



**University of  
Sunderland**

Matheson, Elizabeth C., Thomas, Huw, Case, Marian, Blair, Helen, Jackson, Rosanna K., Masic, Dino, Veal, Gareth, Halsey, Chris, Newell, Herbie, Vormoor, Josef and Irving, Julie A.E. (2019) Glucocorticoids and selumetinib are highly synergistic in RAS pathway mutated childhood acute lymphoblastic leukemia through upregulation of BIM. *Haematologica*, 104. pp. 1804-1811. ISSN 0390-6078

Downloaded from: <http://sure.sunderland.ac.uk/id/eprint/11055/>

**Usage guidelines**

Please refer to the usage guidelines at

<http://sure.sunderland.ac.uk/policies.html> or alternatively contact [sure@sunderland.ac.uk](mailto:sure@sunderland.ac.uk).

**Glucocorticoids and selumetinib are highly synergistic in RAS pathway mutated childhood acute lymphoblastic leukemia through upregulation of BIM.**

Elizabeth C. Matheson<sup>1</sup>, Huw Thomas<sup>1</sup>, Marian Case<sup>1</sup>, Helen Blair<sup>1</sup>, Rosanna K. Jackson<sup>1</sup>, D Masic<sup>1</sup>, Gareth Veal<sup>1</sup>, Chris Halsey<sup>2</sup>, David R Newell<sup>1</sup>, Josef Vormoor<sup>1,3</sup> and Julie A.E Irving<sup>1</sup>

1. Newcastle Cancer Centre at the Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, UK.

2. Wolfson Wohl Cancer Research Centre, Institute of Cancer Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

3. Great North Children's Hospital, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK

**Corresponding author:** Dr Julie Irving, Northern Institute for Cancer Research, Herschel building, Newcastle upon Tyne, Tyne and Wear, UK, NE2 4HH. Tel. 0044-191-208-2234. Fax. 0044-191-208-4301. Email. j.a.e.irving@ncl.ac.uk.

**Key words:** glucocorticoids, MEK inhibitor, childhood acute lymphoblastic leukaemia, RAS signalling.

**Running Title:** Glucocorticoids and selumetinib in RAS pathway mutated ALL

**Word count:** abstract 203, main text 3614

## Abstract

New drugs are needed for relapsed acute lymphoblastic leukemia and preclinical evaluation of the MEK inhibitor, selumetinib, has shown excellent activity in those with RAS pathway mutations. The proapoptotic protein, BIM is pivotal in the induction of cell death by both selumetinib and glucocorticoids, suggesting the potential for synergy. Thus, combination indices for dexamethasone and selumetinib were determined in RAS pathway mutated acute lymphoblastic leukemia primagraft cells *in vitro* and were indicative of strong synergism (CI <0.2; n=5). Associated pharmacodynamic assays were consistent with the hypothesis that the drug combination enhanced BIM upregulation over single drug alone. Dosing of dexamethasone and selumetinib singly, and in combination in mice engrafted with primary derived RAS pathway mutated leukemia cells, resulted in a marked reduction in spleen size which was significantly greater with the drug combination. Assessment of the central nervous system leukaemia burden showed a significant reduction in drug treated mice, with no detectable leukemia in those treated with the drug combination. These data suggest that a selumetinib-dexamethasone combination may be highly effective in RAS pathway mutated acute lymphoblastic leukemia and an international phase I/II clinical trial of dexamethasone and selumetinib (Seludex trial) is underway for children with multiple relapsed/refractory disease.

## Introduction

Progress in the treatment of childhood acute lymphoblastic leukaemia has been exceptional and using contemporary regimens, sustained remission is achievable in almost 90% of children<sup>1,2</sup>. However, the outcome of children who relapse is much poorer and remains a frequent cause of death in children with cancer<sup>3-5</sup>. Since further intensification with traditional agents is often associated with significant toxicity and limited success, new therapies are clearly needed. One promising avenue that may deliver novel drugs comes from our previous work showing that mutation in genes which activate the Ras/Raf/Mek/Erk pathway such as *NRAS*, *KRAS*, *FLT3*, and *PTPN11* are highly prevalent in relapsed ALL and importantly, mutated ALL cells are differentially sensitive to the MEK inhibitor, selumetinib (AZD6244, ARRY-142886)<sup>6-8</sup>. In contrast, RAS pathway wildtype ALL cells were insensitive to MEK inhibition, both *in vitro* and *in vivo*<sup>6</sup>. In the *IBFMREZ2002* clinical trial for relapsed ALL, RAS pathway mutations were associated with high risk features such as early relapse, central nervous system (CNS) disease and chemo-resistance and a poorer overall survival was seen in patients with *KRAS* mutations<sup>6</sup>. In the UKALLR3 trial, a poorer survival was seen in children with *NRAS* mutations<sup>7</sup>. Thus, this genetic subtype of relapsed ALL clearly warrants exploratory therapies.

