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Highlights:

- MLLT1/3 has potential to be a key therapeutic target in acute leukaemias
- KMT2A::AFF1 leukemia cells appear particularly sensitive to MLLT1/3 inhibition
- An MLLT1/3 inhibitor kills leukaemia cells without harming normal HSCs

Title:
Inhibition of MLLT1 limits growth of KMT2A::AFF1 leukaemias without killing healthy haematopoietic stem cells

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Keywords: MLLT1, leukaemia, acute lymphoblastic leukaemia, haematopoietic stem cell, KMT2A::AFF1, MLL:AF4

Short title: MLLT1 inhibition selectively blocks t(4;11) leukaemias

Abstract:
A major challenge in cancer therapeutics has been the identification of targets that are selectively toxic to cancer cells while displaying limited effects on healthy counterparts. Toxicities related to blood production from haematopoietic stem and progenitor cells (HSPCs) can be particularly problematic and can result in patient morbidity and mortality. MLLT1 has been identified as a key potential target in acute myeloid leukaemia. Here we evaluated the sensitivity of an MLLT1 inhibitor, SGC-iMLLT, on a panel of leukaemia cell lines and on healthy haematopoietic stem and progenitor cells (HSPCs). We found that SGC-iMLLT downregulated MLLT1 target genes and strongly inhibited KMT2A::AFF1-driven leukaemia growth *in vitro* and *in vivo*. By contrast, SGC-iMLLT did not alter *in vitro* colony forming potential of human HSPCs or affect long-term *in vivo* function of mouse HSPCs. These results suggest that SGC-iMLLT may have a promising therapeutic window in the treatment of KMT2A::AFF1-driven leukaemias, and that further clinical development is warranted.

Introduction:

A major challenge in cancer therapeutics has been the identification of targets that are selectively toxic to cancer cells while displaying limited effects on healthy counterparts. Toxicities related to blood production from haematopoietic stem and progenitor cells (HSPCs) can be particularly problematic and can result in patient morbidity and mortality. Here we evaluated a novel inhibitor of MLLT1 (ENL) and MLLT3 (AF9), SGC-iMLLT[1], on a panel of leukaemic cell lines and healthy HSPCs. MLLT1 has been recently identified as a key potential target in acute myeloid leukaemia (AML)[2,3]. MLLT1 and MLLT3 are both components of the super elongation complex (SEC), which plays a crucial role in transcriptional elongation[4]. Although the exact mechanisms of MLLT1/3 function have not been worked out, MLLT1 and MLLT3 have very similar structures and act as adapter proteins[5]. MLLT1 is essential for the recruitment of SEC components to gene targets[6]. The acyl-lysine reader (YEATS) domain is essential both proteins, likely acting in complex formation and chromatin anchoring[7,8]. Of the two, only MLLT1 is essential for AML growth[2,3]. However, MLLT3 has been shown to have a key role in fetal haematopoietic stem cell (HSC) self-renewal[9] and erythroid/megakaryocyte differentiation from umbilical cord blood[10].

Because of this potential as a therapeutic target, several small molecule inhibitors including SGC-iMLLT[1] have been developed to bind the YEATS domain, targeting both MLLT1 and MLLT3[11-16] and displaying *in vivo* efficacy in AML[15,16]. MLLT1-specific inhibitors have limited efficacy[17], but recent exciting work has found that MLLT1-specific proteolysis-targeting chimera molecules are effective *in vivo*[6,18], displaying low toxicity[6]. Although this suggests targeting MLLT1 alone may be effective, there is always the possibility that MLLT3 could play an important role in individual patient response as well as relapse. Thus, more needs to be understood about the range of leukaemias sensitive to MLLT1/3 inhibition, and whether targeting both MLLT1 and MLLT3 could have any potential toxicity.

To date, MLLT1 and MLLT3 have primarily been investigated as a therapeutic target in AML. The t(4;11) leukaemias are poor prognosis leukaemias[4,19] caused by chromosome translocations involving the *Lysine Methyltransferase 2A* (*KMT2A*, also known as *MLL*) and *AF4/FMR2 family member 1* (*AFF1,* also known as *AF4*) genes, creating a novel KMT2A::AFF1 fusion protein, primarily associated with acute lymphoblastic leukaemia (ALL)[20]. SEC activity is essential in t(4;11) leukaemias[21,22], so we hypothesised MLLT1/3 function may be essential for this subtype of ALL as well. Interestingly, MLLT3 protein levels are relatively low in t(4;11) leukaemias[23], suggesting that MLLT1 may be the main effector of SEC activity in this leukaemia context.

