saramaki A, Banwell CM, Campbell MJ, Carlberg C. Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. *Nucleic Acids Res* 2006; **34**:543–554.
23 Studzinski GP, Garay E, Patel R, Zhang J, Wang X. Vitamin D receptor signaling of monocytic differentiation in human leukemia cells: role of MAPK pathways in transcription factor activation. *Curr Top Med Chem* 2006; **6**:1267–1271.
24 Wang Q, Salman H, Danilenko M, Studzinski GP. Cooperation between antioxidants and 1,25-dihydroxyvitamin D3 in induction of leukemia HL60 cell differentiation through the JNK/AP-1/Egr-1 pathway. *J Cell Physiol* 2005; **204**:964–974.
25 Wang X, Studzinski GP. Raf-1 signaling is required for the later stages of 1,25-dihydroxyvitamin D3-induced differentiation of HL60 cells but is not mediated by the MEK/ERK module. *J Cell Physiol* 2006; **209**:253–260.
26 Wang X, Wang TT, White JH, Studzinski GP. Induction of kinase suppressor of RAS-1(KSR-1) gene by 1, alpha25-dihydroxyvitamin D3 in human leukemia HL60 cells through a vitamin D response element in the 5′-flanking region. *Oncogene* 2006; **25**:7078–7085.
27 Wang X, Gocek E, Novik V, Harrison JS, Danilenko M, Studzinski GP. Inhibition of Cot1/Tlp2 oncogene in AML cells reduces ERK5 activation and up-regulates p27Kip1 concomitant with enhancement of differentiation and cell cycle arrest induced by silibinin and 1,25-dihydroxyvitamin D(3). *Cell Cycle* 2010; **9**:4542–4551.
28 Zinser GM, Tribble E, Valrance M, Urben CM, Knutson JC, Mazess RB, *et al*. 1,24(S)-dihydroxyvitamin D2, an endogenous vitamin D2 metabolite, inhibits growth of breast cancer cells and tumors. *Anticancer Res* 2005; **25**: 235–241.
29 Verlinden L, Verstuyf A, Van Camp M, Marcelis S, Sabbe K, Zhao XY, *et al*. Two novel 14-Epi-analogues of 1,25-dihydroxyvitamin D3 inhibit the growth of human breast cancer cells in vitro and in vivo. *Cancer Res* 2000; **60**:2673–2679.
30 Posner GH, Crawford KR, Peleg S, Welsh JE, Romu S, Gewirtz DA, *et al*. A non-calcemic sulfone version of the vitamin D(3) analogue seocalcitol (EB 1089): chemical synthesis, biological evaluation and potency enhancement of the anticancer drug adriamycin. *Bioorg Med Chem* 2001; **9**:2365–2371.
31 Chodynski M, Wietrzyk J, Marcinkowska E, Opolski A, Szelejewski W, Kutner A. Synthesis and antiproliferative activity of side-chain unsaturated and homologated analogs of 1,25-dihydroxyvitamin D(2). (24E)-(1S)-24-Dehydro-24a-homo-1,25-dihydroxyergocalciferol and congeners. *Steroids* 2002; **67**:789–798.
32 Wietrzyk J, Chodynski M, Fitak H, Wojdat E, Kutner A, Opolski A. Antitumor properties of diastereomeric and geometric analogs of vitamin D3. *Anticancer Drugs* 2007; **18**:447–457.
33 Filip B, Milczarek M, Wietrzyk J, Chodynski M, Kutner A. Antitumor properties of (5E,7E) analogs of vitamin D3. *J Steroid Biochem Mol Biol* 2010; **121**:399–402.
34 Barycki R, Sicinski RR, Plum LA, Grzywacz P, Clagett-Dame M, DeLuca HF. Removal of the 20-methyl group from 2-methylene-19-nor-(20S)-1alpha,25-dihydroxyvitamin D(3) (2MD) selectively eliminates bone calcium mobilization activity. *Bioorg Med Chem* 2009; **17**:7658–7669.
35 Grzywacz P, Chiellini G, Plum LA, Clagett-Dame M, DeLuca HF. Removal of the 26-methyl group from 19-nor-1alpha,25-dihydroxyvitamin D markedly reduces in vivo calcemic activity without altering in vitro VDR binding, HL-60 cell differentiation, and transcription. *J Med Chem* 2010; **53**:8642–8649.
36 Wietrzyk J, Pelczynska M, Madej J, Dzimira S, Kusnierczyk H, Kutner A, *et al*. Toxicity and antineoplastic effect of (24R)-1,24-dihydroxyvitamin D3 (PRI-2191). *Steroids* 2004; **69**:629–635.
