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ORIGINAL ARTICLE

Using combination therapy to override stromal-mediated chemoresistance in mutant FLT3-positive AML: synergism between FLT3 inhibitors, dasatinib/multi-targeted inhibitors and JAK inhibitors

E Weisberg[1,6], Q Liu[2,6], E Nelson[1], AL Kung[3], AL Christie[3], R Bronson[4], M Sattler[1], T Sanda[1], Z Zhao[5], W Hur[2], C Mitsiades[1], R Smith[1], JF Daley[1], R Stone[1], I Galinsky[1], JD Griffin[1] and N Gray[2]

Acute myeloid leukemia (AML) progenitors are frequently characterized by activating mutations in the receptor tyrosine kinase Fms-like tyrosine kinase-3 (FLT3). Protein tyrosine kinases are integral components of signaling cascades that have a role in both FLT3-mediated transformation as well as viability pathways that are advantageous to leukemic cell survival. The bone marrow microenvironment can diminish AML sensitivity to tyrosine kinase inhibitors. We hypothesized that inhibition of protein kinases in addition to FLT3 may be effective in overriding drug resistance in AML. We used a cell-based model mimicking stromal protection as part of an unbiased high-throughput chemical screen to identify kinase inhibitors with the potential to override microenvironment-mediated drug resistance in mutant FLT3-positive AML. Several related multi-targeted kinase inhibitors, including dasatinib, with the capability of reversing microenvironment-induced resistance to FLT3 inhibition were identified and validated. We validated synergy *in vitro* and demonstrated effective combination potential *in vivo*. In particular Janus kinase inhibitors were effective in overriding stromal protection and potentiating FLT3 inhibition in primary AML and cell lines. These results hint at a novel concept of using combination therapy to override drug resistance in mutant FLT3-positive AML in the bone marrow niche and suppress or eradicate residual disease.

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INTRODUCTION

The development of resistance in acute myeloid leukemia (AML) patients to treatment with targeted tyrosine kinase inhibitors (TKIs) is a growing area of concern. A mutated form of the class III receptor tyrosine kinase, FLT3 (Fms-like tyrosine kinase-3; human stem cell tyrosine kinase-1; or fetal liver kinase-2), is expressed in a subset of AML patients and thus represents an attractive target for small molecule inhibition.[1] Constitutively activated FLT3 occurs most frequently as internal tandem duplications (ITD) within the juxtamembrane domain, and is observed in ~20–25% of AML patients.[2]

One of the most clinically advanced kinase inhibitors for this population of AML patients is the FLT3 inhibitor, PKC412 (midostaurin),[3] which is currently being tested in Phase III clinical trials. In Phase I and II clinical trials, PKC412 was administered in sequential and simultaneous combinations with daunorubicin and cytarabine induction and high-dose cytarabine consolidation and yielded clinical responses with transient and/or reversible side effects.[4] Of the other FLT3 kinase inhibitors that are in early clinical development, particularly promising is the highly selective and potent FLT3 inhibitor, AC220 (quizartinib),[5] which has yielded encouraging results in the form of transient clinical responses as a single agent. The fact, however, that FLT3 inhibitors tested thus far clinically at best induce partial and short-lived responses in patients when used alone suggests a need for development of novel agents that can either be used effectively alone or combined with other agents to suppress disease progression and prolong lifespan.

Clinical trial data with TKIs show that while the peripheral blood of patients responds well, a more modest response is observed in patient bone marrow. It is possible that a small residual pool of leukemic CD34+ cells may persist in the marrow microenvironment of leukemia patients after years of therapy with kinase inhibitors. It is thus likely that the marrow is a sanctuary site for leukemic stem cell, and that the interaction of these quiescent leukemic cells with stroma is sufficient to induce relative drug resistance through activation of alternative viability pathways. While these leukemic cells cannot proliferate in response to oncogene-driven signals, they still survive and form a reservoir of persistent disease.

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA; [2]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA; [3]Department of Pediatric Oncology, Dana-Farber Cancer Institute and Children's Hospital, Boston, MA, USA; [4]Rodent Histopathology Core, Department of Pathology, Harvard Medical School, Boston, MA, USA and [5]High Magnetic Field laboratory, Chinese Academy of Science, Hefei, Anhui, PRC. Correspondence: Professor JD Griffin, Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue Boston, 02215 Mailstop: Mayer 540, Boston, MA 02215, USA.
E-mail: james_griffin@dfci.harvard.edu
or Professor N Gray, Department of Cancer Biology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215, USA.
E-mail: nathanael_gray@dfci.harvard.edu
[6]These authors contributed equally to this work.
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Bone marrow stromal cells have been implicated in drug resistance, as they are thought to have a role in the long-term survival and growth of normal and leukemia cells and other hematological malignancies, including lymphoma and multiple myeloma.[6–12] It was recently shown that the activity of first-generation FLT3 inhibitors, such as SU5614 and sorafenib, is diminished against mutant FLT3-expressing cells by contact with stroma.[13]

We demonstrated stromal protection of leukemic cells from the inhibitory effects of nilotinib, and identified stromal-secreted viability factors, including IL-6 and GM-CSF, as possibly having a role in stromal-mediated cytoprotection of TKI-treated chronic myeloid leukemia (CML) and AML.[14–15] In addition, in TKI-treated mice, we identified high tumor burden/residual disease in tissues characterized as significant sources of hematopoiesis-promoting stroma; the pattern of leukemia distribution in mice was consistent with what is observed in imatinib/nilotinib-treated CML patients.[14]

Protein kinases are intrinsically involved in regulation of signal transduction pathways, and deregulated kinase activity, which affects cell proliferation and apoptosis, often leads to development of cancer. The survival advantage of mutant FLT3-driven leukemia is known to be largely because of mutant FLT3 activation of three major intracellular signaling pathways, PI3K/PTEN/Akt/mTOR, RAS/Raf/MEK/ERK, and Janus kinase/signal transducer and activator of transcription (JAK/STAT).[16] Serine/threonine kinases and dual-specificity kinases, such as those comprising the MAP kinase pathway, non-receptor tyrosine kinases (that is, Src), receptor tyrosine kinases (that is, FLT3) and receptor-associated tyrosine kinases involved in cytokine-mediated signaling (that is, JAK, whose effects are mediated by STAT proteins), all represent putative therapeutic targets. As examples, protein kinases, such as SRC, are often activated in malignant cells and contribute to tumor development and are thus viable candidates for targeted drug development. The cross-talk between the three signaling pathways, coupled with the involvement of a variety of signaling mediators in viability pathways conferring a survival advantage to transformed cells in microenvironmental recesses, strongly warrants the use of multi-targeted therapy to treat leukemia and potentially override drug resistance.

We hypothesized that combination therapy geared toward inhibition of diverse protein kinase targets may be effective in potentiating TKI effects on mutant FLT3-positive leukemia cells cultured in a cytoprotective stromal microenvironment. As such, we performed a screen of a kinase inhibitor focused library to identify agents with demonstrated ability to synergize with PKC412 and related inhibitors against stroma-protected, mutant FLT3-expressing cells. Our screen led to the identification of three structurally-related multi-targeted TKIs, including dasatinib, that were able to override the cytoprotective effect of stromal-derived viability factors on FLT3 inhibitor-treated cells. Synergy between these agents was confirmed *in vitro*, using both stromal-conditioned media (SCM) and adherent stroma, and a positive combination effect was observed *in vivo* with prolonged survival demonstrated in combination-treated mice. We also independently demonstrated the ability of inhibitors of JAK kinase, a mediator of IL-6 and related growth factor signaling, to similarly potentiate the effects of FLT3 inhibitors, as well as dasatinib and related multi-targeted TKIs, and in effect override stromal-mediated chemoresistance.

MATERIALS AND METHODS

Kinase inhibitor focused library

We utilized a focused library of kinase inhibitors to screen for inhibitors showing little-to-no appreciable efficacy as single agents; however, demonstrating the ability to synergize with PKC412 against human FLT3-ITD-expressing MOLM13 cells cultured in the presence of 50% HS-5 SCM. The library is comprised of ~300 publically disclosed kinase inhibitors and ~800 novel ATP competitive kinase inhibitors targeting either active or inactive kinase conformations. The chemical screening concentration was 660 nM. Details regarding this are in the Supplementary data section.

Cell lines and cell culture

Details are provided as Supplementary Material.[17–21]

AML patient cells

Details are provided as Supplementary Material.

