Antileukemic Activity of Lysophosphatidic Acid Acyltransferase-β Inhibitor CT32228 in Chronic Myelogenous Leukemia Sensitive and Resistant to Imatinib

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Abstract

Purpose: Lysophosphatidic acid acyltransferase (LPAAT)-β catalyzes the conversion of lysophosphatidic acid to phosphatidic acid, an essential component of several signaling pathways, including the Ras/mitogen-activated protein kinase pathway. Inhibition of LPAAT-β induces growth arrest and apoptosis in cancer cell lines, implicating LPAAT-β as a potential drug target in neoplasia.

Experimental Design: In this study, we investigated the effects of CT32228, a specific LPAAT-β inhibitor, on BCR-ABL-transformed cell lines and primary cells from patients with chronic myelogenous leukemia.

Results: CT32228 had antiproliferative activity against BCR-ABL-positive cell lines in the nanomolar dose range, evidenced by cell cycle arrest in G2-M and induction of apoptosis. Treatment of K562 cells with CT32228 led to inhibition of extracellular signal-regulated kinase 1/2 phosphorylation, consistent with inhibition of mitogen-activated protein kinase signaling. Importantly, CT32228 was highly active in cell lines resistant to the Bcr-Abl kinase inhibitor imatinib. Combination of CT32228 with imatinib produced additive inhibition of proliferation in cell lines with residual sensitivity toward imatinib. In short-term cultures in the absence of growth factors, CT32228 preferentially inhibited the growth of granulocyte-macrophage colony-forming units from chronic myelogenous leukemia patients compared with healthy controls.

Conclusion: These data establish LPAAT-β as a potential drug target for the treatment of BCR-ABL-positive leukemias.

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell disorder caused by the oncogenic fusion protein Bcr-Abl (1), a chimeric tyrosine kinase resulting from the (9;22) chromosomal translocation (2). The constitutively active Bcr-Abl kinase leads to tyrosine phosphorylation of substrates, thereby stimulating signaling pathways that promote cell growth and survival (3, 4). Leukemogenesis driven by Bcr-Abl depends on activation of the Ras/mitogen-activated protein kinase (MAPK) pathway (1, 5). Active Ras can bind and target the cytoplasmic Raf kinase to the cell membrane, where a kinase cascade involving MAPK kinase 1 (MAPKK1 or MEK1) and MAPK1/2 [extracellular signal-regulated kinase (ERK) 1/2] is initiated (6, 7). Raf activation requires phosphatidic acid, a lipid cofactor derived from the acetylation of lysophosphatidic acid by lysophosphatidic acid acyltransferase (LPAAT; ref. 8). LPAATs are an isoenzyme family, with the β isoenzyme (LPAAT-β) showing limited expression in normal tissues but high expression in a variety of tumor cell lines (9). The fact that LPAAT-β expression is tumor specific and that overexpressed LPAAT-β contributes to transformation in vitro has implicated this enzyme as a potential drug target for malignant diseases (9).

CML can effectively be treated with the Bcr-Abl-directed kinase inhibitor imatinib (Gleevec), with excellent response rates in early-stage CML (10). However, patients in advanced CML relapse with high frequency (11). Moreover, imatinib fails to induce a complete cytogenetic response in ~15% of patients in chronic phase CML, and only a minority of patients achieve complete molecular remission as detected by highly sensitive PCR assays, a situation referred to as disease persistence at the molecular level (12). Several mechanisms causing primary
resistance have been proposed, including quiescence of leukemic progenitor cells (13), drug efflux (14), mutations in the imatinib-binding kinase domain (15), or the activation of alternative pathways, including MAPK (16). It is thought that the latter may be compensating for inactivation of the Bcr-Abl kinase.

