T-cell acute lymphoblastic leukemia (T-ALL) is frequently characterized by glucocorticoid (GC) resistance, which is associated with inferior outcomes, thus highlighting the need for novel therapeutic approaches for GC-resistant T-ALL. The pre-T-cell receptor (pTCR)/TCR signaling pathways play a critical role in cell fate decisions during physiological thymocyte development, with an interplay between TCR and glucocorticoid receptor (GR) signaling determining the T-lymphocyte selection process. We performed an shRNA screen in vitro and in vivo in T-ALL cell lines and patient-derived xenograft (PDX) samples to identify vulnerabilities in the pTCR/TCR pathway and identified a critical role for the lymphocyte cell-specific kinase (LCK) in cell proliferation. LCK knockdown or inhibition with dasatinib (DAS) caused cell cycle arrest. Combination of DAS with dexamethasone (DEX) resulted in significant drug synergy leading to cell death. The efficacy of this drug combination was underscored in a randomized phase II-like murine trial, recapitulating an early phase human clinical trial. T-ALL expansion in immunocompromised mice was significantly impaired using this drug combination, compared to mice receiving control vehicle or single drug treatment, highlighting the immediate clinical relevance of this drug combination for high-risk T-ALL patients. Our results thus provide a strategy to improve the efficacy of current chemotherapy platforms and circumvent GC resistance.
Activation of this complex occurs through the SRC family kinase (SKF) members lymphocyte cell-specific protein-tyrosine kinase (LCK) and FYN. They are critical modulators of T-cell development and activation. LCK phosphorylates the plasma membrane-associated TCR complex and ZAP70. ZAP70 in turn phosphorylates the linker for activation of T cells (LAT) leading to the activation of downstream signaling cascades. The overall activity of LCK is regulated by the phosphorylation status of the activating and inhibitory tyrosine residues 394 and 505, respectively. LCK activation correlates with Y416SRC (also Y394LCK) phosphorylation, as the latter over-rode the inhibitory effects of Y505 phosphorylation.

We hypothesized, therefore, that T-ALL continues to rely on proliferative and survival stimuli inherent to the TCR signaling pathway, which, if inhibited, may enhance GC sensitivity. A targeted shRNA screen directed against components of the TCR signaling initiation complex identified a crucial role for LCK in T-ALL proliferation, both in vitro and in vivo. The anti-proliferative effects of LCK knockdown could be replicated by using the small molecule inhibitor dasatinib (DAS). Drug synergy was observed using DAS in combination with dexamethasone (DEX) on patient-derived xenograft (PDX) cell survival in vitro. Mirroring the design of early phase human trials, a murine phase II-like trial demonstrated significantly impaired leukemia progression in vivo using combination treatment. Our results present a clear rationale for using DAS in conjunction with DEX to enhance conventional chemotherapeutic treatment and revert GC resistance in pediatric T-ALL patients.

Methods

Patient samples

The patient-derived material was collected as part of diagnostic investigations of patients at the Great North Children’s Hospital, Department of Paediatric Haematology and Oncology, Newcastle upon Tyne, UK. The material was collected and stored with informed consent obtained from all subjects in accordance with the Declaration of Helsinki.

Samples with explicit written consent for in vivo studies were requested from the Newcastle Biomedicine Biobank, Newcastle University, UK, and used according to approvals given by the Newcastle Biomedicine Biobank (NHB application NHB-008) and the local institutional review board Newcastle & North Tyneside Ethics Committee (REC reference: 07/H0906/109).

Drug matrix assays

Dasatinib (9 nM – 30 μM) (DC Chemicals, Shanghai, China) was titrated on T-ALL cell lines (4x10^4/well) in 96-well plates (Corning, NY, USA). Cell viability was assessed after 3 days using Cell Counting Kit 8 (NBS Biologicals, Cambridgeshire, UK). Absorbance was measured at OD450 nm using a POLARstar Omega plate reader (BMG LABTECH, Bucks, UK). IC50 values were determined by GraphPad Prism. Assays were performed in triplicate and at least three independent repeats were performed.