The Ras/Raf/Mek/Erk cascade regulates diverse cellular functions, including cell proliferation, survival, differentiation, angiogenesis and migration and is deregulated in numerous cancers, including ALL<sup>9-13</sup>. Classic activation is initiated by ligand binding to receptor tyrosine kinases at the cell surface and via Ras, then Raf activates MEK1/2 which has restricted substrate specificity for extracellular signal-regulated kinase 1 and 2 (Erk). ERK is a potent kinase with over 200 nuclear and cytoplasmic substrates including transcription

factors such as the ETS family and proteins involved in the apoptotic machinery, such as the proapoptotic BIM. Phosphorylation of the predominant form of BIM (BIM<sub>EL</sub>) by ERK1/2, targets it for ubiquitination and proteasomal degradation and may also directly hinder its interactions with Bax<sup>14, 15</sup> and selumetinib-induced apoptosis is associated with BIM induction.<sup>16</sup>

Relapsed ALL is generally more drug resistant than newly diagnosed disease and despite the use of more intensive chemotherapeutic regimens at ALL relapse, there are lower rates of complete remission and end of induction MRD negativity<sup>2,3</sup>. Assessment of *in vitro* drug sensitivity of primary ALL samples have shown that blasts at relapse are significantly more resistant to many of the drugs used in upfront treatment protocols, with the highest level of drug resistance seen to glucocorticoids (GC)<sup>17, 18</sup>. GC, such as dexamethasone, are pivotal agents in the treatment of all lymphoid malignancies due to their ability to specifically induce apoptosis in developing lymphocytes and induction of pro-apoptotic BIM is key to this effect<sup>19</sup>. Thus, BIM is a common effector in both selumetinib and dexamethasone induced apoptosis, suggesting the potential for synergy. In addition, GC resistance in ALL has been associated with enhanced activation of the pathway and its inhibition has led to GC re-sensitisation<sup>20-22</sup>. These effects may be more pronounced in the context of RAS pathway mutated ALL, therefore, we have preclinically evaluated the combination of dexamethasone and selumetinib *in vitro* and in an orthotopic mouse model engrafted with primary-derived ALL cells and shown pronounced drug synergism in RAS pathway mutated ALL. These data suggest that this drug combination may be highly effective in this significant subgroup of patients and has led to the Seludex trial, an international Phase I/II expansion study for the treatment of relapsed/refractory RAS pathway mutated ALL.

## **Methods**

Additional methods are found in the supplementary material.

### **Compounds and formulation**

Selumetinib was kindly provided by AstraZeneca (Cheshire, UK) and for the *in vitro* studies, was dissolved in DMSO to a concentration of 100mM and stored in single use aliquots at -20°C. Dexamethasone was purchased from Sigma-Aldrich (Dorset, UK), dissolved in ethanol at 20mM and stored at -20°C. For *in vivo* studies, selumetinib was prepared as a suspension in 0.5% hydroxypropyl methylcellulose + 0.1% polysorbate 80.

### **Patient Samples**

Primagrafts were generated in NOD SCID  $\gamma$  null (NSG) mice using ALL cells from bone marrow samples of children presenting or relapsing with ALL and accessed through the Newcastle Haematology Biobank, after appropriate consent (reference numbers 2002/111 and 07/H0906). Clinical details of the patients are given in Table 1. Mutational screening for RAS pathway mutations and assessment of pathway activation by western blotting of p-ERK was performed as previously described<sup>8, 23</sup>.

### ***In vitro* drug sensitivity and synergy**

Freshly harvested primagraft cells were suspended in RPMI1640 with 15% fetal bovine serum and plated out in triplicate at a density of  $5 \times 10^5$  cells/100 $\mu$ l/well into 96-well plates and treated with a range of dexamethasone (0.1nM to 10 $\mu$ M) or selumetinib concentrations (1nM to 100 $\mu$ M). After 96 hours, cytotoxicity was assessed using the CellTiter 96 Aqueous One kit

(Promega, Southampton, UK). The results were averaged and expressed as a percentage of the control vehicle. Survival curves were plotted and growth inhibitory (GI50) values calculated using GraphPad Prism software (GraphPad software Inc., San Diego, CA, USA). Drug combination experiments were analysed for synergistic, additive, or antagonistic effects using the combination index method developed by Chou and Talalay<sup>24</sup>. Briefly, primagraft cells were treated with fixed dose ratios based on the GI50 values of each drug (x0.25, x0.5, x1, x2 and x4) and evaluated by median effect analysis using CalcuSyn software (Cambridge, UK). The dose-effect curve for each drug alone is determined using the median-effect principle and is compared to the effect achieved with a combination of the two drugs to derive a combination index (CI) value.