CT32228, an aryldiaminotriazine, is a small-molecule isoenzyme-specific inhibitor of LPAAT-β. The antiproliferative IC_{50} in a screen of multiple solid tumor cell lines was in the low nanomolar range (17). CT32228 and related compounds are highly antiproliferative and proapoptotic in multiple myeloma cells (18). Biochemical studies showed inhibition of Ras/Raf/MAPK and phosphatidylinositol 3-kinase/Akt pathways leading to cell cycle arrest and apoptosis (17). However, little is known about the antileukemic potency of LPAAT-β inhibitors in leukemia.

Given the involvement of MAPK signaling in the pathogenesis of CML and its potential role in imatinib resistance, we decided to evaluate the effects of CT32228 in inhibiting LPAAT-β activity with CT32228 in BCR-ABL-positive cell lines and primary CML cells. Moreover, we explored the activity of this small molecule in cell lines resistant to imatinib.

Materials and Methods

Cell culture and drug preparation. MO7e (19), a human megakaryoblastic cell line, was grown in RPMI 1640 with 20% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA), 2% L-glutamine (Life Technologies), 1% penicillin/streptomycin, and 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Immunex, Seattle, WA). 32D (20), a murine myeloid hematopoietic cell line, was grown in RPMI 1640 with 10% FBS, 2% L-glutamine, 1% penicillin/streptomycin, and 15% WEHI-conditioned medium as the source of interleukin-3 (IL-3). All other cell lines were grown in RPMI 1640 with 10% fetal bovine serum, 2% L-glutamine, and 1% penicillin/streptomycin. The cytokines (IL-3, GM-CSF, SCF, and +/- EPO) with graded concentrations of CT32228. Cytokine concentrations were as given for the semisolid medium protocol (see above). After 3 days, cells were washed and subsequently plated in duplicate in methylcellulose medium as described above. After a 2-week incubation at 37°C, BFU-E and CFU-GM colonies were counted. Results were calculated as the percentage of control. Statistical significance of differences was derived using the t test (SPSS software, SPSS, Inc., Chicago, IL).

Measurement of cell proliferation. The tetrazolium-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assays were done as described previously (26). Exponentially growing cells were plated in triplicate or quadruplicate at 5 × 10^4 per well and exposed to escalating doses of each drug independently or in combination. Cell proliferation was measured with MTT-based (Sigma, St. Louis, MO) or MTS-based viability assay (CellTiter 96 Aqueous One Solution Reagent, Promega, Madison, WI) at 24-hour intervals under exponential growth conditions. Means and SDs generated from three to four independent experiments are reported as the percentage of growth versus control at 72 hours. The IC_{50} and IC_{90} values were derived manually from the dose–response curve generated by Microsoft Excel.

Combination studies were designed according to Chou and Talalay (27): cell lines were incubated with increasing doses of drug dilutions or combinations of drug dilutions. CalcuSoft software (Biosoft, Cambridge, United Kingdom) was used to calculate the combination index at different levels of growth inhibition as a quantitative measure of the degree of drug interaction. Combination index values greater than 1 indicate antagonism, values equal to 1 indicate additivity, and values lower than 1 indicate synergy. Combined drugs were used at fixed molar ratios to accommodate software requirements. Equitoxic drug doses that produced ~50% of growth inhibition in single-agent experiments were chosen to determine an appropriate fixed molar ratio of 2 combined drugs.

Immunoblotting. K562 cells were cultured with increasing concentrations of CT32228 or with 0.01% DMSO as solvent control for up to 72 hours, and aliquots of cultures were harvested at certain intervals. For immunoblot analysis of Bcr-Abl, ERK and phosphorylated ERK, total phosphotyrosine, and Akt and phosphorylated Akt, the cells were...
Cell cycle analysis. K562 cells were incubated in the presence of CT32228 or DMSO only for up to 48 hours. Cells were fixed and stained according to the manufacturer’s instructions and analyzed on a Guava Technologies Personal Cell Analysis instrument equipped with Cytosoft software (Guava Technologies, Hayward, CA). The relative percentages of cells in G1, S, or G2-M phase were calculated from FL-2 histograms using ModFit LT software.

