Biomarker profile for prediction of response to SMAC mimetic monotherapy in pediatric precursor B-cell acute lymphoblastic leukemia

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Second mitochondria-derived activator of caspase (SMAC) mimetics (SMs) targeting inhibitor of apoptosis proteins (IAPs) activate cell death pathways, and are currently being evaluated in clinical trials. Their successful therapeutic implementation requires upfront identification of patients who could benefit from a SM-based treatment but biomarkers for SM sensitivity have not yet been described. Here, we analyzed the intrinsic activity of two monovalent (AT406 and LCL161) and two bivalent (Birinapant and BV6) SMs on unselected patient-derived pediatric precursor B-cell acute lymphoblastic leukemia (BCP-ALL) identifying a subset of patient samples to be particularly sensitive to SM-induced cell death. This subset was defined by a characteristic gene expression signature with 127 differentially regulated genes, amongst them TNFRSF1A encoding TNFR1, and a critical role of TNFR1 in SM-induced cell death in sensitive BCP-ALL was confirmed on the functional level. Interestingly, samples with intermediate or low sensitivity to SMs were sensitized to SM-induced cell death by inhibition of caspases using zVAD.fmK or Emricasan, a pan-caspase inhibitor in clinical trials. When we compared our expression data to published data sets, we identified an overlap of four genes to be commonly differentially regulated in SM-sensitive BCP-ALL, that is, TSPAN7, DIPK1C, MTX2 and, again, TNFRSF1A. Functional testing revealed that this set of genes identified samples with high sensitivity to SM treatment. In summary, our data suggest using this gene signature as biomarker predicting response to SM treatment and point to the development of new combinatorial treatments consisting of SMs and pan-caspase inhibitors for a successful clinical implementation of SMs in treatment of BCP-ALL.

Introduction
Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood. While improved multiagent chemotherapy regimens with individualized risk stratification have led to increased survival rates of approximately 80%,¹² 20% of patients respond poorly to therapy or relapse with eventually poor outcome.³⁴ In addition, cytotoxic drugs have severe short- and long-term side effects. Therefore, novel therapeutic avenues are needed to improve treatment outcome, overcome resistance and reduce side effects. Failure to undergo cell death represents a key survival mechanism of cancer cells and results in drug resistance and clonal escape in many cancers, including leukemia.⁵⁶ Drug-induced cell death involves activation of cell-intrinsic pathways that regulate various forms of cell death.

Additional Supporting Information may be found in the online version of this article.

Key words: BCP-ALL, SMAC mimetic, TNFR1, biomarker, Emricasan

Abbreviations: ADAM: a disintegrin and metalloprotease; ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; BCP-ALL: B-cell precursor acute lymphoblastic leukemia; BIR: baculoviral IAP repeat; cIAP: cellular inhibitor of apoptosis protein; DIPK1C: divergent protein kinase domain 1C; IAP: inhibitor of apoptosis protein; LTA: lymphotoxin alpha; MTX2: metaxin 2; Ph: Philadelphia chromosome; RING: really interesting new gene; RIPK: receptor interacting protein kinase; SMAC: second mitochondria-derived activator of caspase; SMs: SMAC mimetics; TNF: tumor necrosis factor; TNFR: tumor necrosis factor receptor; TNFRSF1A: TNF receptor superfamily member 1A; TSPAN7: tetraspanin 7; XIAP: X-linked inhibitor of apoptosis protein

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SMAC mimetics can activate cell death pathways and are currently undergoing clinical trials for treatment of advanced solid tumors and multiple myeloma. Successful therapeutic implementation would require upfront identification of patients most likely to benefit, but biomarkers for SMAC mimetics sensitivity have not yet been described. Here, the authors identified a highly sensitive subset of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) primografts that showed a characteristic gene expression pattern consisting in high TSPAN7, DIPK1C, and TNFRSF1A and low MTX2. The gene signature could potentially be used in the clinic as a biomarker predicting response to SMAC mimetics treatment.

under physiological and pathophysiological conditions, for example, apoptosis, necroptosis and others.\textsuperscript{7,8}

Cell death pathways are blocked by inhibitor of apoptosis proteins (IAPs) such as cellular IAP1 and 2 (cIAP1 and 2) and X-linked IAP (XIAP), which interfere with the execution phase of apoptosis.\textsuperscript{9} Their mechanism of action includes direct inhibition of caspases via baculoviral IAP repeat (BIR) domains or the binding and ubiquitination of proapoptotic factors via an E3 ligase activity-conferring really interesting new gene (RING) finger domain.\textsuperscript{9,10} IAPs can be antagonized by endogenous counter players, such as second mitochondria-derived activator of caspase (SMAC).\textsuperscript{11} As IAPs are often overexpressed in malignant cells and their overexpression correlates with inferior survival rates,\textsuperscript{12,13} they provide an attractive molecular target for therapeutic intervention. Based on our initial description that inhibition of XIAP strongly sensitizes tumor cells for cell death induction,\textsuperscript{12} small molecule inhibitors have been developed that act as SMAC mimetics (SMs) to counteract the cell death inhibitory function of IAPs.

