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Therapeutic targeting of Polo-like kinase-1 and Aurora kinases in T-cell acute lymphoblastic leukemia

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Polo-like kinases (PLKs) and Aurora kinases (AKs) act as key cell cycle regulators in healthy human cells. In cancer, these protein kinases are often overexpressed and dysregulated, thus contributing to uncontrolled cell proliferation and growth. T-cell acute lymphoblastic leukemia (T-ALL) is a heterogeneous malignancy arising in the thymus from T-cell progenitors. Primary chemoresistant and relapsed T-ALL patients have yet a poor outcome, therefore novel therapies, targeting signaling pathways important for leukemic cell proliferation, are required. Here, we demonstrate the potential therapeutic effects of BI6727, MK-5108, and GSK1070916, three selective inhibitors of PLK1, AK-A, and AK-B/C, respectively, in a panel of T-ALL cell lines and primary cells from T-ALL patients. The drugs were both cytostatic and cytotoxic to T-ALL cells by inducing G_2/M-phase arrest and apoptosis. The drugs retained part of their pro-apoptotic activity in the presence of MS-5 bone marrow stromal cells. Moreover, we document for the first time that BI6727 perturbed both the PI3K/Akt/mTORC2 and the MEK/ERK/mTORC1 signaling pathways, and that a combination of BI6727 with specific inhibitors of the aforementioned pathways (MK-2206, CCI-779) displayed significantly synergistic cytotoxic effects. Taken together, our findings indicate that PLK1 and AK inhibitors display the potential for being employed in innovative therapeutic strategies for improving T-ALL patient outcome.

Introduction

The aberrant growth of cancer cells can often be explained also by a dysregulation of the control of both cell cycle and cell division. Cell proliferation encompasses a tightly controlled set of events that are regulated by several nuclear protein kinases. These include checkpoint kinases (CHK), cyclin-dependent kinases (CDK), Polo-like kinases (PLKs), and Aurora kinases (AKs). In cancer, these protein kinases are often dysregulated and cause uncontrolled cell proliferation and growth.[1]

In humans, 5 members of the PLK family of serine/threonine protein kinases have been identified, and PLK1 is the best characterized.[1] PLK1 plays a fundamental role in cell division and its inhibition leads to a failure in completing mitosis.[2] Moreover, PLK1 strongly promotes cell cycle progression.[3] As PLK1 is overexpressed in a broad range of human tumors, it is currently regarded as a potential target for drug development in cancer therapy.[4] The Aurora kinase (AK) family of serine/threonine kinases comprises 3 members referred to as Aurora kinase-A, -B, and -C (AK-A, AK-B and AK-C, respectively). AKs are involved in centrosome function and mitotic spindle assembly, participate in the kinetochore complex, and favor cytokinesis.[5] However, both AK-A and AK-B have been associated with tumorigenesis and their overexpression is frequently observed in multiple tumor types, where it is correlated with higher grades of malignancy, higher proliferation rates, and poor prognosis.[3,6-8] Unlike AK-A and AK-B that are ubiquitously expressed in all mammalian tissues, AK-C expression is restricted to the testis, where it is involved in meiosis.[9]

As far as malignant blood disorders are concerned, PLK1 is expressed at high levels in chronic myelogenous leukemia (CML),[10] acute myelogenous leukemia (AML),[11] and acute lymphoblastic leukemia (ALL).[12] Overexpression of AK-A and -B has

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also been reported in a number of hematological malignancies, which include CML, AML, and ALL.[13]

Several PLK and AK inhibitors have been synthesized and evaluated in vitro and in animal models, and some of them have reached phase I/II clinical trials for cancer therapy.[14] Regarding leukemias, AT9283, an inhibitor of AK-A and -B, has been tested in patients with relapsed/refractory AML,[15] while barasertib (AZD1152), an inhibitor of AK-B, has been evaluated in a phase II clinical trial in AML patients aged ≥60 y.[16] Moreover, the PLK1 inhibitor BI2536 has been tested in elderly patients with refractory/relapsed AML.[11]

Regarding ALL, preclinical studies on the efficacy of PLK1 and AK inhibitors have mostly focused on B-cell ALL (B-ALL), which is the most common malignancy in children,[17-19] while very little is known about the relevance of PLK1 and AKs as therapeutic targets in T-cell ALL (T-ALL). T-ALL is an aggressive malignant disorder resulting from the occurrence of variable mutations in progenitor cells committed to differentiate into T-cell lineage. Although the prognosis of T-ALL has improved, especially in children, the outcome is still poor for patients refractory to chemotherapy or for those who relapse.[20,21] Therefore, it would be very important to identify novel, specific therapeutic targets and strategies for improving T-ALL prognosis in patients who do not respond to current treatment protocols or relapse.[22,23]

Here, we analyzed the efficacy of BI6727,[24] MK-5108,[25] and GSK1070916,[26] 3 selective inhibitors of PLK1, AK-A, and AK-B/C, respectively, in a panel of T-ALL cell lines and primary cells from T-ALL patients. We observed that these drugs were able to induce mitotic arrest and apoptosis of leukemic cells. However, we demonstrated that BI6727 perturbed both the PI3K/Akt/mTORC2 and the MEK/ERK/mTORC1 signaling pathways, and that a combination of BI6727 with either MK-2206 (an Akt inhibitor[27]) or CCI-779 (temsirolimus, an mTORC1 inhibitor[28]) displayed a significant synergistic cytotoxic effect. Altogether, our data demonstrate the potential of PLK1 and AK inhibitors in T-ALL treatment.

Results

Inhibitors of PLK1, AK-A, and AK-B affect viability of T-ALL cell lines

We first investigated the expression of PLK1, AK-A, and AK-B in a panel of T-ALL cell lines. Western blot analysis demonstrated that the CCRF-CEM cell line expressed the lowest levels of both PLK1 and AK-A proteins. BE-13, MOLT-4, Jurkat, and HPB-ALL cell lines expressed comparable levels of AK-A. PLK1 was expressed at the highest level in Jurkat cells, whereas the expression levels of AK-B were similar among T-ALL cell lines. PLK1, AK-A, and AK-B were phosphorylated at amino acidic residues indicative of their activation (**Fig. 1A**). The effects of the inhibitors on T-ALL cell viability were evaluated by MTT assays after 48 h treatment, and the IC_{50} was calculated for each drug. Given that PLK1 and AKs regulate cell cycle and mitosis, we chose to evaluate drug efficacy at 48 h, which is beyond the replication time of all the tested cell lines. Each drug inhibited the phosphorylation of the respective target (**Fig. 1B**). However, the AK-A inhibitor MK-5108 also decreased the levels of p-PLK1, consistently with AK-A being upstream of PLK1.[29]

Most cell lines displayed an IC_{50} for BI6727 around 0.02–0.15 µM, whereas the IC_{50} of CCRF-CEM cells was 1.0 µM (**Fig. 1C**). As to GSK1070916, the IC_{50} ranged from 0.02 to 11.26 µM, while the IC_{50} for MK-5108 was comprised in a range between 0.6 and 2.23 µM. Overall, these findings documented that the PLK1 inhibitor, BI6727, was more efficacious than the other drugs we tested on this panel of T-ALL cell lines.