Detection of apoptosis and activated caspase-3. K562 cells were cultured at $10^5/mL$ and incubated in the presence of different concentrations of CT32228 or with DMSO alone, as a control, for 72 hours. The cells were harvested, stained with Alexa Fluor 488 Annexin V and propidium iodide (PI; Molecular Probes, Eugene, OR) according to the manufacturer’s instructions, and analyzed on a Becton Dickinson FACSAria flow cytometer. Results based on two independent triplicate experiments are reported as averages ± SE. For flow cytometric detection of activated caspase-3, K562 cells were cultured at $10^5/mL$ and incubated in the presence of different concentrations of CT32228 or with DMSO alone, as a control. The cells were harvested and analyzed at 24, 48, and 72 hours according to the manufacturer’s instructions (Caspase-3 Detection kit, Calbiochem, San Diego CA). Results based on three independent experiments are reported as averages ± SE.

Additionally, cytospin slides of the respective samples were analyzed by Giemsa staining (28).

### Table 1. Inhibitory concentrations of CT32228 in proliferation assays

**A. Antiproliferative activity of CT32228 against a panel of hematopoietic cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC_{50} (nmol/L)</th>
<th>IC_{80} (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32D</td>
<td>60</td>
<td>95</td>
</tr>
<tr>
<td>32D-BCR-ABL</td>
<td>80</td>
<td>112</td>
</tr>
<tr>
<td>Mo7e</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>Mo7e-BCR-ABL</td>
<td>105</td>
<td>165</td>
</tr>
<tr>
<td>AR230-s</td>
<td>23</td>
<td>35</td>
</tr>
<tr>
<td>AR230-r</td>
<td>85</td>
<td>108</td>
</tr>
<tr>
<td>BaF/3</td>
<td>88</td>
<td>125</td>
</tr>
<tr>
<td>BaF/BCR-ABL</td>
<td>85</td>
<td>110</td>
</tr>
<tr>
<td>BaF/BCR-ABL (WEHI)</td>
<td>105</td>
<td>&gt;250</td>
</tr>
<tr>
<td>BaF/BCR-ABL-r1</td>
<td>90</td>
<td>112</td>
</tr>
<tr>
<td>BaF/BCR-ABL-T315I</td>
<td>92</td>
<td>135</td>
</tr>
<tr>
<td>BaF/BCR-ABL-M351T</td>
<td>85</td>
<td>122</td>
</tr>
<tr>
<td>BaF/BCR-ABL-H356R</td>
<td>82</td>
<td>120</td>
</tr>
<tr>
<td>K562</td>
<td>56</td>
<td>115</td>
</tr>
<tr>
<td>KCL-22</td>
<td>90</td>
<td>225</td>
</tr>
<tr>
<td>Lama84</td>
<td>62</td>
<td>120</td>
</tr>
</tbody>
</table>

**B. Antiproliferative activity of CT32228 against primary hematopoietic progenitor cells**

<table>
<thead>
<tr>
<th>IC_{50} (nmol/L)</th>
<th>IC_{80} (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-GM, plus GF</td>
<td>190</td>
</tr>
<tr>
<td>Normal CML</td>
<td>410</td>
</tr>
<tr>
<td>CFU-GM, no GF</td>
<td>170</td>
</tr>
<tr>
<td>Normal CML</td>
<td>&gt;2,000</td>
</tr>
</tbody>
</table>

**NOTE:** (A) Given are drug concentrations that induce 50 (IC_{50}) or 80% (IC_{80}) growth inhibition compared with untreated control as derived from MTS/MTT assays. Given are means of three independent experiments. (B) Given are drug concentrations that induce 50 (IC_{50}) or 80% (IC_{80}) growth inhibition of CFU-GM compared with untreated control as derived from colony-forming assays. Given are means of five independent patient samples (see Fig. 2). Conditions with and without growth factors are listed (plus GF and no GF).