SMs can activate and/or modulate cell death pathways, and are currently being evaluated in clinical trials for both, solid tumors and hematologic malignancies.\textsuperscript{13,14} To date, monovalent and bivalent SMs have been developed and demonstrated to inhibit cell growth and to induce cell death in cancer cell lines.\textsuperscript{15} Bivalent SMs consist of two monovalent subunits tethered together via a linker and have a higher binding affinity toward IAPs.\textsuperscript{15} They are designed to bivalently bind BIR2 and 3 domains,\textsuperscript{16} and are characterized by additional cell death-promoting activity via a tumor necrosis factor (TNF) feed forward loop.\textsuperscript{17,18} Here, binding of SMs results in autoubiquitination of cIAPs and their subsequent proteasomal degradation.\textsuperscript{19} The absence of cIAPs enables noncanonical NF-kB activation and expression of NF-kB target genes, amongst them TNF resulting in stimulation of tumor necrosis factor receptor (TNFR) 1, and possibly also TNFR2, in an autocrine feed-forward loop.\textsuperscript{17,18} TNFR engagement leads to activation of caspase-8 and induction of cell death by complex IIa containing receptor-interacting protein kinase 1 (RIPK1).\textsuperscript{17,20} In addition, SMs were also shown to induce cell death in a TNF-independent manner.\textsuperscript{21–23}

Our group has recently shown that BCP-ALL cell lines and patient-derived BCP-ALL primografts show a heterogeneous response to SM treatment with BV6, a compound in preclinical evaluation.\textsuperscript{24} In addition, efficacy of the SMs LCL161 or Birinapant was assessed in BCP-ALL primografts \textit{ex vivo} and \textit{in vivo} in two additional studies with encouraging results in a subset of patient samples, including relapsed, refractory or high-risk cases, for example, Philadelphia chromosome (Ph)-like ALL.\textsuperscript{25,26} Thus, successful therapeutic implementation of SMs requires identification of patients who respond to SMs ideally before start of therapy.

Here, we evaluated the response of primary patient-derived BCP-ALL samples to SMs AT406, LCL161, Birinapant or BV6 which are currently in preclinical and clinical evaluation.\textsuperscript{13,14} A subset of BCP-ALL samples showed remarkable sensitivity to SMs alone, and inhibition of caspase activity by zVAD.fmk or the FDA-approved drug Emricasan could sensitize samples with intermediate and low sensitivity to SM-induced cell death, a drug combination which has proven to be successful in preclinical studies of acute myeloid leukemia (AML).\textsuperscript{27} Moreover, we identified a characteristic gene expression signature with MTX2 low and TSPAN7, DIPK1C and TNFRSF1A (encoding TNFR1) high in the subset of BCP-ALL samples with high sensitivity to SMs. Using this expression pattern we were able to correctly predict SM sensitivity of additional primary BCP-ALL samples to SM-induced cell death in 89% of the cases. Based on these findings, we propose our identified gene expression pattern as biomarker for upfront selection of patients with a good response to SM treatment and suggest the development of combinatorial treatments consisting of SMs and pan-caspase inhibitors for successful implementation of these compounds into the treatment of BCP-ALL.

Materials and Methods
Reagents
AT406, LCL161, Birinapant and BV6 are available from Selleckchem. Antibodies detecting TNFR1 (Abcam, Cambridge, UK, ab19139), cIAP1 (R&D Systems, Minneapolis, MN, AF8181), cIAP2 (Enzo Life Sciences, Farmingdale, NY, ALX 803341), XIAP (BD Biosciences, San Diego, CA, 610716), RIPK1 (BD, 610459), RIPK3 (Imgenex/Novus Biologicals, Littleton, CO, IMG-5846A/NBP2-24588) and Actin (Sigma Aldrich, St. Louis, MO, A5441) were used for Western Blot. Secondary antibodies for Western Blot were purchased from Southern Bio- tech (Birmingham, AL, rabbit anti-goat IgG Fc-HRP (6163-05); goat anti-rat IgG (H + L), mouse ads-HRP (1080-05)) or Santa Cruz Bio- tech (Birmingham, AL, rabbit anti-goat IgG Fc-HRP (6163-05); goat anti-rat IgG (H + L), mouse ads-HRP (1080-05)) or Santa Cruz Bio- technology (Dallas, TX, mouse anti-rabbit IgG-HRP (sc-2357)). The pan-caspase inhibitor zVAD.fmk and the RIPK1 inhibitor Necrostatin-1 (Nec-1) are available from R&D Systems.

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pan-caspase inhibitor Emricasan is available from Selleckchem and Etanercept and Infliximab from Wyeth Europa Ltd and MSD Sharp & Dohme GmbH, respectively. Blocking antibodies against TNF (anti-hTNF-alpha, AF-210-NA) or LTA (anti-hLymphotoxin-alpha, AF-211-NA) were purchased from R&D Systems. Recombinant human TNF was kindly provided by Henning Walczak.

**Cell lines**

BCP-ALL cell lines were obtained from DSMZ, Braunschweig, Germany. RS4;11 (RRID:CVCL_0093), NALM-6 (RRID:CVCL_0092) and Reh (RRID:CVCL_1650) cells were maintained in RPMI-1640 (Gibco, New York, NY) supplemented with 20% FCS (Gibco), 1% Penicillin/Streptomycin (Gibco) and 2% l-Glutamine (Gibco) at 37°C in a humidified atmosphere with 5% carbon dioxide and split twice weekly at a ratio of 1 in 5–10. Murine bone marrow-derived MS-5 cells (RRID:CVCL_2128) were cultured in alpha-MEM (Gibco) supplemented with 10% FCS and 1% Penicillin/Streptomycin. MS-5 cells were maintained by splitting cells twice per week at a ratio of 1:10. Cell lines were tested mycoplasma-free on a regular basis using the mycoplasma detection kit MycoAlert™ (Lonza). All experiments were performed with mycoplasma-free cells. All human cell lines used in our study have been authenticated by STR profiling within the last 3 years.

**BCP-ALL xenograft samples**

Leukemia samples were obtained from pediatric patients diagnosed with BCP-ALL after informed consent of patients and/or their legal guardians in accordance with the institution’s ethical review board. Primograft leukemia samples were established by transplantation of patient-derived BCP-ALL cells onto female NOD/SCID mice (NOD.CB17-Prkdcscid/ NcrCrl, Charles River, Germany) at the age of 6–10 weeks as described in earlier studies. Immunophenotyping of cells isolated from spleens of leukemia-bearing mice was carried out following standard procedures by analyzing cells on the LSR II flow cytometer (BD Biosciences, Germany). Leukemia cells were subsequently cryopreserved. For ex vivo-testing of xenografts, cells were thawed, washed twice in DPBS (Gibco) and resuspended in StemSpan™ SFEM II medium (STEMCELL Technologies, Vancouver, Canada) supplemented with 20% FCS (Gibco) and 1% Penicillin/Streptomycin (Gibco) for further experiments. All animal experiments were approved by the appropriate authority (Regierungspräsidium Tübingen) and carried out following institutional and national guidelines on care and use of laboratory animals.