Inhibitors of PLK1 and AKs block cells in the G_2/M phase of the cell cycle

In order to better characterize the drug effects on cell cycle progression, CCRF-CEM, HPB-ALL, and MOLT-4 cell lines were incubated for 48 h with either PLK1 or AK inhibitors at their respective IC_{50}. Flow cytometric analysis of PI-stained samples documented a decrease in the percentage of cells in G_0/G_1 phase of the cell cycle and a concomitant increase in the G_2/M phase (**Fig. 2A**). Of note, there also was the appearance of a sub-G_0/G_1 peak, which was indicative of DNA fragmentation and apoptosis. Another interesting consequence of the treatment with AK inhibitors was the increase in hyperdiploid cells, as indicated by the presence of peaks with a DNA content greater than 2n (G_0/G_1) or 4n (G_2/M), an observation which was consistent with the findings previously reported for neuroblastoma cells.[24]

Moreover, BI6727 treatment of MOLT-4 cell line prevented the orderly bipolar mitotic spindle formation, as revealed by immunofluorescence staining with an anti-β-tubulin antibody (**Fig. 2B**). In agreement with recent investigations performed in solid tumors,[30,31] we detected an increase in the expression levels of Ser10 p-histone H3 (an established bio-marker for both PLK1 and AK-A activity) in BE-13 and CCRF-CEM cell lines treated with either BI6727 or MK-5108.

Inhibitors of PLK1 and AKs had pro-apoptotic effects on T-ALL cell lines and synergized in reducing cell viability

It was then investigated whether the drug effects on cell viability could be also related to apoptosis.

Flow cytometric analysis of Annexin V/FITC-stained MOLT-4 and BE-13 cells demonstrated an increase in both early (single-positive for Annexin V) and late (double positive for Annexin V and PI) cells in MOLT-4 cells treated with BI6727 and in BE-13 cells treated with GSK1070916 (**Fig. 3A and B**). We also analyzed caspase-7 and poly (ADP-ribose) polymerase (PARP) cleavage. Western blot analysis documented caspase-7 and PARP cleavage in BE-13, CCRF-CEM, and MOLT-4 cell lines treated with BI6727, GSK1070916, or MK-5108 (**Fig. 3C**). Overall, these findings and those highlighted in **Figure 2** demonstrated that inhibition of either PLK1 or AK-A/-B activity decreased viability of T-ALL cell lines by inducing both a cell cycle arrest in the G_2/M phase of the cell cycle and apoptosis.

PLK1 and AKs directly interact at the molecular level in the regulation of cell cycle progression, and more effective antileukemic effects may thus be achieved when targeting of both enzymes is combined.[32] This could allow the use of lower drug concentrations resulting in less severe side effects. We, therefore, investigated whether the combination of PLK1 and AK-A inhibitors

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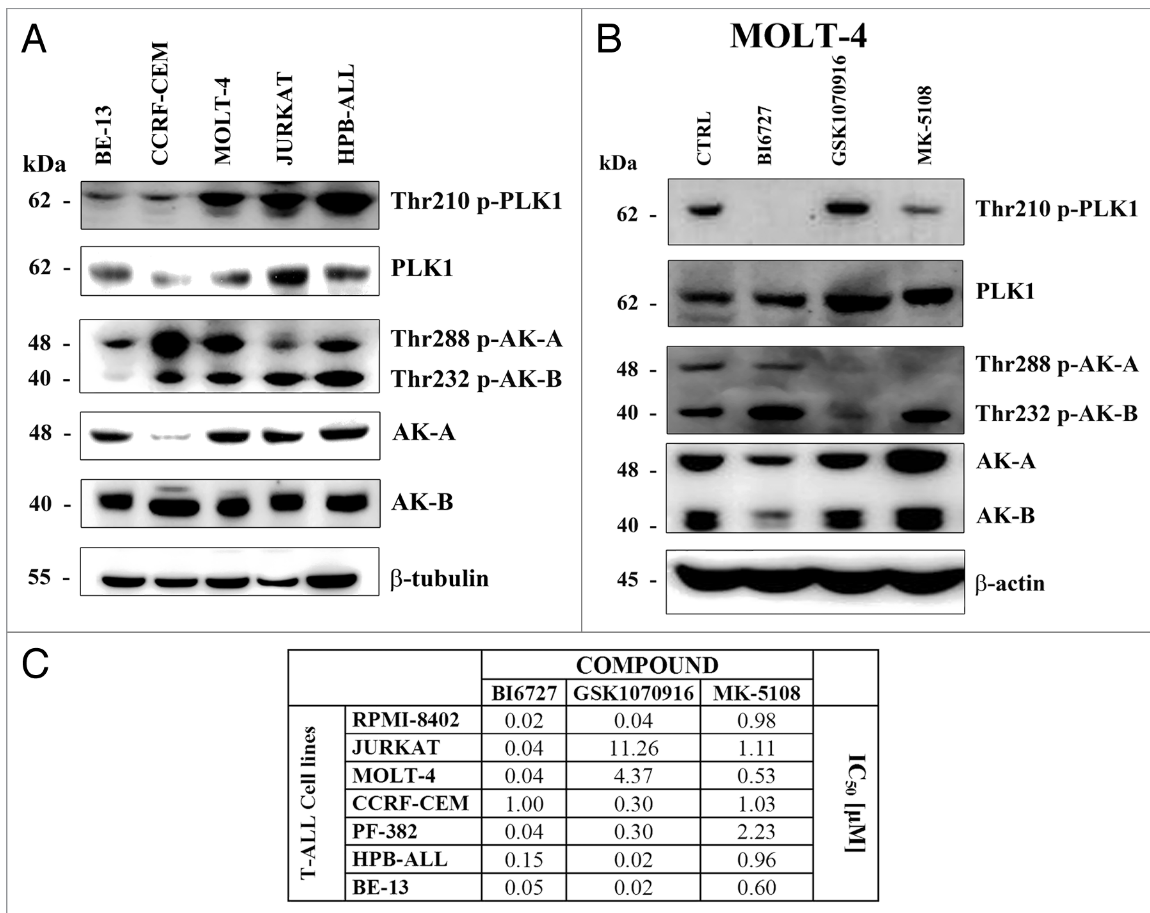
could synergize in T-ALL cells. HPB-ALL cells were treated with increasing concentrations of BI6727 and MK-5108 for 48 h, either alone or in combination. MTT assays documented the existence of a strong synergism (CI < 0.3) at drug concentrations lower than their respective IC_{50}.