For DAS/DEX combination treatments DAS (80 nM – 50 μM) and DEX (0.09 nM – 600 nM) were titrated in 2-dimensions on T-ALL cell lines (4x10^4 cells per well in 96-well plate) or ex vivo expanded PDX cells (8x10^4 cells per well in 96-well plate). Ex vivo expansion was achieved after co-culture with OP9-DL1 for 1 week, after which cells were separated from their feeders by repetitive transfer and subsequently plated. After 72 hours (h) of culture, the plates were developed as above. Drug synergy was determined using Combenefit software (v.2.021).

Phase II-like murine trial

For each of the ten PDX samples, 8x10^6 cells were intrafemoral (IF) injected into four NSG mice (40 mice in total) under isoflurane anesthesia. The four NSG mice derived from one PDX sample were matched for gender and age. T-ALL engraftment in mouse peripheral blood was monitored weekly by tail vein bleeds (20 μl blood/mouse). The four mice of each PDX were randomized to receive control vehicle, DAS (35 mg/kg), DEX (1 mg/kg) or DEX/DAS combination by intraperitoneal (IP) injection upon engraftment, defined as ≥0.5% peripheral blood hCD45^+/hCD7^− cells. The median treatment duration of these mice was 15 days, depending on their clinical status. When any of the four mice displayed signs of ill health or weight loss, all four mice derived from this PDX were killed at the same time to assess leukemia engraftment in bone marrow, blood, spleen, liver and CNS. Spleen size and weight were recorded. Statistical analyses were performed using RStudio (Boston, MA, USA) with linear mix model. The final analysis excluded the four mice derived from patient sample LK214, as all mice succumbed to T-ALL before treatment was initiated.

See the Online Supplementary Appendix for further details of the methods used.

Results

A targeted shRNA screen of T-cell receptor pathway components identifies an essential role for lymphocyte cell-specific kinase in T-cell acute lymphoblastic leukemia cell line and patient-derived xenograft proliferation in vitro

To explore the importance of the pTCR/TCR signaling complex in proliferation and survival of malignant T cells, we performed a limited shRNA screen targeting six genes with three shRNAs per gene, including LCK, ZAP70, PTCRA, FYN, CD3ε and LAT in four T-ALL cell lines (HPB-ALL, CUTLL1, MOLT4, SUPT1), and included 18 control shRNAs (see Online Supplementary Methods and Online Supplementary Table S1A). In silico analysis using the Cancer Cell Line Encyclopedia (CCLE) demonstrated that these six genes are highly expressed in a panel of T-ALL cell lines (Online Supplementary Figure S1A). LCK and PTCRA expression was confirmed by targeted gene expression analysis in T-ALL cell lines and patient samples (Figure 1B) (Online Supplementary Figure S1B). The limited shRNA screen revealed the shLCK#3 construct targeting LCK was the only construct significantly depleted in all four cell lines, when compared with base line shRNA integration, underlining an important role for LCK in T-ALL cell line proliferation and/or survival (Figure 1A and Online Supplementary Figure S1C and D). The shLCK#1, shZAP70#1 and shPTCRA2# constructs were lost in 3 out of 4 cell lines. Constructs targeting FYN, CD3ε, or LAT were significantly depleted in one cell line only, suggesting that these molecules do not play an universal role in T-ALL cell proliferation. ShRNAs against essential ribosomal genes were predictably depleted, whilst all three shRNA constructs targeting the tumor suppressor PTEN were enriched as expected. Repeated sampling at 16, 30 and 40 days after transduction demonstrated progressive depletion of shRNA constructs targeting LCK and ZAP70 (Online Supplementary Table S2 and Online Supplementary Figure S1E). PDX LK203 showed...
good viability (≥75%) and proliferation potential (T₄ = 2.8 days) in co-culture with human mesenchymal stem cells (hMSC), hence subjected to shRNA screening. ShRNA sequencing 30 days after transduction confirmed all constructs targeting LCK were significantly depleted (Figure 1A, Online Supplementary Table S3 and Online Supplementary Figure S1F).