### Results

CT32228 has antiproliferative activity in BCR-ABL-positive and BCR-ABL-negative hematopoietic cell lines. MTS proliferation assays in the presence of increasing concentrations of CT32228 were done comparing three growth factor-dependent cell lines (BaF/3, 32D, and Mo7e) with their growth factor-independent derivatives engineered to express Bcr-Abl (BaF/Bcr-Ab1 3p210, 32Dp210, and Mo7p210). Growth inhibition by CT32228 was independent of BCR-ABL status (Fig. 1), with IC_{50} values in the low nanomolar dose range (50-105 nmol/L; Table 1). This suggested that CT32228 affects pathways...
common to IL-3 (BaF/3 and 32D) or GM-CSF (MO7e) and Bcr-Abl. Consistent with this, IL-3 (WEHI-conditioned medium) failed to rescue BaF/Bcr-Abl 3p210 cells from the effects of LPAAT-β inhibition, although we observed a small but reproducible reduction of CT32228 sensitivity in the presence of IL-3 (Table 1A). Cell lines derived from patients with CML blast crisis (AR230-s, K562, and KCL-22) showed IC_{50} values between 23 and 90 μmol/L in the same range as the values observed in the cell lines engineered to express Bcr-Abl. The fact that the sensitivity of cell lines to CT32228 was independent of their BCR-ABL status suggested that Bcr-Abl expression did not influence LPAAT-β activity. Consistent with this, we detected comparable levels of LPAAT-β activity in BaF/Bcr-Abl 3p210, MO7p210 cells, and their parental lines (data not shown).

Effects of CT32228 on primary hematopoietic cells. Next, we investigated the effects of CT32228 on primary hematopoietic cells from CML patients and healthy bone marrow donors. In the first set of experiments, we assessed colony formation by mononuclear cells grown in semisolid culture in the continuous presence of CT32228. The compound reduced formation of CFU-GM and BFU-E colonies by normal mononuclear cells in a dose-dependent manner, with IC_{50} and IC_{80} values 2 to 10 times higher than for cell lines. However, there was no significant difference between CML patients and healthy donors (Fig. 2A; Table 1B). Because previous studies had shown that inhibition of LPAAT-β activity may be more toxic to proliferating cells than to resting cells (9), we sought to exploit the known capacity of CML progenitor cells to enter the cell cycle in the absence of cytokines (29). Mononuclear cells from CML patients and healthy individuals were cultured in graded concentrations of CT32228, with or without added cytokines.

![Fig. 3. Combined antiproliferative activity of CT32228 with imatinib. BCR-ABL-transduced cell lines sensitive (BaF/BCR-ABL) and resistant (BaF/BCR-ABL-r1, BaF/BCR-ABL_{M351T}, and BaF/BCR-ABL_{T315I}) to the Abl kinase inhibitor imatinib were treated with CT32228 in the presence of increasing concentrations of imatinib (colored lines, concentrations). Percentage inhibition of untreated control (Y-axis) by increasing concentrations of CT32228 (X-axis). Points, mean of three independent experiments.](image)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dose ratio</th>
<th>IC_{50}</th>
<th>IC_{90}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baf/BCR-ABL</td>
<td>2:1</td>
<td>0.97 (0.02)</td>
<td>0.82 (0.05)</td>
</tr>
<tr>
<td>Baf/BCR-ABL_{M351T}</td>
<td>10:1</td>
<td>0.83 (0.79-0.86)</td>
<td>0.73 (0.66-0.79)</td>
</tr>
<tr>
<td>Baf/BCR-ABL_{T315I}</td>
<td>50:1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Baf/BCR-ABL-r1</td>
<td>25:1</td>
<td>0.92 (0.78-1.07)</td>
<td>0.80 (0.62-0.99)</td>
</tr>
<tr>
<td>K562</td>
<td>5:1</td>
<td>1.09 (1.03-1.16)</td>
<td>1.01 (0.94-1.08)</td>
</tr>
<tr>
<td>32Dp210</td>
<td>2:1</td>
<td>0.96 (0.16)</td>
<td>0.87 (0.14)</td>
</tr>
</tbody>
</table>

