Sphingosine Kinase 2 Promotes Acute Lymphoblastic Leukemia by Enhancing MYC Expression

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Abstract

Sphingosine kinase 2 (SK2) may have utility as a prognostic marker in inflammatory diseases such as cancer where it has been rationalized as candidate therapeutic target. Here we show that SK2 has an oncogenic role in acute lymphoblastic leukemia (ALL) by influencing expression of MYC. Genetic ablation of SK2 impaired leukemia development in a mouse model of ALL and pharmacological inhibition extended survival in mouse xenograft models of human disease. SK2 attenuation in both settings reduced MYC expression in leukemic cells, with reduced levels of acetylated histone H3 within the MYC gene associated with reduced levels of MYC protein and expression of MYC regulated genes. Our results demonstrated that SK2 regulates MYC, which has a pivotal role in hematological malignancies, providing a preclinical proof of concept for this pathway as a broad-based therapeutic target in this setting.
Introduction
Sphingosine kinases (SK) 1 and 2 catalyze the synthesis of sphingosine 1-phosphate (S1P) from sphingosine (1). Over-expression of SK1 has indisputable tumor-promoting properties (2) and has been associated with poor survival in solid tumor patients (3-5), resistance to imatinib in chronic myeloid leukemia (CML) (6-8) and to daunorubicin in acute myeloid leukemia (AML) cells (9). In contrast, the role of SK2 in cancer is less clear. While over-expression suppressed cell growth and induced apoptosis (1), knockdown of *SPHK2* in glioblastoma cells inhibited proliferation more effectively than knockdown of *SPHK1*, and *SPHK2*-deficient breast cancer cells proliferated poorly *in vivo* (5, 10, 11). SK2 has basal activity that is enhanced by ERK-mediated phosphorylation (12), placing it downstream of signaling pathways frequently activated in malignancies including B-Raf, Ras, Jak and Flt3 (13, 14) all of which can activate ERK (15).

S1P is best known for its extracellular effects mediated through G protein-coupled receptors, S1P₁ to S1P₅. S1P can also act intracellularly by direct modulation of histone deacetylase activity and the ubiquitin ligase activity of tumor necrosis factor receptor-associated factor 2 (TRAF2) (1). In the nucleus SK2 binds directly to histone deacetylases (HDAC) where locally produced S1P inhibits HDAC1 and 2, increasing acetylation of histone H3 on Lys⁹, H4 on Lys⁵ and H2B on Lys¹² (16). Gene expression of the cell cycle inhibitor p21 and transcriptional regulator c-fos is subsequently increased, providing an explanation for the reported growth inhibitory effects of SK2.

The Myc proto-oncogene protein (c-Myc) is over-expressed across a diverse range of human cancers (17), including hematological malignancies, playing a role in both tumor initiation and maintenance (18). In acute lymphoblastic leukemia (ALL), the role of c-Myc has not been
extensively explored, however, translocations involving MYC have been reported in some cases (19, 20). Normally MYC expression is tightly controlled by transcriptional regulatory motifs in its proximal promoter region (21), however epigenetic modifications can also influence the expression of MYC, including in ALL (22).

We demonstrate that a gene signature consistent with increased SK2 activity is present in ALL patient diagnostic samples and that SK2 protein is over-expressed and activated in ALL cells. Inhibition of SK2 kills B-lineage ALL cells and synergizes with the proteasome inhibitor bortezomib and with imatinib in Philadelphia chromosome positive (Ph⁺) cells in vitro. SK2 inhibition reduced expression of MYC mRNA, protein and c-Myc target genes. Reduced association of acetylated histone H3 with MYC was observed, a finding consistent with the effect being mediated by the role of SK2 in histone acetylation. In vivo, SK2 inhibition showed significant anti-leukemic effects in a human xenograft model of ALL, and enhanced the efficacy of imatinib resulting in increased survival in mice bearing a Ph⁺ xenograft. Furthermore, deletion of Sphk2 significantly reduced leukemia development in a murine model of BCR/ABL-driven ALL.
Methods

Cells

Cell lines used were ALL1 (Dr Radich, Fred Hutchinson Cancer Research Centre, Seattle OR), 2070 (23), TOM1 (Prof Melo, IMVS, Adelaide SA), REH, K562 (both from American Tissue Culture Collection), NALM6 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), LK63 (Prof Boyd, QIMR, Brisbane QLD) and stromal-dependent ALL lines derived in our laboratory from patient samples (2032, 1345, 2070, 1809). Leukemic blasts were obtained from patients with ALL with informed consent and institutional ethics committee approval from the Sydney West Area Health Service Human Research Ethics Committee. Spleen cells from previously xenografted animals were used in the in vivo experiments except sample 2070 where cryopreserved patient cells were used. Patient details are in Table S1 and previous papers (23, 24).