**Determination of EC_{50} values of AT406, LCL161, Birinapant or BV6**

To determine EC_{50} values of AT406, LCL161, Birinapant or BV6, xenograft cells were thawed, washed twice in DPBS and resuspended in StemSpan™ SFEM II medium (STEMCELL Technologies) supplemented with 20% FCS (Gibco) and 1% Penicillin/Streptomycin (Gibco), counted and seeded at a density of 5 × 10^4–1 × 10^5 cells/well into 96-well plates. Cells were subsequently treated with increasing concentrations of the respective SM. Cell death was determined by staining for propidium iodide (PI)-positive cells after 24 hr and automated sampling at the Attune NXT Flow Cytometer and Autosampler. EC_{50} values were determined using GraphPad Prism 8 by creating a XY data table, transforming concentrations into logs and calculating EC_{50} values choosing nonlinear regression, then dose–response curves—stimulation and log(agonist) vs. normalized response.

**Classification of SM-sensitive and SM-insensitive samples**

Samples were ranked according to their EC_{50} values (Supporting Information Table S2). Samples were classified as sensitive (blue) if EC_{50} values appeared in the upper third of the list of EC_{50} values of all four compounds (AT406, LCL161, Birinapant and BV6), and as insensitive (red) if EC_{50} values appeared in the lower third of the list of EC_{50} values of all four compounds. Samples which did not appear in the top or bottom third of the list of all four compounds were not classified as overall sensitive or insensitive and marked in gray. Via this approach 8 out of 29 samples were classified as sensitive and six samples as insensitive to SM-induced cell death.

**Western blot**

Cells were washed twice with ice-cold DPBS (Gibco) before lysis in lysis buffer containing 30 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM KCl, 10% Glycerol, 1% Triton X-100, 1× complete EDTA-free protease-inhibitor mix (Roche) and 1× phosphatase-inhibitor cocktail 2 (Sigma). Protein concentration of lysates was determined using BCA protein assay (Thermo Fisher Scientific, Waltham, MA). Lysates were denatured in 10× Bolt™ sample reducing agent (Invitrogen, Carlsbad, CA) and 4× Bolt™ LDS sample buffer (Invitrogen) at 90°C for 10 min before separation on Bolt™ 4–12% Bis-Tris Gels (Invitrogen) and transfer on nitrocellulose membranes using the iBlot gel transfer device. Membranes were incubated in milk (Sigma) for 1 hr at room temperature to block nonspecific binding, and subsequently incubated with primary antibodies at 4°C overnight or for 1 hr at room temperature. Washing of membranes was performed in 1× PBS containing 0.1% Tween-20 (Sigma-Aldrich) for 3 × 10 min before incubation with the secondary antibody for 1 hr at room temperature. Membranes were developed using Pierce™ ECL Western Bloting Substrate (ThermoFisher) or Western Lightning Plus-ECL (Perkin Elmer, Waltham, MA) on Amersham Hyperfilm ECL (GE Healthcare, Chicago, IL) or using the ChemiDoc™ gel imaging system.

**Gene expression profiles**

Basal gene expression of eight SM-sensitive (X068, X074, X093, X112, X134, X139, X17 and X187) and six SM-insensitive (X015, X018, X024, X036, X116 and X142) samples was analyzed using Human Genome U133 Plus 2.0 Arrays. RNA was...
quality-analyzed using the Agilent 2100 Bioanalyzer from Agilent Technologies and arrayed using Affymetrix Human Genome U133 Plus 2.0 with the Affymetrix 3’-IVT Express kit according to manufacturer’s instructions. Then, 127 differentially regulated genes (see Supporting Information Table S3) were identified with a false discovery rate (FDR) of <0.2 as cut-off.

**RNA isolation and qRT-PCR**

RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. cDNA was synthesized using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) with random primers (Thermo Fisher Scientific). qRT-PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a Bio-Rad CFX Connect Real-Time PCR Detection System with the following protocol: 95°C for 30 sec, then 39 cycles of 95°C for 5 sec followed by 60°C for 30 sec. Primer sequences are provided in Supporting Information Table S5.

**Cell death blocking experiments**

Xenograft samples were incubated with Etanercept (100 μg/ml), Infliximab (100 μg/ml) or blocking antibodies against TNF (1 μg/ml or 10 μg/ml) or LTA (1 μg/ml or 10 μg/ml) 2 hr prior to stimulation with 1 μM AT406, LCL161, Birinapant or BV6. Cell death was assessed by PI positivity after 24 hr at the Attune NxT Cytometer and Autosampler. BCP-ALL samples were incubated with the pan-caspase inhibitor zVAD.fmk (20 μM), the RIP1K-inhibitor Nec-1 (30 μM) or a combination thereof 2 hr prior to stimulation with 1 μM AT406, LCL161, Birinapant or BV6. SM-insensitive BCP-ALL samples were also preincubated with Emricasan (5 μM), Nec-1 (30 μM) or a combination thereof prior to stimulation with 1 μM Birinapant. Cell death was assessed by PI positivity after 24 hr at the Attune NxT Cytometer and Autosampler.

**TNF stimulation of MS-5 cells in presence or absence of SMs**

MS-5 cells were seeded at a density of 5 x 10^4 cells/well in 96-well plates 1 day prior to stimulation with increasing concentrations of TNF in absence or presence of 1 μM AT406, LCL161 or Birinapant. Cell viability was determined by CellTiter-Glo® assay after 24 hr.