Here we evaluated the sensitivity of SGC-iMLLT on a panel of leukaemia cell lines and on healthy HSPCs. We found that SGC-iMLLT strongly inhibited t(4;11) leukaemia growth *in vitro* and *in vivo*. By contrast, SGC-iMLLT did not alter *in vitro* colony forming potential of human HSPCs or affect long-term *in vivo* function of mouse HSPCs. These results suggest that SGC-iMLLT may have a promising therapeutic window in the treatment of KMT2A::AFF1-driven leukaemias, and that further clinical development is warranted.

Results and Conclusions:

To initially test the hypothesis that t(4;11) leukaemias may be sensitive to specific loss of MLLT1, we engineered the human KMT2A::AFF1-driven SEM ALL cell line by inserting a

FKBP12^{F36V} degron tag[24] into the N-terminus of MLLT1 (**Figure S1a-c**). In these cells, MLLT1 is degraded in the presence of dTAG-13 (**Figure S1d**). The FKBP-MLLT1 SEM lines proliferated and formed colony forming units (CFUs) comparable to parental SEM cells (**Figure 1a**). However, when SEM FKBP-MLLT1 lines were grown in the presence of dTAG-13, essentially all growth potential was lost (**Figure 1a**).

Given that the degron-mediated depletion of MLLT1 impaired the growth of SEM cells, we tested the effects of various MLLT1/3 small molecule inhibitors in MV4;11 cells, another human (AML) cell line driven by KMT2A::AFF1 (**Figure S1e-g)**. The results show that MV4;11 cells were indeed sensitive to MLLT1/3 inhibition within clinically relevant concentrations. SGC-iMLLT displayed an IC50 of 195 nM (**Figure S1e**). This was similar to a second MLLT1/3 inhibitor, NVS-MLLT-1, with an IC50 of 168 nM (**Figure S1f**) although less potent than the Song MLLT1/3 PROTAC[18] with an IC50 of 31 nM (**Figure S1g**). As SGC-iMLLT displayed an IC90 of 1 µM, we selected this dose for further studies.

We explored the effects of SGC-iMLLT on the growth of several leukaemic cell lines (**Figure 1b**). In line with the degron experiment (**Figure 1a**), SEM cells again lost growth potential when MLLT1/3 was inhibited (**Figure 1b**). A second human cell line carrying the KMT2A::AFF1 translocation, AML MV4;11 cells, also demonstrated high sensitivity (**Figure 1b**). Other human AML cell lines and one ALL cell line, including those harbouring MLL fusion protein KMT2A::AF6, *NPM1* mutation and ETV6::RUNX1 fusion also displayed growth inhibition **(Figure 1b)**. However, statistically significant reductions were not observed for the AML cell line THP1 (KMT2A::MLLT3), the CML cell line K562 (BCR::ABL) or the ALL cell line RCH-ACV (E2A::PBX1) (**Figure 1b**). This indicates that MLLT1/3 inhibition may have activity against several leukaemia types, particularly those carrying *KMT2A* rearrangements.

To better understand the molecular consequences of SGC-iMLLT on KMT2A::AFF1 leukaemias, we analysed the transcriptional consequences of 24-hour SGC-iMLLT treatment. We initially focused on *MYC* expression, a known MLLT1 target, by RT-qPCR, and could confirm significant reductions in *MYC* expression in sensitive cell lines **(Figure 1c**). We next used nascent RNA-seq to understand the global transcriptional consequences in MV4;11 cells. We observed 965 DEGs with the majority of these being downregulated (**Table S1**), consistent with the expected role for the SEC in promoting transcription. This included reduced expression of known targets of MLLT1[21] **(Figure 1d).** Gene set enrichment analysis confirmed downregulation of MYC targets, alongside cell cycle/cell growth processes, including E2F targets and ribosome biogenesis **(Figure 1e).** Together, this aligns with the disrupted colony forming potential observed following MLLT1/3 inhibition.

