Potentiation of antileukemic therapies by Smac mimetic, LBW242: effects on mutant FLT3-expressing cells

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Abstract

Members of the inhibitor of apoptosis protein (IAP) family play a role in mediating apoptosis. Studies suggest that these proteins may be a viable target in leukemia because they have been found to be variably expressed in acute leukemias and are associated with chemosensitivity, chemoresistance, disease progression, remission, and patient survival. Another promising therapeutic target, FLT3, is mutated in about one third of acute myelogenous leukemia (AML) patients; promising results have recently been achieved in clinical trials investigating the effects of the protein tyrosine kinase inhibitor PKC412 on AML patients harboring mutations in the FLT3 protein. Of growing concern, however, is the development of drug resistance resulting from the emergence of point mutations in targeted tyrosine kinases used for treatment of acute leukemia patients. One approach to overriding resistance is to combine structurally unrelated inhibitors and/or inhibitors of different signaling pathways. The proapoptotic IAP inhibitor, LBW242, was shown in proliferation studies done *in vitro* to enhance the killing of PKC412-sensitive and PKC412-resistant cell lines expressing mutant FLT3 when combined with either PKC412 or standard cytotoxic agents (doxorubicin and Ara-c). In addition, in an *in vivo* imaging assay using bioluminescence as a measure of tumor burden, a total of 12 male NCr-nude mice were treated for 10 days with p.o. administration of vehicle, LBW242 (50 mg/kg/day), PKC412 (40 mg/kg/day), or a combination of LBW242 and PKC412; the lowest tumor burden was observed in the drug combination group. Finally, the combination of LBW242 and PKC412 was sufficient to override stromal-mediated viability signaling conferring resistance to PKC412. [Mol Cancer Ther 2007;6(7):1951–61]

Introduction

Acute myelogenous leukemia (AML) is a hematologic malignancy characterized by a block in cellular differentiation and aberrant growth of myeloid precursor cells. Approximately 30% of AML patients and a portion of acute lymphoblastic leukemia (ALL) patients express a mutated form of the class III receptor tyrosine kinase, FLT3 [Fms-like tyrosine kinase-3; STK-1 (human stem cell tyrosine kinase-1); or FLK-2 (fetal liver kinase-2); ref. 1]. Constitutively activated FLT3 occurs most often as internal tandem duplications within the juxtamembrane domain (2) and is observed in ~20–25% of AML patients, but in <5% of patients with myelodysplastic syndrome (MDS; refs. 2–6). The transplantation of murine bone marrow cells infected with a retrovirus expressing a FLT3-ITD mutant has been shown to lead to the development of a rapidly lethal myeloproliferative disease in mice (7). Gain-of-function FLT3 occurs less often as point mutations in the activation loop (in ~7% of AML cases) and is often characterized by an aspartate (D) residue at position 835 (8). Occurring less frequently are additional point mutations in the kinase domain, including N841I (9) and Y842C (10).

The broad spectrum, small-molecule inhibitor, PKC412, was discovered to inhibit the growth and viability of mutant FLT3-expressing cells *in vitro* and also to extend the life span of mice harboring bone marrow transduced with mutant FLT3 (11). A recent phase Ib clinical trial was carried out involving treatment of newly diagnosed AML patients with PKC412 (at 50 mg p.o. b.i.d.) in sequential and simultaneous combinations with daunorubicin and cytarabine induction and high-dose cytarabine consolidation (12). Side effects were transient and/or reversible, and clinical responses were observed, with 100% of patients harboring mutant FLT3 achieving clinical complete response (12).

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Although small-molecule inhibitors that inhibit protein tyrosine kinases are showing promise in the clinic, a growing problem for the treatment of acute leukemia patients is the development of resistance resulting from acquired point mutations in the targeted kinases (13, 14). In addition, clinical trial data with PKC412 show that peripheral blood of AML patients responds well to the inhibitor, whereas bone marrow responds less well. Similarly, quiescent Bcr-Abl–positive stem cells are often found in imatinib-treated chronic myelogenous leukemia (CML) patients and are unresponsive to imatinib therapy. Based on these findings, our hypothesis is that stromal cells provide viability signals to leukemic cells that protect them from the effects of the inhibitor. One possible strategy to prevent relapse due to the emergence of point mutations in target proteins, as well as to overcome drug resistance believed to be caused by stromal-mediated viability signals, would be combined therapy with small-molecule inhibitors that interact with key components of different signaling pathways.

Mediators of apoptotic signaling represent an attractive target for therapeutic intervention. Smac (Second mitochondria-derived activator of caspase) mediates apoptosis occurring through the intrinsic apoptotic pathway (15) and binds to and inhibits the inhibitor of apoptosis protein (IAP) family of proteins (16, 17). Smac is likely the functional equivalent of *Drosophila* Reaper, Hid, and Grim (18–20); the mouse Smac ortholog is DIABLO (21). Identified human IAPs [c-IAP-1, c-IAP-2, and X-chromosome–linked IAP (XIAP)]. IAPs also directly bind and inhibit active caspases (22–24); the BIR (baculovirus IAP repeat) domain is responsible for the antiapoptotic activity of IAPs (25).

The inherent resistance of AML to apoptosis has led to the hypothesis that IAP is a good therapeutic target for molecular intervention. The development of the proapoptotic IAP inhibitor, LBW242, a 3-mer and Smac mimetic, was based on the ability of the NH_2-terminal seven amino acids of Smac to neutralize the BIR3 domain of XIAP (16, 17). LBW242 was the result of a rational drug design effort based on Smac 4-mer and has been found to display nanomoles-per-liter potency against XIAP and c-IAP-1 in a competitive binding assay with Smac 7-mer. LBW242 also kills cells in a manner strictly dependent on caspases, and death is accompanied by PARP cleavage, Annexin positivity, and accumulation of cells in sub-G_1. Here, we investigated the activity of LBW242, alone and in combination with PKC412, against a variety of PKC412-sensitive and PKC412-resistant cell lines both *in vitro* and *in vivo*. We also examined the ability of LBW242 to enhance the antiproliferative effects of standard chemotherapeutic agents such as doxorubicin and Ara-c. Finally, we investigated the ability of LBW242 to override stromal-mediated chemoresistance.

Materials and Methods

Cell Lines and Cell Culture

FLT3-ITD– and FLT3-D835Y–containing murine stem cell virus (MSCV) retroviruses were transfected into the interleukin-3 (IL-3)–dependent murine hematopoietic cell line Ba/F3 as previously described (7), and PKC412-resistant Ba/F3 cell lines expressing FLT3-harboring mutations in the ATP-binding pocket were developed as previously described (26). The human AML-derived, FLT3-ITD–expressing cell lines, MV4;11 (27) and Molm14 (28), were provided to us by Dr. Scott Armstrong (Dana-Farber Cancer Institute, Boston, MA). Ba/F3-FLT3-ITD cells were transduced with a vesicular stomatitis virus glycoprotein (VSVG)–pseudotyped retrovirus comprised of the firefly luciferase–coding region (from pGL3-basic; Promega) cloned into PMSCV puro (Clonetech), and the human AML-derived, FLT3-ITD–expressing cell line, MOLM-13, was engineered to express luciferase fused to neomycin phosphotransferase (pMMP-LucNeo) by transduction with a VSVG-pseudotyped retrovirus as previously described (29). The human CML cell lines, K562 and KU812, were purchased from the American Type Culture Collection. The human B cell precursor leukemia cell line, BV-173, was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures). Murine 32D.p210 cells were developed as previously described (30).