BI6727 also displays pro-apoptotic effects in the presence of mouse stromal cells

It is well documented how leukemic cells interact with cells of the bone marrow microenvironment that provides a protection from chemotherapy treatment and induces a drug-resistant phenotype.[33] MS-5 stromal cells have been reported to secrete a variety of growth factors and cytokines as well as extracellular matrix proteins[34] that could recreate in vitro most of the in vivo interactions between leukemic cells and the bone marrow microenvironment.[35] We used a Transwell system that allowed a cross-talk between MOLT-4 cells and the mouse stromal cells MS-5 by the diffusion of small molecules. We analyzed the difference of viability of MOLT-4 and MS-5 cells treated with BI6727 when grown alone or co-cultured. Cell viability, as assessed by MTT assays, decreased ($P = 0.0087$) when MOLT-4 cells were cultured alone if compared with MOLT-4 cells co-cultured with MS-5 cells (**Fig. 4A**). However, the drug retained part of its pro-apoptotic activity, as indicated by PARP cleavage, which was detected by western blot (**Fig. 4B**). Moreover, flow cytometric analysis on $CD45^+$-gated cells confirmed that BI6727 retained its pro-apoptotic effect even when MOLT-4 cells were directly added to MS-5 monolayers. (**Fig. 4C**).

PLK1 inhibition influences both PI3K/Akt/mTORC2 and MEK/ERK /mTORC1 signaling pathways in T-ALL cells

It is now emerging that PLK1 functions could be intertwined with both PI3K/Akt and MEK/ERK signaling.[36,37] Therefore, we tested whether BI6727 could modulate either of these signaling pathways. BI6727 increased the phosphorylation levels of Ser473 p-Akt (an mTORC2 substrate) as well as those of both Thr389 p-p70S6K and Ser235/236 p-S6RP, two mTORC1 down-stream substrates. In contrast, the total expression levels of these proteins were unaffected by the drug (**Fig. 5A**). Similar results were detected with CCFR-CEM cells (not shown). Therefore, we treated MOLT-4 cells with CCI-779 used either as a single agent or in combination with BI6727. Although CCI-779 is mainly considered to be an mTORC1 inhibitor, it is also capable of inhibiting mTORC2 activity, especially when used in cells of hematopoietic



T-ALL Cell lines		COMPOUND			
		BI6727	GSK1070916	MK-5108	IC_{50} [μM]
	RPMI-8402	0.02	0.04	0.98	
	JURKAT	0.04	11.26	1.11	
	MOLT-4	0.04	4.37	0.53	
	CCRF-CEM	1.00	0.30	1.03	
	PF-382	0.04	0.30	2.23	
	HPB-ALL	0.15	0.02	0.96	
	BE-13	0.05	0.02	0.60	

Figure 1. Effects of PLK1 and AK-A/-B inhibitors on T-ALL cell viability. (**A**) BE-13, CCRF-CEM, MOLT-4, Jurkat, and HPB-ALL cell lines were collected, lysed, and analyzed by western blot for the expression of PLK1 and AK-A/-B and of their phosphorylated forms. Molecular weights are indicated on the left. (**B**) MOLT-4 cells were treated with BI6727, GSK1070916, and MK-2206 at the respective IC_{50} for 48 h; next they were collected, lysed, and analyzed by western blot. Molecular weights are indicated on the left. CTRL, untreated cells. (**C**) T-ALL cell lines were treated with the drugs for 48 h. Next the rates of survival were evaluated by MTT assays. Data are representative of 3 independent experiments and SD was less than 10%.

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lineage.[38] Consistently, CCI-779, when used either as single agent or in combination with BI6727, blocked the upregulation of p-Akt, whereas total levels of expression were unaffected by the drugs (**Fig. 5A**). Overall, these results suggested that inhibition of PLK1 may led to upregulated mTORC1/mTORC2 signaling. Nevertheless, it should be considered that mTORC1 activity could also be regulated through MEK/ERK signaling.[39] Accordingly, treatment of MOLT-4 cells with BI6727 resulted in increased phosphorylation levels of Thr202/Tyr204 p-ERK and of its downstream substrate, Thr573 p-p90^{RSK} (**Fig. 5B**; refs. 40–42). Treatment with the MEK inhibitor U0126 blunted the phosphorylation of both ERK and p90^{RSK}. Intriguingly, U0126 did not affect the basal levels of Ser235/236 p-S6RP; however, it completely blocked S6RP phosphorylation induced by BI6727. Overall, these findings demonstrated that increased S6RP phosphorylation, which was detected in MOLT-4 in response to PLK1 inhibition, was dependent on aberrantly activated MEK/ERK signaling.

The relevance of both Akt and mTORC1 activation induced by BI6727 was investigated using MTT assays in MOLT-4 cells treated with BI6727 and either MK-2206 (an Akt inhibitor) or CCI-779, used as single agents or in combination. Both of these drugs are now being tested in phase I/II clinical trials for patients with leukemias (ClinicalTrials.gov: NCT01369849; NCT01403415). Result analysis documented that both the drug combinations were synergistic (**Fig. 5C and D**).

Inhibitors of PLK1 and AKs displayed cytotoxic effects on T-ALL patient samples

To better assess the effectiveness of PLK1 and AK inhibitors as a potential therapeutic agents in T-ALL, we examined 6 pediatric T-ALL patient samples isolated from bone marrow or peripheral blood. Samples expressed variable amount of PLK1 and AK-A/-B, as documented by western blot analysis (data not shown). The effects of the inhibitors on T-ALL lymphoblast samples were evaluated first by treating the cells with increasing concentrations of the inhibitors and then analyzing the rates of viability by MTT assays after 96 h. A marked reduction in leukemia cell viability was detected. The patient IC_{50} for BI6727 ranged between 0.11 and 0.48 μM, while for the AK inhibitors, it ranged between 0.12 and 1.39 μM (**Fig. 6A**). Induction of apoptosis in primary T-ALL cells treated with BI6727 (0.5 μM) was suggested by flow cytometric analysis of Annexin V–FITC/PI-stained samples (**Fig. 6B**). Moreover, western blot analysis of samples treated for 48 h with BI6727, GSK1070916, and MK-5108, documented a significant accumulation of the caspase-7 cleaved form, as well as of cleaved PARP (**Fig. 6C**). Interestingly, and similarly to the MOLT-4 cell line, western blotting analysis on