Knockdown of lymphocyte cell-specific kinase in T-cell acute lymphoblastic leukemia cell lines confirms an essential role for lymphocyte cell-specific kinase in vitro propagation

To confirm the role of LCK and other components of the pTCR/TCR signaling complex in cell proliferation, competitive outgrowth assays were performed. SUPT1, MOLT4 and CUTLL1 cells were transduced with lentiviral shRNAs targeting LCK, ZAP70, FYN, PTCRA or non-targeting control shRNAs. Successfully transduced cells expressing green fluorescence protein (GFP) were seeded in a 1:1 ratio with parental cells.

Three shRNAs were used to silence LCK, of which shLCK#3 achieved the greatest degree of knockdown. Lentiviral knockdown with shLCK#3 led to significant reduction in mRNA in SUPT1 (75%KD), MOLT4 (55% KD), and CUTLL1 (45% KD) cells (Figure 2A). In general, greater knockdown was associated with more pronounced impairment of in vitro proliferation (Online Supplementary Figure S2A). LCK expression was confirmed at protein level, demonstrating ubiquitous expression of LCK in cell lines (Online Supplementary Figure S1G). In line with mRNA downregulation, knockdown of LCK led to a decrease in total LCK protein expression (Figure 2A). Non-transduced cells consistently outcompeted LCK knockdown cells resulting in a pronounced loss of over 70% transduced GFP⁺ cells in all three cell lines, underlining the critical and universal role of LCK in T-ALL cell line maintenance (Figure 2B and C, and Online Supplementary Figure S2A and C).

A similar but less significant observation was made for ZAP70 knockdown in SUPT1, MOLT4 and CUTLL1 cells. Efficient ZAP70 knockdown correlated with a pronounced proliferation defect (Online Supplementary Figure S2A and C). Knockdown of PTCRA affected proliferation in pTCR⁺ MOLT4 and SUPT1, but not in pTCR⁻ CUTLL1 (Online Supplementary Figure S2A and C). Moreover, FYN knockdown did not affect proliferation in any of the cell lines despite efficient knockdown (Online Supplementary Figure S2B).

Knockdown of lymphocyte cell-specific kinase in T-cell acute lymphoblastic leukemia cell lines and patient-derived xenograft samples impairs leukemia propagation in vivo

To confirm a functional role for LCK in vivo, PDX L963 cells were transduced with our shRNA library and transplanted into six NSG mice (Figure 3A and Online Figure S1A).
DEX and DAS impair in vivo T-ALL propagation

Supplementary Figure S3A). Genomic DNA (gDNA) was extracted from L963 cells isolated from bone marrow and spleen after mice became symptomatic (week 11). ShRNA sequencing indicated that shLCK#3 represented the most significantly depleted shRNA construct in vivo (Figure 3A, Online Supplementary Table S3 and Online Supplementary Figure S3B).

To assess the effect of LCK knockdown on engraftment fitness, MOLT4 cells were transduced with lentiviral vectors encoding either red fluorescent protein RFP/shNTC (non-targeting control) or GFP/shLCK#3. Equal proportions of cell populations were transplanted into NSG mice (n=5). Leukemia cells were isolated from spleen, bone marrow and liver once mice were symptomatic (day 26). Flow cytometric analysis of the leukemic cell population established that cells carrying shNTC had a clear competitive engraftment advantage over cells with LCK knockdown in all mice tissues sampled (Figure 3B and Online Supplementary Figure S3C).