All cell lines were cultured with 5% CO_2 at 37°C in RPMI (Mediatech, Inc.) with 10% FCS and supplemented with 1% glutamine. Parental Ba/F3 cells expressing wild-type FLT3 were similarly cultured with 15% WEHI-conditioned medium as a source of IL-3. All transfected cell lines, with the exception of 32D.p210, were cultured in media supplemented with 1 mg/mL G418.

Chemical Compounds and Biological Reagents

PKC412 and LBW242 were synthesized by Novartis Pharma AG and were dissolved in DMSO to make 10 mmol/L stock solutions. Serial dilutions were then made, also in DMSO, to obtain final dilutions for cellular assays.

Antibodies and Immunoblotting

Antibodies to XIAP (clone 28; BD Sciences), c-IAP-1 (Cell Signaling Technology), Bcl-2 (c-2), and Bcl-XL (H-5; Santa Cruz Biotechnology) were each used at a dilution of 1:200. The monoclonal anti–β-actin antibody (clone AC-15; Sigma-Aldrich) and α-tubulin antibody (clone DM1A; Sigma-Aldrich) were each used at a 1:2,000 dilution. Protein lysate preparation and immunoblotting were carried out as previously described (11).

Cell Viability, Apoptosis, and Caspase Activity Analysis

Cell counts were obtained using the trypan blue exclusion assay as previously described (11). Cell viability is reported as percentage of control (untreated) cells. Error bars represent the SE for each data point. Apoptosis of drug-treated cells was measured using the Annexin-V–Fluos Staining Kit (Boehringer Mannheim) as previously described (11). Caspase assays were done as described (Supplementary data).[7]

[7] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Human Stroma Experiments

HS-5 human stromal cells (10,000 per well) were seeded 24 h in advance of seeding MOLM13-luc+ cells (40,000 per well), followed by treatment with a range of concentrations of LBW242 and PKC412, alone and combined. Cells were then analyzed for luciferase expression using a Xenogen imager.

Drug Combination Studies

For drug combination studies, PKC412 and LBW242 were added simultaneously at fixed ratios to FLT3-ITD-Ba/F3 cells and PKC412-resistant mutant FLT3-expressing cells. Cell viability was determined using the trypan blue exclusion assay and expressed as the function of growth-affected (FA) drug-treated versus control cells. Data were analyzed by Calcusyn software (Biosoft) using the Chou-Talalay method (31). The combination index = $[D]_1\,[D_x]_1 + [D]_2/[D_x]_2$, where $[D]_1$ and $[D]_2$ are the concentrations required by each drug in combination to achieve the same effect as concentrations $[D_x]_1$ and $[D_x]_2$ of each drug alone. Values <1 indicate synergy, whereas values >1 indicate antagonism.

Mouse Studies and *In vivo* Imaging

FLT3-ITD-Ba/F3-luc+ cells free of *Mycoplasma* and viral contamination were resuspended in HBSS (Mediatech) before i.v. administration to mice. About 6% w/w PKC412 in Gelucire 44/14 (Gattefosse) was diluted with 1× PBS and warmed in a 42°C water bath until liquid. The solution was then stored at 4°C until used for gavage treatment of mice. LBW242 was prepared by first wetting 10 mg powder stock with 30 μL water and then dissolving in two equivalents (0.73 μL/mg of compound) of 6.0 N HCl. The resulting (clear) solution was brought up to 1 mL in acetate buffer (pH, 4.6), and the resulting stock was stored frozen at −20°C until used for gavage treatment of mice.

Male NCr-nude mice (5–6 weeks of age; Taconic) were given a total of 800,000 FLT3-ITD-Ba/F3-luc+ cells by tail vein injection. Mice were imaged, and total body luminescence was quantified as previously described (29). Baseline imaging 1 day after tumor cell inoculation was used to establish treatment cohorts with matched tumor burden. Cohorts of mice were treated with p.o. administration of vehicle, 40 mg/kg/day PKC412 (formulated as above), 50 mg/kg LBW242 (formulated as above), or a combination of PKC412 and LBW242. Imaging was done at various intervals. At the planned end of the first of two studies (8 days following initial i.v. injection of FLT3-ITD-Ba/F3-luc+ cells), mice were sacrificed, body and spleen weights were recorded, and tissues were preserved in 10% formalin for histopathologic analysis. For the second study, mice were sacrificed on the last imaging day.

Results

Effects of LBW242 on Proliferation and Apoptosis of PKC412-Sensitive and Resistant Mutant FLT3-Expressing Cells

We randomly screened LBW242 (Fig. 1E) against a panel of leukemia cell lines expressing either Bcr-Abl or mutant FLT3. None of the Bcr-Abl–expressing lines (K562, 32D.p210, KU812, BV-173) showed appreciable responsiveness to 1 μmol/L LBW242 as a single agent (data not shown). In contrast, the growth of mutant FLT3-expressing lines, such as MV4;11, was partially inhibited at 1 μmol/L LBW242 (data not shown).

The results of this preliminary screen prompted further investigation of LBW242 against a panel of PKC412-sensitive or PKC412-resistant, mutant FLT3-expressing Ba/F3 lines (26). PKC412 shows variable activity against each of the resistant lines at concentrations that are physiologically relevant (Fig. 1). IC_{50} values for LBW242 ranged from 0.5 to >1 μmol/L following 3 days of incubation (Fig. 1A and B). The PKC412-resistant N676D- and G697R-Ba/F3 lines showed higher sensitivity to the inhibitor at 1 μmol/L LBW242 (Fig. 1B), and proliferation of cells expressing the point mutant FLT3-D835Y was similarly suppressed at 1 μmol/L LBW242 (Fig. 1C). There was no inhibitory effect of LBW242 on cell growth of wild-type FLT3-expressing Ba/F3 cells at concentrations ≤1 μmol/L; however, concentrations of LBW242 >1 μmol/L led to death of these cells (Fig. 1C).

Consistent with what was observed in proliferation studies, no significant induction of apoptosis was observed in the majority of mutant FLT3-expressing cell lines treated with 1 μmol/L LBW242 as a single agent for 2 days, with greater (10–50%) induction of apoptosis in the G697R- and N676D-FLT3 lines, respectively (Supplementary data).[7] Caspase activity was highest in N676D-FLT3-Ba/F3 and G697R-FLT3-Ba/F3 cells treated with 5 and 25 μmol/L LBW242 for 3 days (Supplementary data).[7]

We investigated the treatment of N676D-Ba/F3 cells with 1 μmol/L LBW242 in the presence and absence of WEHI (used as a source of IL-3). WEHI did not rescue cells cultured simultaneously in the presence of LBW242 (Fig. 1D), suggesting that LBW242, consistent with its proposed mechanism of inhibition of IAP, does not selectively inhibit mutant FLT3, but generally interferes with viability.