**NOTE:** Indicated cell lines were treated with a fixed ratio of increasing concentrations of either drug and analyzed by the MTS assay. Data analysis was done by the CalcuSyn software. Only cells with significant growth modulation by either drug alone can be analyzed (all except Baf/BCR-ABL_{T315I}, which is completely resistant to imatinib). Combination index values are given at different levels of growth inhibition (IC_{50} and IC_{90}) and indicate antagonism (>1), additivity (1), or synergy (<1) of combined treatment. Reported values represent mean of two or three independent experiments. The range of individual results (two experiments) or the SD (three experiments) is indicated in the parenthesis.

**Abbreviation:** NA, not analyzed.

After 72 hours, the cells were plated in semisolid medium supplemented with cytokines, and CFU-GM and BFU-E were counted after 14 days. At 0.125 μmol/L CT32228, the lowest concentration used in these experiments, BFU-E and CFU-GM derived from CML patients were reduced to 18% and 10% of controls, respectively, whereas colony formation by normal MNC was much less affected (~60% of controls; P = 0.0001). Moreover, whereas there was practically complete inhibition of colony formation by CML cells at concentrations above 0.5 μmol/L, inhibition of normal colonies plateaued at ~70% at concentrations of 0.5 μmol/L, with no increase in toxicity at higher concentrations (Fig. 2B; Table 1B).

**Effects of CT32228 on cell lines resistant to imatinib.** Resistance to imatinib as a result of increased Bcr-Abl expression or mutations in the kinase domain is a significant clinical problem (30). We therefore tested the activity of CT32228 against cell lines representing these common mechanisms of imatinib resistance. Our panel included BaF3 cells expressing several Bcr-Abl kinase domain mutants frequently detected in patients (BaF/BCR-ABL_{T315I}, BaF/BCR-ABL_{Y253F}, BaF/BCR-ABL_{M351T}, and BaF/BCR-ABL_{T315I}) (31) and sublines with significantly increased levels of Bcr-Abl (AR230-r1 and BaF3/p210-r1) (25). Only AR230-r1 cells were less sensitive to CT32228 than the parental line, whereas all others showed very similar sensitivity (Table 1).

LPAAT-β inhibitors may eventually be combined with imatinib to overcome drug resistance. We therefore tested the combination of imatinib and CT32228 for its activity in BaF/3 cells expressing wild-type Bcr-Abl, the T315I and M351T mutants, as well as a subclone with increased protein expression. We observed additive to synergistic effects in all cell lines with residual sensitivity to imatinib, but not in the T315I mutant, which is completely resistant to the drug (Fig. 3; Table 2). These results suggest that combinations of
LPAAT-β inhibitors and imatinib may be clinically beneficial in patients with imatinib resistance, as long as there is residual sensitivity to imatinib. This is consistent with our previous observations (26).

**CT32228 induces a G2-M arrest and apoptosis.** To investigate the cellular mechanisms leading to growth inhibition in the presence of CT32228, we treated K562 cells with CT32228 for 1, 6, 12, and 24 hours and did cell cycle analysis by PI staining. For these experiments, a dose of 115 nmol/L was chosen, corresponding to the IC80 in the MTS assays (Table 1). As shown in Fig. 4, K562 cells started to accumulate in the G2-M phase after 6 hours of incubation. At 24 hours, ~50% of the cells were in G2-M, and an increase of cells in the sub-G1 (hypodiploid) compartment was evident, indicating cell death (Fig. 4). Giemsa staining of K562 cells treated with 115 nmol/L CT32228 for 24 hours showed morphologic evidence of mitotic catastrophe as well as apoptosis (Fig. 5A).

To verify specificity of the cell cycle effect, this experiment was also done using another CML cell line (Lama84). As seen with K562 cells, treatment with CT32228 leads to G2-M arrest of Lama84 cells with similar kinetics shown with K562 (data not shown).