Antibodies and Reagents

ABC294640 was obtained from Enzo Life Sciences (Basel, Switzerland), the SK1 inhibitor PF543 was obtained from Merck Millipore (Kilsyth, Australia) and SK1-I from Tocris Bioscience (Bristol, UK), the c-Myc inhibitor, 10058-F4, and 3-methyladenine (3MA) from Sigma Aldrich (St. Louis, MO), bortezomib from Selleck Chemicals (Houston, TX) and Z-VAD-FMK from BD Biosciences (San Jose, CA). The specificity of ABC294640 and PF543 was determined in isoform specific assays (Figure S1), Antibodies to the following were purchased: cleaved caspase-3-FITC, CD19-PE, mCD45R/B220-PE-Cy5, mCD19-PE, mCD11b-FITC (BD Biosciences), mCD45-FITC (Invitrogen), LC3, histone H3 acetylated on Lys\textsuperscript{9}, c-Myc from Cell Signaling (Beverly, MA) and SK2, phospho(Thr\textsuperscript{578})-SK2 from ECM Biosciences (Versailles, KY).
Gene Expression Analysis

Total RNA extracted from cells using the Qiagen RNeasy Mini Kit (Valencia, CA) was amplified and biotinylated with the TargetAmpTM-Nano Labeling Kit (Epicenter Biotechnologies, Madison WI), and gene expression analyzed using the Illumina HumanHT-12 v4.0 Whole-Genome Gene Expression BeadChip and GenomeStudio software (San Diego, CA).

Generation of Gene Signatures

Gene signatures were generated using normalized data (GenomeStudio) from cell lines treated with 60 μM (ALL1, TOM1, NALM6) or 80 μM (REH) ABC2924640 for 24 h. Data was filtered using a Diff p value of <0.01 or 2-fold regulation followed by principal component analysis (Qlucore, Lund, Sweden) (p<0.005, false discovery rate <0.01). The validation cohort was generated using 80 μM ABC294640-treated 2032, 1345, 2070, 1809, and LK63 cells. Hierarchical Clustering was performed using the GenePattern web site and Pearson’s or Spearman’s correlation for the column distance measure and Pairwise complete-linkage method, on data normalized for each row and centered on the mean. Heat maps were generated using Java Treeview. The signal intensity for genes was extracted from GSE28497. The mean of the log2-transformed data of the up-regulated genes was calculated and the signature intensity expressed as the change in this value in patient samples relative to that in the normal controls.

S1P Quantitation and Western Blotting

S1P concentrations in mouse plasma and cell lysates were determined using an S1P ELISA kit (Echelon Biosciences Inc., Salt Lake City, UT) according to the manufacturer’s instructions. Western blotting was performed as previously described (24).
Flow Cytometry, Viability and Proliferation Assays

Viability was measured using propidium iodide (PI) and annexin V-FITC (BD Biosciences) staining and proliferation measured by $^3$H-thymidine incorporation both as previously described (25). Intracellular caspase-3 staining was performed as previously described (26), using FACSCanto or LSRFortessa flow cytometers (BD Biosciences).

Assessment of Drug Interactions

Cells were cultured alone or with IC$_{50}$ concentrations of agents to be tested when used singly and viability assessed by flow cytometry. The proportion of surviving cells expected if no interactive effects were observed was calculated according to the method of Webb (27).

Chromatin Immunoprecipitation (ChIP) Assay

Protein was cross-linked to DNA for 10 min at 37°C with 1% formaldehyde then quenched with 125 mM glycine. Cells were lysed in 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8 for 10 min on ice and sonicated using five 1 min cycles at 40% amplitude with 60% duty from a Branson sonicator. Lysates were clarified by centrifugation at 10 000 x g for 10 min at 4°C and diluted 10 fold in IP buffer (0.5% NP-40; 50 mM Tris, pH 8; 120 mM NaCl; 0.5mM PMSF; Complete protease inhibitor cocktail (Roche, Castle Hill NSW, Australia)). Lysates were pre-cleared for 1 h at 4°C with protein G magnetic beads (Invitrogen) then incubated with primary antibody and protein G magnetic beads overnight at 4°C. Beads were sequentially washed with IP buffer, high salt wash buffer (IP buffer containing 500 mM NaCl), lithium wash buffer (250 mM LiCl; 0.5% NP-40; 1% sodium deoxycholate; 1 mM EDTA, 10 mM Tris-HCl, pH 8) and Tris/EDTA (10 mM Tris, 1 mM EDTA, pH 8). The protein–DNA complexes were eluted with 1% SDS in 0.1 M NaHCO$_3$ at room temperature.
for 15 min, crosslinks reversed in 50 mM NaCl at 65°C for 6 h and DNA purified using Qiaquick PCR columns (Qiagen).

**PCR, qPCR and qRT-PCR**

*BCR/ABL* was amplified from genomic DNA using Takara Taq TM HS (Takara Bio Inc.) and primers specific for the e1a2 transcript of *BCR/ABL* and *Sphk2* using primers (see supplementary information). Products were separated on agarose gels and stained with ethidium bromide (Amresco, Solon, OH) (*BCR/ABL*) or Midori Green (Nippon Genetics, Duren Germany) and visualized using Molecular Imager Gel Doc TM XR (Bio-Rad, Hercules, CA).