Effects of LBW242 Plus PKC412 against PKC412-Sensitive and PKC412-Resistant Cells

LBW242 was tested in combination with PKC412 against FLT3-ITD-Ba/F3 cells, and positive cooperativity was observed between the two agents (Fig. 2A, Table 1). Analysis of the combined effects of LBW242 and PKC412 using Calcusyn software suggests nearly additive to synergistic effects across a range of doses [effective dose 50 (ED50)–ED90; Table 1]. The combination of LBW242 and PKC412 was also found to enhance inhibition of proliferation of the human acute leukemia, FLT3-ITD–expressing cell line, MV4;11. Moderate synergy to synergy was observed only at higher doses as compared with each drug alone (Supplementary data).[7]

The combination of LBW242 and PKC412 was additionally investigated against the PKC412-resistant, mutant FLT3-expressing cell lines, A627T-FLT3-Ba/F3, F691I-FLT3-Ba/F3, G697R-FLT3-Ba/F3, and N676D-FLT3-Ba/F3. Nearly additive to synergistic effects were observed for the

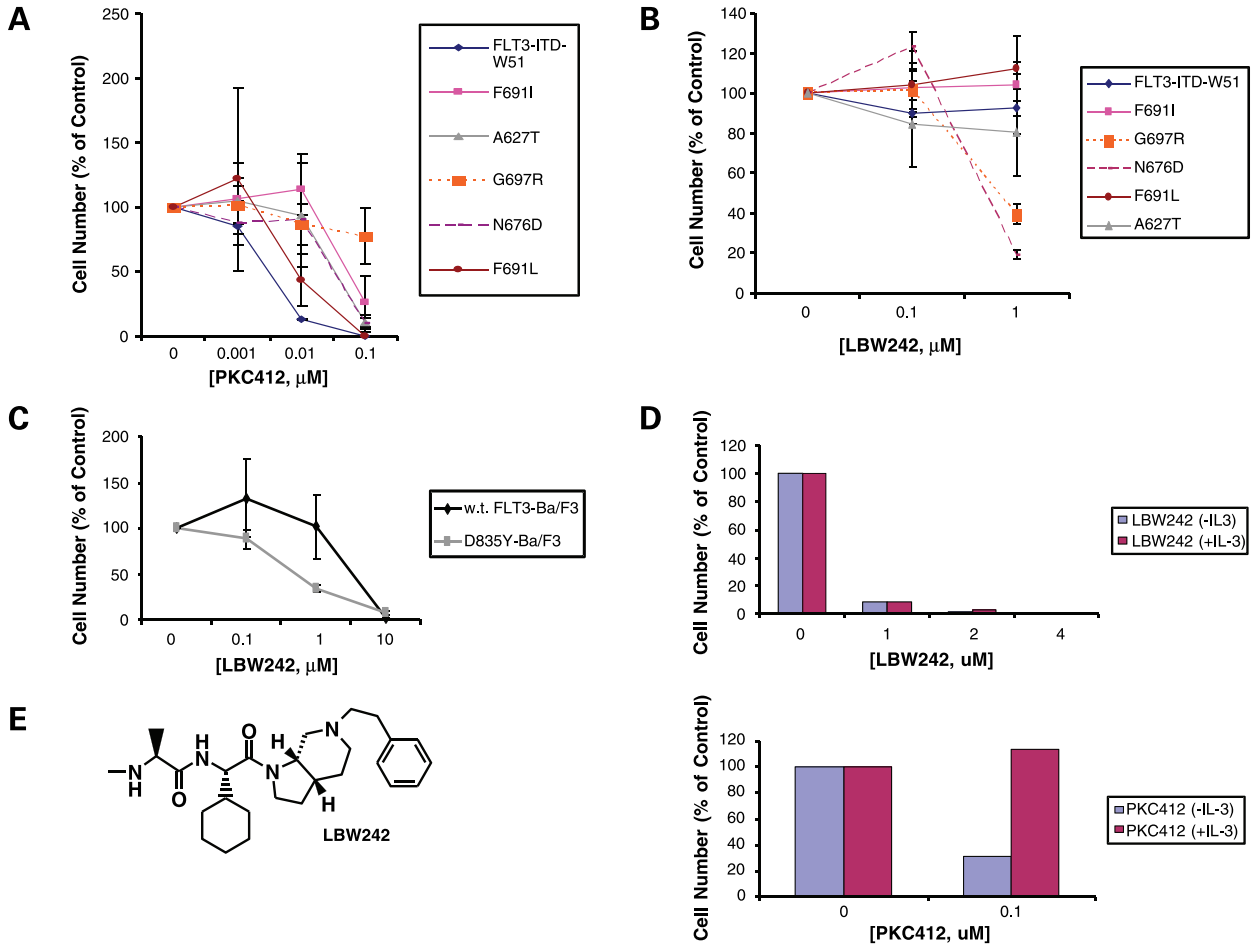

Figure 1. Effects of LBW242 on proliferation of PKC412-sensitive and PKC412-resistant mutant FLT3-expressing cells. **A,** 2-d treatment of FLT3-ITD-Ba/F3 and PKC412-resistant Ba/F3-derived mutant FLT3 cells with PKC412 ($n = 2$). **B,** 3-d treatment of FLT3-ITD-Ba/F3 and PKC412-resistant Ba/F3-derived mutant FLT3 cells with LBW242 ($n = 2$). **C,** 3-d treatment of wild-type FLT3-Ba/F3 and D835Y-Ba/F3 cells with LBW242 ($n = 2$). **D,** IL-3 rescue of N676D-Ba/F3 cells treated in parallel for 2 d with LBW242 ($n = 2$) or PKC412 ($n = 1$). **E,** chemical structure of LBW242.

A627T-FLT3-Ba/F3 and F691I-FLT3-Ba/F3 lines (ED50–ED90), and synergy was observed for the G697R-FLT3-Ba/F3 line (ED50–ED90; Fig. 2B–D and Table 1). A nearly additive effect (ED50–ED90) was observed for N676D-FLT3-Ba/F3 cells (Supplementary data).[7]

For comparison, we investigated the combined effects between PKC412 and the standard chemotherapy agents, doxorubicin and Ara-c, against FLT3-ITD-Ba/F3 cells. Synergy was observed for PKC412 and doxorubicin across a range of doses (ED50–ED90; Supplementary data). In contrast, the combination of PKC412 and Ara-c resulted in slight antagonism to antagonism against FLT3-ITD-Ba/F3 cells (ED50–ED75), although with nearly additive effects observed at ED90 (Supplementary data).[7]

We were interested next in investigating the combined effects of LBW242 plus doxorubicin. For FLT3-ITD-Ba/F3 cells, LBW242 plus doxorubicin was nearly additive to synergistic across a range of doses (ED50-ED90; Fig. 3A, Table 1). For A627T-FLT3-Ba/F3 cells, LBW242 plus doxorubicin acted in synergy (ED50–ED90; Fig. 3B and Table 1). For F691I-FLT3-Ba/F3 cells, LBW242 plus doxorubicin was nearly additive to synergistic only across the higher range of doses (ED75–ED90; Supplementary data).[7]

The combination of LBW242 and Ara-c was also investigated. For FLT3-ITD-Ba/F3 cells, moderate synergy to synergy was observed between LBW242 and Ara-c (ED50–ED90; Fig. 3C and Table 1). For F691I-FLT3-Ba/F3 cells and A627T-Ba/F3 cells, results ranged from nearly additive to synergistic (ED50–ED90; Fig. 3D, Table 1, and Supplementary data).[7]

In vivo Investigation of Effects of LBW242 and PKC412, Alone and Combined

To directly assess the *in vivo* antitumor efficacy of LBW242 alone, PKC412 alone, and the combination of LBW242 plus PKC412, we tested a mouse model of acute leukemia in which tumor burden was quantified by noninvasive imaging of luminescent tumor cells (Fig. 4). NCr nude mice were inoculated with FLT3-ITD-Ba/F3 cells

engineered to stably express firefly luciferase. Noninvasive imaging was used to assess tumor burden, and mice with established leukemia were divided into cohorts with similar tumor burden. PKC412, LBW242, or PKC412 plus LBW242 were then given via oral gavage, as was vehicle. Administration of both agents together was done by gavaging one agent ~20–30 min before gavaging the other.