Knockdown of lymphocyte cell-specific kinase leads to cell cycle arrest in T-cell acute lymphoblastic leukemia cell lines and patient-derived xenograft samples

Next we investigated the mechanisms underlying the defect in proliferation, survival and engraftment observed after LCK knockdown. Jurkat, MOLT4 and SUPT1 cells were transduced with shLCK#1/#3 and cell cycle analyses performed. In all cell lines, we observed significant cell cycle arrest with an increase in G0/G1 phase and decrease in S phase after LCK knockdown (Figure 4A-C and Online Supplementary Figure S4A).

ShLCK#3 led to decreased protein levels of total LCK and activated p-Y416SRC in cell lines, suggesting activation status of LCK is associated with cell cycle arrest. In PDX L963, LCK knockdown led to a 45% reduction in total LCK expression, as well as a 71% reduction in p-Y416SRC as assessed by Phosflow (Figure 4D). This knockdown resulted in a decrease in S phase over time compared to control (Online Supplementary Figure S4B). The proliferative behavior of PDX cells was analyzed after labeling with cell trace violet (CTV). PDX L963 cells were transduced with shRNA constructs targeting LCK or a non-targeting control (NTC) and co-cultured with OP9-DL1 feeder cells for 13 days. The LCK knockdown cells showed restricted proliferation compared to the control cells (Figure 4E). Confirmatory siRNA knockdown of LCK was undertaken in PDX samples LK203 and L963. Knockdown of total and activated LCK was confirmed by Phosflow. Cell cycle
arrest was observed, corroborating our earlier findings (Online Supplementary Figure S4C and D).

Knockdown of LCK was analyzed for early apoptosis induction in CUTLL1, MOLT4, SUPT1 and Jurkat. Although a clear increase in Annexin V staining was observed in MOLT4, suggesting LCK knockdown led to apoptosis, this was not observed in CUTLL1, Jurkat or SUPT1 (Online Supplementary Figure S4E). This suggests that cell cycle arrest, rather than apoptosis induction, is the predominant effect leading to diminished cell expansion in vitro and reduced propagation in vivo after LCK knockdown.

Tyrosine kinase inhibitor dasatinib blocks lymphocyte cell-specific kinase cell-specific kinase function and leads to cell cycle arrest while lymphocyte cell-specific kinase activation levels predict response to its inhibition

The tyrosine kinase inhibitor Dasatinib is a dual SRC/ABL inhibitor known to effectively inhibit LCK.21 The effect of Dasatinib on LCK protein expression and activation status was assessed by western blot, after demonstrating near universal LCK activation as evidenced by tyrosine residue 416 phosphorylation in cell lines (Online Supplementary Figure S1G). We confirmed that Dasatinib effectively abolished activated p-Y416SRC in all four T-ALL cell lines tested, whilst slightly decreasing total LCK protein levels. Furthermore, dephosphorylation of inhibitory Y505LCK was noted, as well as a decrease in p-Y783PLCγ1 and p-Y493ZAP70, two downstream targets of LCK (Figure 5A and Online Supplementary Figure S5A). As knockdown of LCK leads to cell cycle arrest, we performed cell cycle analyses after administration of Dasatinib. Cell cycle arrest was observed in all six T-ALL cell lines tested, with a significant increase in G0/G1 and decrease in S phase (Figure 5A and Online Supplementary Figure S5B). In parallel, PDX cells supported by in vitro co-culture with OP9-DL1 were exposed to Dasatinib. In line with our cell line data, Dasatinib abolished activated p-Y416SRC levels in all six PDX samples (Figure 5B and Online Supplementary Figure S5C) and cell cycle arrest was observed in all three PDX samples tested (Figure 5B and Online Supplementary Figure S5D).