### **Pharmacokinetic analyses**

Plasma pharmacokinetics of selumetinib and dexamethasone were determined using non compartmental analysis in female CD1 mice after oral dosing. Plasma concentrations of both drugs were measured by LCMS (API4000 LCMS/MS, Applied Biosystems, California, USA), attached to a Perkin Elmer chromatography system (Perkin Elmer Ltd, Beckonsfield, UK) and calibrated using standards prepared in blank mouse plasma. In both cases separation was performed using a Gemini 3 $\mu$  C18 110A column (50x3mm) fitted with a 4x2mm C18 cartridge (Phenomenex, Macclesfield, UK).

### ***In vivo* experiments**

All experiments were performed under the UK home Office NCL- PLL60/4552. Drug efficacy studies were performed as previously described<sup>6</sup>. Briefly, primagraft cells were

injected intrafemorally and mice were monitored for engraftment every 3-4 weeks by tail vein bleed. Blood was red cell lysed and analysed by flow cytometry on a BD FACSCanto II, using anti human CD10, CD34 and CD19 and anti-mouse CD45 antibodies. Human leukaemia cells were gated and expressed as a % of the total number of nucleated cells. Once the level of human leukaemia cells reached >1% of total cells, mice were randomised into control vehicle (0.5% hydroxypropyl methylcellulose + 0.1% polysorbate 80) and drug treatment groups (6 mice per group) were dosed with dexamethasone, selumetinib or both, by oral gavage. Selumetinib was dosed at 25mg/kg BID, while the dexamethasone dosing varied in each study. Tumour burden was monitored weekly by flow cytometry. Pharmacodynamic studies were performed in highly engrafted mice which were dosed for 72 hours. Spleens were removed following euthanasia and assessed by flow cytometry to confirm an engraftment of >85%. Cells were lysed and analysed by western blotting for levels of p-ERK, ERK2, BIM, MCL1 and  $\alpha$ -Tubulin, as described above.

## Results

### **Selumetinib and dexamethasone show synergy *in vitro* in RAS pathway mutated ALL and is associated with enhanced induction of BIM.**

To investigate possible synergism, the R3F9 cell line and primagraft ALL cells, with and without Ras pathway mutations (n=8) were treated with dexamethasone, selumetinib and the drug combination at 0.25x, 0.5x, 1x, 2x and 4x their respective GI<sub>50</sub> concentrations and viability data evaluated by median effect analysis. CI for all RAS pathway samples were indicative of strong synergy with a mean of 0.1 (range, 0.02-0.15) (Figure 1A, B and Supplementary Figure 1). Synergism was not observed in ALL primagraft cells without RAS pathway activation, CI >1.2. Mechanistic assessments were performed with GI<sub>50</sub> concentration of both drugs for 24 hours. As expected, ALL cells treated with selumetinib resulted in almost complete inhibition of ERK phosphorylation and downregulation of MCL1 levels. Dexamethasone treatment also downregulated pERK levels. Treatment with dexamethasone or selumetinib was associated with increased levels of BIM which was further enhanced with the drug combination. A representative western blot and a histogram of the combined densitometry values (n=4 PDX) are shown in Figures 1C and Supplementary Figure 2A. The apoptotic marker, cleaved PARP was enhanced with the drug combination in some, but not all PDX samples, at this time point (Supplementary Figure 2B). While the loss in cell viability in non-dividing PDX ALL cells must be due to increased cell death, we also showed for the *NRAS* mutated R3F9 cell line, enhanced apoptosis with the drug combination (Supplementary Figures 2 C and D) and BIM knockdown reduced the effect (Supplementary Figures 2E and F). There were similar levels of induction of the GR target gene, GILZ in both dexamethasone and drug combination treated cells suggesting that enhanced GR transcriptional activity is not a component of the synergism (supplementary Figure 3). Synergism between selumetinib and other drugs for example, gemcitabine, is highly schedule

dependent and sequential rather than simultaneous dosing appears optimal<sup>25</sup>. Thus, we assessed synergism in primagraft ALL cells dosed simultaneously or with only selumetinib or dexamethasone for 24 hours followed by both drugs for an additional 72 hours, prior to cell viability assessments. We saw similar synergism across all experimental parameters (Figure 1D) and thus we selected simultaneous drug administration in subsequent *in vivo* studies.