37 Siwinska A, Opolski A, Chrobak A, Wietrzyk J, Wojdat E, Kutner A, *et al*. Potentiation of the antiproliferative effect in vitro of doxorubicin, cisplatin and genistein by new analogues of vitamin D. *Anticancer Res* 2001; **21**:1925–1929.
38 Pelczynska M, Wietrzyk J, Jaroszewicz I, Nevozhay D, Switalska M, Kutner A, *et al*. Correlation between VDR expression and antiproliferative activity of vitamin D3 compounds in combination with cytostatics. *Anticancer Res* 2005; **25**:2235–2240.
39 Opolski A, Wietrzyk J, Siwinska A, Marcinkowska E, Chrobak A, Radzikowski C, *et al*. Biological activity in vitro of side-chain modified analogues of calcitriol. *Curr Pharm Des* 2000; **6**:755–765.
40 Chou TC, Talalay P. Generalized equations for the analysis of inhibitions of Michaelis-Menten and higher-order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. *Eur J Biochem* 1981; **115**:207–216.
41 Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984; **22**:27–55.
42 Yardley DA, Burris HA III, Markus T, Spigel DR, Greco FA, Mainwaring M, *et al*. Phase II trial of docetaxal plus imatinib mesylate in the treatment of patients with metastatic breast cancer. *Clin Breast Cancer* 2009; **9**:237–242.
43 Huang CH, Williamson SK, Van Veldhuizen PJ, Hsueh CT, Allen A, Tawfik O, *et al*. Potential role of platelet-derived growth factor receptor inhibition using imatinib in combination with docetaxel in the treatment of recurrent non-small cell lung cancer. *J Thorac Oncol* 2011; **6**:372–377.
44 Skorta I, Oren M, Markwardt C, Gutekunst M, Aulitzky WE, Van der KH. Imatinib mesylate induces cisplatin hypersensitivity in Bcr-Abl+ cells by differential modulation of p53 transcriptional and proapoptotic activity. *Cancer Res* 2009; **69**:9337–9345.
45 Ghosal N, Mais K, Shenjere P, Julyan P, Hastings D, Ward T, *et al*. Phase II study of cisplatin and imatinib in advanced salivary adenoid cystic carcinoma. *Br J Oral Maxillofac Surg* 2010 [Epub ahead of print].
46 Quintas-Cardama A, Kantarjian H, Garcia-Manero G, O'Brien S, Faderl S, Ravandi F, *et al*. A pilot study of imatinib, low-dose cytarabine and idarubicin for patients with chronic myeloid leukemia in myeloid blast phase. *Leuk Lymphoma* 2007; **48**:283–289.

47 Gucluler G, Baran Y. Docetaxel enhances the cytotoxic effects of imatinib on Philadelphia positive human chronic myeloid leukemia cells. *Hematology* 2009; **14**:139–144.
48 Seiter K, Katragadda S, Ponce D, Rasul M, Ahmed N. Temozolomide and cisplatin in relapsed/refractory acute leukemia. *J Hematol Oncol* 2009; **2**:21.
49 Moore DC, Carter DL, Bhandal AK, Studzinski GP. Inhibition by 1,25 dihydroxyvitamin D3 of chemically induced erythroid differentiation of K562 leukemia cells. *Blood* 1991; **77**:1452–1461.
50 Yee SW, Campbell MJ, Simons C. Inhibition of Vitamin D3 metabolism enhances VDR signalling in androgen-independent prostate cancer cells. *J Steroid Biochem Mol Biol* 2006; **98**:228–235.
51 Kizildag S, Ates H, Kizildag S. Treatment of K562 cells with 1,25-dihydroxy-vitamin D(3) induces distinct alterations in the expression of apoptosis-related genes BCL2, BAX, BCL(XL), and p21. *Ann Hematol* 2010; **89**:1–7.
52 Wang X, Studzinski GP. Activation of extracellular signal-regulated kinases (ERKs) defines the first phase of 1,25-dihydroxyvitamin D3-induced differentiation of HL60 cells. *J Cell Biochem* 2001; **80**:471–482.
53 Wietrzyk J, Nevozhay D, Milczarek M, Filip B, Kutner A. Toxicity and antitumor activity of the vitamin D analogs PRI-1906 and PRI-1907 in combined treatment with cyclophosphamide in a mouse mammary cancer model. *Cancer Chemother Pharmacol* 2008; **62**:787–797.
54 Lennartsson J, Jelacic T, Linnekin D, Shivakrupa R. Normal and oncogenic forms of the receptor tyrosine kinase kit. *Stem Cells* 2005; **23**:16–43.