**Multiplex ligation-dependent probe amplification**

Copy number alterations of genes and chromosomal arms were assessed by Multiplex Ligation-dependent Probe Amplification (MLPA) with SALSA MLPA P335-B2 ALL-IKZF1 probemix and SALSA MLPA P181-B1 Centromere probemix (MRX-Holland, The Netherlands), according to manufacturer’s instructions. The presence of fusion transcripts was analyzed by RT-PCR as previously described. For BCP-ALL cell lines expression levels of the 4 genes were calculated as log2 fold change (log2fc) relative to the mean ΔCT value of the respective gene. Expression levels of the four different genes in xenograft samples were calculated as log2fc relative to the mean ΔCT-value of the samples of the initial cohort validated by qRT-PCR (Figs. 2b and 4c; mean ΔCT-values: 9.49 (DIPK1C), 13.10 (MTX2), 8.95 (TSPAN7) and 5.80 (TNFRSF1A)). A log2fc above or below 0 was considered as high (= T) or low (= F) expression of the respective gene. With this we could derive the following classification rules:

1. (TNFRSF1A = T) AND (DIPK1C = T) AND (TSPAN7 = T) AND (MTX2 = F) = “SM-sensitive”;
2. (TNFRSF1A = F) AND (DIPK1C = F) AND (TSPAN7 = F) AND (MTX2 = T) = “SM-insensitive”.

Samples with an expression pattern of DIPK1C, TSPAN7 and TNFRSF1A high and MTX2 low were predicted to be SM-sensitive whereas samples with the opposite pattern (DIPK1C, TSPAN7 and TNFRSF1A low and MTX2 high) were predicted to be SM-insensitive. To test this hypothesis samples which either fulfilled the criteria of SM insensitivity or SM sensitivity were then stimulated with increasing concentrations of the SMs AT406, LCL161, Birinapant or BV6 and EC50 values were determined by FACS analysis.

Next, hierarchical clustering (average linkage) was performed on the treatment’s EC50 values from all four SM treatments (AT406, LCL161, Birinapant and BV6). Accuracy was calculated in the confusion matrix for sensitivity/insensitivity predicted by the classifier and verified by the four treatments. Binarization of the treatment’s EC50 values was performed to assess for categorization into SM-sensitive/SM-insensitive.

**Statistical analysis**

Data were analyzed using Prism 8 (GraphPad Software, La Jolla, CA). Statistical significance between groups was determined using an appropriate statistical test. p < 0.05 was considered significant (*p < 0.05; **p < 0.01; and ***p < 0.001).

**Data availability**

Gene expression datasets generated in our study are accessible at the Gene Expression Omnibus database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/geo, accession no. GSE140556). Gene expression datasets from Richmond et al. can be accessed at www.ncbi.nlm.nih.gov/geo (accession no. GSE52991, GSE57795, GSE74460). Other data will be made available upon reasonable request.

**Results**

To evaluate the intrinsic activity of SM compounds in childhood BCP-ALL, we stimulated a cohort of 29 unselected primary patient-derived BCP-ALL samples (Supporting Information Table S1) with increasing concentrations of monovalent SMs AT406 or LCL161 (Fig. 1a) or bivalent SMs Birinapant or BV6 (Fig. 1b). Their response to SM-induced cell death was variable.

**Expression of DIPK1C, MTX2, TSPAN7 and TNFRSF1A as biomarker of SM sensitivity**

Expression of DIPK1C, MTX2, TSPAN7 and TNFRSF1A was assessed by qRT-PCR in BCP-ALL cell lines and xenografts.
Figure 1. A subset of primary BCP-ALL samples is sensitive to SMs. Primary patient-derived BCP-ALL samples \( (n = 29) \) were stimulated in duplicates with increasing concentrations \( (0, 0.012, 0.037, 0.1, 0.3, 1, 3 \text{ and } 9 \mu \text{M}) \) of the monovalent SMs \( \text{AT406} \) or \( \text{LCL161} \) (a) or the bivalent SMs Birinapant or \( \text{BV6} \) (b). \( \text{EC}_{50} \) curves are depicted; blue: SM-sensitive, red: SM-insensitive, dashed line: sample with intermediate sensitivity to SMs. (c) Six sensitive and six insensitive samples were lysed and subjected to analysis by Western Blot. Protein expression of \( \text{cIAP1}, \text{cIAP2} \) and \( \text{XIAP} \) was determined. Actin serves as loading control. (d) Three sensitive samples (X068, X112, X187) and three insensitive samples (X024, X116, X142) were stimulated with \( 1 \mu \text{M} \text{AT406}, \text{LCL161}, \text{Birinapant} \) or \( \text{BV6} \) for 2 hr or left untreated. Cells were subsequently lysed and protein expression of \( \text{cIAP1}, \text{cIAP2} \) and \( \text{XIAP} \) was analyzed by Western Blot. Actin serves as loading control. [Color figure can be viewed at wileyonlinelibrary.com]
over a broad range with EC50 values from 7.99 nM up to 11 mM (Figs. 1a and 1b; Supporting Information Table S2). Importantly, response to SMs was independent of spontaneous cell death rates of primograft samples (Supporting Information Fig. S1) previously shown to be associated with prognosis.\textsuperscript{29,33} Interestingly, we identified a subset of samples with high

![Image](image-url)

Figure 2. Legend on next page.
sensitivity to SM-induced cell death with EC_{50} values from 7.99 nM up to 6.5 μM. Of note, bivalent SMs showed an overall higher intrinsic activity than monovalent compounds in BCP-ALL primografts in line with previous reports. We next ranked the samples according to their EC_{50} values for the individual compounds (Supporting Information Table S2) thereby defining samples as SM-sensitive when they appeared in the top third of EC_{50} values of all four compounds and as SM-insensitive when samples were listed in the bottom third of EC_{50} values of all four compounds.