RNA was extracted, reverse transcribed and quantitative RT-PCR performed using SYBER Green Real-Time PCR Master Mix (Invitrogen, Carlsbad, CA). Products were normalized to GAPDH and analyzed on Rotor-Gene software. Further details are in supplementary information and Table S2.

**Leukemia Models**

Mice were used with permission from the Westmead Hospital Animal Ethics committee and experiments performed under guidelines from the Australian code of practice for the care and use of animals for scientific purposes 7th Edition, 2004.

**Induction of BCR/ABL-Positive ALL in Mice** - pMSCV retroviral vectors containing a 5’ LTR-driven p185 *BCR/ABL* and internal ribosome entry site (IRES) enhanced GFP (provided by Dr Mullighan, St Jude Children’s Research Hospital, TN), were used to generate helper-free retrovirus after transient transfection of HEK293T cells using Fugene 6 transfection
reagent (Roche). B-cell progenitors isolated by FACS from WT and B6N.129S6-Sphk2<sup>tm1Rlp</sup> (SK2<sup><sup>−/−</sup></sup>) (Prof Proia, Bethesda, MD) C57Bl6 mouse bone marrow (BM) (Figure S2) and number did not significantly different between WT and SK2<sup><sup>−/−</sup></sup> mice (Figure S3). B-cell progenitors were transduced with 4 additions of retroviral supernatants over 24 h on OP9 murine BM stroma in the presence of 20 ng/mL IL-3, 30 ng/mL IL-7, 20 ng/mL SCF and 10 ng/mL Flt-3 ligand (R&D Systems, Minneapolis MN). Transduced cells were injected into sub-lethally (5 Gy) irradiated WT mice (ARC, Perth WA, Australia). The transduction efficiency of WT and SK2<sup><sup>−/−</sup></sup> B cell progenitors using the p185 <em>BCR/ABL</em> viral particles was 13.2±4.3% and 13.6±3.8 respectively.

**Assessment of In Vivo ABC294640 Efficacy** - NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ (NOD/SCIDγ<sup>−/−</sup>) mice were given 2-5 x 10<sup>6</sup> ALL cells by tail vein injection. Three (xenograft 1345) or 7 (xenografts 1999 and 2070) days post injection of leukemic cells, 50 mg/kg (xenograft 2070), 100 mg/kg ABC294640 (xenografts 1345 and 1999) or vehicle (PBS) were administered daily for 21 days by intra-peritoneal injection and the animals sacrificed. The percentage of human cells in the peripheral blood (assessed weekly), BM and spleen was assessed by flow cytometry as described (28). Total leukemia was calculated based on the BM from one femur representing 5.8% of the total BM and a blood volume of 80 mL/kg of body weight.

For survival studies mice engrafted with ALL as above were treated for 21 days with 100 mg/kg of ABC294640 when approximately 1% leukemia was detected in the blood. Animals were sacrifice as required due to deteriorating health scores.

**Statistical Analysis**
The expression of the gene signatures in ALL cells was performed using the Mann-Whitney test. The Fractional product method (27) was used to discriminate between synergistic and antagonistic effects of drug combinations with a value of >0.1 indicating antagonism and <-0.1 synergy. Comparisons between two groups were performed using the Student’s t test and between multiple groups using ANOVA analysis. A level of significance of <0.05 was deemed significant. For survival studies Kaplan-Meier analysis was performed and a Log-rank test used to determine significance with Bonferoni’s correction for multiple comparisons.
Results

Pharmacological Inhibition of SK2 Reduces ALL Cell Proliferation and Survival in Vitro and Induces Caspase-Independent Cell Death

Here we show that ABC294640 reduced SK2 activity and S1P production in leukemic cell lines but did not affect SK1 activity or protein expression (Figure 1A, S1 and S4A). SK2 inhibition consistently reduced cell proliferation (Figure 1B and data not shown), and induced cell death (Figure 1C) in a time dependent manner (Figure 1E) with IC$_{50}$ values at 24 h being significantly higher than those measured at later time points (p<0.001, ANOVA test with repeated measures). Furthermore, SK2 inhibition also induced cell death in patient-ALL cells (Figure 1D) while IC$_{50}$ concentrations for normal bone marrow mononuclear cells and B-cell progenitors were not achieved even after 72 h (Figure S5).