Chemical compounds and biological reagents

PKC412 and AUZ454 were synthesized by Novartis Pharma AG, Basel, Switzerland, and were dissolved in DMSO to obtain 10 mM stock solutions. Serial dilutions were then made, to obtain final dilutions for cellular assays with a final concentration of DMSO not exceeding 0.1%.

Dasatinib, AZD-1480, AZD-0530, INCB-18 424, CYT387, AC220 and KIN040 were purchased from Haoyuan Chemexpress (Shanghai, China; KIN112, KIN113, were developed in Dr Gray's lab (DFCI). Chemical structures are shown in Supplementary Figure 1.

Molecular modeling

Details are provided as Supplementary Material.

Cell proliferation, cell cycle and viability analysis

The trypan blue exclusion assay (for proliferation), Annexin-V-Fluos Staining Kit (Boehringer Mannheim, Indianapolis, IN, USA) (for apoptosis), and cell cycle analysis were carried out as previously described.[3] The Cell Titer Glo assay (Promega, Madison, WI, USA) (for proliferation) was used where indicated, and carried out according to manufacturer instructions.

Antibodies

All antibodies used were purchased from Cell Signaling Technology, Danvers, MA, USA. Phospho-STAT5 Tyr694 (rabbit, #9351S) was used at 1:1000. Total STAT5 (3H7) (rabbit, #9358 mAb) was used at 1:1000. Phospho-AKT (Ser 473) (rabbit, #9271) and total AKT (rabbit, #9272) were used at 1:2500. Anti p-Tyr (clone 4G10, Upstate Biotechnology, Lake Placid, NY, USA) was used at 1:1000 for immunoblotting.

Immunoblotting

Protein lysis preparation, immunoprecipitation and immunoblotting were carried out as previously described.[3]

Drug combination studies

For drug combination studies, single agents were added simultaneously at fixed ratios to mutant FLT3-expressing cells. Cell viability was determined using the trypan blue exclusion assay, and expressed as the function of growth affected drug-treated versus control cells; data were analyzed by Calcusyn software (Biosoft, Ferguson, MO, USA and Cambridge, UK), using the Chou-Talalay method (Chou and Talalay, 1984).[22] 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. Calcusyn combination indices can be interpreted as follows: CI < 0.1 indicate very strong synergism (a). Values 0.1–0.3 indicate strong synergism (b). Values 0.3–0.7 indicate synergism (c). Values 0.7–0.85 indicate moderate synergism (d). Values 0.85–0.90 indicate slight synergism (e). Values 0.9–1.1 indicate nearly additive effects (f). Values 1.10–1.20 indicate slight antagonism (g). Values 1.20–1.45 indicate moderate antagonism (h). Values 1.45–3.3 indicate antagonism (i). Values 3.3–10 indicate strong antagonism (j). Values >10 indicate very strong antagonism. Note: for some experiments, namely those in which there was no observed single-agent activity because of stromal protection, combination indices were not able to be reliably calculated using the Calcusyn software.

Human stroma experiments

Details are provided as Supplementary Material.

Bioluminescent model of progressive FLT3-ITD-driven AML

For administration to 30 female Nu/Nu NCR-nude mice (8 weeks of age; Charles River Laboratories, Wilmington, MA, USA), virus- and Mycoplasma-free Ba/F3-FLT3-ITD-luc+ cells were washed in Hank's Balanced Salt Solution (HBSS; Mediatech, Inc., Cellgro, Manassas, VA, USA), resuspended in PBS for injection, and administered via intravenous tail vein injection (250 μl, 1.5×10^6 cells). Following administration of Ba/F3-FLT3-ITD-luc+ cells, mice were then imaged 2 days later to determine baseline bioluminescence and quantify tumor burden as previously described.[19] Mice were then divided into four treatment groups ($n = 7$ or 8 per group): vehicle (PKC412 pre-concentrate; 10 μl/g), PKC412 (50 mg/kg), dasatinib (10 mg/kg) and combination. All treatments were dosed PO once daily; mice were not treated on days 8–9 to allow them to gain weight after a significant initial drop (particularly in the dasatinib-treated groups). Groups were imaged every 3–5 days for 2 weeks, at which time mice started to become moribund. Survival data were collected for all mice. Four whole mice from each group were fixed in 10% neutral-buffered formalin upon death and sent for histopathological analysis.

Histopathology

Formalin-fixed tissues were dissected and processed routinely for paraffin embedding and sectioning. Micron sections were stained with hematoxylin and eosin.

Drug formulations for *in vivo* studies

PKC412, synthesized by Novartis Pharma AG was supplied as a micro-emulsion pre-concentrate (5% w/v) and diluted with water to achieve the desired final concentrations. Dasatinib was obtained from Haoyuan Chemexpress as a powder. Dasatinib was formulated in 50% propylene glycol, 50% water for *in vivo* administration.

RESULTS

In vitro chemical screen to identify protein kinase inhibitors able to potentiate the effects of stromal-protected TKIs aimed at AML

In an attempt to identify protein kinase inhibitors that are able to effectively synergize with standard TKIs, the inhibitory activity of which is diminished in the presence of adherent stroma or stromal-secreted factors, we conducted a combinatorial kinase inhibitor screen *in vitro* using a chemical library consisting of early-in-development- and FDA-approved kinase inhibitors. As the activity of imatinib and nilotinib against KU812F-luc+ cells has been shown to be diminished in the presence of plated HS-5 stromal cells[23] and SCM,[24] we decided to use this assay system to test the efficacy of PK412, as a single agent, as compared with PKC412 in combination with library compounds. In an unbiased screen of 1100 kinase inhibitors, three library-derived kinase