IAPs are regarded as attractive target in anticancer therapy because of their high expression in cancer cells. Thus, we went on to determine IAP expression levels in SM-sensitive and SM-insensitive BCP-ALL primograft samples (Fig. 1c). Both, cIAP1 and XIAP were similarly expressed in all samples whereas expression of cIAP2 was only detectable in a subset of samples independent of their SM sensitivity (Fig. 1c). Furthermore, SMs are known to result in degradation of cIAP1 and 2. To assess whether degradation of IAP proteins upon SM treatment was affected by intrinsic SM sensitivity, we analyzed protein expression of cIAP1, cIAP2 and XIAP upon treatment with SMs (Fig. 1d). cIAP1, and, if expressed, also cIAP2 were efficiently degraded upon incubation with each of the four compounds irrespective of a sample’s sensitivity to SMs (Fig. 1d). Of note, bivalent SMs Birinapant or BV6 were more effective in degrading cIAP1 than monovalent compounds AT406 or LCL161. Levels of XIAP were not affected by SM treatment. These results suggest that intrinsic resistance mechanisms of BCP-ALL to SMs are to be found downstream of SM-induced IAP degradation.

In order to identify possible candidates that might confer sensitivity to SMs, we compared basal gene expression profiles of eight overall SM-sensitive samples (EC_{50} AT406: 88.5 nM–6.5 μM; EC_{50} LCL161: 37.3 nM–4.6 μM; EC_{50} Birinapant: 8 nM–1.1 μM; EC_{50} BV6: 15.1 nM–1.5 μM) and six overall SM-insensitive samples (EC_{50} AT406: 85 μM–11 mM; EC_{50} LCL161: 81.0 μM–1.2 mM; EC_{50} Birinapant: 21.6 μM–99.5 μM; EC_{50} BV6: 9.8 μM–70.4 μM). Via this approach, we identified a characteristic gene signature of SM sensitivity with 127 differentially regulated genes between SM-sensitive and SM-insensitive samples (Fig. 2a and Supporting Information Table S3).

Interestingly, among the 127 differentially regulated genes we found TNF Receptor Superfamily Member 1A (TNFRSF1A) encoding TNFR1 to be upregulated in the group of SM-sensitive samples (Fig. 2a and Supporting Information Table S3) in line with previous reports. Both, lymphotoxin alpha (LTA) and TNF bind to TNFR1 and are capable of inducing cell death. SMs were suggested to mediate cell death via induction of autocrine TNF in several publications. In BCP-ALL, both, specific TNF-blocking antibodies or Etanercept, a TNFR2-Fc fusion protein, inhibited SM-induced cell death ex vivo and in vivo, albeit to variable extent. Thus, we aimed to validate the differential expression of TNFRSF1A in SM-sensitive samples as compared to SM-insensitive samples on mRNA (Fig. 2b) and protein (Fig. 2c) level. Next, and to assess whether the TNF/TNFRI-axis plays a functional role in SM-induced cell death ex vivo, we incubated SM-sensitive xenograft samples with Etanercept prior to stimulation with SMs (Fig. 2d and Supporting Information Fig. S2a). Coincubation with Etanercept significantly blocked cell death induced by SMs (Fig. 1d) pointing toward a critical role of the TNF/TNFRI-axis in SM-induced cell death in line with previous reports. However, Etanercept is the only clinically available anti-TNF therapy which, in addition to TNF, also blocks LTA. To exclude a potential role of LTA in SM-induced cell death, we incubated SM-sensitive samples with blocking antibodies for TNF, LTA or both at concentrations of 1 and 10 μg/ml supposed to efficiently capture LTA and/or TNF in comparison to 100 μg/ml Etanercept (Supporting Information Fig. S2b). Despite using at least 10 times higher concentrations of blocking antibodies than indicated by the manufacturer, we did not observe any blockage.

Figure 2. The TNF/TNFRI-axis plays a critical role in SM-induced cell death in BCP-ALL. (a) Basal gene expression of eight SM-sensitive and six SM-insensitive samples analyzed by Affymetrix Human Genome U133 Plus 2.0 Array was assessed and 127 differentially regulated genes with a false discovery rate (FDR) of <0.2 are depicted. (b) Expression of TNFRSF1A was confirmed by qRT-PCR in the eight SM-sensitive and six SM-insensitive samples analyzed in (a). Data are presented as mean ± SD of eight SM-sensitive and six SM-insensitive samples measured in triplicates. Mann–Whitney test; *p < 0.05. (c) Six SM-sensitive and six SM-insensitive samples were lysed and protein expression of TNFR1 was analyzed by Western Blot. Actin serves as loading control. (d) Eight SM-sensitive BCP-ALL samples from (a) were incubated or not with 100 μg/ml Etanercept prior to stimulation with 1 μM AT406, LCL161, Birinapant or BV6. Cell death was determined by staining for PI-positive cells after 24 hr. Data are presented as mean ± SD of eight samples measured in duplicates. Unpaired Student’s t-test; **p < 0.01. (e) Three SM-sensitive (X068, X074 and X187) xenograft samples were incubated with 100 μg/ml Etanercept, 100 μg/ml Infliximab or left untreated prior to stimulation with 1 μM AT406, LCL161, Birinapant or BV6. Cell death was measured by staining for PI-positive cells after 24 hr. Data are presented as mean ± SD of three SM-sensitive samples measured in triplicates. Friedman’s test and Dunn’s multiple comparison test; ns, nonsignificant; **p < 0.01. (f) Three SM-sensitive (X068, X074 and X187; upper panel) or three SM-insensitive (X024, X036 and X116; lower panel) patient-derived xenograft samples were stimulated with 1 μM of LCL161 or Birinapant for 4 hr or left untreated. Relative expression of LTA or TNF mRNA is shown. Data are presented as mean ± SD of three SM-sensitive samples and three SM-insensitive samples measured in triplicates. Friedman’s test and Dunn’s multiple comparison test; ns, nonsignificant; *p < 0.05. (g) Relative expression of TNFRSF1A of eight SM-sensitive and six SM-insensitive samples and their corresponding EC_{50} values for AT406, LCL161, Birinapant and BV6 are depicted. The nonparametric Spearman correlation coefficient was calculated for relative expression of TNFRSF1A vs. EC_{50} values of AT406, LCL161, Birinapant or BV6. [Color figure can be viewed at wileyonlinelibrary.com]
of SM-induced cell death for neither the LTA- nor the TNF-blocking antibody nor a combination of the two whereas Etanercept very efficiently prevented SM-induced cell death (Supporting Information Fig. S2b). We reasoned that the concentration of blocking antibodies validated with exogenous TNF or LTA may not be sufficient to block autocrine TNF or LTA in our experimental setting. As Infliximab, in comparison to Etanercept, blocks only TNF but with similar affinity, we compared efficacy of the two compounds in preventing SM-induced cell death (Fig. 2e). Etanercept was more efficient in blocking SM-induced cell death than Infliximab indicating that, indeed, LTA may add to SM-induced cell death in BCP-ALL in some samples (Fig. 2e). To address this question further, we also evaluated basal expression levels of LTA and TNF in SM-sensitive and SM-insensitive primografts (Supporting Information Fig. S2c) which was similar in both groups. We found, however, significant upregulation of TNF, but not LTA, upon stimulation with Birinapant, but not LCL161, in both, SM-sensitive and SM-insensitive samples (Fig. 2f). This suggests that resistance mechanisms are to be found downstream of autocrine TNF.