In line with our cell line data, Dasatinib sensitized a panel of nine T-ALL cell lines to Dasatinib. The IC50 observed ranged from 5 nM (HSB2) to 15 μM (MOLT16) (Figure S5C). The cell line HSB2 not only demonstrated the highest sensitivity to Dasatinib but also the highest p-Y416SRC activation level as determined by Phosflow. This observation can be explained by the presence of a t(1;7)(p34;q34) translocation leading to LCK activation by TCRβ enhancer elements in HSB2. We thus hypothesized that the level of activated LCK might represent a biomarker for Dasatinib responsiveness. Phosflow was used to quantify and calculate the ratio between p-Y416SRC and total LCK. A strong and significant correlation was observed between the IC50 for Dasatinib and the ratio of activated Y416SRC in T-ALL cell lines (R2=0.778, P=0.004) (Figure S5C). The sensitivity of PDX cells to Dasatinib ranged from GI50 of 23.8 nM to 19.7 nM (MOLT4) to 15 μM (MOLT16) (Figure S5C). The cell line HSB2 not only demonstrated the highest sensitivity to Dasatinib but also the highest p-Y416SRC activation level as determined by Phosflow. This observation can be explained by the presence of a t(1;7)(p34;q34) translocation leading to LCK activation by TCRβ enhancer elements in HSB2. We thus hypothesized that the level of activated LCK might represent a biomarker for Dasatinib responsiveness. Phosflow was used to quantify and calculate the ratio between p-Y416SRC and total LCK. A strong and significant correlation was observed between the IC50 for Dasatinib and the ratio of activated Y416SRC in T-ALL cell lines (R2=0.778, P=0.004) (Figure S5C). The sensitivity of PDX cells to Dasatinib ranged from GI50 of 23.8 nM to 19.7 μM (median of 1.2 μM). However, in this setting, no significant correlation between the GI50 and p-Y416SRC/LCK ratio was identified, suggesting that Dasatinib sensitivity of patient-derived cells is dependent on additional factors (Online Supplementary Figure S5E).
Dasatinib re-sensitizes dexamethasone resistance in T-cell acute lymphoblastic leukemia cell lines and patient-derived xenograft samples

DAS leads to complete inhibition of p-Y416SRC and cell cycle arrest in T-ALL cell lines and PDX cells, suggesting that DAS treatment of T-ALL has a cytostatic effect. In clinical practice, effective eradication of T-ALL relies on the application of combinatorial treatment. LCK inhibition has previously been shown to sensitize chronic lymphoid leukemia (CLL) to DEX and induce cell death.23 We thus went on to investigate potential synergy between LCK inhibition and DEX, as DEX is universally used for treatment of ALL. The cell viability of SUPT1 and CUTLL1, in the presence of DEX, was evaluated after knockdown of LCK. Whereas the cell viability of mock transduced and non-targeting control cells was minimally affected by DEX treatment, LCK knockdown increased DEX sensitivity suggesting that LCK protein and/or activity levels play a crucial role in GC resistance (Figure 6A and Online Supplementary Figure S6A).

A more detailed analysis of the potency of the combination of DEX and LCK inhibition was examined by using DAS instead of the LCK knockdown. DEX (0-600 nM) and DAS (0-50 μM) were titrated along a dose matrix and cell viability was determined. Synergy for individual drug combinations was determined using Combenefit.22 The matrix revealed drug synergy at concentrations which are clinically achieved, i.e., 100 nM for DEX and 264 nM for DAS (Figure 6A and Online Supplementary Figure S6B).24 Bioinformatic analysis of all ten T-ALL cell lines revealed a statistically significant enrichment of drug synergy at clinically relevant concentrations. This synergy was observed at 8-110 nM of DEX and 0.223-4.5 μM of DAS (Online Supplementary Figure S6C and D).

Subsequently, PDX cells were expanded ex vivo for 1 week and exposed to the same drug combinations in dose matrices. These assays verified the synergistic action of DEX+DAS in a wide range of PDX cells, whilst confirming that increased DAS concentrations and resultant LCK inhibition augmented the response to DEX (Figure 6C and Online Supplementary Figure S6E). Combined analysis of all drug matrices with PDX cells again revealed a statistically significant enrichment of drug synergy at clinically relevant concentrations (Online Supplementary Figure S6F). Moreover, the combination of DEX+DAS induced more cell death compared with control vehicle or single drugs as revealed by Annexin V/PI staining (Figure 6C).