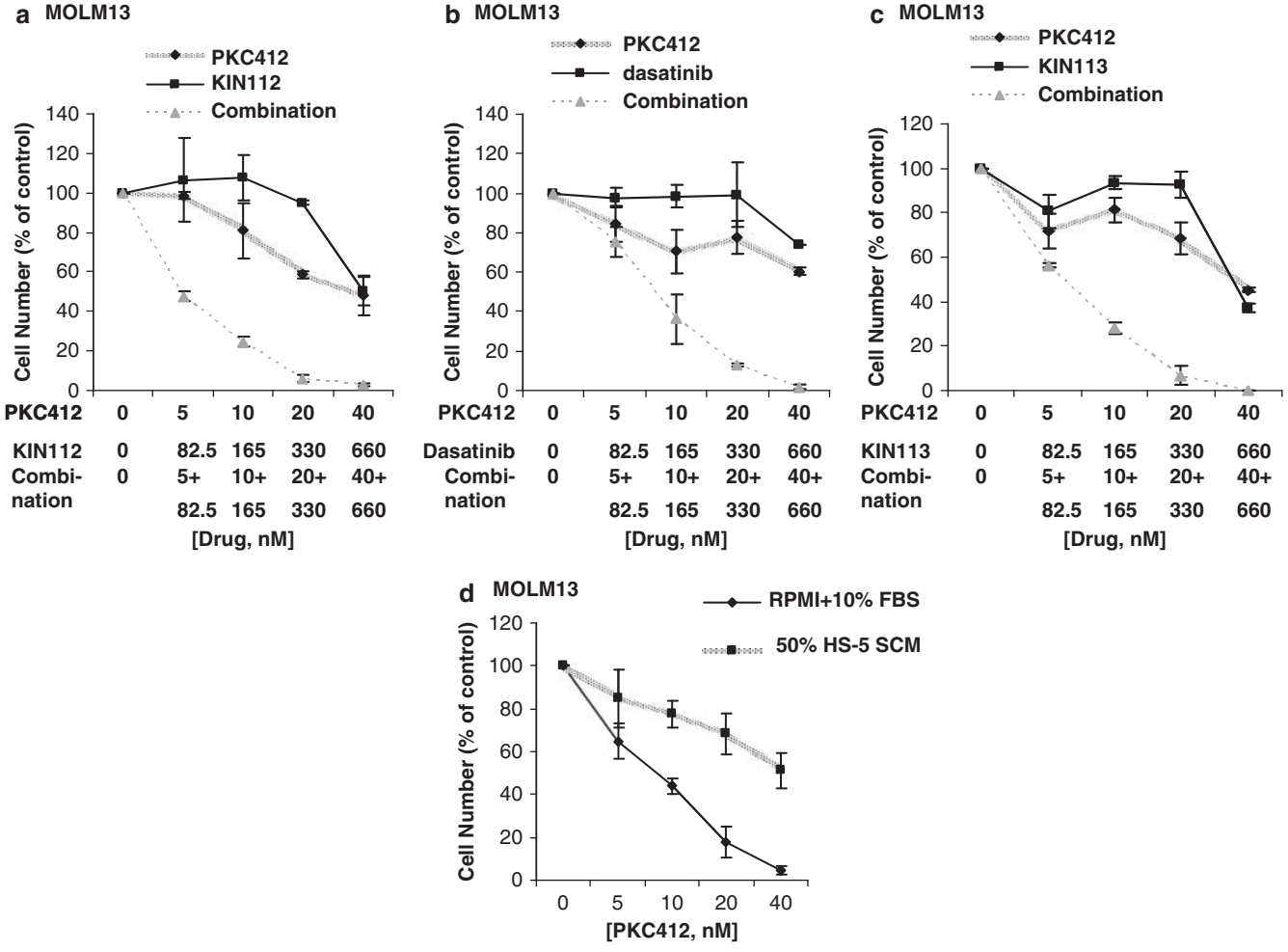

Figure 1. Proliferation studies performed with selected chemical library compounds in combination with PKC412 in the presence of HS-5 SCM. Proliferation studies (trypan blue exclusion assay): approximately 2-day treatment of MOLM13 cells, in the presence of 50% HS-5 SCM, with PKC412, KIN112, or a combination of the two agents (**a**), PKC412, dasatinib, or a combination of the two agents (**b**), or PKC412, KIN113, or a combination of the two agents (**c**). Shown are averages of 2–4 separate calculations of cell viability for each data point. These data are representative of two independent sets of experiments in which similar results were observed (the second data set is shown as Supplementary data). (**d**) Proliferation studies (trypan blue exclusion assay): approximately 2-day treatment of MOLM13 cells, in the absence and presence of 50% HS-5 SCM, with PKC412 ($n = 3$).

inhibitors were identified, including the BCR/ABL and Src-family inhibitor, dasatinib, and the related multi-targeted kinase inhibitors, KIN112 and KIN113. These drugs showed little-to-no single-agent activity at the chemical screen concentration of 660 nM; however, they were able to synergize with PKC412 against MOLM13 cells cultured in the presence of SCM (Figures 1a-c and Supplementary Figure 2). Synergy between PKC412 + dasatinib, PKC412 + KIN112 and PKC412 + KIN113 can be appreciated by the nearly 100% inhibition of cell growth at the highest concentrations of each drug combined, compared with ~50% killing of cells by each individual drug at the highest concentrations. The cytoprotective effect of conditioned media derived from two independent human stromal cell lines, HS-5- and HS27a, on PKC412-treated leukemic cells was confirmed in this screening, as was the expected potency of PKC412 in the absence of SCM. Increased inhibition of cell proliferation by drug combination treatments, as compared with single-agent treatments, correlated with increased percentages of apoptotic and necrotic cell populations (up to 40% higher in drug combination groups) as well as a higher percentage of cells in the G1 phase of the cell

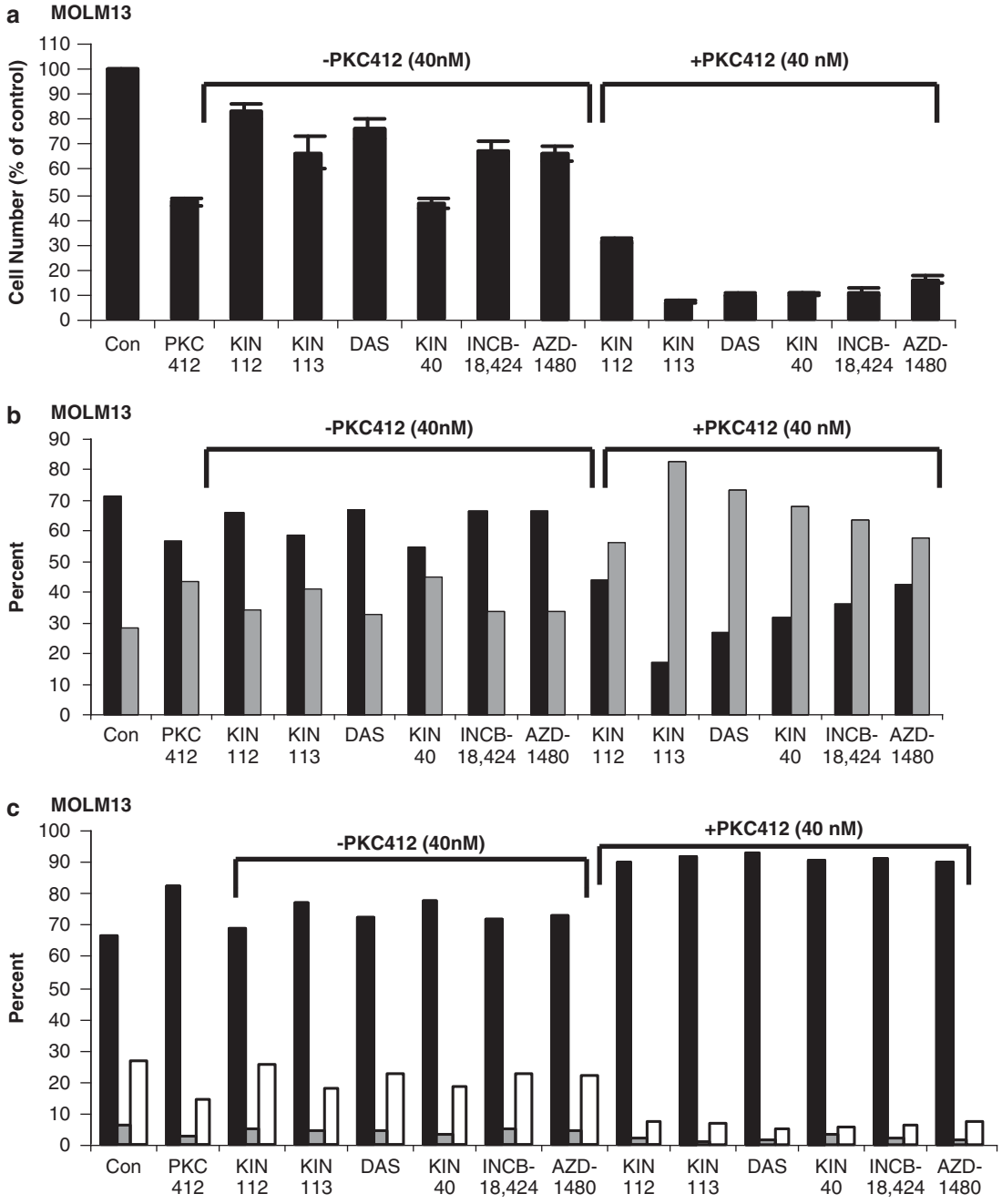

Figure 2. Proliferation studies, viability studies and cell cycle analysis performed with selected chemical library compounds in combination with PKC412 in the presence of HS-5 SCM. (**a**) Proliferation studies (Cell Titer Glo assay): approximately 2-day treatment of MOLM13 cells, in the presence of 50% HS-5 SCM. PKC412 was tested at 40 nM. KIN112, KIN113, dasatinib ('DAS'), KIN40, INCB-18 424 and AZD-1480 were tested at 660 nM. Values were calculated as percent of untreated controls and were the average + s.e.m. of duplicate wells per treatment. (**b**) Viability assays (Annexin-V-Fluos staining). Black bars represent percent viable. Gray bars represent percent apoptotic/necrotic. (**c**) Cell cycle analysis. Black bars represent percent G1. Gray bars represent percent G2. White bars represent percent S.

cycle (up to 10–20% higher in drug combination groups) (Figure 2), suggesting that observed synergy is due to both cell killing as well as cell cycle arrest.

Inhibitors were able to similarly synergize in the presence of SCM with the highly potent and relatively selective FLT3 inhibitors, AUZ454[ref. 25] and AC220 (Figure 3 and Supplementary Figure 3). Synergy was observed in the presence of SCM derived from HS27a stroma as well as HS-5 stroma (Supplementary Figure 4). Synergy observed between KIN112/KIN113/dasatinib and PKC412 was more pronounced in the presence of SCM than in its absence (Figure 3), suggesting that factors mediating the observed synergy may have a significant role in SCM. Importantly, none of the three identified inhibitors showed appreciable cytotoxicity toward human HS-5 or HS27a bone marrow stromal cells (Supplementary Figure 5).

Synergy between KIN112/KIN113/dasatinib and PKC412 was also observed against MOLM14-luc+ cells co-cultured with adherent HS27a stromal cells (Supplementary Figure 6). Adherent stromal cells conferred a cytoprotective effect on PKC412-treated MOLM14-luc+ cells.