Because the hierarchical activation of caspasas by proteolytic cleavage is a key event in the extrinsic and intrinsic pathways of apoptosis, we analyzed caspase activation in cellular lysates prepared from K562 exposed to graded concentrations of CT32228. Caspase-3, caspase-8, and caspase-9 were detected by immunoblotting with specific antibodies. However, in multiple repeat experiments and despite using multiple different antibodies, we were unable to detect significant cleavage of any of these caspasas on immunoblots even at time points when a considerable proportion of cells were apoptotic as assessed by Annexin V/PI staining (data not shown). Hideshima et al. reported that the levels of cleaved caspase-3 in multiple myeloma cells treated with a CT32228 analogue are relatively low, suggesting that our failure to detect cleavage in the K562 cells was related to the limited sensitivity of the immunoblots. We therefore analyzed caspase-3 activation by fluorescence-activated cell sorting. Cells were exposed to 115 nmol/L CT32228 for up to 72 hours and analyzed daily after staining with Annexin V/PI or anti–active caspase-3 (Fig. 5B).

After 24 hours, the Annexin V-positive/PI-negative cell fraction was ~12%, whereas caspase-3 activation became detectable after 48 hours. Immunoblot detection of cleaved PARP, a signaling marker for caspase-dependent apoptosis and, as recently recognized (32), caspase-independent apoptosis, was positive after 24 hours (Fig. 5C).

**CT32228 inhibits MAPK signaling in BCR-ABL-positive cells.** To exclude any nonspecific effects of CT32228 on Bcr-Abl kinase activity or protein levels, we did immunoblots on lysates from K562 cells treated with graded concentrations of CT32228 for up to 48 hours. As expected, CT32228 did not affect Bcr-Abl protein levels or phosphorylation (Fig. 6). We next examined effects of CT32228 on pathways downstream of
Bcr-Abl. CT32228 has been shown to inhibit translocation of Raβ1 to the plasma membrane (9). We reasoned that this might interfere with signal transduction from Ras to MAPK. Consistent with this hypothesis, a significant reduction of ERK phosphorylation was detectable at 24 hours and was more pronounced with higher concentrations of CT32228 (Fig. 6). At 48 hours, phosphorylation was partially restored in cells treated with 56 nmol/L CT32228, the concentration corresponding to the IC₅₀ in cell proliferation assays. In contrast to ERK, phosphorylation of Akt was not altered by treatment with CT32228, suggesting that this compound does not affect signaling via the phosphatidylinositol 3-kinase pathway in BCR-ABL-positive cells (Fig. 6).

**Discussion**

To assess the potential of LPAAT-β inhibition as a treatment of BCR-ABL-positive leukemias, we tested the effects of CT32228, a specific inhibitor of LPAAT-β in a panel of BCR-ABL-positive and BCR-ABL-negative cell lines as well as primary cells from CML patients. We found that CT32228 has strong antiproliferative activity in the nanomolar dose range against all cell lines tested, regardless of their BCR-ABL status. Consistent with this lack of differential activity, LPAAT-β activity was not significantly different between Mo7e and Ba/F3 cells transduced with BCR-ABL and the parental lines, and CT32228 did not have detectable effects on Bcr-Abl expression or tyrosine phosphorylation.

Effective and tolerable treatment of leukemia depends on differential activity of the antileukemic agent, which should preferentially kill leukemic cells while sparing normal tissues from cytotoxic effects. Our experiments using short-term liquid cultures followed by colony-forming assays reveal differential activity of CT32228 in CML cells compared with normal progenitor cells only when cultured in the absence of growth factors. Under these conditions, normal cells were protected, whereas in the presence of cytokines, no differential effect was observed. Of note, at increasing concentrations of CT32228, growth factor-independent CML progenitor colonies were practically eradicated, whereas healthy progenitor colonies plateaued at 70% growth inhibition. The difference seen in the absence of cytokines may reflect the fact that only CML cells are capable of entering the cell cycle in the absence of cytokines and could evidently be exploited for in vitro purging of CML bone marrow (29). Of note, Hideshima et al. (18) reported that a CT32228 analogue had only minor effects on the growth of normal mononuclear cells. Whether a differential effect of CT32228 against CML versus normal progenitor cells will be seen in vivo may ultimately depend on local cytokine concentrations in the bone marrow.