We next asked whether SGC-iMLLT treatment could inhibit tumour growth *in vivo* by tracking tumour burden in immunodeficient NOD-SCID mice subcutaneously inoculated with MV4;11 cells. Daily (*Quaque die*) treatment with 100 mg/kg SGC-iMLLT significantly inhibited tumour growth over 20 days (**Figure 1f**). Additionally, analysis of the MV4;11 cells isolated at the assay endpoint displayed significant reductions in expression of known MLLT1 target (including *MYC* and *BCL2*) (**Figure 1g**). Together, these results confirm the therapeutic potential of SGC-iMLLT in t(4;11) leukaemias. However, additional *in vivo* efficacy could be confirmed in future studies using additional models such as orthotropic transplantation or patient-derived xenograft models.

An optimal cancer therapy needs to selectively target diseased cells while showing limited toxicity for normal healthy cells. Loss of normal blood production in standard cancer treatments is particularly dangerous and is a major cause of therapy-related morbidity and mortality. Given the described functions of MLLT3 in HSC regulation[9,10], we investigated the effect of SGC-iMLLT on healthy human and mouse HSPCs to determine the potential therapeutic window of SGC-iMLLT.

We initially assessed the *in vitro* colony forming potential of human $CD34^+$ HSPCs, with and without SGC-iMLLT. No significant changes in colony potential were observed with SGC-iMLLT (**Figure 2a, S2a**), suggesting human HSPC differentiation potential was not acutely sensitive to MLLT1/3 inhibition. Next, we assessed mouse HSCs in an *ex vivo* expansion culture assay. Again, no significant changes in growth were observed, and similar numbers of immunophenotypic $CD201^+CD150^+$c-$Kit^+Sca1^+Lineage^-$ HSCs were generated (**Figure 2b**). To understand how this compared to other leukaemia drugs, we tested the Menin inhibitor (SNDX-5613[25]) and the CDK9 inhibitor (AZD4573[26]) in these mouse HSC cultures. Menin inhibition was well tolerated but we saw high toxicity from the CDK9 inhibitor (**Figure S2b**).

Finally, to confirm that HSC function was unaffected, we performed competition transplantation assays with HSCs treated with SGC-iMLLT for 7 days *ex vivo* (**Figure 2c**). While reduced peripheral blood chimerism was observed at early time points, no significant difference was observed at 16-weeks post-transplantation (**Figure 2d**). Similar lineage output was also observed to controls (**Figure 2d**). Additionally, similar high-level donor chimerism was observed in bone marrow HSPCs at the endpoint (**Figure 2e**). Together, these results confirm SGC-iMLLT has minimal toxicity on HSC activity and suggest SGC-iMLLT may have a good therapeutic window in the treatment of leukaemic cells, particularly KMT2A::AFF1-driven leukaemias. However, further work will be needed to understand the consequence of this inhibitor on *in vivo* haematopoiesis.

Recent studies have focused on developing MLLT1-specific targeting molecules[6,17,18], as MLLT1 appears to be a main effector of leukaemic growth[2,3]. However, even with highly effective targeted therapies such as Menin inhibitors, there are often patients that do not respond[25]. Expression of MLLT3 could provide an escape mechanism by which leukaemia cells can bypass the requirement for MLLT1. Thus, successful treatment may require drugs that are able to target both MLLT1 and MLLT3, which brings with it the possibility of increased toxicity. Here, we have demonstrated that MLLT1 is required for human t(4;11) leukaemia cell lines to grow in vitro and that SGC-iMLLT also limits growth in vivo. We further confirmed that SGC-iMLLT displays limited toxicity to healthy human and mouse HSPCs. This suggests that MLLT1/3 dual inhibitors may have potential as selective inhibitors of t(4;11) leukaemias, as well as having a potential other leukaemias.

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Data availability:
Next generation sequencing data has been deposited on GEO (GSE326010; reviewer token: cxwbwouubfgdzef). Other datasets available upon reasonable request from the corresponding authors.