55 Reiterer G, Yen A. Platelet-derived growth factor receptor regulates myeloid and monocytic differentiation of HL-60 cells. *Cancer Res* 2007; **67**:7765–7772.
56 Gianni M, Kalac Y, Ponzanelli I, Rambaldi A, Terao M, Garattini E. Tyrosine kinase inhibitor STI571 potentiates the pharmacologic activity of retinoic acid in acute promyelocytic leukemia cells: effects on the degradation of RARalpha and PML-RARalpha. *Blood* 2001; **97**:3234–3243.
57 Johnson CS, Hershberger PA, Bernardi RJ, McGuire TF, Trump DL. Vitamin D receptor: a potential target for intervention. *Urology* 2002; **60**:123–130.
58 Cho YL, Christensen C, Saunders DE, Lawrence WD, Deppe G, Malviya VK, *et al*. Combined effects of 1,25-dihydroxyvitamin D3 and platinum drugs on the growth of MCF-7 cells. *Cancer Res* 1991; **51**:2848–2853.
59 Donaldson KL, Goolsby GL, Wahl AF. Cytotoxicity of the anticancer agents cisplatin and taxol during cell proliferation and the cell cycle. *Int J Cancer* 1994; **57**:847–855.
60 Gaczynski M, Briggs JA, Wedrychowski A, Olinski R, Uskokovic M, Lian JB, *et al*. cis-diamminedichloroplatinum(II) cross-linking of the human myeloid cell nuclear differentiation antigen to DNA in HL-60 cells following 1,25-dihydroxy vitamin D3-induced monocyte differentiation. *Cancer Res* 1990; **50**:1183–1188.
61 Hershberger PA, McGuire TF, Yu WD, Zuhowski EG, Schellens JH, Egorin MJ, *et al*. Cisplatin potentiates 1,25-dihydroxyvitamin D3-induced apoptosis in association with increased mitogen-activated protein kinase kinase kinase 1 (MEKK-1) expression. *Mol Cancer Ther* 2002; **1**:821–829.
62 Koshizuka K, Koike M, Kubota T, Said J, Binderup L, Koeffler HP. Novel vitamin D3 analog (CB1093) when combined with paclitaxel and cisplatin inhibit growth of MCF-7 human breast cancer cells in vivo. *Int J Oncol* 1998; **13**:421–428.
63 Moffatt KA, Johannes WU, Miller GJ. 1Alpha,25dihydroxyvitamin D3 and platinum drugs act synergistically to inhibit the growth of prostate cancer cell lines. *Clin Cancer Res* 1999; **5**:695–703.
64 Qin X, Liu C, Zhou Y, Wang G. Cisplatin induces programmed death-1-ligand 1(PD-L1) over-expression in hepatoma H22 cells via Erk /MAPK signaling pathway. *Cell Mol Biol (Noisy-le-grand)* 2010; **56 (Suppl):**OL1366–OL1372.
65 Sanchez-Perez I, Perona R. Lack of c-Jun activity increases survival to cisplatin. *FEBS Lett* 1999; **453**:151–158.
66 Johansen C, Kragballe K, Henningsen J, Westergaard M, Kristiansen K, Iversen L. 1alpha,25-dihydroxyvitamin D3 stimulates activator protein 1 DNA-binding activity by a phosphatidylinositol 3-kinase/Ras/MEK/extracellular signal regulated kinase 1/2 and c-Jun N-terminal kinase 1-dependent increase in c-Fos, Fra1, and c-Jun expression in human keratinocytes. *J Invest Dermatol* 2003; **120**:561–570.
67 Chrobak A, Radzikowski C, Opolski A. Side-chain-modified analogs of calcitriol cause resistance of human HL-60 promyelocytic leukemia cells to drug-induced apoptosis. *Steroids* 2005; **70**:19–27.
68 Del Bino G, Li X, Traganos F, Darzynkiewicz Z. Altered susceptibility of differentiating HL-60 cells to apoptosis induced by antitumor drugs. *Leukemia* 1994; **8**:281–288.
69 Kleuser B, Cuvillier O, Spiegel S. 1Alpha,25-dihydroxyvitamin D3 inhibits programmed cell death in HL-60 cells by activation of sphingosine kinase. *Cancer Res* 1998; **58**:1817–1824.
70 Wang X, Studzinski GP. Antiapoptotic action of 1,25-dihydroxyvitamin D3 is associated with increased mitochondrial MCL-1 and RAF-1 proteins and reduced release of cytochrome c. *Exp Cell Res* 1997; **235**:210–217.
71 Wietrzyk J, Milczarek M, Kutner A. The effect of combined treatment on head and neck human cancer cell lines with novel analogs of calcitriol and s. *Oncol Res* 2007; **16**:517–525.