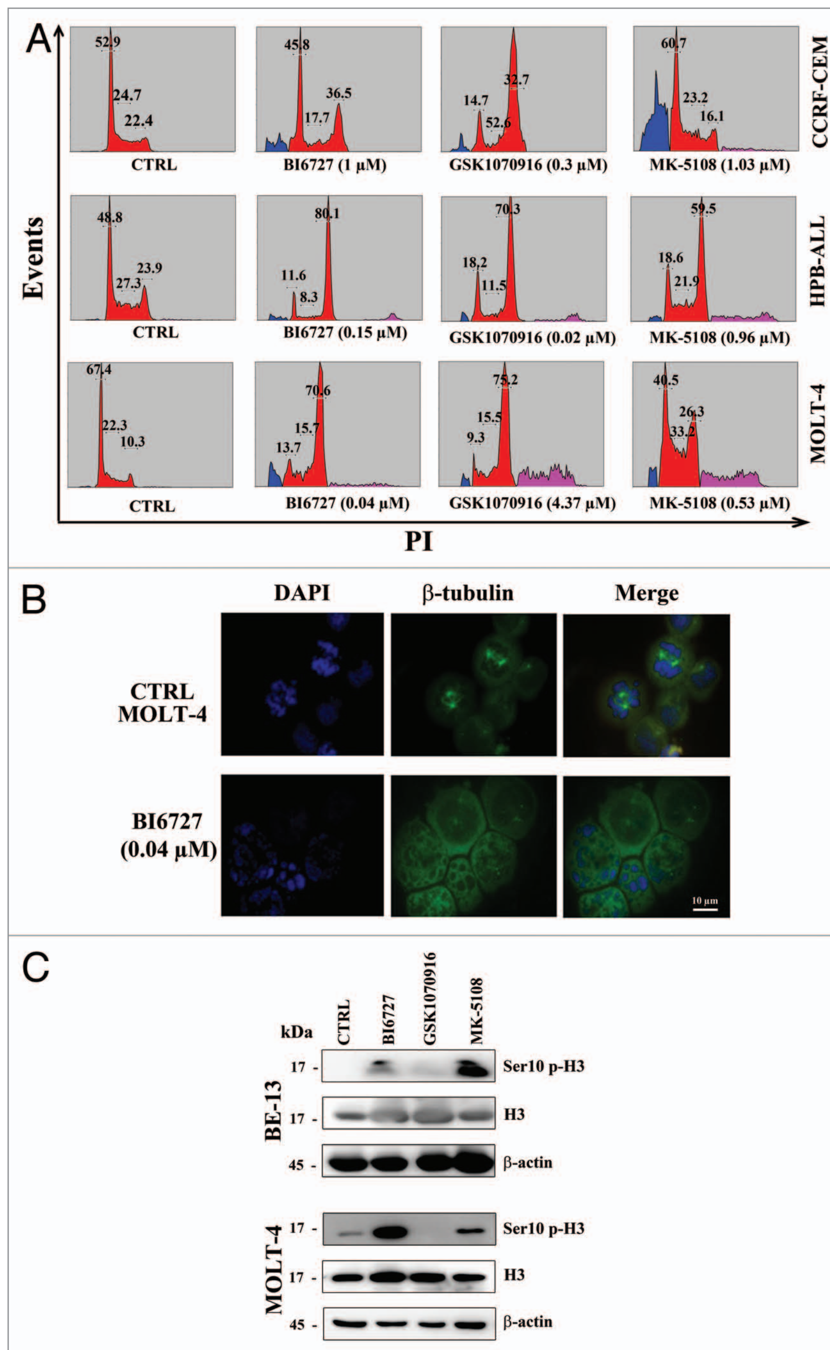

Figure 2. Effects of PLK1 and AK inhibitors on cell cycle progression of T-ALL cell lines. (**A**) CCRF-CEM, HPB-ALL, and MOLT-4 cell lines were treated for 48 h with BI6727, GSK1070916, and MK-5108. Then, cell cycle analysis was performed by flow cytometry. One representative of 3 separate experiments is shown, which yielded similar results. The percentages of the cells in the various phases of the cell cycle are indicated. (**B**) MOLT-4 cells were treated for 48 h with BI6727 (0.04 μM). Then, cells were spun down, fixed, permeabilized, and stained with antibody to β-tubulin (green). Nuclei were counterstained with DAPI (blue). (**C**) T-ALL cell lines were treated with BI6727, GSK1070916, and MK-5108 at their respective IC_{50s} for 48 h, then they were collected, lysed, and analyzed by western blot. Molecular weights are indicated on the left. CTRL, untreated cells.

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patient samples treated with BI6727 confirmed an increase in Ser473 p-Akt, Ser235/236 p-S6RP, and Thr202/Tyr204 p-ERK 1/2 levels (**Fig. 6D**).

Discussion

Primary chemoresistant and relapsed T-ALL patients have yet a poor outcome; therefore novel therapies, capable of hitting signaling pathways critical for T-ALL proliferation, are required. Traditional anti-cancer therapies rely on drugs that interfere with cell cycle progression. In particular, vinca alkaloids that interact with tubulin, preventing the microtubules polymerization in mitotic spindles,[43] and taxanes, which bind to tubulin molecules and impair the formation of the mitotic spindle,[44] block cells in the G_2/M phase of the cell cycle. However, it is well known that these compounds cause significant side effects, including neurotoxicity.[45] Therefore, more specific mitosis-targeting drugs with enhanced therapeutic efficacy and fewer side effects should be developed. Mitosis-specific kinases, such as members of PLK and AK families, were identified as potential targets for cancer treatment. Accordingly, several PLK and AK inhibitors have been developed over the last years.[14] Promising results were obtained by targeting PLK1, as its inhibition preferably kills cancer cells compared with normal cells.[46] The aim of this study was to test the therapeutic potential of PLK1 and AK inhibitors (BI6727, MK-5108, and GSK1070916) on T-ALL cell lines and primary T-ALL lymphoblasts. All these inhibitors are able to interfere with cell cycle progression, reducing drastically the cellular proliferation followed by the induction of apoptosis.

Indeed, we have documented that, even if they displayed a different efficacy, the PLK1 and AK inhibitors that we tested decreased the viability of T-ALL cell lines and primary T-ALL lymphoblasts. Among the drugs we tested, BI6727 proved to be the most efficacious in terms of IC_{50}. Only the CCRF-CEM cell line seemed to be less sensitive to BI6727 (IC_{50} = 1 μM). Interestingly, western blotting analysis documented that this cell line expressed lower levels of PLK1. Overall, our findings suggested that PLK and AK inhibitors reduced viability of most of the T-ALL cell lines and primary samples we studied, acting at submicromolar concentrations. Reduction in cell viability was due to both a block in the G_2/M phase of the cell cycle and induction of apoptosis. BI6727 and MK-5108 could be employed