### **Pharmacokinetic studies define clinically relevant oral dose and exclude drug-drug interactions**

To determine the optimal oral dose of dexamethasone that will achieve clinically relevant serum levels, pharmacokinetic studies were performed in CD1 mice. Mice (n=27) were dosed with 0.5, 1 and 5mg/kg dexamethasone by oral gavage and blood samples taken at 15min, 30min, 1hr, 3hr, 6hr and 24hr and serum dexamethasone levels analysed (Supplementary Figure 4A). A T<sub>max</sub> of 60 minutes was observed, with C<sub>max</sub> values of 48.9, 94.7 and 766.5 ng/ml following 0.5, 1 and 5mg/kg doses, respectively. Given the reported C<sub>max</sub> average of 40-90 ng/ml in recent UK and American ALL trials, 1mg/kg was deemed the most appropriate dose level<sup>26, 27</sup>.

Dexamethasone can induce cytochrome P450 forms, including CYP3A4, the principal isoform responsible for selumetinib oxidative metabolism, therefore we performed selumetinib pharmacokinetic analyses, alone (25mg/kg) and after co-administration of 1mg/kg dexamethasone (Supplementary Figures 4B and C). A T<sub>max</sub> of 60 minutes was observed, with C<sub>max</sub> values for selumetinib of 4.74ug/ml compared to 5.49 ug/ml, respectively (p>0.05, student t test). Other parameters were also similar (Supplementary Figure 4C), indicative of no drug-drug interaction (p>0.05 for all).

## Selumetinib and dexamethasone show synergy *in vivo* and clear CNS disease

The drug combination was evaluated *in vivo* and compared to single drug and control vehicle in primagrafts derived from diagnostic ALL (*NRAS Q61R* and *KRAS G12D*) and a relapse (*KRAS G13D*). Scheduling and dosing was by oral gavage and is shown in Figure 2 A-G. Due to significant weight loss (>20%) associated with dexamethasone, dosing could not be prolonged, even when the dose was lowered from 1mg/kg bid to 0.25mg/kg sid. There was no additional toxicity observed in mice given the drug combination. Nevertheless, at the end of the dosing period, there was a significant reduction in spleen size with selumetinib and dexamethasone alone but was statistically lower in mice given the drug combination, with spleen weights approaching those of healthy mice (combined data are shown in Figure 2G) ( $p < 0.001$ ). In addition, brains were assessed for the depth of leukaemia infiltration in the leptomeninges. For mice engrafted with L897 and L779 primagraft cells, there was a significant reduction in leukaemic infiltration in drug treated mice, with a mean and SD of  $66.3\mu \pm 100.6$  for CV, compared to  $3.1\mu \pm 12.5$  for dexamethasone and  $5.37\mu \pm 21.475$  for selumetinib (Supplementary Figure 5A). Mice treated with the drug combination showed no leukaemic infiltration ( $p < 0.05$  for all by student t test). For L779, there was demonstrable CNS disease once peripheral ALL exceeded 1% i.e. pre-dosing (Supplementary Figure 5B). Clearance of CNS disease in mice engrafted with L829R cells was unevaluable due to minimal CNS leukaemia in both CV and drug treated mice. Pharmacodynamic assessment of engrafted spleens after short term dosing were consistent with observations *in vitro*; inhibition of ERK phosphorylation and lower MCL1 levels associated with selumetinib dosing, similar induction of GILZ with dexamethasone dosing, and modest enhancement of BIM levels with the drug combination (Figure 3A-D). Annexin V binding in circulating ALL cells, as detected by multi-parameter flow cytometry, increased in all drug treated mice and was highest for the drug combination at both 24 hours and 48 hours (Figure 3E).

## Discussion

Selumetinib is a potent, selective, allosteric inhibitor of MEK1/2 with demonstrated anti-tumour activity and a favourable toxicity profile. It has progressed to phase III clinical trial for several types of adult solid cancers<sup>28-30</sup>. In the paediatric setting, selumetinib has recently undergone phase I clinic testing as a monotherapy in children with *BRAF*-driven recurrent/refractory paediatric low grade glioma which defined a maximum tolerated dose of 25 mg/m<sup>2</sup>/dose BID<sup>31</sup>. Sustained responses (1 complete, 7 partial) were observed in some children and selumetinib was well tolerated, with the most common toxicity being rash. In addition, a phase I trial of selumetinib in children with neurofibromatosis type 1 and inoperable plexiform neurofibromas, showed partial responses in 17 of 24 children (71%) and reported no excess toxicity<sup>32</sup>.