Inhibition of SK2 resulted in externalization of phosphatidylserine (Figure 1F), however a transition from annexin V$^+$/PI$^-$ (early apoptosis) to annexin V$^+$/PI$^+$ (late apoptosis) was not clear (Figure 1F). Apoptotic cells, as defined by nuclear condensation and apoptotic bodies on examination by light microscopy, were largely absent in ABC294640-treated cultures (Figure S6) despite cleavage of caspase-3 (Figure 1G). While caspase inhibition significantly inhibited apoptosis induced by doxorubicin, it did not significantly reduce ABC294640-induced cell death (p=0.18, n=8, Figure 1H). Although LC3-I was processed to LC3-II following SK2 inhibition (Figure 1I) the autophagy inhibitor 3MA did not diminish ABC294640-induced cell death (Figure 1J), despite reducing LC3 processing (data not shown). Overall, cell death following SK2 inhibition was largely caspase-independent and lacked morphological features of apoptosis. Although autophagy was occurring following ABC294640 treatment, this was not the mechanism of cell death.
Sphingosine Kinase Expression in ALL

To establish a gene signature for activation of SK2, we treated ALL cell lines with the SK2 specific inhibitor ABC294640 and analyzed gene expression by microarray. A Diff p value filter produced a list of 12 probes representing 11 genes, and principal component analysis identified 40 probes representing 35 genes (Figure 2A). Notably, a gene signature could not be generated from cells treated with the SK1 specific inhibitor SK1-I (29) and the genes regulated by the SK2 inhibitor were not altered in cells treated with the SK1 inhibitor SK1-I (Figure S7) demonstrating specificity of the signature to SK2. Regulation of selected genes was confirmed by qRT-PCR (Figure S8). Both SK2 gene signatures segregated control and ABC294640 treated cell lines using Hierarchical Clustering and Qlucore software (Figure 2A and data not shown) and were validated using an additional 5 cell lines (Figure S9). In contrast, these signatures could not separate cells treated with SK1-I from control treated cells (Figure S7). The SK2 signatures were used to interrogate a large publicly available gene expression dataset obtained from pediatric ALL patients at the time of diagnosis (GSE28497) (30). SK2 activity signatures were higher in ALL samples (p=0.001 and p=0.027) (Figure 2B and data not shown) than normal B-cell progenitors. The $\text{SHPK2}$ gene was not over-expressed, however, increased SK2 protein was detected (Figure 2C) with increased phosphorylation, suggesting increased enzymatic activity (Figure 2C). Together these data show activation of SK2 without increased gene expression.

SK2 Inhibition in ALL Inhibits Expression of MYC and c-Myc Regulated Genes

In order to determine the mechanism by which inhibition of SK2 induced cell death in ALL we undertook microarray analysis of control and ABC294640-treated cells at the earlier time point of 6 hr. Only 5 genes were consistently regulated across all cell lines (Figure 3A) and these genes were not consistently regulated in SK1-I treated cells (data not shown).
Examination of the diagnostic pediatric ALL gene expression dataset, GSE28497(30) revealed MYC to be more than 2-fold over-expressed (p=0.03) affecting all sub-classifications (Figure 3B). FAM129A was slightly over-expressed (log2 fold change 0.84, p=0.0001) and DDIT3 under-expressed (log2 fold change -0.73, p=0.001) while TSC22D3 and TRIB3 were within normal limits. MYC remained significantly decreased at 24 h in all 9 cell lines and metacore analysis identified c-Myc as the transcription factor most significantly associated with the changes in gene expression in these cell lines (p<1.0e-250, z-Score=90.57, g-Score=93.07) (Table S3). Down regulation of MYC expression was confirmed by qRT-PCR (Figure 3C) but was not observed following treatment with the SK1-selective inhibitor PF543 (Figure S10). c-Myc protein was also markedly reduced by ABC294640 (Figure 3D) and was also decreased following knockdown of SK2 by siRNA (Figure S11). Despite recent reports of increased Sphk1 expression in the absence of Sphk2 (31), SPHK1 gene expression was not increased by SK2 inhibition as determined by microarray analysis.

We questioned whether changes in c-Myc regulated genes could segregate SK2 inhibitor-treated ALL cells from their respective controls. To this end we extracted three previously published c-Myc gene signatures (22, 32, 33) from the original and the validation microarray data sets. Unsupervised hierarchical clustering separated the SK2 inhibitor treated cells from control treated cells (Figure 3E and Figure S12). Gene Set Enrichment Analysis (GSEA) demonstrated a significant negative enrichment of both the Bild and Schuhmacher c-Myc signatures in SK2 inhibited cells. The specificity of this effect was apparent from the lack of any consistent association between NF-κB or PI-3K/AKT pathway members or SP1 targets (Figure 3F and Table S4) and the absence of these signatures in SK1-I treated cells (p=0.17 and 0.08 respectively) (Figure S10). This suggests that regulation of MYC plays a significant role in the response of ALL cells to SK2 but not SK1 inhibition. Interrogation of the
GSE28497 dataset using the Schuhmacher et al (33) c-Myc signature found c-Myc targets to be increased in diagnostic patient samples (p=0.019) consistent with the over-expression of MYC in these patient samples (Figure 3G).