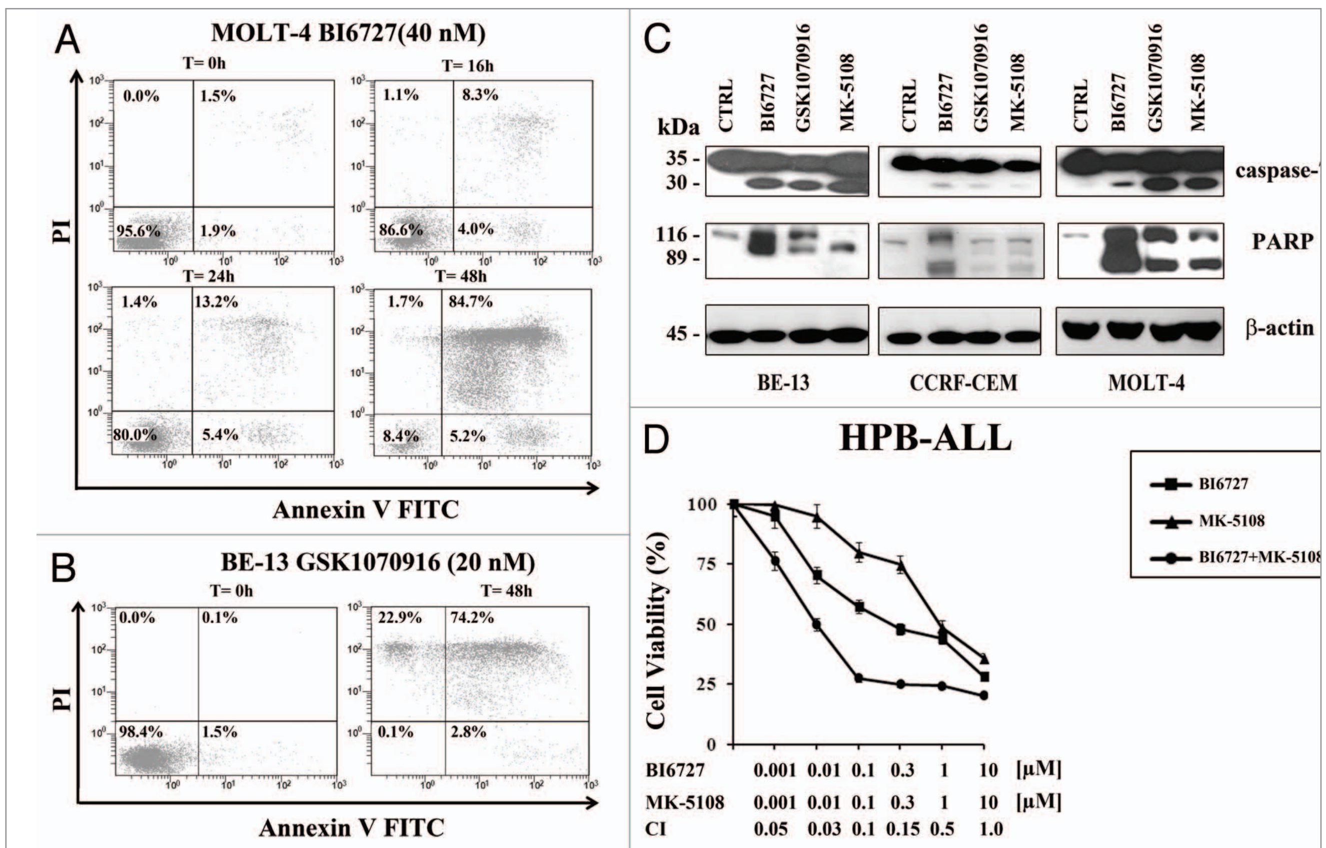

Figure 3. Inhibition of PLK1 and AK-A/-B induced apoptosis in T-ALL cell lines. (**A**) MOLT-4 cell line was treated with BI6727 (40 nM) for the indicated times, then they were collected and stained with Annexin V-FITC/PI and analyzed by flow-cytometry. (**B**) BE-13 cells were treated with GSK1070916 (20 nM) for 48 h, then they were collected, stained with Annexin V-FITC/PI, and analyzed by flow-cytometry. (**C**) T-ALL cell lines were treated for 48 h with BI6727, GSK1070916, and MK-5108 at their respective IC_{50}, then they were collected, lysed, and analyzed by western blot. Molecular weights are indicated on the left. CTRL, untreated cells. (**D**) HPB-ALL cells were treated for 48 h with BI6727 and MK-5108, used either alone or in combination. Cell viability was analyzed by MTT assays. Results are the mean of at least 3 different experiments ± SD. The combination index (CI) value for each data point was calculated with the appropriate software for dose effect analysis (CalcuSyn).

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together, and the combined treatment resulted in a synergistic reduction of cell viability.

Remarkably, BI6727 retained at least part of its pro-apoptotic activity also when MOLT-4 cells were co-cultured with MS-5 stromal cells, which partly mimic the leukemic bone marrow microenvironment that is known to promote leukemic cell proliferation and survival.[47]

An entirely novel finding that emerged from our study is that PLK1 inhibition interferes with both PI3K/Akt/mTORC2 and MEK/ERK/mTORC1, which are 2 signaling pathways frequently hyperactivated in T-ALL.[48] Indeed, we documented that when MOLT-4 cells were treated with BI6727, Ser473 p-Akt levels increased, which is indicative of upregulated mTORC2 activity. There also was an increase in the phosphorylation levels of p-70S6K and p-S6RP, two downstream targets of mTORC1. Co-treatment with CCI-779 blunted increased phosphorylation of Akt, p70S6K, and S6RP. It should be recalled here that CCI-779, besides blocking mTORC1 activity, acts as an inhibitor of mTORC2, especially when administered for extended periods of time (48 h in our case) in cells of hematopoietic lineage.[38]

Previous studies indicated that MEK/ERK signaling was upregulated in PLK1-depleted cells.[49] Accordingly, in response to BI6727, we detected increased phosphorylation of ERK and of its downstream target p90^{RSK}, an activator of mTORC1.[50] Interestingly, treatment with the MEK inhibitor U0126, besides reducing ERK and p90^{RSK} phosphorylation levels, prevented the BI6727-induced, but not the basal, S6RP phosphorylation. This indicated that in cells treated with BI6727, mTORC1 was activated by the MEK/ERK/ p90^{RSK} axis. Similar findings were also observed with T-ALL primary samples.