![Figure 3](image-url) SM-sensitive samples die in a RIPK1-dependent manner whereas insensitive samples are sensitized to SM-induced cell death by concomitant inhibition of caspase activity. (a) SM-sensitive samples (n = 8) were incubated or not with 20 μM zVAD.fm.k, 30 μM Nec-1 or a combination thereof prior to stimulation with 1 μM AT406 or LCL161 in (a) or 1 μM Birinapant or BV6 in (b). Cell death was assessed by staining for PI-positive cells after 24 hr. Data are presented as mean ± SD of eight BCP-ALL samples measured in duplicates. (c) SM-insensitive samples (n = 6) were incubated or not with 20 μM zVAD.fm.k, 30 μM Nec-1 or a combination thereof prior to stimulation with 1 μM Birinapant or BV6 and cell death was assessed by PI positivity after 24 hr. Data are presented as mean ± SD of six BCP-ALL samples measured in duplicates. (d) SM-insensitive samples (n = 5) were incubated or not with 5 μM Emricasan alone or in combination with 30 μM Nec-1 prior to stimulation with 1 μM Birinapant. Cell death was assessed by staining for PI-positive cells after 24 hr. Data are presented as mean ± SD of five BCP-ALL samples measured in duplicates. RM one-way ANOVA, Dunnett’s multiple comparison’s test; *p < 0.05; **p < 0.01; ***p < 0.001. [Color figure can be viewed at wileyonlinelibrary.com]
production in BCP-ALL. Next, we reasoned that if TNFR1 expression was decisive for SM sensitivity, then SM-insensitive samples showing low expression of TNFR1 should not be sensitized to exogenous TNF-induced cell death in the presence of SMs. Indeed, we did not detect any significant sensitization to TNF-induced cell death in SM-insensitive xenografts in the presence of SMs (Supporting Information Fig. S2d). In contrast, bone marrow-derived MS-5 cells were strongly sensitized to TNF-induced killing in presence of SMs proving effectivity of the recombinant TNF (Supporting Information Fig. S2e).

While these data indicate that TNFR1 may be a decisive factor determining SM sensitivity in BCP-ALL, we did not find any significant correlation between EC50 values of AT406, LCL161, Birinapant and BV6 and TNFR1 mRNA expression as such (Fig. 2g).

Response to SM-induced cell death has recently been identified to be dependent on a cell’s capability of undergoing both, apoptosis and necroptosis in a receptor interacting protein kinase (RIPK)1-dependent manner. Analysis of RIPK1 and RIPK3 protein expression in SM-sensitive and SM-insensitive samples did not reveal any differences in expression levels between the two groups (Supporting Information Fig. S3a). Next, we evaluated SM-induced cell death in presence of either the pan-caspase inhibitor zVAD.fmk supposed to block apoptosis or/and the RIPK1 inhibitor Necrostatin-1 (Nec-1) preventing RIP1K-dependent cell death. We found that both, monovalent (Fig. 3a) and bivalent (Fig. 3b) compounds induced cell death in SM-sensitive samples that was not significantly prevented by caspase inhibition but inhibited by the addition of Nec-1 alone or in combination with zVAD.fmk indicating that cell death induced by SMs in sensitive samples is mainly dependent on RIPK1 activity (Figs. 3a and 3b). When we next assessed the effect of pan-caspase or/and RIPK1 inhibition in SM-insensitive samples, we surprisingly found a sensitization to bivalent SM-induced cell death (Fig. 3c) and, to a lesser extent, also to LCL161-induced cell death (Supporting Information Fig. S3b) in the presence of the pan-caspase inhibitor zVAD.fmk. The