In the first of two independent studies, mice were given vehicle alone, LBW242 (50 mg/kg), PKC412 (40 mg/kg), or a combination of both agents (Fig. 4A–C). The lowest tumor burden as assessed by bioluminescence was observed to be in the drug combination group on days 5 and 7 post-i.v. injection of FLT3-ITD-Ba/F3-luc+ cells (and corresponding to 4 and 6 days of drug treatment, respectively; Fig. 4A and B and Supplementary data).[7] The Student's *t* test was used for statistical evaluation of bioluminescence results as observed on day 7 post-i.v. injection: $P \leq 0.056247$ (vehicle versus LBW242 alone); $P \leq 0.048250$ (vehicle versus PKC412 alone); $P \leq 0.043290$ (vehicle versus LBW242 and PKC412, combined); $P \leq 0.132180$ (LBW242 versus LBW242 and PKC412, combined); $P \leq 0.401261$ (LBW242 versus PKC412); $P \leq 0.245418$ (PKC412 versus LBW242 and PKC412, combined). Statistical evaluation (via Student's *t* test) for day 5 post-i.v. injection yielded $P \leq 0.077299$ (vehicle versus LBW242 alone); $P \leq 0.075852$ (vehicle versus PKC412 alone); $P \leq 0.06826$ (vehicle versus LBW242 and PKC412, combined); $P \leq 0.0.020204$ (LBW242 versus LBW242 and PKC412, combined); $P \leq 0.778690$ (LBW242 versus PKC412); $P \leq 0.245417$ (PKC412 versus LBW242 and PKC412, combined). All treatment groups thus seem to be effective in reducing tumor burden as compared with vehicle controls ($P \leq 0.05$). An ANOVA test was also done with repeated measures and yielded $P \leq 0.03$ for day 5 post-i.v. injection for all group comparisons and $P \leq 0.01$ on day 7 post-i.v. injection for all group comparisons.

Compared with vehicle-treated mice, the percent spleen weights of drug-treated mice were observed to be lower following sacrifice 8 days after the last imaging day (Fig. 4C). The results of a *t* test suggest significant

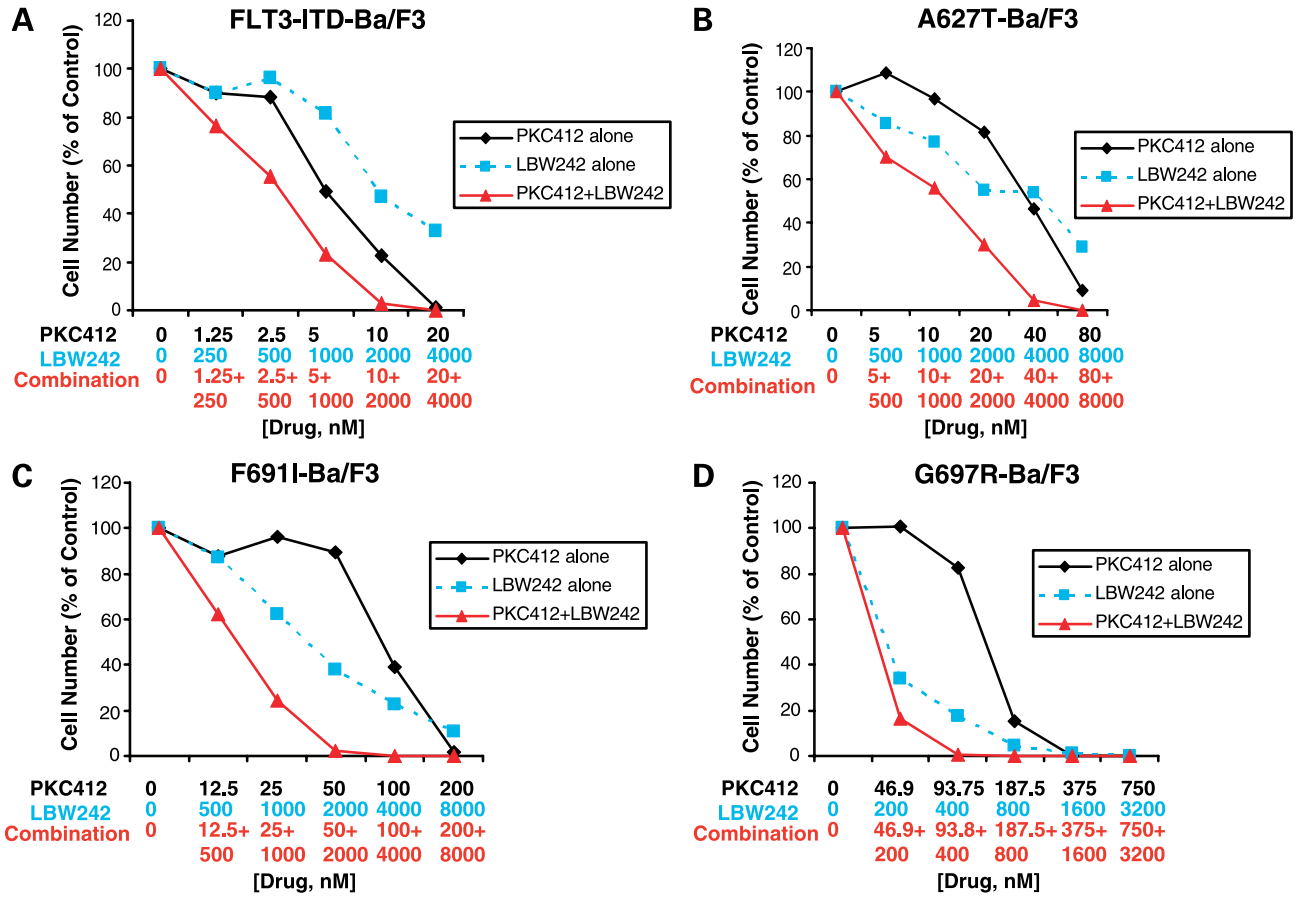

Figure 2. Effects of LBW242 plus PKC412 on proliferation of PKC412-sensitive and PKC412-resistant mutant FLT3-expressing cells. **A,** proliferation study showing 3-d treatments of FLT3-ITD-W51-Ba/F3 cells with PKC412, LBW242, or a combination of PKC412 and LBW242 (study shown is representative of three independent experiments). **B,** proliferation study showing 3-d treatments of A627T-Ba/F3 cells with PKC412, LBW242, or a combination of PKC412 and LBW242 ($n = 1$). **C,** proliferation study showing 3-d treatments of F691I-Ba/F3 cells with PKC412, LBW242, or a combination of PKC412 and LBW242 ($n = 1$). **D,** proliferation study showing 3-d treatments of G697R-Ba/F3 cells with PKC412, LBW242, or a combination of PKC412 and LBW242 (study shown is representative of two independent experiments).