While we have previously shown single agent preclinical activity in ALL, selumetinib like other MEKi, is likely to show maximal therapeutic benefit in combination. Therefore, in phase III clinical trials for advanced non-small-cell lung cancer and uveal melanoma, selumetinib has been evaluated in combination with docetaxel and dacarbazine, respectively<sup>33</sup>. In this current study, we show significant synergy of selumetinib with the synthetic glucocorticoid dexamethasone *in vitro* and in an orthotopic mouse model engrafted with RAS pathway activated primary-derived ALL cells. Importantly, we demonstrate this across a range of cytogenetic subgroups, including high hyperdiploidy, B-other, t(17;19) and t(1;19). Pharmacokinetic data shows clinically relevant drug levels and optimal scheduling and *in vivo* pharmacodynamic analyses confirmed impact on drug targets and apoptosis. Mechanistically, the synergism was associated with enhanced induction of the proapoptotic, BIM and decreases in the anti-apoptotic BH3 only protein MCL1. BIM is a BH3 only protein

that binds to anti apoptotic BCL2 family members, including MCL1 and BCL2, to liberate and directly activate bax and bak which then elicit caspase dependent apoptosis. BIM is an effector protein in both GC and MEK inhibitor response and reducing pERK activity enhances BIM levels as well as decreasing MCL1 protein levels by increasing its turnover<sup>34</sup>. Therefore, we propose that the drug combination enables BIM to more completely inhibit anti-apoptotic BH3 only proteins and directly activate BAX and BAK. Others have reported a direct effect of MEK inhibition on GR transcriptional activity which may also contribute to the synergism, but this did not appear significant in our experiments at the time point chosen<sup>22</sup>. Epigenetic regulation of the *BIM* locus due to acetylation has been described in a subgroup of GC resistant ALLs and is associated with BIM under expression. Such individuals may be expected to have a suboptimal response to the selumetinib/dexamethasone drug combination<sup>35</sup>. However, the incidence of acetylated *BIM* in the relapsed setting and in the context of RAS pathway mutations has not been described to date. Our synergism data are supported by a study from Jones et al., who used an integrated approach to understand GC resistance and relapse and identified MAPK pathways as a contributory factor<sup>22</sup>. In this study, knockdown of MEK2 or MEK inhibition enhanced response not only to GC but to other chemotherapeutics and was not dependent on the presence of RAS pathway mutations, suggestive of activation of the pathway through alternative routes. We have previously shown excellent correlation between pERK activation and the presence of RAS pathway mutations, although we too noted some rare exceptions which in our study were in part explained by the presence of chromosomal translocations, including Ph+ and 11q23<sup>6</sup>.

Drug synergies have also been shown for MEK inhibitors with both traditional chemotherapeutics such as gemcitabine and targeted agents including PI3K/AKT inhibitors

<sup>36, 37</sup> and the BCL-XL inhibitor, Navitoclax (ABT263)<sup>38</sup>. Inhibiting the other effector pathways of RAS is clearly a rational strategy, however, while we have observed synergism of MEK and AKT inhibition in RAS pathway mutated ALL *in vitro*, the synergism was considerably weaker than that observed with dexamethasone (unpublished observations). In solid cancers, increased levels of BIM protein are also observed with MEK inhibition, but is inactive due to sequestration by high levels of BCL-XL. In the presence of Navitoclax, BIM is released, triggering an apoptotic response <sup>38</sup>.

We have previously reported a reduction of CNS leukaemia in selumetinib treated mice and now confirm this in additional primagraft samples and show complete absence of leukaemic infiltrate in the leptomeninges of mice treated with the selumetinib/dexamethasone drug combination <sup>6</sup>. The identification of CNS disease in mice with similar levels of ALL engraftment prior to drug dosing, suggests that the drug combination completely eradicated the leukaemia *in situ*. This is a highly significant finding given the association of RAS pathway mutations and CNS disease at relapse that we previously reported in the *IBFMREZ2002* clinical trial and the fact that in contemporary regimens, the proportion of CNS relapses is increasing<sup>39</sup>.

A key question, relevant to MEKi therapy, is whether Ras pathway mutations are initiating events in ALL or secondary, cooperating genetic events and there is evidence for both (reviewed in <sup>13</sup>). However, for targeted therapies to be successful, the target is ideally present on all tumour cells and we and others have reported that mutations can be subclonal, particularly at diagnosis, and can be gained or lost at relapse <sup>6, 40-42</sup>. Importantly, we have also shown that mutations at relapse are in the major ALL clone, are often selected from a

minor subclone at diagnosis and that apparent ‘loss’ of a Ras pathway mutation can be ‘replacement’ of one for another<sup>8, 43, 6</sup>. This suggests a dependence on the pathway that can be exploited by MEK inhibition and as we show here, is enhanced with dexamethasone co-exposure.