Both CCI-779 and MK-2206, an Akt inhibitor,[27] synergized with BI6727 in reducing the viability of MOLT-4 cells. Interestingly, a functional link between PLK1 and mTOR signaling pathway has been recently reported,[51] as a combination of BI2536 (a PLK1 inhibitor) and PI3K-mTOR dual inhibitors eradicated colon cancer stem cells, which overcome conventional

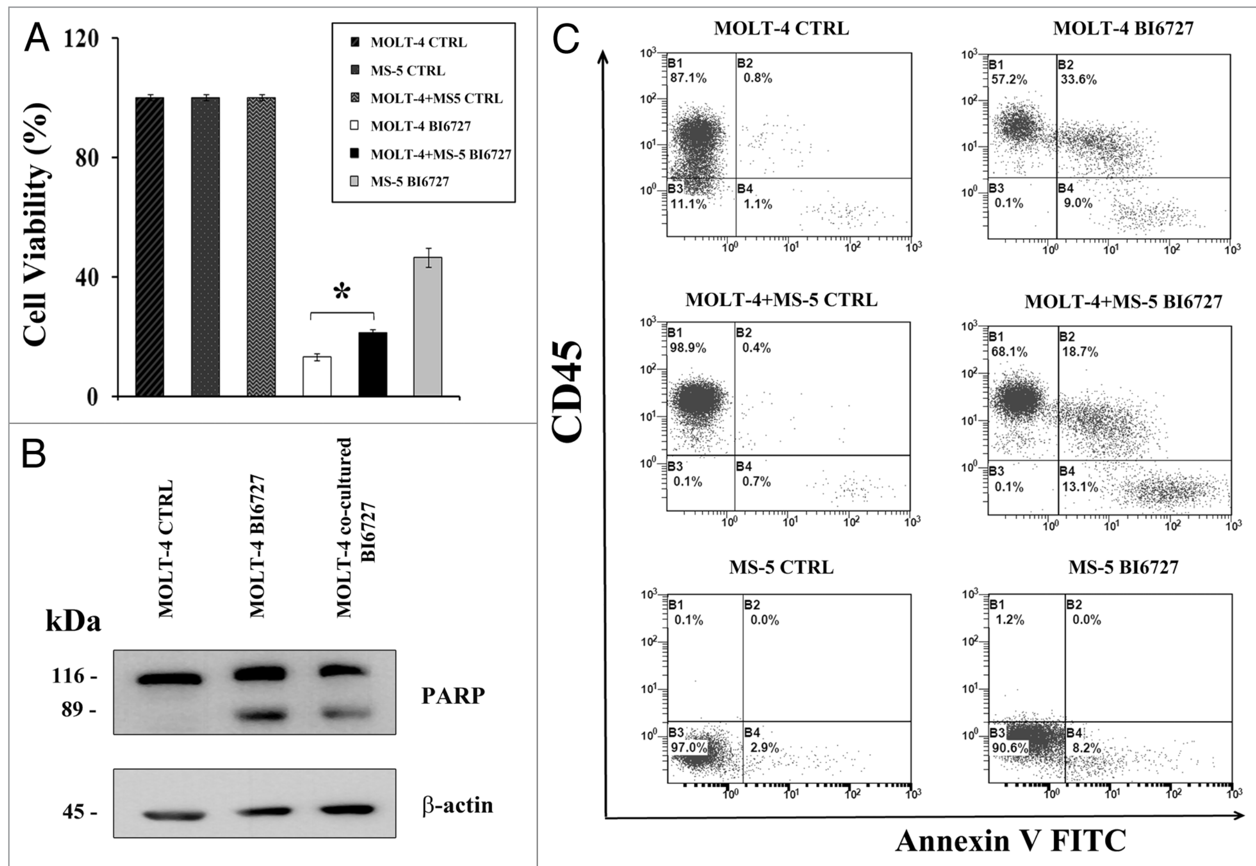

Figure 4. BI6727 retains pro-apoptotic effects also in the presence of a microenvironment of bone marrow stromal cells. (**A**) The MS-5 cell line was grown in the lower chamber of Transwell® 6-well plates, than MOLT-4 cells were added to the upper chamber containing a 0.4-µm-polyester membrane and treated with BI6727 (40 nM) for 48 h. The viability of treated cell lines grown alone and co-coltured was then evaluated by MTT assays. CTRL, untreated cells. (**B**) The MS-5 cell line was grown in the lower chamber of Transwell® 6-well plates, then MOLT-4 cells were added to the upper chamber containing a 0.4-µm-polyester membrane and treated with BI6727 (40 nM) for 48 h. Then, cells were separately collected, lysed, and analyzed by western blot for PARP cleavage. Molecular weights are indicated on the left. CTRL, untreated cells. (**C**) MOLT-4 cells were directly seeded on top of MS-5 cells and treated with BI6727 (40 nM). After 48 h, cells were harvested with trypsin/EDTA, washed, and resuspended in binding buffer containing Annexin V-FITC. Cells were counterstained with either a PE-conjugated anti-CD45 antibody or with an irrelevant isotypic control antibody and analyzed by flow cytometry after electronic gating on CD45. CTRL, untreated cells.

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cancer therapies and are involved in relapse and in tumor regeneration by switching off c-Myc protein.

Therefore, also in T-ALL, the efficacy of PLK1 inhibition could be limited by the presence of multiple feedback loops and/or cross-talk with alternative oncogenic signaling pathways.[52]

In summary, we characterized the effects of PLK1 and AK inhibitors on T-ALL cells, confirming that they are potentially useful drugs in the treatment of this disorder. The identification of a link between PLK1 and PI3K/Akt/mTORC2 and PLK1 and MEK/ERK/mTORC1 signaling pathways supports the use of a combined therapy. Hence, our data not only provided insight into the effects of PLK1 and AK inhibitors, but may also be important in the design of therapeutic protocols that involve targeting of both PLK1 and other pathways.

Materials and Methods

Materials

BI6727, GSK1070916, MK-5108, CCI-779, and UO126 were from Selleck Chemicals. For western blotting and fluorescence microscopy, primary antibodies were bought from Cell