Table 1. Combination indices calculated for dose-response curves for combination studies described in results and shown in Figs. 2 and 3

Cell lines	Combination indices		
	ED50	ED76	ED90
FLT3-ITD-Ba/F3 (LBW242 + PKC412)	0.74780	0.58808	0.47158
A627T-Ba/F3 (LBW242 + PKC412)	055582	0.40946	0.36244
F691l-Ba/F3 (LBW242 + PKC412)	0.63409	0.43267	0.30145
G697r-Ba/F3 (LBW242 + PKC412)	0.39783	0.43210	0.47464
FLT3-ITD-Ba/F3 (LBW242 + doxorubicin)	0.89723	0.81295	0.73688
A627T-Ba/F3 (LBW242 + doxorubicin)	0.38613	0.35638	0.35964
FLT3-ITD-Ba/F3 (LBW242 + Ara-c)	0.60784	0.45149	0.33535
F691l-Ba/F3 (LBW242 + Ara-c)	0.60377	0.47024	0.41785

differences between percent spleen weights of vehicle controls and those of the PKC412-treated mice ($P \leq 0.012070$), LBW242-treated mice ($P \leq 0.010584$), and drug combination–treated mice ($P \leq 0.004206$). A significant difference was also found between the percent spleen weights of PKC412-treated mice and drug combination–treated mice ($P \leq 0.001983$). P values corresponding to percent spleen weight differences were >0.05 between LBW242-treated mice and drug combination–treated mice ($P \leq 0.070595$) and between PKC412-treated mice and LBW242-treated mice ($P \leq 0.378882$).

Similar bioluminescence results were observed in the second of two *in vivo* imaging studies, in which mice were treated for a total of 6 days with vehicle, LBW242 (50 mg/kg, formulated as above), PKC412 (40 mg/kg, formulated as above), or a combination of LBW242 and PKC412 at the respective doses (Fig. 4D and E). The lowest average bioluminescence values were observed in the mice treated with LBW243 plus PKC412 (Fig. 4D and E). Student's t test results for bioluminescence values on day 7 post-i.v. injection were as follows: $P \leq 0.022239$ (vehicle versus LBW242 alone); $P \leq 0.001628$ (vehicle versus PKC412 alone); $P \leq 0.000031$ (vehicle versus LBW242 and PKC412, combined); $P \leq 0.301642$ (PKC412 versus LBW242); $P \leq 0.134141$ (PKC412 versus LBW242 and PKC412, combined); $P \leq 0.205895$ (LBW242 versus LBW242 and PKC412, combined). Statistics were also analyzed with a Kruskal-Wallis nonparametric ANOVA, followed by a Dunn's multiple comparisons test for selected pairs. The Kruskal-Wallis Test (for nonmatched samples) yielded $P = 0.0259$, with $P \leq 0.05$ for the post hoc test between drug combination and vehicle-treated groups.

Effects of LBW242 on Stromal-Mediated Resistance of Mutant FLT3-Expressing Cells to PKC412

Clinical trial data with PKC412 show that whereas good responses are achievable in AML patient peripheral blood, patient bone marrow responses are more modest. Based on this, our hypothesis is that stromal cells provide viability signals to AML or CML cells that protect the cells from the effects of the selective inhibitor being used. We investigated the responsiveness of Ba/F3-FLT3-ITD cells cultured in the presence and absence of WEHI to the cytotoxic effects of PKC412, and we found that the presence of IL-3 completely protected cells from PKC412 inhibition of cellular proliferation (Supplementary data).[7] In contrast to PKC412, LBW242 inhibited Ba/F3-FLT3-ITD proliferation in both the absence and the presence of IL-3 (Supplementary data).[7] Interestingly, LBW242 plus PKC412 inhibited the growth of Ba/F3-FLT3-ITD cells cultured in the presence of IL-3 to a greater extent than either agent alone; this inhibition was similar to that achieved with the coadministration of both agents in the absence of IL-3 (Supplementary data),[7] suggesting that an IAP inhibitor is able to enhance the effects of a FLT3 inhibitor and override chemoresistance due to the provision of viability signals. In an attempt to more closely model putative stromal-AML cell interactions and stromal-mediated viability signaling effects on the cytotoxic effects of PKC412, we used the human stromal cell line, HS-5, in combination with the mutant FLT3 AML line, MOLM-13. To measure only the leukemic component, we introduced luciferase into MOLM-13 so that we could specifically quantitate the viable cell number using light emission. In the study shown (Fig. 5), MOLM-13-luc+ cells (40,000 per well) were exposed to PKC412 in the presence or absence of a near-confluent monolayer of HS-5. The stromal cells enhanced growth and were partially protective against the inhibitory effects of PKC412 (Fig. 5A). LBW242 enhanced the cytotoxic effects of PKC412 against MOLM13-luc+ cells in both the absence of HS-5 human stromal cells as well as their presence (Fig. 5B). The results shown in Fig. 5A support the notion that stromal-mediated viability signals may contribute to chemoresistance to FLT3 inhibitors (such as PKC412) observed in marrow. The results shown in Fig. 5B support the idea that such resistance may be overcome by the inclusion of IAP inhibitor treatment.

Mutant FLT3 Regulation of IAPs

To better understand the individual and combined antiproliferative and proapoptotic effects of a protein tyrosine kinase inhibitor agent and a proapoptotic agent against mutant FLT3-transformed cells, we investigated the expression of a panel of antiapoptotic signaling factors, including IAP substrates of LBW242, and Bcl-2 and Bcl-XL, in mutant FLT3-expressing cells. We observed only modest fluctuations in XIAP, Bcl-2, and Bcl-XL protein expression in FLT3-ITD–expressing Ba/F3 cells, as compared with parental Ba/F3 cells, with no detectable change in c-IAP-1 expression, and no effect of IL-3 on protein levels following 2 days of exposure of cells to growth factor (Supplementary data).[7] Treatment of FLT3-ITD-Ba/F3 cells with 0.1 μmol/L PKC412 for 22 h led to a partial decrease in Bcl-XL and XIAP protein expression, suggesting that FLT3 regulates the expression of these proteins (Supplementary data).[7]

Discussion

Drug resistance and treatment-induced mortality are primary reasons underlying the failure of current, conventional therapies for AML (32). The use of standard

chemotherapeutic agents independently is associated with a low treatment-induced mortality but a high risk of relapse (33). Allogeneic transplantation (alloBMT), on the other hand, is associated with a lower risk of relapse but a high treatment-induced mortality (33). Although alloBMT results in 25–30% 10-year survival for young patients, it is less effective in patients near the age of 60; because 64 years is the median age of AML patients, the influence of alloBMT on the majority of patients with this disease is limited (34). Thus, there exists a need for improved treatment modalities for AML.