Based on these promising data, an international phase I/II clinical trial of oral dexamethasone and selumetinib (Seludex) is underway in RAS pathway mutated, multiple relapse/refractory ALL. A parallel, national study in adult disease at first relapse is also ongoing, since the prevalence of RAS pathway mutations and association with poor prognosis has also been noted<sup>44</sup>. One relevant observation from selumetinib and other Mek inhibitor trials is that the most common toxicity is inflammatory rash. In severe cases, the recommended treatment is oral glucocorticoids and no adverse effects of drug co-administration have been reported<sup>45</sup>. Thus, if efficacy is seen in the proposed clinical trials, selumetinib and other Mek inhibitors may be a much needed novel therapy for a substantial number of children with high risk, relapsed disease. There may also be a role for the drug combination in the upfront treatment of RAS-driven, high risk ALL, to avert relapse.

**Acknowledgements:** The authors gratefully acknowledge Cancer Research UK (project grant to JAEI, HN and JV, number 18780), Bloodwise (previously known as the Leukaemia and Lymphoma Research Fund, project grant to JAEI, number 11007), the North of England Children's Cancer Research Fund and the Newcastle Haematology Biobank for ALL samples. We are grateful to AstraZeneca for their kind donation of selumetinib. CH is funded by Chief Scientist Office (ETM/374). We thank Clare Orange and Lynn Stevenson, University of Glasgow and Think Pink, Scotland for help with histology and slide scanning.

**Authorship:** Contribution: JAEI was principal investigator and conceived and gained funding for the study, with input from HN, JV and LM. All authors designed, performed research and analysed and/or interpreted data. JI and LM drafted the article and all authors critically appraised and approved the final version.

**Conflict of interest disclosure:** The authors declare no competing financial interests.

## References

1. Vora A, Goulden N, Mitchell C, et al. Augmented post-remission therapy for a minimal residual disease-defined high-risk subgroup of children and young people with clinical standard-risk and intermediate-risk acute lymphoblastic leukaemia (UKALL 2003): a randomised controlled trial. *Lancet Oncol.* 2014;15(8):809-818.
2. Vora A, Goulden N, Wade R, et al. Treatment reduction for children and young adults with low-risk acute lymphoblastic leukaemia defined by minimal residual disease (UKALL 2003): a randomised controlled trial. *Lancet Oncol.* 2013;14(3):199-209.
3. Parker C, Waters R, Leighton C, et al. Effect of mitoxantrone on outcome of children with first relapse of acute lymphoblastic leukaemia (ALL R3): an open-label randomised trial. *Lancet.* 2010;376(9757):2009-2017.
4. Hof J, Krentz S, van Schewick C, et al. Mutations and deletions of the TP53 gene predict nonresponse to treatment and poor outcome in first relapse of childhood acute lymphoblastic leukemia. *J Clin Oncol.* 2011;29(23):3185-3193.
5. Malempati S, Gaynon PS, Sather H, La MK, Stork LC, Children's Oncology G. Outcome after relapse among children with standard-risk acute lymphoblastic leukemia: Children's Oncology Group study CCG-1952. *J Clin Oncol.* 2007;25(36):5800-5807.
6. Irving J, Matheson E, Minto L, et al. Ras pathway mutations are prevalent in relapsed childhood acute lymphoblastic leukemia and confer sensitivity to MEK inhibition. *Blood.* 2014;124(23):3420-3430.
7. Moorman AV, Irving J, Enshaei A, et al. Composite Index for Risk Prediction in Relapsed Childhood Acute Lymphoblastic Leukaemia. *European Haematology Association; 2015 2015; Vienna; 2015.*
8. Case M, Matheson E, Minto L, et al. Mutation of genes affecting the RAS pathway is common in childhood acute lymphoblastic leukemia. *Cancer Res.* 2008;68(16):6803-6809.
9. Chung E, Kondo M. Role of Ras/Raf/MEK/ERK signaling in physiological hematopoiesis and leukemia development. *Immunol Res.* 2011;49(1-3):248-268.
10. Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer.* 2011;11(11):761-774.
11. Ahearn IM, Haigis K, Bar-Sagi D, Philips MR. Regulating the regulator: post-translational modification of RAS. *Nat Rev Mol Cell Biol.* 2012;13(1):39-51.
12. Ward AF, Braun BS, Shannon KM. Targeting oncogenic Ras signaling in hematologic malignancies. *Blood.* 2012;120(17):3397-3406.
13. Knight T, Irving JA. Ras/Raf/MEK/ERK Pathway Activation in Childhood Acute Lymphoblastic Leukemia and Its Therapeutic Targeting. *Front Oncol.* 2014;4(160).
14. Balmanno K, Cook SJ. Tumour cell survival signalling by the ERK1/2 pathway. *Cell Death Differ.* 2009;16(3):368-377.
15. Ley R, Ewings KE, Hadfield K, Cook SJ. Regulatory phosphorylation of Bim: sorting out the ERK from the JNK. *Cell Death Differ.* 2005;12(8):1008-1014.
16. Meng J, Fang B, Liao Y, Chresta CM, Smith PD, Roth JA. Apoptosis induction by MEK inhibition in human lung cancer cells is mediated by Bim. *PLoS One.* 2010;5(9):e13026.
17. Hongo T, Fujii Y. In vitro chemosensitivity of lymphoblasts at relapse in childhood leukemia using the MTT assay. *Int J Hematol.* 1991;54(3):219-230.
18. Klumper E, Pieters R, Veerman AJ, et al. In vitro cellular drug resistance in children with relapsed/refractory acute lymphoblastic leukemia. *Blood.* 1995;86(10):3861-3868.
19. Lu J, Quearry B, Harada H. p38-MAP kinase activation followed by BIM induction is essential for glucocorticoid-induced apoptosis in lymphoblastic leukemia cells. *FEBS Lett.* 2006;580(14):3539-3544.