DEX has a wide range of actions, including genomic and non-genomic effects. Genomic effects are the result of nuclear translocation of the GC receptor and subsequent transactivation or repression of genes containing a GC response element (GRE), as exemplified by the Glucocorticoid-Induced Leucine Zipper (GILZ) gene. Accordingly, we observed strong induction of GILZ gene expression after DEX exposure in the T-ALL cell line Jurkat and five PDX samples tested (Figure 6A and Online Supplementary Figure S6A). This response was significantly enhanced when combining DEX with knockdown of LCK (Figure 6C) or DEX+DAS in a range of T-ALL cell lines and PDX samples, suggesting that LCK inactivation augments DEX-induced gene transcription and reverses DEX resistance (Figure 6D and Online Supplementary Figure S6G).
Phase II-like trial in vivo demonstrates significant reduction in leukemia burden after combination treatment with dexamethasone and dasatinib

To test the efficacy of DEX and DAS in vivo, we conducted a phase II-like trial in mice (Figure 7A).26 Ten PDXs were engrafted in four mice each. The four mice derived from one single patient sample were randomly assigned to treatment arms, namely control vehicle, DEX (1 mg/kg), DAS (35 mg/kg) or DEX+DAS (1 mg/kg DEX + 35 mg/kg DAS). After IF injection, mice tail vein blood was monitored weekly for human CD7/CD45 and murine CD45 expression to monitor peripheral blood engraftment. Representative PDX L809 commenced treatment 46 days after injection for a total duration of 3 weeks; the four mice were culled 72 days after injection (Figure 7B). L809 cells engrafted in the spleens of the four mice showed greatly reduced levels of total LCK and dephosphorylation of LCK (p-Y416<sup>SRC</sup> and p-Y505<sup>LCK</sup>) after DAS or DEX+DAS combination treatment (Figure 7C). Western blot analysis of positively selected viable human cells again demonstrated decreased protein expression of LCK and p-Y416<sup>SRC</sup> after DAS treatment. The number of residual viable human cells after effective DEX+DAS treatment was not sufficient to categorically confirm reduced protein expression (Figure 5F). One mouse in the DAS arm (LK080) developed uterine prolapse before dosing commenced and the mice derived from PDX LK214 succumbed during the first week of treatment. These five were excluded from the final analysis. Combining the results of 35 mice derived from nine patient samples, DEX+DAS treatment significantly impaired leukemia progression more than single drug DEX, DAS or control vehicle.
DEX and DAS impair in vivo T-ALL propagation

Figure 6. Dexamethasone (DEX) and dasatinib (DAS) act synergistically to induce cell death in T-cell acute lymphoblastic leukemia (T-ALL). (A) Cell viability of parental SUPT1 cells (mock), shCtrl (NTC), shLCK#1 or shLCK#3 transduced SUPT1 cells upon treatment with increasing DEX concentrations (0-1699 nM). (B) Cell viability of SUPT1 with and without DAS (left; black line, no DAS; blue line, 0.8 µM; red line, 2.0 µM) in combination with increasing concentrations of DEX (0-600 nM) as derived from the drug matrix with titration of DEX (0-600 nM) and DAS (0 - 50 uM; right). (Right) Combenefit analysis of drug matrix demonstrates drug synergy in SUPT1 cells at clinically relevant drug concentrations. (C) (Left) LK203 cells were expanded ex vivo on OP9-DL1 feeder cells for 1 week prior to treatment with and without DAS (black line, no DAS; blue line, 0.08 µM; red line, 2.0 µM; orange line, 10 µM) in combination with increasing concentrations of DEX (0-600 nM) as derived from a drug matrix with DEX (0-600 nM) and DAS (0 - 50 µM) (Online Supplementary Figure S6E). (Right) Cell death analysis in LK203 cells exposed to control (Ctrl) conditions, DAS (1 µM), DEX (100 nM) or DAS+DEX combination treatment. (D) (Left) Normalized GILZ mRNA expression in Jurkat cells after transduction with shNTC or shLCK#3 with or without DEX exposure (100 nM). (Right) Normalized GILZ mRNA expression in Jurkat cells after exposure to Ctrl conditions, DAS (2 µM), DEX (100 nM) or DAS+DEX combination treatment at the same concentrations. Student’s t-test: **P<0.01, ***P<0.005, ****P<0.001.