Positive clinical responses have been observed in wild-type FLT3-expressing AML patients treated with the small-molecule inhibitor, PKC412 (12). Recent results with PKC412 have been especially promising in PKC412-treated AML patients harboring mutations in the tyrosine receptor kinase FLT3 (12). The risk, however, of development of drug resistance in acute leukemia resulting from the emergence of point mutations in target kinases prompted the investigation of the combined effects of PKC412 and agents known to work through mutant FLT3-independent mechanisms.

The inherent resistance of AML cells to apoptosis suggests that signaling components associated with apoptotic signaling are aberrantly active or expressed in transformed cells and, therefore, may be an effective target for therapy. Members of the antiapoptotic IAP family of gene products are involved in the regulation of programmed cell death and prevent cell death by inhibiting caspases (22–24). Their expression levels have been found to be increased in various malignancies, including ovarian and breast cancer, hepatocellular carcinoma, adult T-cell leukemia, and chronic neutrophilic leukemia (35–39); this overexpression has also been linked to UV and chemoresistance and a poor prognosis (35, 40–43).

Despite the fact that there is some evidence that IAPs significantly contribute to drug resistance of leukemia cell lines, little is known about the role of IAPs in

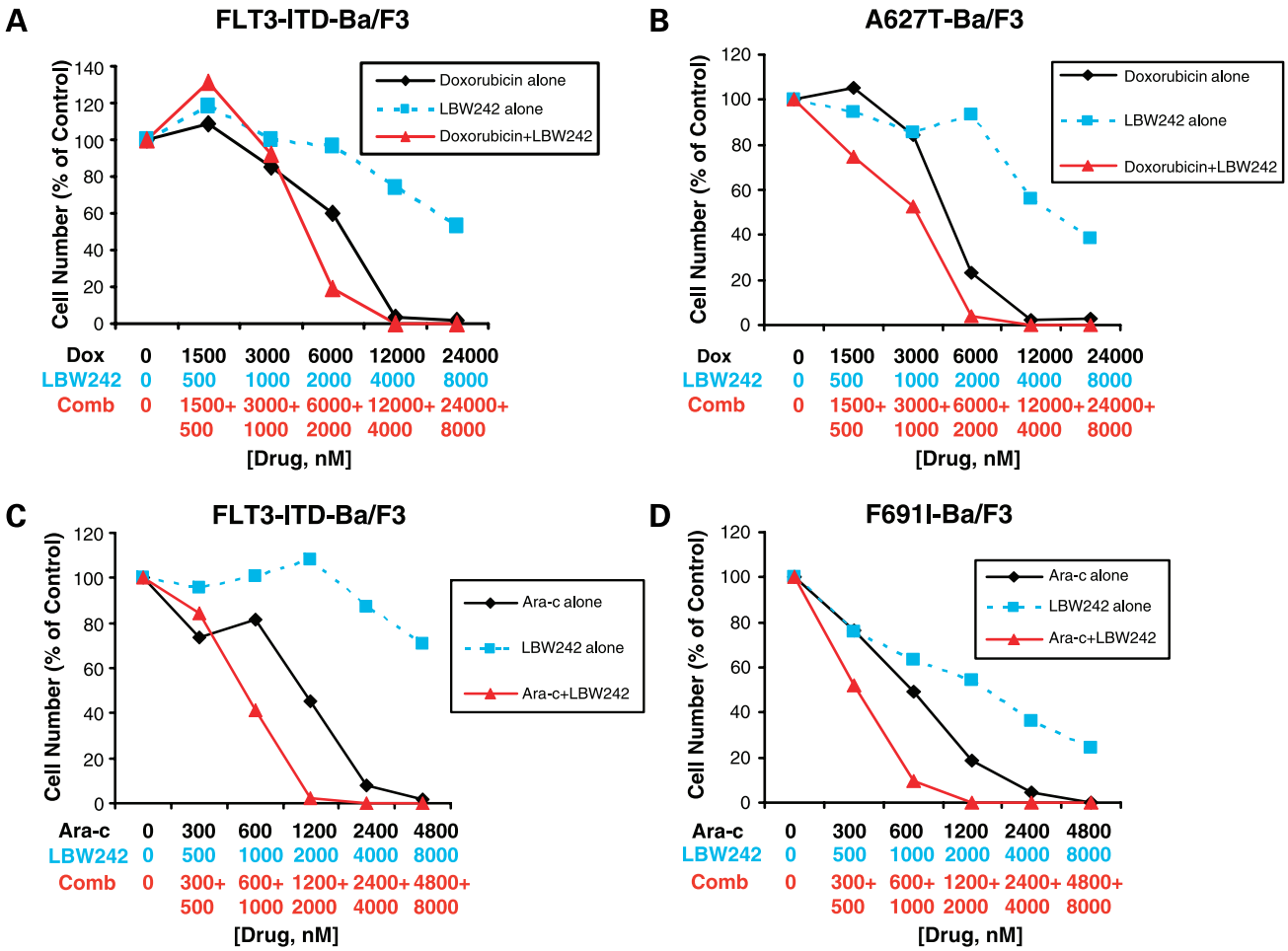

Figure 3. Effects of LBW242 plus doxorubicin or Ara-c, respectively, on proliferation of PKC412-sensitive and PKC412-resistant mutant FLT3-expressing cells. **A,** proliferation study showing 3-d treatments of FLT3-ITD-Ba/F3 cells with doxorubicin, LBW242, or a combination of doxorubicin and LBW242 (n = 1). **B,** proliferation study showing 3-d treatments of A627T-Ba/F3 cells with doxorubicin, LBW242, or a combination of doxorubicin and LBW242 (n = 1). **C,** proliferation study showing 3-d treatments of FLT3-ITD-Ba/F3 cells with Ara-c, LBW242, or a combination of Ara-c and LBW242 (n = 1). **D,** proliferation study showing 3-d treatments of F691I-Ba/F3 cells with Ara-c, LBW242, or a combination of Ara-c and LBW242 (n = 1).