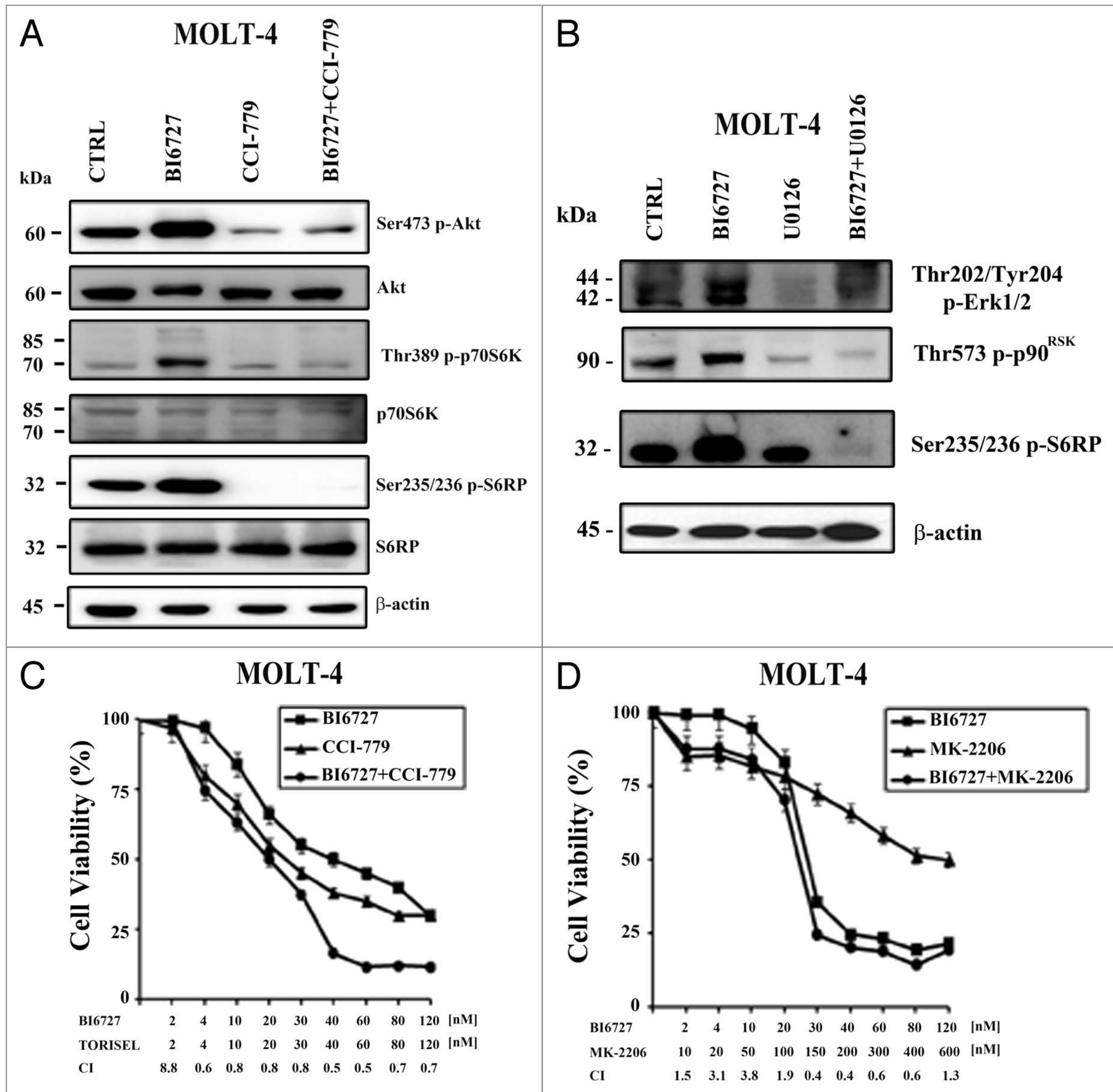

Figure 5. BI6727 upregulates PI3K/Akt/mTORC2 and MEK/ERK/mTORC1 signaling in MOLT-4 cells. (**A**) Cells were treated for 48 h with BI6727 (0.04 μM), CCI-779 (0.1 μM), and the combination of the 2 drugs, then they were collected, lysed, and analyzed by western blot. Molecular weights are indicated on the left. CTRL, untreated cells. (**B**) Cells were treated for 48 h with BI6727 (0.04 μM), U0126 (10 μM), or the 2 drugs combined, then they were collected, lysed, and analyzed by western blot. Molecular weights are indicated on the left. CTRL, untreated cells. (**C**) Cells were treated for 48 h with BI6727 and CCI-779, used either alone or in combination. The combination index (CI) value for each data point was calculated with the appropriate software for dose effect analysis (CalcuSyn). Results are the mean of at least 3 different experiments ± SD. (**D**) Cells were treated for 48 h with BI6727 and MK-2206, used either alone or in combination. The combination index (CI) value for each data point was calculated with the appropriate software for dose effect analysis (CalcuSyn). Results are the mean of at least 3 different experiments ± SD.

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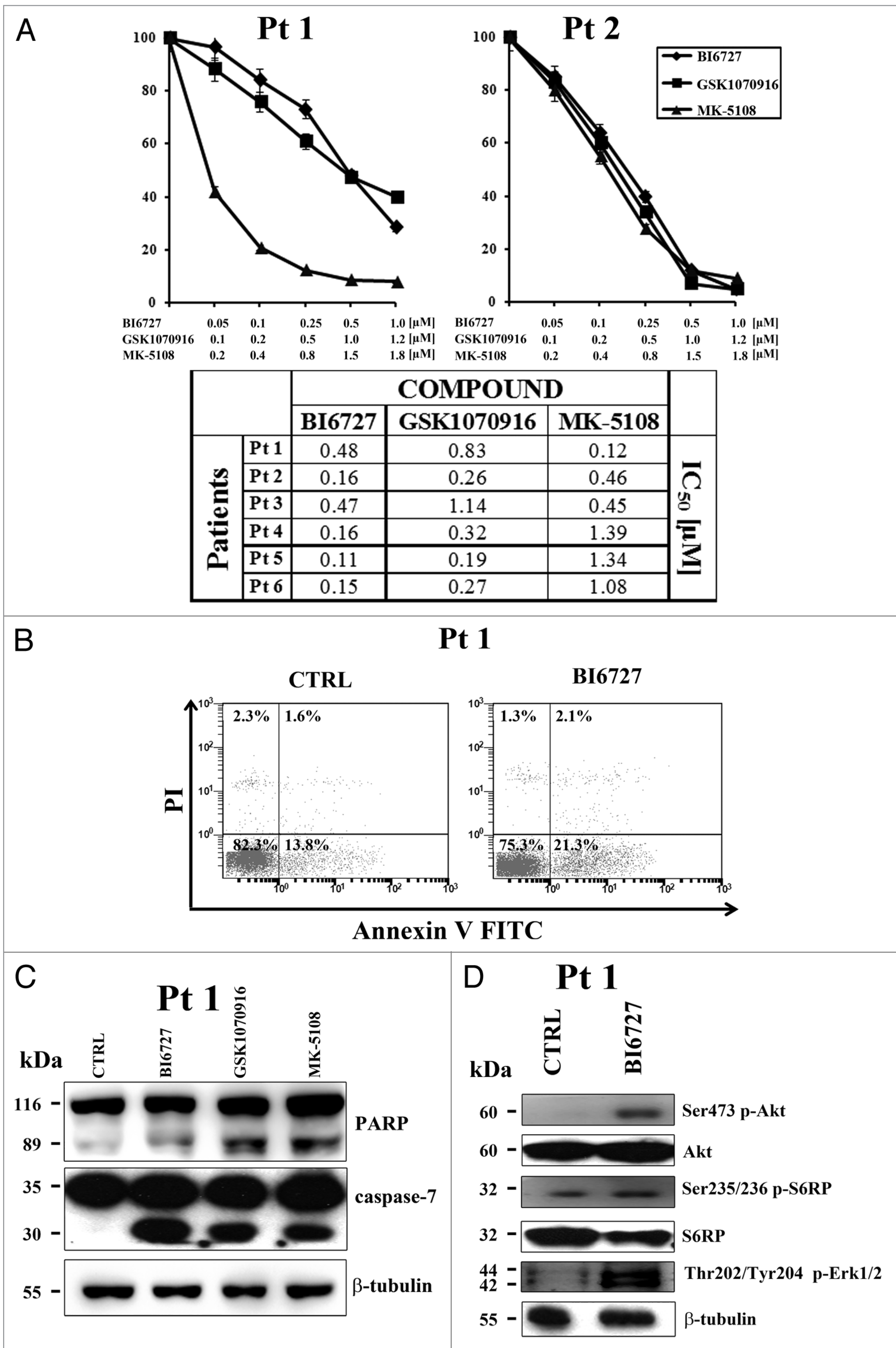