In vivo investigation of the combination of PKC412 and dasatinib against progressive mutant FLT3-positive leukemia

An *in vivo* study was carried out to investigate both the therapeutic potential as well as toxicity/side effects of the combination of dasatinib and PKC412 in mice-harboring mutant FLT3-positive leukemia. This murine model utilized cells expressing FLT3-ITD (which leads to aggressive disease and poor prognosis in AML patients) and was designed to mimic progressive, FLT3-ITD-driven disease.

Before injection into mice, Ba/F3-FLT3-ITD-luc+ cells were first tested for responsiveness to PKC412, as well as growth factor protection from the cytotoxic effects of PKC412. WEHI-conditioned media (used as a source of IL-3) significantly protected or 'rescued' Ba/F3-FLT3-ITD-luc+ cells from the inhibitory effects of PKC412 (Figure 4a and Supplementary Figure 7a). Dasatinib as a single agent does not inhibit growth of this cell line when cultured in the presence of IL-3 (Figure 4b and Supplementary Figure 7b). However, the combination of dasatinib and PKC412 led to substantially more cell killing than either agent alone under the same conditions (Figure 4b and Supplementary Figure 7b).

Compared with mice treated for 13 days with vehicle, dasatinib alone, or PKC412 alone, mice treated with a combination of dasatinib and PKC412 trended towards lower tumor burden (bioluminescence) (Figure 4c). As results for the imaging at this point did not reach statistical significance, drug treatments were continued to the time of killing. Whereas monotherapy with PKC412 or dasatinib had no appreciable effects, PKC412+ dasatinib combination treatment significantly prolonged survival (Figure 4d) (*P*-value: 0.0255). These data suggest that the combination of PKC412 and dasatinib may have potential clinical benefit and translate into increased survival.

All mice at the time of killing were determined by histopathology to harbor leukemia. Most of the mice had large numbers of tumor cells in meninges, bone marrow, liver, spleen and occasionally ovary. As all mice included in this study died from disease and not drug-related toxicities, PKC412 and dasatinib as single agents and in combination were considered to be generally well tolerated. However, initial weight loss was observed in those treatment groups receiving dasatinib (Supplementary Figure 7c).

Structural information and comparisons between KIN112, dasatinib and KIN113

Structural information and comparisons between KIN112, dasatinib and KIN113. The three identified inhibitors, dasatinib, KIN112 and KIN113, from the chemical library screen are all primarily classified as Src-family kinase inhibitors, though additional kinase selectivity profiling using KinomeScan against a panel of 442-kinases reveals many additional potential kinase targets (Supplementary Figure 8).[26,27] KIN112 possesses a chemical structure that is predicted to be isosteric with that of dasatinib. KIN112 and KIN113 are very potent SRC inhibitors, with an IC_{50} for KIN112 against c-SRC of 6 nM, and an IC_{50} for KIN113 against c-SRC of 102 nM. Molecular modeling studies predict that KIN112 would preferentially bind to SRC in active conformation (DFG-in), analogously to the crystallographic co-structure of dasatinib bound to Src (PDB ID 3G5D), whereas KIN113 is predicted to preferentially bind to the inactive conformation (DFG-out) of Src (Supplementary Figure 9). The highly multi-targeted nature of KIN112, KIN113 and dasatinib make it very difficult to establish whether a particular kinase or a combination of kinases is pharmacologically relevant to the observed synergy with FLT3 inhibition.

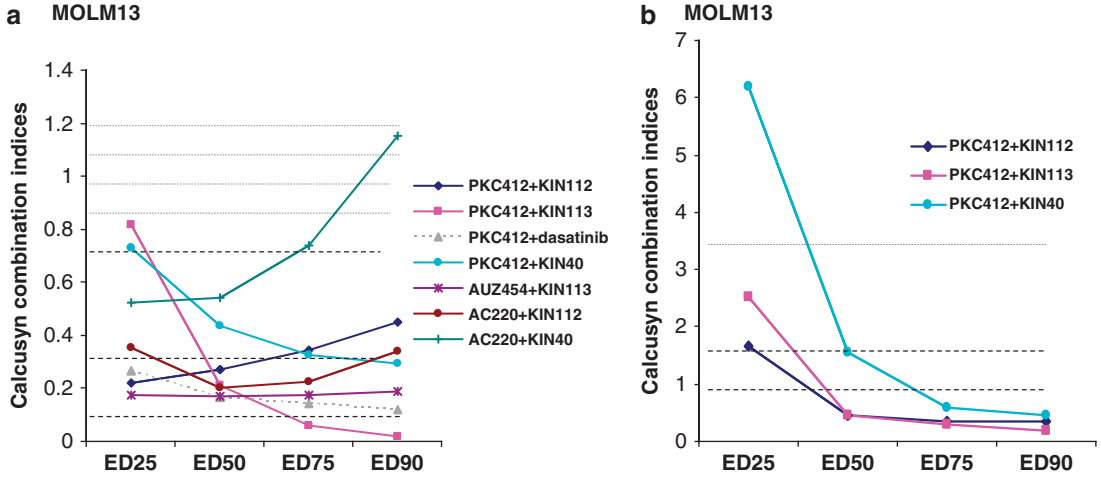

Figure 3. Calcusyn combination indices generated for combination experiments with FLT3 inhibitors and selected library compounds. (**a**) Calcusyn combination indices calculated for proliferation studies performed with FLT3 inhibitors combined with chemical library compounds (KIN112, dasatinib, KIN113) or the pan-Jak inhibitor, KIN40, in the presence of SCM. (**b**) Calcusyn combination indices calculated for proliferation studies performed with PKC412 combined with chemical library compounds (KIN112, KIN113) or KIN40 in the presence of RPMI + 10% FBS.

Investigation of synergizing potential of JAK inhibitors combined with TKIs against AML

IL-6 and related cytokines exert their effects via by binding to receptors (that is, glycoprotein 130, OSM receptor and LIF receptor) that transduce signals to the activation of JAK/STAT and mitogen-activated protein kinase cascades. We hypothesized that inhibition of JAK/STAT signaling may be effective in overriding the viability-inducing effects of stromal-secreted IL-6 and related cytokines on kinase inhibitor-treated leukemic cells. Accordingly, we tested in combination with PKC412 several selective JAK inhibitors, including the JAK1/2 inhibitor, INCB-18 424, selective JAK2 inhibitor, AZD-1480, selective JAK3 inhibitor, Tofacitinib (CP-690550) and the JAK1/2 inhibitor, CYT387.[28] We also tested in combination with PKC412 the pan-JAK inhibitor, KIN40, and in combination with PKC412 the selective SRC inhibitor, AZD-0530. KinomeScan kinase selectivity profiles for KIN40 and the selective JAK and SRC inhibitors are shown in Supplementary Figure 8.[26,27] The JAK inhibitors significantly enhanced the activity of PKC412 in the presence of SCM and led to more cell killing (Figure 5; Supplementary Figure 10). A more modest positive combination effect was observed between PKC412 and INCB-18 424 with FLT3-ITD-positive MV4, 11 cells treated in the presence of SCM (Supplementary Figure 10). Synergy observed between the pan-JAK inhibitor, KIN40, and PKC412 against MOLM13 cells was more pronounced in the presence of SCM than in its absence (Figure 3), suggesting that factors mediating the observed synergy may have a significant role in stromal-mediated chemoresistance.