Figure 4. Identification of DIPK1C, MTX2, TSPAN7 and TNFRSF1A as potential biomarker genes for prediction of SM sensitivity in BCP-ALL. (a) Overlap was assessed of 127 differentially regulated genes between SM-sensitive and SM-insensitive samples of our cohort and 64 differentially regulated genes between SM responders and nonresponders identified in an independent cohort by Richmond et al. DIPK1C, MTX2, TSPAN7 and TNFRSF1A are commonly differentially regulated between the two cohorts. (b) Log2 fold change (log2fc) of DIPK1C, MTX2, TSPAN7 and TNFRSF1A from Richmond et al. (SM responder vs. nonresponder) and our cohort (SM-sensitive vs. SM-insensitive) is depicted. (c) Relative mRNA expression of DIPK1C, MTX2 and TSPAN7 in eight SM-sensitive and six SM-insensitive samples is depicted. Data are presented as mean ± SD of eight SM-sensitive and six SM-insensitive samples measured in triplicates. Mann–Whitney test; *p < 0.05. [Color figure can be viewed at wileyonlinelibrary.com]
very same effect was observed for some BCP-ALL primograft samples with intermediate sensitivity to SMs (Supporting Information Fig. S4c). Thus, we reasoned that a combination of pan-caspase inhibitor and SMs may be more effective in samples with intermediate or low sensitivity to SMs. Thus, we titrated Emricasan, a pan-caspase inhibitor in clinical trials for patients with cirrhosis,40 on SM-insensitive xenograft samples in comparison to zVAD.fmk (Supporting Information Fig. S3c). When SM-insensitive samples were stimulated with Birinapant alone or in combination with either

Figure 5. Legend on next page.
Emricasan alone or together with Nec-1, we could confirm a sensitization to SM-induced cell death by Emricasan (Fig. 3d and Supporting Information Fig. S3d).

Although identified by our gene expression screen for SM sensitivity and functionally proven, TNFR1 mRNA expression alone did not correlate with response to SMs (Fig. 2g). When we compared our list of 127 differentially regulated genes between SM-sensitive and SM-insensitive samples with the list of differentially regulated genes between SM responders and SM nonresponders of a recently published cohort of BCP-ALL which we refer to as “Richmond cohort” (Supporting Information Table S4), we interestingly found a total of 4 genes to overlap in these two lists, that is, Tetraspanin 7 (TSPAN7), Divergent protein kinase domain 1C (DIPK1C), Metaxin 2 (MTX2) and, again, TNFRSF1A (Fig. 4a). Of note, all four genes were similarly regulated in SM-sensitive samples of our cohort and SM responders of the Richmond cohort with TSPAN7, DIPK1C and TNFRSF1A high and MTX2 low (Fig. 4b). In addition, expression levels of 64 differentially regulated genes of the Richmond cohort correlated with expression levels of these genes in our cohort (Supporting Information Fig. S5a). Next, we validated differential miRNA expression levels of TSPAN7, DIPK1C and MTX2 in SM-sensitive and SM-insensitive samples of our cohort (Fig. 4c). Richmond et al. suggested Ph-like ALL to be particularly sensitive to SM treatment.26 Ph-like ALL is a high-risk subset of BCP-ALL defined by a gene expression signature resembling that of Ph-positive ALL with BCR-ABL fusion gene but carrying other kinase-activating fusions including P2RY8-CRLF2.41 Thus, when testing two additional samples from our biobank carrying this fusion transcript, we found them to be particularly sensitive to SM-induced cell death (Supporting Information Figs. S5b and S5c) which was prevented by Etanercept (Supporting Information Fig. S5d). Comparing additional recurrent genetic aberrations between SM-sensitive and SM-insensitive samples, we did not detect any additional mutations indicative for SM sensitivity in our cohort (Supporting Information Table S1).

In both, our cohort and the cohort published by Richmond et al. a pattern of TNFRSF1A, TSPAN7, DIPK1C high and MTX2 low was indicative of SM sensitivity (Fig. 4b). We found 4/8 samples with this expression pattern in our SM-sensitive samples and 3/6 samples with the opposite pattern in our group of SM-insensitive samples (Supporting Information Fig. S6a). Thus, we hypothesized that this expression pattern of TNFRSF1A, TSPAN7, DIPK1C high and MTX2 low or TNFRSF1A, TSPAN7, DIPK1C low and MTX2 high was predictive of SM sensitivity or SM insensitivity, respectively. To test this hypothesis, we next determined mRNA expression levels of the four genes in three BCP-ALL cell lines (NALM-6, Reh and RS4;11). Whereas NALM-6 cells met the expression criteria of SM-insensitive samples, Reh cells complied with the requirements of SM sensitivity (Fig. 5a). RS4;11 cells did not show any pattern. We next evaluated the response of NALM-6 and Reh cells to AT406, LCL161, Birinapant or BV6 and, indeed, found NALM-6 cells to be insensitive and Reh cells to be sensitive to SM-induced cell death as predicted by the expression pattern (Fig. 5b). Next, we tested additional BCP-ALL xenograft samples from our biobank (Supporting Information Table S1, marked as additional samples) for their relative expression levels of TNFRSF1A, TSPAN7, DIPK1C and MTX2. Then, 6/17 samples showed the expression profile of SM sensitivity with TNFRSF1A, TSPAN7, DIPK1C high and MTX2 low (Fig. 5c) whereas 3/17 samples matched the criteria of SM insensitivity with TNFRSF1A, TSPAN7, DIPK1C low and MTX2 high (Fig. 5c). Next, we evaluated the response of these nine samples (see Supporting Information Fig. S6b for rates of spontaneous cell death) to monovalent SMs AT406 and LCL161 (Fig. 5d) and to bivalent SMs Birinapant and BV6 (Fig. 5e). Of note, SM-induced cell death was again prevented by Etanercept indicating that functional TNFR1 was crucial for the response also in these additional BCP-ALL samples (Supporting Information Fig. S6c). To test whether our classifiers of SM sensitivity with TNFRSF1A, TSPAN7, DIPK1C high and MTX2 low and of SM insensitivity with TNFRSF1A, TSPAN7, DIPK1C low and MTX2 high were able to significantly predict a sample’s response to SM treatment, we next performed hierarchical clustering (average linkage) on the treatment’s EC50 values from all four treatments. Of note, the partitioning into two clusters...
(topmost split) shows a high concordance with the label predicted by the classifier (Fig. 5f, left panel). Moreover, the confusion matrix for sensitivity/insensitivity to SM treatment predicted by the classifier and verified by the four different SM treatments AT406, LCL161, Birinapant and BV6 shows an accuracy of 89% indicating that 89% of samples were correctly predicted by our classifiers of SM sensitivity and SM insensitivity (Fig. 5f, right panel). In addition, we found that AT406 allows a significant binarization (BASCA)\textsuperscript{31,32} of the treatment’s EC\textsubscript{50} values into categories sensitive/insensitive with a p-value of 0.001 (Supporting Information Fig. S6d) indicating that this two-class split is in high concordance with the categorization predicted by the classifiers (blue/red).