Patients	COMPOUND: BI6727	COMPOUND: GSK1070916	COMPOUND: MK-5108
Pt 1	0.48	0.83	0.12
Pt 2	0.16	0.26	0.46
Pt 3	0.47	1.14	0.45
Pt 4	0.16	0.32	1.39
Pt 5	0.11	0.19	1.34
Pt 6	0.15	0.27	1.08

IC$_{50}$ [μM]

Figure 6. For figure legend, see page 9.

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Figure 6 (see previous page). PLK1 and AK-A/-B inhibitors are cytotoxic to T-ALL primary cells. (**A**) Lymphoblasts from T-ALL patients were cultured in RPMI1640 medium supplemented with 20% FBS, insulin-transferrin-sodium selenite, and 10 ng/ml interleukin-7. MTT assays were performed on samples treated for 96 h with BI6727, GSK1070916, and MK-5108. Results are the mean of at least 2 different experiments ± SD. (**B**) Patient samples were treated with BI6727 (0.5 μM) for 48 h, then cells were collected, stained with Annexin V-FITC/PI, and analyzed by flow-cytometry for apoptosis. A representative sample is shown. CTRL, untreated cells. (**C**) Western blot analysis of a patient sample treated for 48 h with 0.5 μM BI6727, 0.5 μM GSK1070916, and 0.8 μM MK-5108. Thirty micrograms (30 μg) of protein/lane were electrophoresed on SDS-PAGE, transferred to nitrocellulose membrane, and probed with the appropriate antibodies. One representative of 2 different experiments is shown. Molecular weights are indicated on the left. CTRL, untreated cells. (**D**) Western blot analysis of a patient sample treated for 48 h with 0.5 μM BI6727. Thirty micrograms (30 μg) of protein/lane were electrophoresed on SDS-PAGE, transferred to nitrocellulose membrane, and probed with the appropriate antibodies. One representative of 2 different experiments is shown. Molecular weights are indicated on the left. CTRL, untreated cells.

Signaling Technology. A FITC-conjugated anti-rabbit IgG antibody was purchased from Sigma-Aldrich. Anti-CD45-APC-conjugated antibody for flow cytometry was obtained from Beckton-Dickinson.

Cell culture and primary T-ALL samples

The T-ALL cell lines RPMI-8402, Jurkat, MOLT-4, CCRF-CEM, BE-13, PF-382, and HPB-ALL were grown in RPMI 1640, supplemented with either 10% or 20% fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin. Samples from T-ALL pediatric patients, obtained after informed consent according to the Ethics Committee of Human Experimentation guidelines, were isolated using Ficoll-Paque (GE Healthcare Bio-Sciences AB), and grown as previously described.[53] MS-5 mouse stromal cells were grown in MEM Alpha medium supplemented with 10% FBS.

Cell viability analysis

MTT (3-[4,5-Dimethylthythiazol-2-yl]-2,5-Diphenyltetrazolium Bromide) assays were performed to assess the sensitivity of cells to drugs, as previously described.[54,55]

Cell cycle and apoptosis analysis

Flow cytometric analysis of the cell cycle was performed using a propidium iodide (PI)/RNase A staining according to standard procedures, as described previously.[56] Samples were analyzed on a FC500 flow cytometer (Beckman Coulter) with the appropriate software (CXP, Beckman Coulter). For analysis of apoptosis induction, T-ALL cell lines grown alone or co-cultured with MS-5 cells were washed twice in PBS, labeled with Annexin V/FITC in binding buffer, and then analyzed on an FC500 flow cytometer after electronic gating on $CD45^+$ leukemic cells.[53]

Western blot analysis

This was performed by standard methods, as previously reported.[57] Analysis with an antibody to either β-actin or β-tubulin demonstrated equal protein loading.

Fluorescence microscope analysis

Cells were cytocentrifuged to coverslips (200 g, 5 min), fixed, and permeabilized with 100% methanol for 3 min on ice. Samples were stained with an anti-β-tubulin antibody (1:100), followed by FITC-conjugated anti-rabbit IgG (1:500). Slides were then treated with ProLong® Gold antifade reagent (Life Technologies Italia), containing 4›,6-diamidino-2-phenylindole (DAPI). The analysis was performed on an Axio Imager Microscope (Zeiss) equipped with an Apotome module. Images were acquired with AxioVision software (Zeiss).

Combined drug effect analysis

The combination effect and potential synergy were evaluated from quantitative analysis of dose-effect relationships, as described previously.[58] For each combination experiment, a combination index (CI) number was calculated using the CalcuSyn software (Biosoft). This method of analysis generally defines CI values of 0.9 to 1.1 as additive, 0.3 to 0.9 as synergistic, and <0.3 as strongly synergistic, whereas values >1.1 are considered antagonistic.

T-ALL cell co-culture with MS-5 mouse stromal cells

MOLT-4 cells were seeded at 2.5 ´ 10^5/ml and, after an overnight incubation, cell suspension was transferred on the top of MS-5 mouse stromal cells (at 70% confluence) and treated with BI6727 (40 nM). After 48 h, MOLT-4 cells were collected, washed, and incubated with an APC-conjugated anti-CD45 antibody or with an irrelevant isotypic control antibody. After a 30-min incubation, cells were resuspended in binding buffer containing Annexin V-FITC and analyzed by flow cytometry after electronic gating on $CD45^+$ leukemic cells. In another set of experiments, MS-5 cells were grown in the lower chamber of Transwell® 6-well plates (Corning) containing a 0.4-μm polyester membrane, then MOLT-4 cells were added to the upper chamber and treated with BI6727 (40 nM). After 48 h, the viability of treated cell lines grown either alone or co-cultured was evaluated. Furthermore, cells were collected, lysed, and analyzed by western blot.

Statistical analysis

The data are presented as mean values from 3 separate experiments ± SD. Data were statistically analyzed by a Dunnet test after one-way analysis of variance (ANOVA) at a level of significance of $P < 0.05$ vs. control samples.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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