We showed via p-TYR immunoprecipitation and whole-cell lysate immunoblots that SCM treatment of MOLM13 cells serum-starved for varying lengths of time leads to an increase in tyrosine phosphorylation of STAT5 (Figures 6a-d), suggesting that STAT5

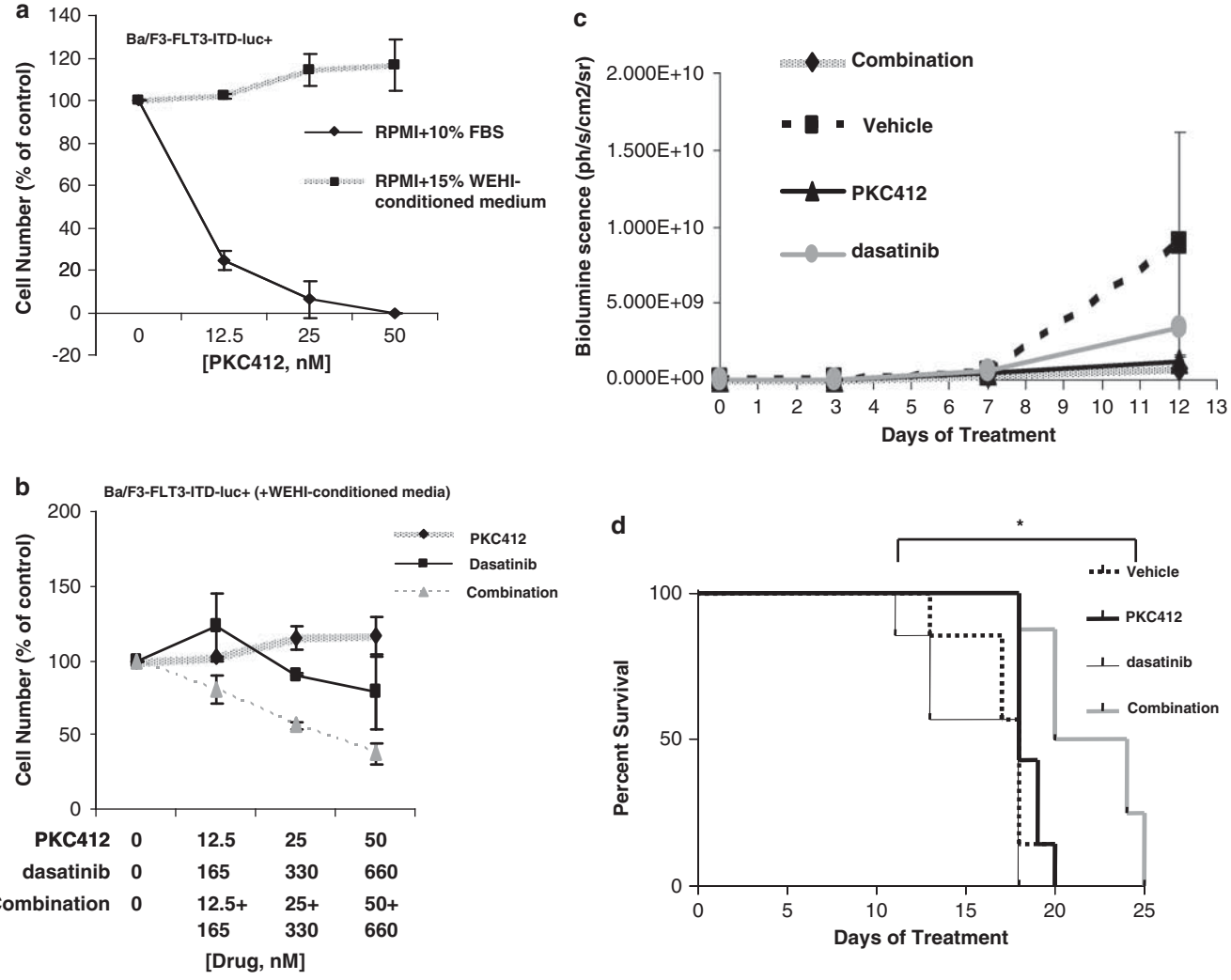

Figure 4. Investigation of the combination of PKC412 and dasatinib against Ba/F3-FLT3-ITD-luc+ cells in mutant FLT3-harboring mice. (**a**) Proliferation study performed *in vitro* (trypan blue exclusion assay), testing PKC412 against Ba/F3-FLT3-ITD-luc+ cells in the presence and absence of 15% WEHI-conditioned media (used as a source of IL-3). Shown are averages of 2–4 separate calculations of cell viability for each data point. These data are representative of two independent sets of experiments in which similar results were observed (the second data set is shown as Supplementary data). (**b**) Proliferation study performed *in vitro* (trypan blue exclusion assay), testing the combination of PKC412 and dasatinib against Ba/F3-FLT3-ITD-luc+ cells in the presence of 15% WEHI-conditioned media. Shown are averages of 2–4 separate calculations of cell viability for each data point. These data are representative of two independent sets of experiments in which similar results were observed (the second data set is shown as Supplementary data). (**c**) Total body luminescence: drug treatment effect on tumor burden. Two-way ANOVA analysis revealed no significant difference between groups. (**d**) Survival curves. *The PKC412+ dasatinib combination treatment significantly prolonged survival of mice as compared with the other treatment groups (*P*-value: 0.0255).

activation may have a role in stromal-mediated chemoresistance. The combination of PKC412 and pan-Jak inhibitor, KIN40, was accompanied by a decrease in levels of phospho-STAT5 that was greater than either drug alone (Figure 6e). This suggests that STAT5, as a key component of both the JAK/STAT-signaling pathway and downstream mediator of FLT3 signaling, likely has a role in synergy between PKC412 and KIN40. Similar results were observed with phospho-STAT5 in MOLM13 cells treated with a combination of dasatinib and PKC412, as compared with either drug alone (Figure 6f). In addition, pAKT levels were inhibited to a greater extent in MOLM cells treated with a combination of dasatinib and PKC412, as compared with either drug alone (Supplementary Figure 11), further suggesting that downstream mediators of FLT3 signaling likely participate in the synergy observed between FLT3 inhibitors combined with dasatinib and related multi-kinase inhibitors.

Similar to PKC412, AC220 synergized with INCB-18 424, AZD-1480 and Tofacitinib in the presence of SCM (Figure 5). The inhibitory effects of AC220 as a single agent were substantially diminished by the addition of SCM (data not shown).

Modest synergy was observed between PKC412 and the selective SRC inhibitor, AZD-0530 (Supplementary Figure 12). In contrast, no apparent positive combination effect was observed between AZD-0530 and the selective JAK inhibitors, AZD-1480 and INCB-18 424 (Supplementary Figure 12). However, the multi-targeted TKIs, dasatinib and KIN113, were observed to synergize with JAK inhibitors (Figure 7 and Supplementary Figure 13). These results suggest that the multiple targets of dasatinib and KIN113