**Discussion**

SMs AT406, LCL161 and Birinapant are currently undergoing clinical evaluation in advanced solid tumors and a range of hematological malignancies, for example, multiple myeloma or B-cell lymphoma. In pediatric BCP-ALL, SMs are still in preclinical evaluation with so far encouraging results as subsets of primary BCP-ALL showed high sensitivity to SM-induced cell death \textit{ex vivo} and \textit{in vivo}.\textsuperscript{24–26} As stand-alone agents, SMs proved limited efficacy in patients with other cancer entities\textsuperscript{13,14} which is why a successful clinical implementation of these compounds is likely to require (i) combinational treatment regimens that enhance SM-induced killing of cancer cells and (ii) identification of those patients with SM-sensitive tumors before start of therapy, ideally via biomarkers.

We identify SMs to be potent inducers of cell death in a subset of BCP-ALL samples in line with previous reports.\textsuperscript{24–26} Interestingly, we also found that BCP-ALL primograft samples with low or intermediate sensitivity to SM treatment are sensitized to SM-induced killing in presence of an inhibitor of caspase activity, including Emricasan, a pan-caspase inhibitor in clinical trials for liver cirrhosis.\textsuperscript{40} This suggests that SM insensitivity in BCP-ALL is due to a caspase-mediated block of necroptosis in these samples. Given the fact that caspase inhibition did not result in blockage of SM-induced cell death in sensitive samples, but, instead, sensitized samples with intermediate or low sensitivity to SM-induced cell death, we propose a combination of SM and Emricasan as potential application in a clinical setting of pediatric BCP-ALL. This data is in line with a preclinical study of this drug combination in AML in which the authors convincingly demonstrated both, antileukemic efficacy and safety of the combinational treatment regimen of Emricasan and Birinapant \textit{in vivo}.\textsuperscript{27}

Comparing basal gene expression profiles of SM-sensitive and SM-insensitive samples, we identified a characteristic gene expression signature indicating high SM sensitivity in BCP-ALL. Comparing our set of differentially regulated genes with that of a published cohort,\textsuperscript{26} we, interestingly, identified 4 genes to overlap in these two sets of genes, that is, \textit{DIPK1C}, \textit{TSPAN7}, \textit{TNFRSF1A} and \textit{MTX2}. Whereas a functional role of TNFR1, which is encoded by \textit{TNFRSF1A}, in SM response of BCP-ALL samples has been demonstrated in recent publications,\textsuperscript{26} it is not exactly clear to which extent SM-induced cell death is induced by autocrine TNF as also ligand-independent function of TNFR1 in SM-induced cell death have been reported.\textsuperscript{25} Differences in the observed dependency of SM-induced cell death on autocrine TNF might also be due to the fact that blocking antibodies against TNF have been used in one study and Etanercept, a clinically available anti-TNF therapeutic, in others.\textsuperscript{24–26} This is of importance as Etanercept is the only anti-TNF therapeutic known to not only bind to TNF but also to LTA, both known to be able to induce TNFR1-dependent cell death.\textsuperscript{34,36} Thus, we addressed this further and confirmed a role for TNF in SM-induced cell death although at this point it cannot be excluded that LTA adds to SM-induced cytotoxicity in some samples of BCP-ALL.

Apart from TNFR1 also \textit{DIPK1C} and \textit{TSPAN7} were higher expressed in SM-sensitive samples. Little is known about \textit{DIPK1C} and its role in SM-induced cell death is unclear to date. The same holds true for \textit{TSPAN7} although tetraspanin-27, tetraspanin-28 and tetraspanin-29 have been implicated in shedding of membrane-bound TNF \textit{via} a disintegrin and metalloprotease (ADAM) 10.\textsuperscript{42} Interestingly, the fourth and only downregulated candidate in SM-sensitive samples is \textit{MTX2} encoding Metaxin-2. Metaxin-2 was identified to be involved in transport of proteins into the mitochondrion and to interact with Metaxin-1 when overexpressed in insect cells.\textsuperscript{43} The exact role of Metaxin-2 has not yet been defined but, interestingly, it was implicated to be involved in TNF-induced apoptosis together with Metaxin-1 in glioma.\textsuperscript{44}

Our identified classifiers of SM sensitivity with \textit{TNFRSF1A}, \textit{TSPAN7}, \textit{DIPK1C} high and \textit{MTX2} low or of SM insensitivity with \textit{TNFRSF1A}, \textit{TSPAN7}, \textit{DIPK1C} low and \textit{MTX2} high were able to correctly predict sensitivity to SM treatment of additional BCP-ALL primograft samples in 89% of the cases suggesting that this set of genes may serve as biomarker for identification of SM-sensitive BCP-ALL in patients.

In summary, we propose a combinational treatment regimen of SMs and Emricasan, a clinically available pan-caspase inhibitor, to enhance SM-induced killing of BCP-ALL cells, and provide a biomarker gene set of \textit{TNFRSF1A}, \textit{TSPAN7}, \textit{DIPK1C} and \textit{MTX2} for identification of BCP-ALL samples with high sensitivity to SM treatment. Our results pave the way to a more personalized treatment approach using SMs in pediatric BCP-ALL.

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