**SK2 Inhibition Reduces the Association of Histone H3 Acetylated on Lysine 9 with the MYC Promoter**

It has been reported that SK2 binds to HDAC in the nucleus and that locally produced S1P inhibits HDAC activity, resulting in increased acetylation of histones including histone H3 on Lys\(^9\) (H3K9ac) (16). We confirmed this data, showing that SK2 inhibition reduced the amount of S1P associated with HDAC2 (Figure S13A). ENCODE/Broad (34) report that H3K9ac associates with MYC (Figure 4A) and using ChIP followed by qPCR of MYC promoter elements, SK2 inhibition was found to reduce the association of MYC with H3K9ac (Figure 4B). In support of a role for c-Myc suppression in ALL cell death following SK2 inhibition, the c-Myc inhibitor 10058-F4 induced cell death in ALL cell lines in a time dependent manner similar to that observed following SK2 inhibition (Figure 4C). Exogenous expression of MYC also provided a degree of resistance to ABC294640 (Figure S13B). The association of NME1 and NUP62 promoters (Figure 4D) with H3K9ac was significantly reduced following inhibition of SK2 (Figure 4F) consistent with their reduced expression.

**Concurrent Bcr/Abl and SK2 inhibition Produces Synergistic Effects in Ph\(^+\) ALL**

New therapies for ALL will almost certainly be employed in combination with established chemotherapy regimens. No significant interactive effects between SK2 inhibition and vincristine or doxorubicin were observed, with the fractional product method showing at best additive effects (Figure 5A and Figure S14). In contrast, synergistic cell death was observed
at all time points when inhibition of SK2 was combined with the proteosome inhibitor bortezomib (Figure 5B).

Imatinib inhibited proliferation, but had little effect on the viability of Ph⁺ ALL cell lines (Figure 5C and D). However, known resistance-inducing mutations in BCR/ABL were not detected. Resistance to imatinib-induced cell death was overcome by concurrent SK2 inhibition and clear synergistic interactions were observed in almost all conditions tested (Figure 5E and Figure S14).

SK2 Inhibition is Effective in a Human Xenograft Model of B-Lineage ALL

Intra-peritoneal injection of NOD/SCID IL2γc⁻/⁻ mice with ABC294640 reduced plasma S1P concentrations by 23% after 2 h, with a further decrease (45%) by 6 h, and levels remained significantly suppressed for at least 24 h (Table S5). This indicates that ABC294640 significantly inhibits SK2 activity, reducing S1P plasma levels in vivo, consistent with findings by Beljanski et al (35). NOD/SCID IL2γc⁻/⁻ mice engrafted with human ALL cells were treated with 100 mg/kg/day ABC294640 or vehicle by intra-peritoneal injection for 21 days after which all animals were sacrificed. The percentage of ALL cells in the BM and blood was significantly reduced in SK2 inhibitor-treated mice, while the contribution to the spleen was significantly reduced in xenograft 1999 only (Figure 6A-C upper panels). Average absolute levels of leukemia in the BM of SK2 inhibitor-treated mice were reduced by between 40 and 60% (p<0.004) (Figure 6A lower panel). Reductions in the blood were between 60% and 67% (p<0.002) and in the spleen by 38% and 79% (p<0.0001) for xenografts 1999 and 1345 (Figures 6B and C lower panels). Overall, inhibition of SK2 decreased the level of disease in all xenografts between 40 and 78% (p<0.00005) (Figure 6E). No overt toxicity was noted, with the murine WBC being higher in SK2 inhibitor-treated animals bearing xenograft
1999 (p=0.018), consistent with reduced disease, and not significantly different in the other xenografts (Figure 6D).

Inhibition of SK2 significantly slowed the increase in circulating ALL cells, prolonging the survival of mice bearing xenograft 1999 (p=0.0012, Figure 6F and G) from a median of 30 days for control mice (95% CI 29-30 days) to 41 days (95% CI 32-38 days) for the treated group. Concurrent inhibition of SK2 and Bcr/Abl with imatinib also significantly improved the survival of mice engrafted with a Ph⁺ ALL (2070) (Figure 6G). In this experiment the median survival of control mice was 49 days (95% CI 47.5-50.5 days) with imatinib extending survival to a median of 65 days (95% CI 63.4-96.6 days). Concurrent inhibition of SK2 further extended survival to a median of 104.5 days (95% CI 91.1-117.9 days, p=0.004 compared to imatinib alone and p=0.044 compared to ABC294640 alone).

SK2 Contributes to ALL Development in Mice

Twenty-two of 29 mice receiving BCR/ABL-transduced B-cell progenitors from WT animals developed ALL, with a median survival of 42 days (95% CI 32-52 days). Absence of SK2 reduced the incidence of BCR/ABL-driven ALL to 16 of 29 animals (median survival of 58 days, 95% CI 47-69 days) (p=0.002) (Figure 7A). In contrast to reports by Liang et al (31), the absence of SK2 did not result in increased SK1 expression (Figure S4B). The presence of BCR/ABL and the expected deletion of Sphk2 were confirmed by PCR in all murine leukemias examined (Figure 7B and C). All leukemias demonstrated a B-cell progenitor phenotype (Figure 7D), with the majority being pre-B-ALL, lacking CD43 expression. Lymphoblasts were present in blood films and livers from all animals (Figure 7E and F). Cells recovered from the spleens of leukemic mice demonstrated reduced c-Myc expression in leukemias arising in SK2⁻ cells as compared to those derived from WT B-cell progenitors (Figure 7G).
These data demonstrate a role for SK2 in the development of BCR/ABL-driven ALL. ALL cells recovered from mice receiving BCR/ABL-transduced WT B-cell progenitors were sensitive to ABC294640 while those from SK2\(^{-/-}\) B-cell progenitors were resistant, confirming that the cytotoxic effects of ABC294640 were mediated through SK2 (Figure 7H).
Discussion