20. Rambal AA, Panaguiton ZL, Kramer L, Grant S, Harada H. MEK inhibitors potentiate dexamethasone lethality in acute lymphoblastic leukemia cells through the pro-apoptotic molecule BIM. *Leukemia*. 2009;23(10):1744-1754.
21. Polak A, Kiliszek P, Sewastianik T, et al. MEK Inhibition Sensitizes Precursor B-Cell Acute Lymphoblastic Leukemia (B-ALL) Cells to Dexamethasone through Modulation of mTOR Activity and Stimulation of Autophagy. *PLoS One*. 2016;11(5):e0155893.
22. Jones CL, Gearheart CM, Fosmire S, et al. MAPK signaling cascades mediate distinct glucocorticoid resistance mechanisms in pediatric leukemia. *Blood*. 2015;126(19):2202-2212.
23. Nicholson L, Knight T, Matheson E, et al. Casitas B lymphoma mutations in childhood acute lymphoblastic leukemia. *Genes Chromosomes Cancer*. 2012;51(3):250-256.
24. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul*. 1984;22(27-55).
25. Xu J, Knox JJ, Ibrahimov E, et al. Sequence dependence of MEK inhibitor AZD6244 combined with gemcitabine for the treatment of biliary cancer. *Clin Cancer Res*. 2013;19(1):118-127.
26. Yang L, Panetta JC, Cai X, et al. Asparaginase may influence dexamethasone pharmacokinetics in acute lymphoblastic leukemia. *J Clin Oncol*. 2008;26(12):1932-1939.
27. Jackson RK, Irving JAE, Veal GJ. Abstract CT115: Pharmacokinetics of standard versus short high-dose dexamethasone therapy in childhood acute lymphoblastic leukemia: results from the UKALL 2011 trial. *Cancer Research*. 2016;76(14 Supplement):CT115-CT115.
28. Bennouna J, Lang I, Valladares-Ayerbes M, et al. A Phase II, open-label, randomised study to assess the efficacy and safety of the MEK1/2 inhibitor AZD6244 (ARRY-142886) versus capecitabine monotherapy in patients with colorectal cancer who have failed one or two prior chemotherapeutic regimens. *Invest New Drugs*.
29. Davies BR, Logie A, McKay JS, et al. AZD6244 (ARRY-142886), a potent inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 kinases: mechanism of action in vivo, pharmacokinetic/pharmacodynamic relationship, and potential for combination in preclinical models. *Mol Cancer Ther*. 2007;6(8):2209-2219.
30. Janne PA, Shaw AT, Pereira JR, et al. Selumetinib plus docetaxel for KRAS-mutant advanced non-small-cell lung cancer: a randomised, multicentre, placebo-controlled, phase 2 study. *Lancet Oncol*. 2013;14(1):38-47.
31. Anuradha Banerjee RJ, Arzu Onar-Thomas, Shengjie Wu, Theodore Nicolaidis, David Turner, Stacye Richardson, Tina Young-Poussaint, Joanna J Phillips, Michael Prados, Roger Packer, Ibrahim A Qaddoumi, Sridharan Gururangan, Stewart Goldman, Ian Pollack, L. Austin Doyle, Clinton F. Stewart, James M. Boyett, Maryam Fouladi. A phase 1 study of AZD6244 in children with recurrent or refractory low-grade gliomas: A Pediatric Brain Tumor Consortium report. *J Clin Oncol* 32:5s abstr 10065, 2014.
32. Dombi E, Baldwin A, Marcus LJ, et al. Activity of Selumetinib in Neurofibromatosis Type 1-Related Plexiform Neurofibromas. *N Engl J Med*. 2016;375(26):2550-2560.
33. [Trials.gov](https://www.trials.gov) C. [Clinical Trials.gov](https://www.clinicaltrials.gov).
34. Domina AM, Vrana JA, Gregory MA, Hann SR, Craig RW. MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol. *Oncogene*. 2004;23(31):5301-5315.
35. Bachmann PS, Piazza RG, Janes ME, et al. Epigenetic silencing of BIM in glucocorticoid poor-responsive pediatric acute lymphoblastic leukemia, and its reversal by histone deacetylase inhibition. *Blood*. 2010;116(16):3013-3022.
36. Engelman JA, Chen L, Tan X, et al. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. *Nat Med*. 2008;14(12):1351-1356.
37. Haagensen EJ, Kyle S, Beale GS, Maxwell RJ, Newell DR. The synergistic interaction of MEK and PI3K inhibitors is modulated by mTOR inhibition. *Br J Cancer*. 2012;