Combinations of CT32228 with imatinib revealed additivity or synergism both in imatinib-sensitive and imatinib-resistant cell lines. However, this was limited to cells with residual sensitivity (i.e., sensitive to dose escalation) to imatinib, whereas no synergism or additivity was seen in the T315I mutant (i.e., insensitive to dose escalation). These data are consistent with our previous observations using combinations of imatinib and decitabine or arsenic trioxide (26). Taken together, the results of the present study show that CT32228 has considerable antileukemic activity as a single agent and in combination with imatinib.

Inhibition of proliferation of K562 cells by CT32228 was associated with blockade of the cell cycle in G₂-M (Fig. 4). This observation is in line with data reported by Hideshima et al. (18), who analyzed CT3228-related compounds with anti-LPAAT-β activity in multiple myeloma cells. As early as 24 hours after start of treatment with CT32228, K562 showed...
morphologic changes consistent with mitotic catastrophe, a finding that is consistent with the cell cycle analysis. The mechanism responsible for the G2-M arrest in the K562 cells is unclear. Hideshima et al. (18) suggested that the G2-M arrest may be triggered through a p53-dependent DNA damage response involving up-regulation of p21. However, as K562 cells lack functional p53 (33), this mechanism cannot account for the G2-M arrest in this cell line. Alternatively, a p53-independent mechanism via p14(ARF) may account for the G2-M arrest in our hands (34). Further experiments will be necessary to determine the underlying mechanism.

FACS analysis with Annexin V/PI-negative staining showed that K562 cells treated with CT32228 die via apoptosis (Fig. 5). Consistent with this, we were able to show cleavage/activation of caspase-3 and cleavage of PARP. It should be noted that the extent of caspase-3 cleavage, detectable by FACS but not on immunoblots, was disproportional and delayed in relation to the significant percentage of apoptotic cells appearing as early as 24 hours after start of treatment. This suggests that a caspase-3-independent mechanism must be responsible for apoptosis induction, at least early after initiating treatment. In line with this observation, cleavage of PARP, a readout molecule for caspase-dependent and caspase-independent apoptosis, was detectable before caspase-3 activation (32).

Because lysophosphatidic acid is required for the activation of Raf1, a key intermediate in the Ras/MAPK pathway, depletion of lysophosphatidic acid by LPAAT-β inhibition was expected to inhibit ERK1/2. Consistent with this, ERK1/2 phosphorylation was reduced in cells exposed to CT32228 (Fig. 6). As activation of MAPK signaling has been reported in response to imatinib treatment as a potential resistance-inducing pathway (16), inhibitors thatcounteract MAPK activity may be of clinical value to overcome imatinib resistance. In contrast to ERK inhibition, LPAAT-β inhibition did not affect phosphatidylinositol 3-kinase/Akt signaling in K562 cells (Fig. 6), although Coon et al. (17) observed a significant effect on Akt signaling in vascular smooth muscle cells and a minor effect in a lymphoma cell line. The biochemical basis for this apparent cell line specificity remains elusive.

In summary our data implicate LPAAT-β as a therapeutic target in BCR-ABL-positive leukemias. Although the clinical usefulness of CT32228 is limited by its low bioavailability due to hydrophobicity, the development of compounds with more favorable pharmacokinetic properties is ongoing. Given the excellent activity of CT32228 in solid tumor cell lines (9), inhibition of LPAAT-β may be a broadly applicable therapeutic strategy in malignant diseases.

References
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