The oncogenic role of SK1 has been demonstrated in a range of malignancies, however the role of SK2 in cancer is largely unknown. Over-expression of SK2 can be pro-apoptotic via binding to BclXL (36) and by its role in sphingolipid metabolism resulting in increased levels of pro-apoptotic ceramide (37). Recently sphingolipid metabolism was shown to be required for activation of BAK and BAX and apoptosis induction (38). However, recent studies have demonstrated a role for SK2 in tumor promotion (11, 35) and demonstrated that inhibition of SK2 can inhibit in vitro and in vivo growth of the myeloid leukemia cell line, U937 (39). We examined the effects of Sphk2 deletion in the development of BCR/ABL-driven ALL, and SK2 inhibition in ALL cells in vitro and in vivo. Despite a recent report showing increased SK1 in Sphk2−/− mice (31), SK1 was not increased in Sphk2−/− ALL cells or following SK2 inhibition (Figure S4). We also explored the mechanism by which SK2 inhibition mediates its anti-cancer activity, revealing suppression of c-Myc as a key mechanism.

A role for SK2 in ALL biology was suggested by the presence of a gene signature consistent with increased SK2 activity in a large cohort of pediatric ALL samples collected at diagnosis. This was supported by the reduced incidence of ALL in a BCR/ABL-driven model of murine ALL in the absence of Sphk2, suggesting that SK2 plays a role in disease initiation in this setting. However, the efficacy of SK2 inhibition in established human leukemia indicates that it is also important in disease progression. Overall this is the first report demonstrating the importance of SK2 in acute lymphoblastic leukemia.

While over-expression of SK2 has been reported to produce anti-proliferative effects and result in cell death, it is clear that SK2 can also promote cell growth. The reasons for these discrepant effects are not entirely clear. The sub-cellular localization of SK2 may be
important for the ultimate function of this enzyme (40). A recent study showed that siRNA knockdown of SPHK2 inhibited breast cancer cell proliferation despite increasing S1P production (41), speaking strongly to the importance of the localized activity of these enzymes over global S1P production. When associated with the endoplasmic reticulum SK2 induced apoptosis via production of ceramide (42), while translocation to the cytoplasm resulted in survival-promoting effects in colon cancer cells (43). SK2 also has demonstrated protective effects at the mitochondria following ischemia reperfusion injury in a number of tissues including cardiomyocytes (44). In this study it appears that the nuclear localization of SK2 and its known effects on histone H3-histone deacetylase (HDAC)1/2 (16) were the dominant mechanism of action. In ALL cells SK2 increases expression of oncogenic c-Myc, presumably through localized SIP production, promoting survival and proliferation. Consistent with this, ALL patient samples have increased MYC expression, increased expression of c-Myc regulated genes and are killed by c-Myc inhibition. Furthermore, exogenous expression of MYC resulted in a degree of resistance to SK2 inhibition by ABC294640.

SK2 inhibition has been associated with both caspase-dependent (45) and -independent cell death (46) with autophagy being involved in the latter. Autophagy also has cell survival roles (47) and although SK2 inhibition induced autophagy in ALL cells, this was not the cause of cell death, a situation similar to that following FTY720 exposure (48). Consistent with the lack of apoptosis, genes involved in apoptosis were not over-represented in the microarray data (data not shown). Inhibition of SK2 has been shown to synergize with chemotherapeutic agents in breast cancer cell lines in vitro, via inhibition of NF-κB mediated survival signals (49). However, our attempts at combining conventional chemotherapeutic agents such as vincristine and doxorubicin were disappointing with no significant synergy seen. The lack of
an effect on NF-κB signalling in ALL cells when SK2 is inhibited (Table S4) may explain this result. We therefore turned our attention to the biological response modifier bortezomib, after reports of efficacy in lymphoid malignancies (50, 51) and found the combination of ABC294640 with this agent to be superior to conventional drugs. In addition, inhibition of SK2 also synergized with imatinib in Ph⁺ disease, reminiscent of work published with SK1 inhibitors (7). Overall this suggests that combination treatments including SK2 inhibitors may have potential as therapeutic strategies.