Author Contributions:
Shivani Rajhansa: investigation; data analysis; writing and editing. **Nicholas T. Crump:** conceptualisation; investigation; data analysis; writing and editing. **Hwei Minn Khoo:** investigation; data analysis. Ana M. Dopico-Fernandez: data analysis. **Yavor Bozhilov:** investigation. **Paul E. Brennan:** funding; supervision; review and editing. **Oleg Fedorov:** funding; supervision; review and editing. **Cassandra Adams:** funding; supervision; review and editing. **Gillian Farnie:** funding; supervision; review and editing. **Thomas A. Milne:** conceptualisation; funding; data analysis; supervision; writing and editing. **Adam C. Wilkinson:** conceptualisation; funding; data analysis; supervision; writing and editing.

Conflict of interest statement:
TAM, NTC, PEB, OF, CA and GF are former shareholders in and current paid consultants for Dark Blue Therapeutics Ltd., a company dedicated to creating novel compounds directed at MLLT1 and other factors, now owned by Amgen. ACW is a consultant for ImmuneBRIDGE.

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Figure Legends:

Figure 1

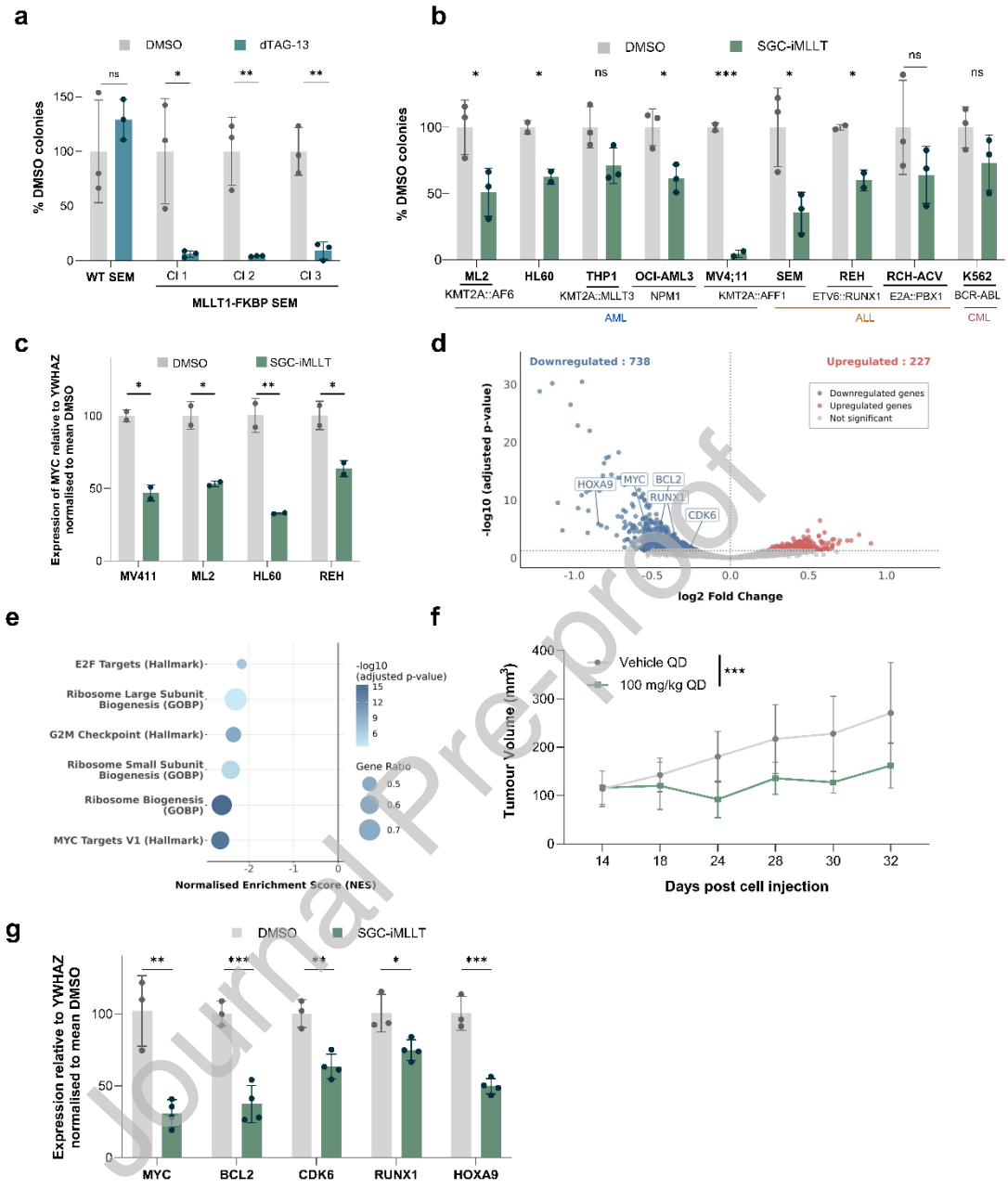

Figure 1: MLLT1 inhibition reduces leukaemogenic potential in vitro and in vivo.