Discussion

Using a phase II-like murine trial, we demonstrate here the efficacy of the drug combination DEX+DAS in impairing expansion of human T-ALL samples. This effect is apparent in an unselected, biologically heterogeneous, cohort of PDX samples. This trial format recapitulates early phase human clinical trials and indicates that this drug combination could be widely applicable in the treatment of T-ALL. Studies by Serafin et al. first proposed a role for this drug combination.28 Our murine trial extends these initial observations with an extensive cohort consisting of nine different PDX demonstrating treatment advantage for both DEX sensitive and resistant T-ALL. Significant superiority of DEX+DAS was demonstrated even after exclusion of mice who reached their clinical end points prematurely. These untoward events highlight the practicalities of performing murine trials.

We propose that the impaired in vivo expansion results from a combination of cell cycle arrest as well as cell death. Several mechanisms could provide plausible explanations for the occurrence of cell cycle arrest. DAS is a protein tyrosine kinase inhibitor which targets Abl and SFK family members. We have confirmed that DAS effectively inhibits activity of the SFK member LCK by preventing phosphorylation, leading to G0/G1 arrest. DAS has previously been shown to inhibit cyclin dependent kinase 1 (CDK1), which plays a central role in G1/S and G2/M

Discussion

Using a phase II-like murine trial, we demonstrate here the efficacy of the drug combination DEX+DAS in impairing expansion of human T-ALL samples. This effect is apparent in an unselected, biologically heterogeneous, cohort of PDX samples. This trial format recapitulates early phase human clinical trials and indicates that this drug combination could be widely applicable in the treatment of T-ALL. Studies by Serafin et al. first proposed a role for this drug combination.28 Our murine trial extends these initial observations with an extensive cohort consisting of nine different PDX demonstrating treatment advantage for both DEX sensitive and resistant T-ALL.

Significant superiority of DEX+DAS was demonstrated even after exclusion of mice who reached their clinical end points prematurely. These untoward events highlight the practicalities of performing murine trials.

We propose that the impaired in vivo expansion results from a combination of cell cycle arrest as well as cell death. Several mechanisms could provide plausible explanations for the occurrence of cell cycle arrest. DAS is a protein tyrosine kinase inhibitor which targets Abl and SFK family members. We have confirmed that DAS effectively inhibits activity of the SFK member LCK by preventing phosphorylation, leading to G0/G1 arrest. DAS has previously been shown to inhibit cyclin dependent kinase 1 (CDK1), which plays a central role in G1/S and G2/M
Furthermore, G1 cell cycle arrest, through upregulation of the cyclin-dependent kinase inhibitors p21CIP1 (CDKN1A) and p27KIP1 (CDKN1B), has been observed after DAS treatment in acute myeloid leukemia. We propose that LCK is the predominant target of DAS in this disease setting, as our shRNA screen identified a critical role for LCK in cell proliferation in cell lines and PDX samples. Moreover, LCK is the proposed DAS target when blocking T-cell activation. Competitive assays confirmed defective proliferation of T-ALL cells after LCK knockdown in vitro and in vivo. We have shown that LCK knockdown leads to G0/G1 cell cycle arrest in cell lines and PDX. This effect was more pronounced using DAS, a finding which could potentially be explained by incomplete knockdown of LCK or the wide spectrum of kinases targeted by DAS.