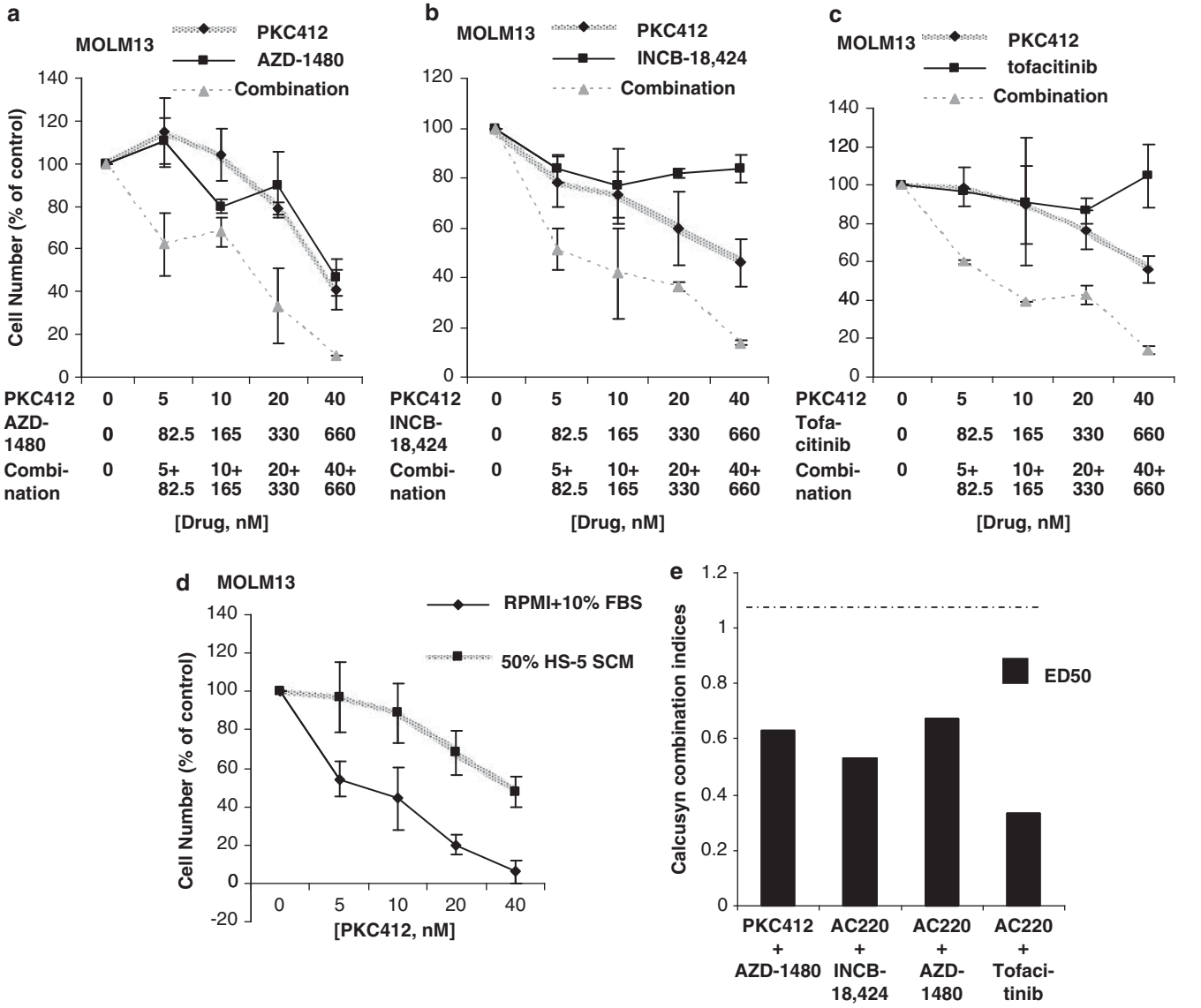

Figure 5. Proliferation studies performed with FLT3 inhibitors and Jak inhibitors. Trypan blue exclusion assay: approximately 2-day treatment of MOLM13 cells, in the presence of 50% SCM consisting of 25% HS-5 SCM and 25% HS27a SCM, with PKC412, AZD-1480, or a combination of the two agents (**a**), PKC412, INCB-18 424, or a combination of the two agents (**b**) and PKC412, Tofacitinib, or a combination of the two agents (**c**). Shown are the averages of 2–4 separate calculations of cell viability for each data point. Data are representative of two independent sets of experiments in which similar results were observed (the second data set is shown as Supplementary data). (**d**) Trypan blue exclusion assay: approximately 2-day treatment of MOLM13 cells, in the absence and presence of 50% SCM consisting of 25% HS-5 SCM and 25% HS27a SCM, with PKC412 for each respective study ($n = 3$). (**e**) Calcusyn combination indices (ED50) calculated for proliferation studies performed with FLT3 inhibitors combined with selective Jak or Src inhibitors. The cutoff for nearly additive effects (CI: 1.1) is marked by a dashed line.

may contribute to the impressive synergy observed with other TKIs in a cytoprotective stromal microenvironment, whereas selective inhibition of SRC may not be sufficient to achieve comparable synergy.

Investigation of drug combination effects against mutant FLT3-positive AML patient cells

PKC412 was tested alone or combined with either multi-targeted TKIs identified in the chemical screen or JAK inhibitors against peripheral blood or bone marrow samples derived from seven AML patients harboring FLT3-ITD. Despite PKC412 responsiveness alone being low in patients that were relapsed or refractory following previous therapies (that is, AML 5, AML 6), more AML patient cell killing was still observed when drug combinations, tested in the presence of either RPMI + 10% FBS or 50–80% SCM, were used as opposed to single agents (Figure 8). Calcusyn analysis revealed additive to synergistic effects for various combinations, including PKC412 + KIN113, PKC412 + dasatinib and PKC412 + INCB-18 424 (Figure 8). These results support the positive drug combination effects observed in mutant FLT3-positive cell lines and suggest the possibility of clinical benefit arising from such drug combinations. Patient information is provided in Supplementary Table I.

DISCUSSION

The communication between leukemia cells and cells comprising the bone marrow microenvironment influences the growth of transformed cells, as well as their responsiveness to anticancer therapy. There is thus a need for development of novel therapeutic approaches that target not only the leukemic cell, but also the surrounding protective stromal microenvironment.

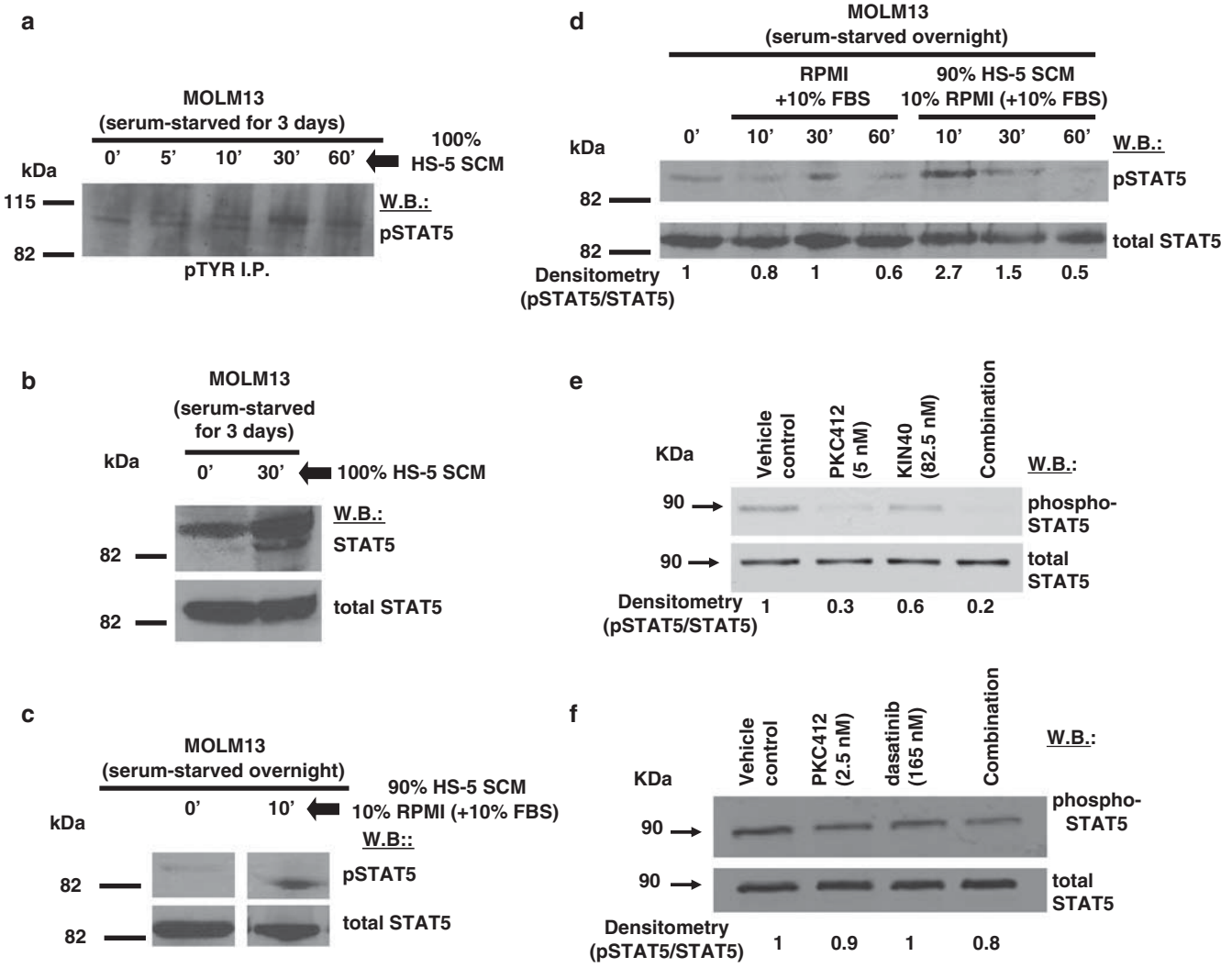

Figure 6. SCM stimulation of phospho-STAT5 and drug combination effect on phosphorylation of phospho-STAT5. (**a**) p-TYR immunoprecipitation (I.P.) followed by phospho-STAT5 western blot (w.b.), showing HS-5 SCM stimulation of phospho-STAT5. (**b**) phospho-STAT5 and total STAT5 whole-cell lysate western blots of MOLM13 cells serum-starved for 3 days and treated with 100% HS-5 SCM, showing HS-5 SCM stimulation of phospho-STAT5 activity. (**c**) phospho-STAT5 and total STAT5 whole-cell lysate western blots of MOLM13 cells serum-starved overnight and treated with 90% HS-5 SCM, showing HS-5 SCM stimulation of phospho-STAT5 activity. (**d**) Comparison of effects of RPMI + 10% FBS versus 90% HS-5 SCM on phospho-STAT5 in MOLM13 cells serum-starved overnight. (**e**) Expression of phospho-STAT5 and total STAT5 in MOLM13 cells treated for 1.5 h with DMSO vehicle, PKC412 (5 nM), KIN40 (82.5 nM) or a combination of both. Results shown are representative of two independent experiments in which similar results were observed. (**f**) Expression of phospho-STAT5 and total STAT5 in MOLM13 cells treated for 15 min with DMSO vehicle, PKC412 (2.5 nM), dasatinib (165 nM) or a combination of both. Results shown are representative of two independent experiments in which similar results were observed. For all experiments, protein lysates were prepared from MOLM13 cells, and were analyzed via immunoblotting with antibodies to phospho-STAT5 and total STAT5.