a) Percentage of colonies formed from SEM and FKBP-MLLT1 SEM cell lines in the presence of 1 µM dTAG-13 relative to DMSO control in three different FKBP-MLLT1 SEM cell line clones (Cl). Error bars represent s.d. of three biological replicates. Statistical analysis performed using t-tests.

b) Percentage of colonies formed from indicated human leukaemia cell lines in the presence of 1 µM SGC-iMLLT relative to DMSO control. Error bars represent s.d. of three biological replicates. Statistical analysis performed using t-tests.

c) RT-qPCR showing MYC expression in human leukaemia cell lines following 24 h treatment with DMSO or 1 µM SGC-iMLLT. Statistical analysis performed using t-tests.

d) Volcano plot of nascent RNA-seq after 24 h treatment of MV4;11 cells with DMSO or 1 µM SGC-iMLLT. Data are the average of three biological replicates.

e) Dot plot of Gene Set Enrichment Analysis (GSEA) showing normalized enrichment scores for significantly enriched "Hallmark" and "Gene Ontology: Biological Process (GOBP)" gene sets following 24h treatment of MV4;11 cells with 1 µM SGC-iMLLT.

f) Tumour volume in MV4;11 xenograft model mice treated with SGC-iMLLT once a day (QD; at 100 mg/kg) for 20 days (n=9-11 mice per group). Statistical analysis performed using two-way ANOVA.

g) RT-qPCR of MLLT1 target genes showing expression in MV4;11 xenograft-derived cells described in (c). Statistical analysis performed using t-tests.

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = no significant difference.