As reported earlier and confirmed in our studies, DAS is cytotoxic to a small subset of T-ALL samples with IC50 values in the low nanomolar range. These observations were made in T-ALL samples without kinase activating mutations, which are seen very infrequently in T-ALL. To the best of our knowledge, our cohort includes only one...
PDX with such an activating genetic lesion (LK287, FIP1L1-PDGFRα). Cytotoxicity to DAS is significantly increased upon combination with DEX. Our data indicate drug synergy between DAS and DEX at clinically relevant concentrations. A previous, mostly in vitro, study advocated the use of DEX+DAS in GC resistant T-ALL.28 Our extended studies indicate DEX+DAS act synergistically in the majority of cell lines and PDX tested independent of their prior sensitivity to DEX. The potential of DEX+DAS to revert GC resistance is an exciting observation. GC resistance is frequently observed in relapsed/refractory T-ALL,3 and DEX+DAS provide a clinically actionable approach to re-sensitize T-ALL resistant to DEX.

The implementation of DAS into clinical management would benefit from the identification of a reliable response biomarker. Although LCK activation status (ratio p-Y416SRC/LCK) strongly correlates with DAS sensitivity in cell lines, we were unable to corroborate this observation in PDX cells. Sample size and intricacies of in vitro assays using PDX cells could provide possible explanations for these inconsistencies. Nevertheless, in vivo drug synergy was observed in the majority of samples tested. Of interest, drug response profiling of T-ALL samples suggested SRC pathway activation may represent a response biomarker.31

The mechanism underlying the observed drug synergy remains to be fully elucidated. T-cell activation can be blocked by using clinically relevant concentrations of the tyrosine kinase inhibitor DAS, which binds to the ATP-binding pocket of LCK thereby preventing the phosphorylation of the activating loop of the kinase domain p-Y416SRC.21,32 When DEX is combined with DAS, physiological CD3+ T-cell proliferation is reduced in an additive way.33,34 Furthermore, it has been previously suggested that the Calcineurin/NFAT/IL-4 axis is activated in patients exhibiting a prednisone poor response.9 We have shown here that combination of DEX+DAS significantly increases GILZ gene expression, reflecting increased transcriptional activity of the GC receptor. We thus hypothesize that inhibition of LCK disrupts the TCR-GR complex and established crosstalk between the TCR and GR pathways leading to dissociation and transcriptional activation of the GR.15

To conclude, drug resistant T-ALL continues to represent an unmet clinical need. We provide further support for the inclusion of DAS in the treatment of T-ALL. It has been reported that DAS in combination with conventional chemotherapy is safe and well tolerated in children and young adults, although hematologic toxicity was significant.35 Thus, the DEX+DAS combination should be considered in the early phase setting to evaluate toxicity and efficacy in patients with GC resistant disease with or without cerebral spinal fluid involvement.

Disclosures
No conflicts of interest to disclose.

Contributions
FWvD, AKH and YS designed the research; YS performed the research; YS, MCB, HJB, OH and RT designed and performed the in vivo experiments; FWvD, AKH and YS analyzed the data and wrote the paper; SN and AE performed the bioinformatics analysis; CH performed brain histology and imaging; JV, OH and CH reviewed the manuscript.

Acknowledgments
The authors would like to thank patients, parents, and hospital staff at the Great North Children’s Hospital, Newcastle upon Tyne, UK, for their valuable collaboration. The authors would like to thank Lynn Stevenson and Clare Orange, University of Glasgow, for brain histology and imaging. The brain histology slides were scanned by Glasgow University slide scanning and image analysis service at the Queen Elizabeth University Hospital, Glasgow. CH was funded by the Chief Scientist Office (ETM/374).

Funding
This work was supported by a Newcastle University Research Fellowship (to FWvD), Chinese Scholarship Council (CSC) (to YS), JGW Patterson Foundation (to MCB), North of England Children’s Cancer Research, Action Medical Research (to PWvD).

References