The association between FLT3-ITD mutations and poor AML patient prognosis[29,30] highlights the potential of FLT3-ITD as an important therapeutic target. In addition, FLT3-ITD activation of signal transduction pathways dependent on the interactions of numerous protein kinases underscores the potential therapeutic value of additionally targeting kinase mediators acting downstream of the receptor. Indeed, phosphoproteomic analysis of FLT3 signaling in human leukemia cells performed on primary AML patient bone marrow samples led to identification of over 800 serine/threonine phosphorylation sites and over 200 tyrosine phosphorylation sites,[31] suggesting the potential importance of a large number of protein kinases as therapeutic targets for overriding cytoprotection conferred by the bone marrow microenvironment of AML patients. In this study, stable isotope labeling by amino acids in cell culture revealed over 400 tyrosine and serine/threonine phosphorylation sites to be responsive to FLT3 inhibition, as well.

Recent studies have implicated kinase activity in stromal-mediated cytoprotection of leukemia cells. For example, in chronic lymphocytic leukemia (CLL), bone marrow stroma, via B-cell receptor activation and induction of chemokines,[32] protects against purine analog chemotherapy-induced apoptosis.[33–34] Pharmacological inhibition of the non-receptor cytoplasmic tyrosine kinase, spleen tyrosine kinase, which mediates B-cell receptor signaling-induced survival signaling, inhibits the bone marrow survival signals that protect CLL cells.[35] Another study demonstrated the ability of a selective JAK3 inhibitor to reverse stromal-mediated chemoresistance of CLL cells.[36]

We have previously shown that inhibitors of PI3K signaling have the ability to effectively combine with PKC412 to effectively kill drug-sensitive and insensitive mutant FLT3-expressing cells.[37] We have also previously demonstrated the ability of small molecule inhibitors to potentiate the effects of targeted TKIs and in effect override stromal-mediated chemoresistance in leukemia models,

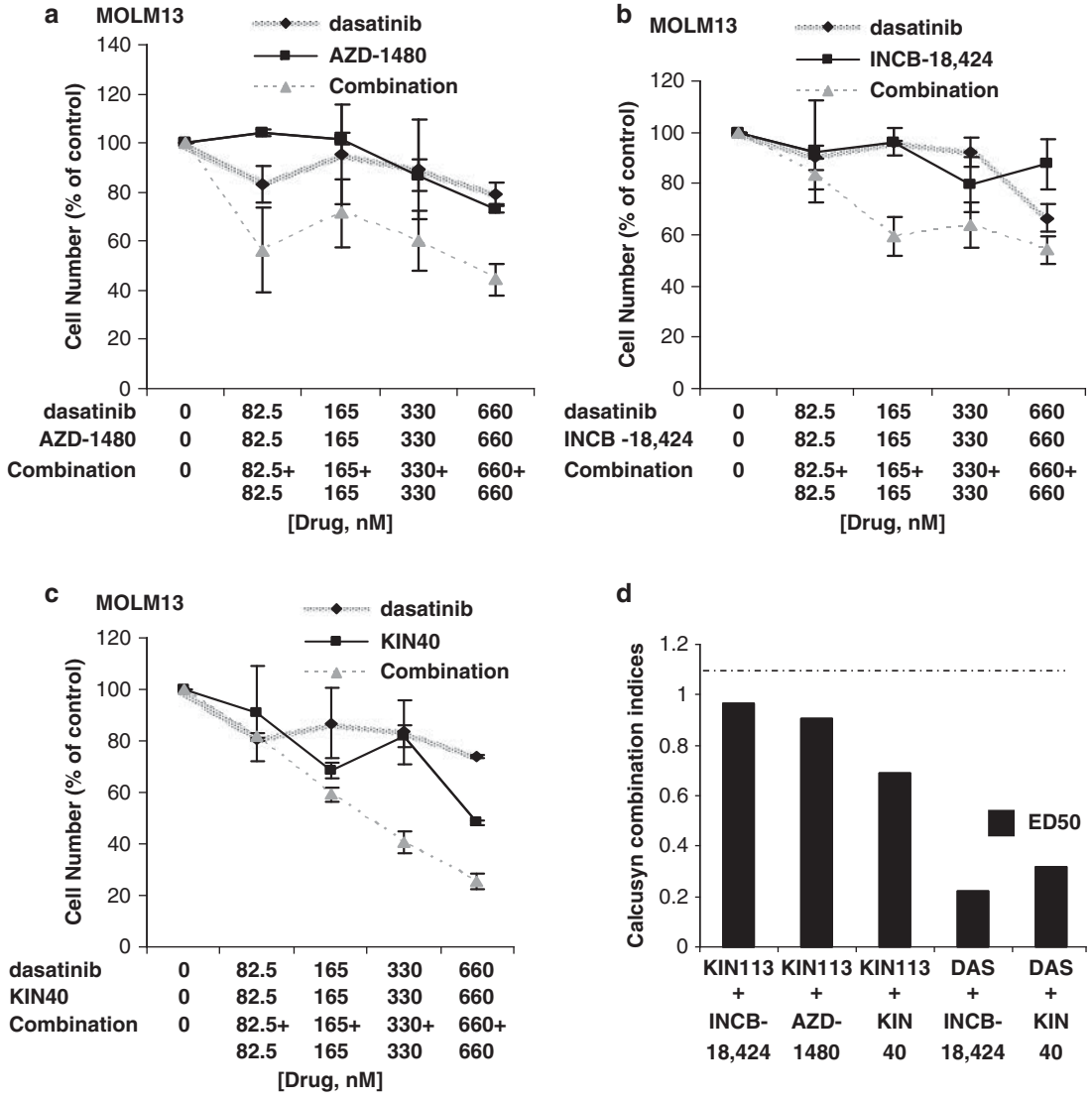

Figure 7. Proliferation studies performed with library-derived multi-targeted inhibitors and Jak inhibitors. (**a–c**) Trypan blue exclusion assay: Dasatinib and Jak inhibitor combination studies performed in the presence of 50% HS-5 + HS27a SCM. Approximately 2-day treatments of MOLM13 cells, in the presence of 50% SCM consisting of 25% HS-5 SCM and 25% HS27a SCM, with dasatinib, AZD-1480, or a combination of the two agents (**a**), with dasatinib, INCB-18 424, or a combination of the two agents (**b**), or with dasatinib, KIN40, or a combination of the two agents (**c**). Shown are averages of 2–4 separate calculations of cell viability for each data point. These data are representative of several independent sets of experiments in which similar results were observed (the second data set is shown as Supplementary data). (**d**) Calcusyn combination indices (ED50) calculated for proliferation studies performed with chemical library compounds combined with selective Jak inhibitors or KIN40. The cutoff for nearly additive effects (CI: 1.1) is marked by a dashed line.

such as inhibitor of apoptosis[24,38] and the CXCR4 antagonist, plerixafor.[39] Thus, TKI-dependent combination therapy appears to represent a potentially effective approach to overriding drug resistance in leukemia patients, due to both factors contributing to aberrant intracellular signaling as well as factors associated with the cytoprotective microenvironment.

Here, in a chemical screen, we identified the dual Src/Abl inhibitor, dasatinib (BMS-354825) and two related multi-targeted TKIs as being able to synergize with FLT3 inhibitors against mutant FLT3-expressing cells cultured in the presence of stroma or SCM. SRC-family kinases, which are targets of the three compounds, in addition to displaying increased activity and expression in transformed cells,[40] have been suggested to have a role in the tumor microenvironment, namely VEGF-induced angiogenesis and vascular permeability.[41–43]

Dasatinib is also an inhibitor of c-KIT, platelet-derived growth factor receptor and EphA2 signaling, and was recently FDA-approved for newly diagnosed CML.[44–46] Dasatinib has been reported to inhibit multiple myeloma-derived angiogenesis via targeting of platelet-derived growth factor receptor beta and Src,[47] and inhibits endothelial and myeloid cell function necessary for sustaining tumor cell growth *in vivo*.[48] A recent study showed that the Src-family kinase Lyn, in association with CXCR4, is involved in stromal microenvironment resistance of CML to imatinib.[49] In this report, imatinib enhancement of CML migration toward stromal cell layers was blocked by inhibition of Lyn or cholesterol depletion, and dasatinib was reported to cause comparatively less CML migration to stroma.