A major novel finding in this study is the down-regulation of the MYC gene and c-Myc target genes as a result of SK2 inhibition. This effect on MYC expression was specific for SK2 with SK1 inhibition failing to influence expression of MYC or c-myc target genes in ALL cells. Increased MYC and c-Myc target gene expression in ALL samples from patients and the ability of a c-Myc inhibitor to kill ALL cells provides evidence for a role for c-Myc in ALL cell biology. These data potentially explain the often opposing roles of SK2 in cell growth and survival, inhibiting growth and survival via effects on p21 and fos, while promoting these features by increasing expression of c-Myc. They also highlight a potentially under-appreciated role of c-Myc in ALL biology and identify potential therapeutic strategies for the treatment of ALL and other c-Myc-driven malignancies.
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Authorship Contributions

CWB performed research and contributed to analysis and interpretation of data, statistical analysis, writing the manuscript and research design, KFB contributed to research design and the writing of the manuscript, LJB contributed to the analysis and interpretation of data,
statistical analysis, writing of the manuscript and research design. SMP performed research and contributed to analysis and interpretation of data. JAP and DT performed experiments.
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Figure Legends

Figure 1. SK2 inhibition suppresses proliferation and induces caspase-independent cell death in ALL cells. (A) ALL cell lines were cultured with 60 μM ABC294640 or vehicle for 24 or 48 h and cellular S1P concentrations determined. (Mean ± SD from 3 cell lines). *p<0.05. (B - E) ALL cell lines (B, C & E) or patient cells (D) were cultured with ABC294640 or vehicle for 48 h (B), 72 h (C) or 16 h (D). (B) The proliferation (B) or viability (C - E), was normalized to control cultures. (F) ALL cell lines were cultured with 75 μM (REH) or 100 μM (ALL1) ABC294640 for 24 h and annexin V and PI staining assessed. (G) ALL cell lines were cultured with 100 μM ABC294640 or vehicle for 72 h, except for NALM6 cells (analyzed at 24 h), and caspase-3 cleavage measured. isotype control, cleaved caspase-3. (H) ALL cell lines were cultured with 0.25 μg/mL of doxorubicin, 80 μM or 90 μM ABC294640 or vehicle with or without pre-incubation with 100 μM Z-VAD-FMK and viability assessed after 16 h. (Mean ± SD from ≥ 3 experiments). *p<0.05 comparing cells pre-incubated with Z-VAD to those without. (I) Cell lines were treated with ABC294640 and cell lysates analyzed for LC3 protein. (J) ALL cell lines were treated with vehicle (Control) or 50 μM ABC294640 for 24 h with or without 5 mM 3MA, except for ALL1 where 48 h treatment with ABC294640 was used. (Mean ± SD).

Figure 2. Expression and Activity of SKs in ALL. (A) Hierarchical clustering of control and SK2 inhibitor treated ALL cell lines using gene expression changes resulting from inhibition of SK2 detected by filtering on the Diff p value (left panel) or principal component analysis (right panel). (B) Gene expression array data from ALL samples were interrogated for the presence of the SPHK2 gene signature relative to normal B-cell progenitors. The signature intensity is shown and patient subtypes are indicated. (C) Expression of phosphorylated and total SK2 in ALL cell lines, 5 patient samples, peripheral blood
mononuclear cells (PBMC) from normal donors and normal CD34⁺CD19⁺ bone marrow B-cell progenitors.

**Figure 3. Inhibition of SK2 suppresses expression of MYC and c-Myc regulated genes.**

(A) Changes in gene expression in NAM6, ALL1, REH and TOM1 cells lines following 6 h of SK2 inhibition. (Mean ± SD) (B) Gene expression array data from ALL samples were interrogated for MYC expression. The signal intensity is shown for each patient and 4 normal donors and patient subtypes are indicated. (C) qRT-PCR analysis of MYC expression in ALL cell lines following a 24 h incubation with 60 μM ABC294640 or vehicle. The mean ± SD of 3 experiments is shown. (D) Western blots showing expression of c-Myc following incubation with ABC294640 at the indicated concentrations and time periods. (E) Hierarchical clustering of control and SK2 inhibitor treated ALL cell lines using a c-Myc gene signature. (F) Gene set enrichment plots were generated using the indicated gene sets and the array data from each of the stromal-dependent cell lines treated with ABC294640. NES=normalized enrichment score, q=false discovery rate, p=significance. (G) Gene expression array data from ALL samples interrogated for the presence of the MYC gene signature(33) relative to normal B-cell progenitors. The signature intensity is shown and patient subtypes are indicated.

**Figure 4. SK2 inhibition regulates MYC expression via deacetylation of histones.**

(A) Schematic diagram showing the H3K9ac track on the MYC gene as determined in GM12878 cells, a B-lymphoblastoid cell line. (B) REH and TOM1 cells were treated with 80 μM ABC294640 for the indicated times. ChIP analysis of the MYC promoter was performed using anti-H3K9ac as the precipitating antibody. Primers in the MYC promoter or an unrelated intergenic region were used to amplify co-precipitated DNA. The mean ± SD of 3 experiments is
shown. *p<0.05. (C) TOM1 and REH cells were cultured with the indicated concentrations of the c-Myc inhibitor 10058-F4 for the indicated times and analyzed for viability using annexin V/PI staining and flow cytometry. The mean ± SD of duplicates for each cell lines are shown. (D) Schematic diagram showing the H3K9ac track on the NME1, SLC29A1 and NUP62 genes as determined in GM12878 cells (Note NUP62 is encoded on the reverse strand). (E) ChIP analysis of NME1 and NUP62 in REH and TOM1 cells treated with 80 μM ABC294640 for the indicated times using anti-H3K9ac as the precipitating antibody. The mean ± SD of 3 experiments is shown. *p<0.05.