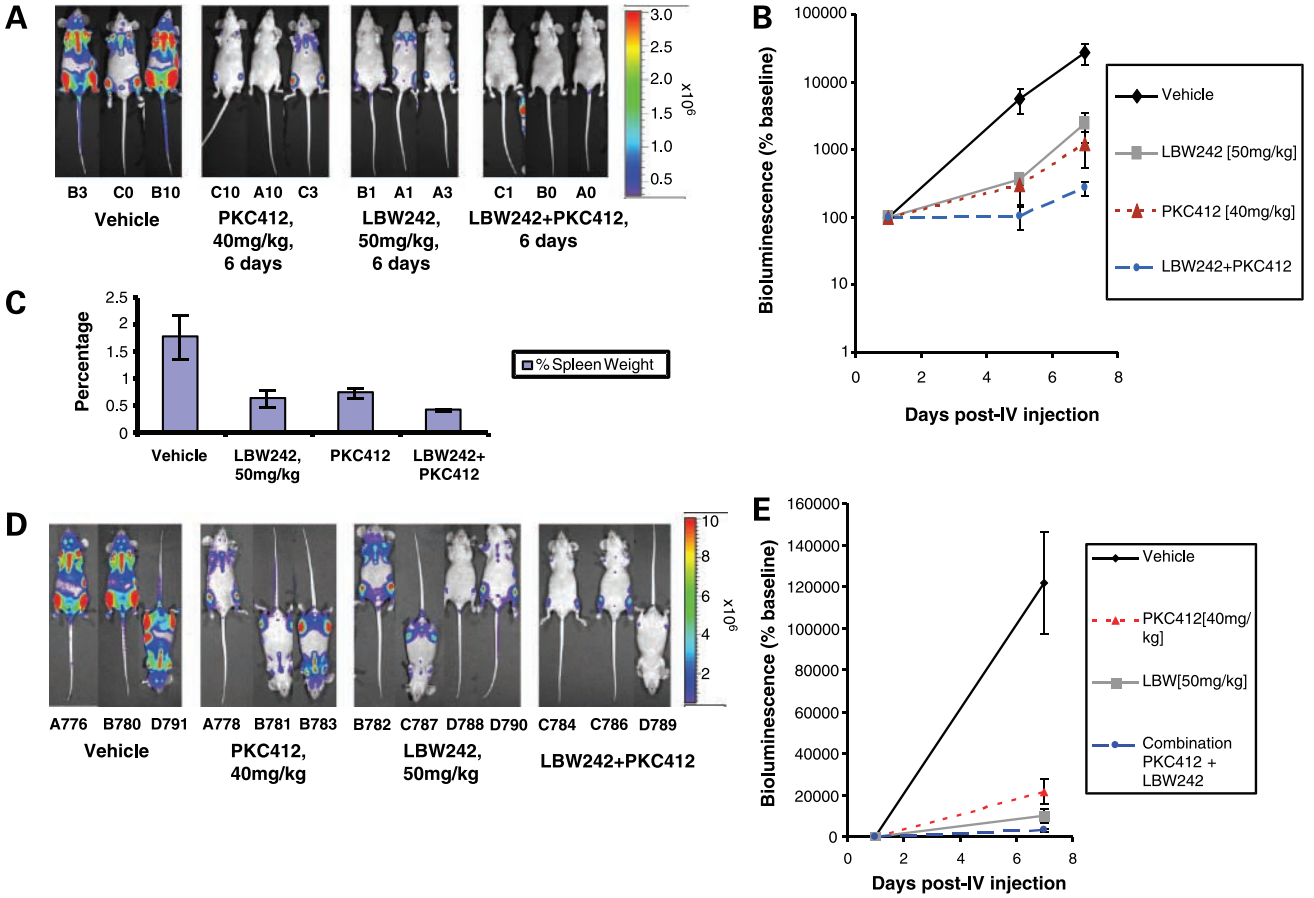

Figure 4. *In vivo* investigation of combined effects of PKC412 and LBW242. **A – C,** NCR nude mice injected with 800,000 Ba/F3-FLT3-ITD-luc+ cells via i.v. tail vein and then treated for up to 10 d by oral gavage with vehicle (NMP + PEG300), LBW242 (50 mg/kg) alone, PKC412 (40 mg/kg) alone, or a combination of LBW242 (50 mg/kg) and PKC412 (40 mg/kg). Mice were sacrificed 8 d after the last imaging day, spleen and total weights were measured, and mice were preserved in 10% formalin for histopathologic analysis. **A,** mouse photos show bioluminescence of mice following 7 d post-i.v. injection. **B,** bioluminescence, as shown in **A,** presented as percent baseline. **C,** percent spleen weights of mice treated for 10 d with LBW242, PKC412, or a combination of LBW242 and PKC412. **D** and **E,** NCR nude mice injected with 800,000 Ba/F3-FLT3-ITD-luc+ cells via i.v. tail vein and then treated for up to 6 d by oral gavage with vehicle (NMP + PEG300), LBW242 (50 mg/kg) alone, PKC412 (40 mg/kg) alone, or a combination of LBW242 (50 mg/kg) and PKC412 (40 mg/kg). Mice were sacrificed on the last imaging day (day 7 post-i.v. injection) and preserved in 10% formalin for histopathologic analysis. **D,** mouse photos show bioluminescence of mice following 7 d post-i.v. injection. **E,** bioluminescence, as shown in **D,** presented as percent baseline.

chemoresistance in human acute leukemia. The exact role of IAPs in acute leukemia is complicated by the fact that they have been found to display variable expression and to play different roles in chemosensitivity and chemoresistance. IAP expression, determined by quantitative reverse transcription-PCR, was found to be higher in drug-resistant adult acute mixed lineage leukemia (AMLL) than in ALL and AML cells, and the expression of survivin, NAIP, and XIAP was higher in AMLL than ALL (44). Other investigators found XIAP and c-IAP-1 to be differentially expressed among AML patients with an absence of NAIP mRNA (45). High levels of CIAP protein have been found to correlate with chemoresistance, high levels of XIAP correlate with drug sensitivity, and low XIAP expression with longer survival and longer remission (45, 46).

The association between IAPs and transformation provides rationale for the development of IAP-related therapies, including small molecules of polyphenylurea-based XIAP inhibitors, as well as antisense oligonucleotides of XIAP or survivin. Small-molecule agents called ''Smac mimetics'' have been developed based on the structure and function of the mitochondrial proapoptotic peptide, Smac (15), and have been shown to enhance the cytotoxic effects of chemotherapeutic agents in different tumor models. Caspase activity and apoptosis are regulated by the interaction between Smac and the BIR3 domain of XIAP and also between caspase-9 and the BIR3 domain of XIAP; both interactions are mutually exclusive, which suggests that Smac binding disrupts the interaction between caspase-9 and XIAP (47). The development of the small molecule, LBW242, was based on the structure and inhibitory function of Smac (16, 17). LBW242 has recently been shown to have activity against multiple myeloma (48).

Considering the proapoptotic nature of the Smac mimetic, LBW242, we studied the activity of this compound against cells transformed by the constitutive tyrosine kinase activity of Bcr-Abl or mutant FLT3. Although no apparent inhibition of Bcr-Abl–expressing cells was observed, LBW242 as a single agent did modestly inhibit PKC412-sensitive and PKC412-resistant mutant FLT3-expressing cells *in vitro*. Although the mechanism is at present unclear, the most responsive of the panel of PKC412-resistant FLT3 mutants were N676D-FLT3 and G697R-FLT3. LBW242 induction of apoptosis, as measured via Annexin-pi staining and caspase assays, was observed with effective concentrations in the micromolar range.

Because of the distinct and independent signaling pathways targeted by LBW242 and PKC412, we then investigated the ability of LBW242 to synergistically enhance the antiproliferative effects of PKC412 against mutant FLT3-expressing cells. Additive to synergistic effects between LBW242 and PKC412 were achieved when both were tested in combination against a variety of PKC412-sensitive and PKC412-resistant cell lines *in vitro*. These results are consistent with what we would expect based on the different mechanisms of action of both agents.