KIN112, another identified multi-targeted TKI possessing a pharmacophore that is isosteric with dasatinib, binds to the active conformation of SRC, whereas the third multi-targeted TKI, KIN113 binds to the inactive conformation of SRC. Interestingly, despite the distinct predicted binding modes of KIN112 and KIN113, both compounds exhibited a similar ability to synergize with FLT3 inhibitors in the presence of a cytoprotective stromal microenvironment.

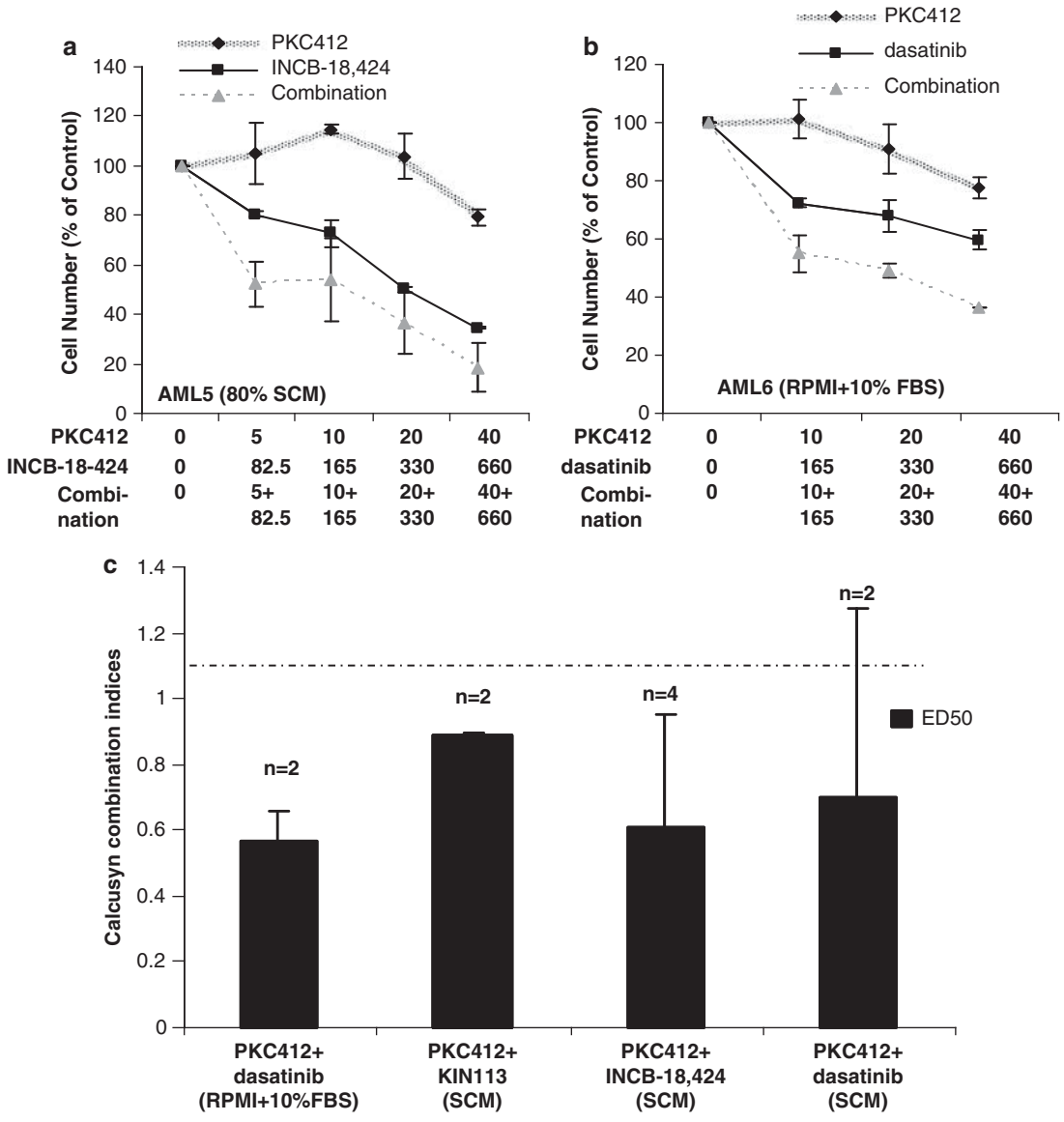

Figure 8. Combination studies performed with primary mutant FLT3-positive AML patient samples performed in the presence of RPMI + 10% FBS or HS-5 SCM. Cell Titer Glo assay: AML patients were treated in the presence of HS-5 SCM or RPMI + 10% FBS. (**a–b**) Representative dose-response curves. (**c**) Calcusyn combination indices (ED50) showing effects ranging from nearly additive (1.1) to synergistic (<0.9) are shown for the different combinations. The cutoff for nearly additive effects (CI: 1.1) is marked by a dashed line. Patient information is provided as Supplementary Table 1. In addition, AML1 treated in the presence of 50% SCM with PKC412 + KIN40 yielded CI (ED50) = 0.98283 (suggesting additive effects). AML6 treated in the presence of 80% SCM with PKC412 + KIN112 yielded a CI (ED50) = 0.76190 (suggesting synergy).

As the effects of stromal-secreted cytokines, including IL-6, are known to activate JAK/STAT signaling, we investigated the ability of a panel of JAK inhibitors to potentiate the effects of FLT3 inhibition as an attempt to override cytoprotection due to stromal-derived cytokine secretion. We observed that JAK inhibitors synergize with FLT3 inhibitors against mutant FLT3-expressing cells in the presence of stroma or SCM. This appears to involve enhanced inhibition of STAT5 phosphorylation, a finding consistent with the fact that STAT5 has been identified as one of the most important mediators of FLT3-ITD-mediated signaling, and it can also be phosphorylated by JAK.[50–53] The finding that PKC412 combined with dasatinib similarly causes a more pronounced decrease in STAT5 phosphorylation levels in mutant FLT3-expressing cells is again consistent with the fact that STAT5 is an integral component of mutant FLT3-mediated signaling, as well as the fact that STAT5 can be phosphorylated by SRC kinase.[52]

In addition, we observed that JAK inhibitors plus dasatinib or KIN113 synergize against mutant FLT3-expressing cells in the presence of a cytoprotective microenvironment. In contrast, a selective SRC inhibitor, AZD-0530, was unable to synergize with JAK inhibitors under similar conditions, suggesting that Src inhibition likely needs to be accompanied by inhibition of additional kinase targets for effective reversal of stromal-mediated drug resistance.

Our results are consistent with those of a study that demonstrated increased survival of AML leukemic and progenitor cells in response to the FLT3 inhibitor, AG1296, when cells were cultured under 'niche-like' conditions (a microenvironment comprised of serum-free medium supplemented with IL-3, IL-6, SCF and Ang-1, and including fibronectin).[54] In this study, AML cells were more sensitive to inhibitors of signaling pathways, including the JAK/STAT inhibitor, AG490. Our observations also support recent findings showing that JAK2 inhibition may be effective in overriding viability signaling in CML.[55] Ours, however, is the first to implicate the potential usefulness of JAK1, JAK2 and JAK3 inhibitors—as well as dasatinib and related multi-kinase inhibitors—as part of combination therapy to override stromal-derived viability signaling in AML.

We thus present for the first time a cell-based chemical screening model designed to mimic bone marrow stroma protection of leukemia. This model was successful in identifying dasatinib and related mutli-targeted inhibitors as potential candidates for clinical testing as part of combination therapy for effectiveness against mutant FLT3-positive leukemia, as well as possible suppression/eradication of minimal residual disease because of stromal protection. In support of this was PKC412 + dasatinib-induced prolongation of survival in a mouse model of aggressive, progressive FLT3-ITD-leukemic disease and enhanced killing of stromal-protected primary AML cells by drug combinations versus single agents. We also demonstrated the ability of JAK inhibitors to synergize with FLT3 inhibitors or dasatinib/multi-targeted TKIs against stroma-protected, mutant FLT3-positive AML cells. In conclusion, our data suggest that the drug combinations introduced herein may be beneficial in potentiating the effects of FLT3 inhibitors against mutant FLT3-positive leukemic disease, as well as in potentially overriding drug resistance because of to the provision of viability signals from the bone marrow microenvironment.

CONFLICT OF INTEREST

JDG has a financial interest with Novartis Pharma AG. JDG and ALK have a financial interest with Novartis Pharma AG. The other authors declare no conflict of interest.

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