Figure 5. SK2 inhibition synergizes with bortezomib and imatinib. The indicated cell lines were cultured with (A) vehicle, 31.25 ng/mL doxorubicin or 1 nM vincristine or (B) vehicle or bortezomib (7.5 nM for NALM6, 5 nM for ALL1), with or without the addition of 50 μM ABC294640 for 24 h (A) or the indicated time (B) and analyzed for viability. The mean and standard deviation 2 experimental replicates is shown. The indicated cell lines were incubated with increasing concentrations of imatinib for 72 h and assessed for proliferation (C) or survival (D). The mean and standard deviation of four (C) and two (D) experimental replicates is shown. (E) The indicated cell lines were cultured with vehicle, or 50 μM ABC294640 with or without the addition of 5 μM imatinib for 48 h or 72 h and analyzed for viability. The mean and standard deviation of 2 experimental replicates is shown. In A, B and E the hatched bar represents the calculated additive effect based on the effect of each agent alone. # Indicates a synergistic interaction between the agents as determined by a fractional product method.

Figure 6. ABC294640 shows in vivo efficacy and in Ph+ and Ph− ALL. (A-E) Groups of 6 IL2γc−/− NOD/SCID mice engrafted with xenografts 1999, 2070 or 1345 were treated with 100
mg/kg of ABC294640 daily by IP injection commencing 3 days for 1345 or 7 days for 1999 and 2070 after receiving ALL cells and continuing for 21 days when all animals were culled. The percentage (upper panels) and absolute number (lower panels) of ALL cells detected in the BM (A), spleen (B) and peripheral blood (C) are shown. The murine WBC is shown in (D) and the total leukemic burden (E) are also shown. Each dot indicates data from an individual animal and the line the mean of the cohort. Significance levels comparing vehicle to ABC294640-treated mice are indicated on the graphs. (F and G) Groups of 6 NOD/SCID IL2γc−/− mice were engrafted with ALL xenografts 1999 (F) or 2070 (G). 100 mg/kg of ABC294640 was administered for 21 days by IP injection and in xenograft 2070 100 mg/kg of imatinib was given alone or in addition to ABC294640 by daily intra-peritoneal injection. The shaded area on each graph indicates the period of treatment. At the end of the treatment period mice were monitored for disease and culled as required.

Figure 7. SKs are important in murine ALL development. (A) Kaplan-Meier survival analysis of WT mice injected with BCR/ABL-transduced B-cell progenitors from a total of nineteen WT and SK2−/− animals. Significance using Cox Regression analysis relative to mice receiving WT cells is indicated. The experiment was run on three separate occasions. (B) PCR detection of BCR/ABL in the leukemias harvested from mice. The positive and negative controls consisted of p190 positive and negative ALL cell lines. (C) PCR assessment of Sphk2 deletion in murine ALL cells. The PCR product from WT cells is 680 bp in WT and 310 bp in SK2−/− cells (right panel). Note the WT band is detected in residual normal cells in the harvested tissue containing ALL. The heavy band in the 100 bp DNA ladder is 500 bp. (D) Representative immunophenotype of a murine ALL sample showing B220 expression and lack of surface IgM. (E) Romanowsky stain of peripheral blood from a mouse with BCR/ABL-driven ALL. Original magnification 400x. Bar indicates 50 μm. (F) Representative
haematoxylin and eosin stained, paraffin embedded liver sections showing intra- and perivascular ALL infiltration. Original magnification 200x (upper panel) 400x (lower panel). Bar indicates 100 μm. (G) Western blot of c-Myc in murine ALL recovered from secondary recipients. Values below the gels indicate the density of the c-Myc band relative to β-actin. Spleen cells recovered from 3 independent leukemias is shown. The mean±SD of the data from the gel is shown in the graph. *p<0.05 compared to WT ALL. (H) ALL cells recovered from the spleens of mice receiving WT or SK2−/− ALL were plated on OP9 stroma to minimize cell death. Cells were treated with the indicated concentrations of ABC294640 for 24 h and viability assessed by annexin V/PI staining. The mean ± SD of duplicates is shown.
Figure 1

A. S1P (μM) at 24 and 48 hours.

B. Relative proliferation of different cell lines.

C. Relative viability of different cell lines.

D. ABC294640 (μM) viability.

E. IC50 values for different cell lines.

F. Annexin V FITC and PI staining for different cell lines.

G. Cleaved Caspase-3 FITC staining for different cell lines.

H. Viability of different treatments.

I. LC3 expression in different treatments.

J. Viability of ALL1 and NALM6 with different treatments.
Figure 2
Figure 4
Figure 5
Figure 6
Sphingosine Kinase 2 Promotes Acute Lymphoblastic Leukemia by Enhancing MYC Expression

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