For comparison, LBW242 was also tested in combination with the standard chemotherapy agents doxorubicin and Ara-c. The ability of LBW242 to enhance the antiproliferative effects of doxorubicin or Ara-c was observed *in vitro* in PKC412-sensitive and PKC412-resistant cells. Again, these results are not surprising considering the different modes of action of LBW242 and cytotoxic agents like doxorubicin and Ara-c in malignant cells.

Furthermore, in an *in vivo* imaging model of acute leukemia, well-tolerated doses of PKC412 (40 mg/kg) alone, and LBW242 (50 mg/kg) alone, resulted in a lowering of tumor burden as compared with vehicle control-treated mice. Similarly, the combination of LBW242 and PKC412 led to a significant suppression of tumor burden in mice, as compared with vehicle-treated controls, which suggests a nonantagonistic combination of the two agents *in vivo*; tumor burden was observed to be lowest in NCr nude mice i.v. injected with FLT3-ITD-Ba/F3-luc+ expressing cells and treated with the combination of agents, as compared with vehicle or each agent alone.

There is a growing level of interest in determining the role of the microenvironment in regulating growth, self-renewal, and drug resistance of leukemic stem cells. This is likely to be of more than passing interest because it seems possible that small numbers of leukemic $CD34^+$ cells can persist in the marrow microenvironment of patients with CML following years of therapy with imatinib. Similarly,

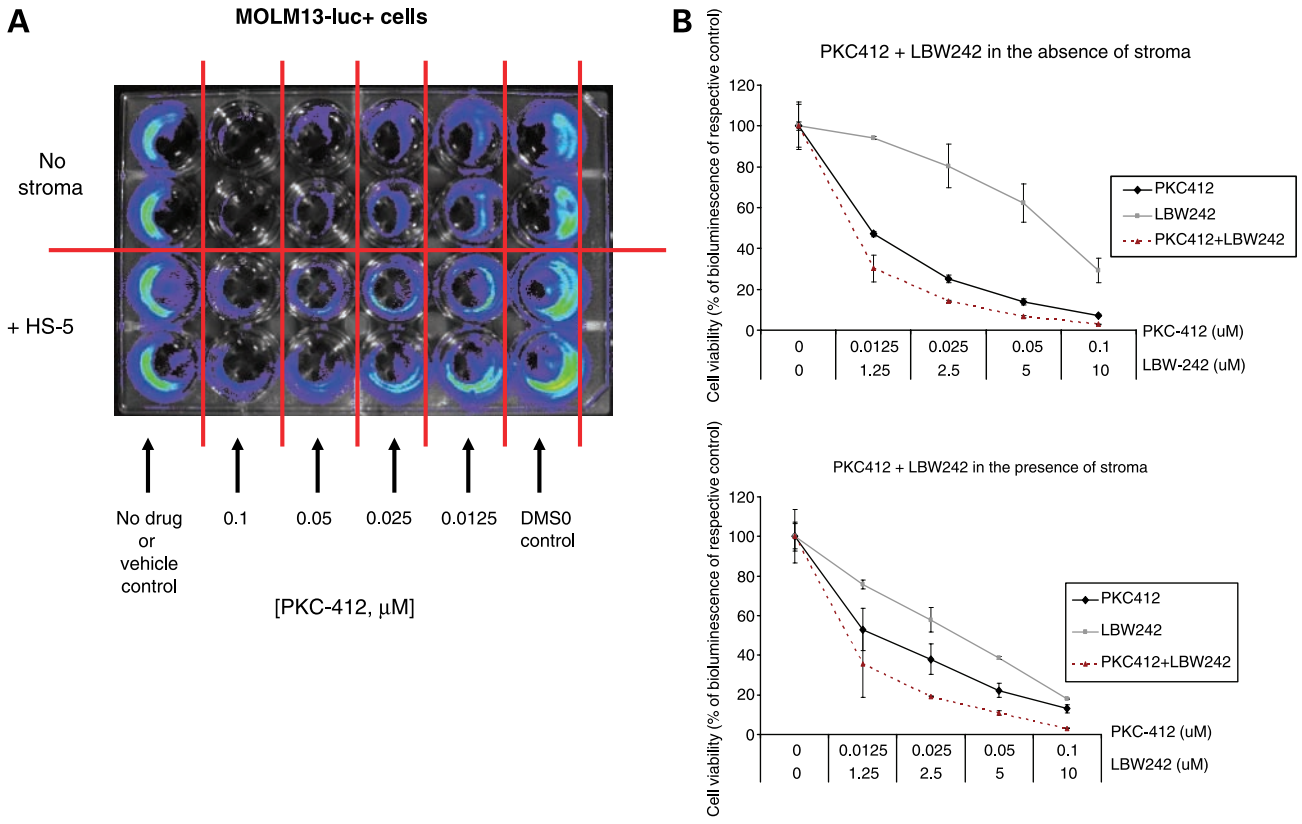

Figure 5. Effects of LBW242 plus PKC412 on stromal-mediated chemoresistance of FLT3-ITD-Ba/F3 cells. **A,** treatment of MOLM13-luc+ cells with different concentrations of PKC412 in the absence and the presence of the human stromal cell line, HS-5. **B,** PKC412 + LBW242 combination experiment using MOLM13-luc+ cells in the absence of stroma (*top*) and the presence of stroma (*bottom*).

clinical studies of patients with advanced AML receiving FLT3 kinase inhibitors revealed that a common pattern of response was a dramatic decrease in the circulating population of blasts, with minimal or delayed decrease in marrow blasts, suggesting a protective environment. These observations suggest that a greater understanding of the interaction of stromal cells with leukemic cells is essential. Here, we have shown that stromal-mediated chemoresistance of mutant FLT3-expressing cells to PKC412 can be overcome by treatment of cells with an IAP inhibitor, suggesting a possible role of IAPs in growth factor–mediated signaling leading to PKC412 resistance.

We studied FLT3 regulation of a panel of apoptosis signaling proteins, including XIAP, c-IAP-1, Bcl-2, and Bcl-XL, and we observed only modest changes in XIAP, Bcl-2, and Bcl-XL in FLT3-transformed cells as compared with parental cells. These small changes in protein expression may have some significance in the susceptibility of FLT3-transformed cells to the cytotoxic effects of an inhibitor of protein tyrosine kinases, such as PKC412, or an IAP inhibitor, such as LBW242, as well as the enhanced cytotoxic effects of the two agents combined. Alternatively, there may be other IAP family members/LBW242 substrates, or alternative apoptotic signaling factors, which are modulated to a greater extent by FLT3 signaling and which consequently may increase susceptibility of cells to the cytotoxic effects of PKC412, LBW242, or the combination of the two compounds.

In conclusion, LBW242 is effective against mutant FLT3 at doses that are physiologically achievable and well tolerated *in vivo*. LBW242 also enhances the inhibitory effects of PKC412, as well as standard chemotherapeutic agents such as doxorubicin and Ara-c, by acting in an additive-synergistic fashion against mutant FLT3-expressing cells *in vitro*. The ability of LBW242 and PKC412 (or doxorubicin or Ara-c) to combine additively or synergistically in PKC412-resistant mutant FLT3-expressing cells suggests that IAP inhibitors like LBW242 could potentially be used in conjunction with other agents to achieve a higher degree of patient responsiveness by suppressing the emergence of drug-resistant FLT3 mutations.

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