Figure 2

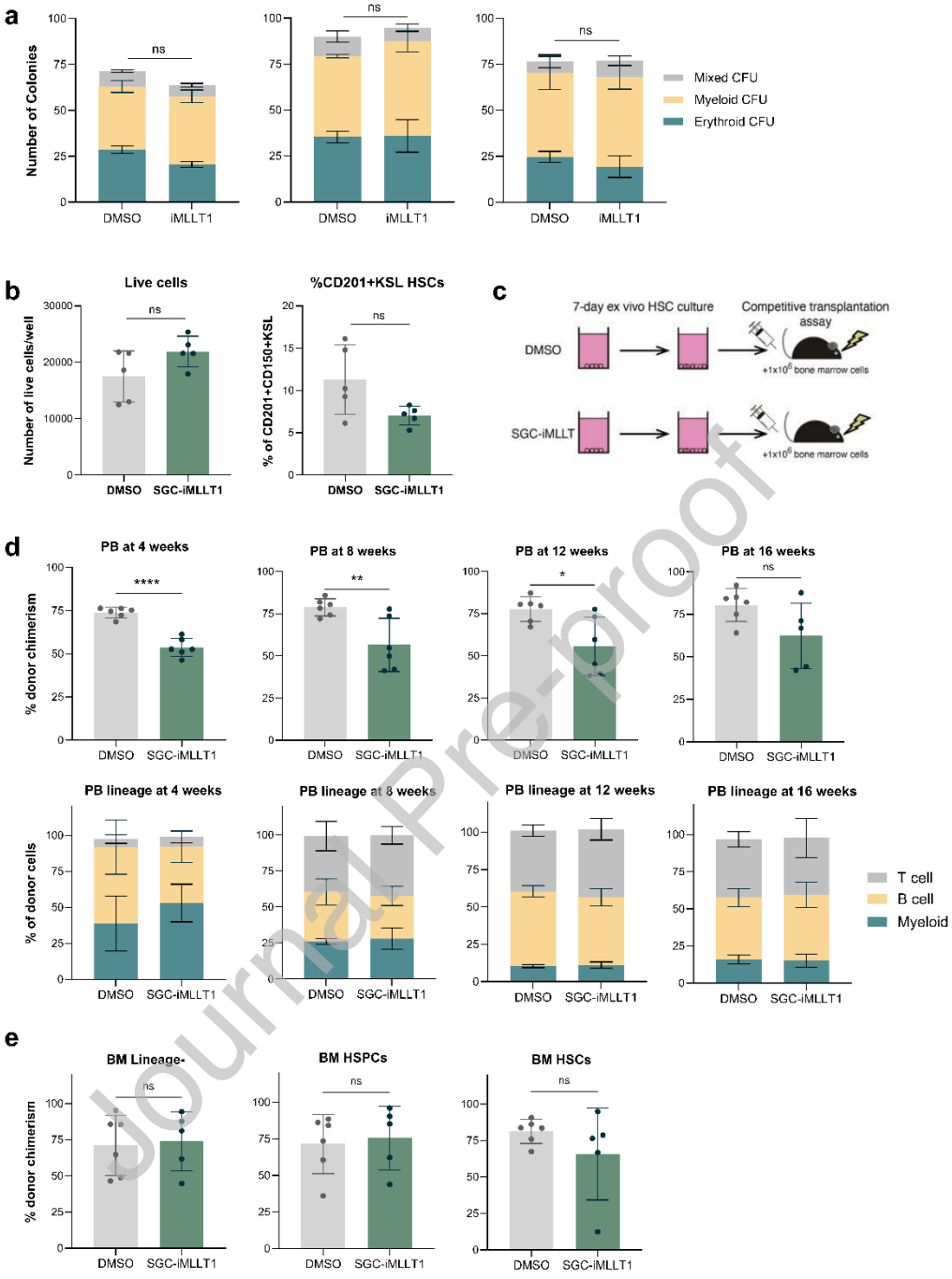

Figure 2: Healthy HSPCs survive MLLT1 inhibition

a) Number of colonies formed from 500 human $CD34^+$ HSPCs grown in 1 µM SGC-iMLLT or DMSO. Erythroid, myeloid, and mixed CFUs were distinguished and counted, see **Figure S2a** for representative images. Experiments performed using 3 different donors with technical triplicates.

b) Live cell numbers and frequency of $CD201^+CD150^+Kit^+Sca1^+Lineage^-$ immunophenotypic mouse HSCs derived from day-7 HSC cultures (n=5) in the presence of 1 µM SGC-iMLLT or DMSO.

c) Schematic of mouse HSC culture and transplantation assay to assess the effect of SGC-iMLLT on HSC activity.

d) 4-16-week peripheral blood chimerism from 100 HSCs cultured for 7-days with 1 µM SGC-iMLLT or DMSO, transplanted alongside 1 x 10^6 whole bone marrow competitors into lethally-irradiated recipients. Error bars represent s.d. from 5-6 recipient mice.

e) 16-week bone marrow chimerism from 100 HSCs cultured for 7-days with 1 µM SGC-iMLLT or DMSO, and then transplanted alongside 1 x 10^6 whole bone marrow competitor cells into lethally-irradiated recipients. Error bars represent s.d. from 5-6 recipient mice.

All statistical analyses performed using t-tests: * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$, ns = no significant difference.

TeaserAbstract (1)

A major challenge in cancer therapeutics has been the identification of targets that are selectively toxic to cancer cells while displaying limited effects on healthy counterparts. We evaluated a novel MLLT1 inhibitor, SGC-iMLLT, on a panel of leukaemia cell lines and on healthy haematopoietic stem and progenitor cells (HSPCs). SGC-iMLLT strongly inhibited MLL-AF4-driven leukaemia growth in vitro and in vivo, did not alter in vitro colony forming potential of human HSPCs or affect long-term in vivo function of mouse HSPCs. SGC-iMLLT may have a promising therapeutic window in the treatment of MLL-AF4-driven leukaemias.