38. Corcoran RB, Cheng KA, Hata AN, et al. Synthetic lethal interaction of combined BCL-XL and MEK inhibition promotes tumor regressions in KRAS mutant cancer models. *Cancer Cell*. 2013;23(1):121-128.
39. Krishnan S, Wade R, Moorman AV, et al. Temporal changes in the incidence and pattern of central nervous system relapses in children with acute lymphoblastic leukaemia treated on four consecutive Medical Research Council trials, 1985-2001. *Leukemia*. 2010;24(2):450-459.
40. Molteni CG, Te Kronnie G, Bicciato S, et al. PTPN11 mutations in childhood acute lymphoblastic leukemia occur as a secondary event associated with high hyperdiploidy. *Leukemia*. 2010;24(1):232-235.
41. Mullighan CG, Zhang J, Kasper LH, et al. CREBBP mutations in relapsed acute lymphoblastic leukaemia. *Nature*. 2011;471(7337):235-239.
42. Ma X, Edmonson M, Yergeau D, et al. Rise and fall of subclones from diagnosis to relapse in pediatric B-acute lymphoblastic leukaemia. *Nat Commun*. 2015;6(6604).
43. Tartaglia M, Martinelli S, Cazzaniga G, et al. Genetic evidence for lineage-related and differentiation stage-related contribution of somatic PTPN11 mutations to leukemogenesis in childhood acute leukemia. *Blood*. 2004;104(2):307-313.
44. Trinquand A, Tanguy-Schmidt A, Ben Abdelali R, et al. Toward a NOTCH1/FBXW7/RAS/PTEN-based oncogenetic risk classification of adult T-cell acute lymphoblastic leukemia: a Group for Research in Adult Acute Lymphoblastic Leukemia study. *J Clin Oncol*. 2013;31(34):4333-4342.
45. Welsh SJ, Corrie PG. Management of BRAF and MEK inhibitor toxicities in patients with metastatic melanoma. *Ther Adv Med Oncol*. 2015;7(2):122-136.

Table 1. Clinical features of patients and characterisation of PDX.

Patient ID	Sex	Age at Diagnosis (years)	Cytogenetics	End of Induction MRD	Ras pathway mutation	Clonality	pERK
L779	M	5.5	High Hyperdiploid	Intermediate	NRAS (Q61R)	Clonal	Positive
L897 <sup>a</sup>	M	16.8	B other	High risk	KRAS (G12D)	Clonal	Positive
L914	F	7.3	High Hyperdiploid	Low risk	CBL/FLT3 Large del/Δ836	Clonal	Positive
L829 <sup>b</sup> relapse	F	3.1	High Hyperdiploid	High risk	KRAS (G13D)	Clonal	Positive
L707 <sup>c</sup>	F	16.5	t(17;19)	High risk	KRAS (insertion)	Clonal	Positive
LX825	F	14.7	B other	High risk	Wildtype	N/A	Negative
L920	F	4.4	B other	Low risk	Wildtype	N/A	Negative
L848	M	2.5	t(12;21)	Low risk	Wildtype	N/A	Negative

<sup>a</sup> patient suffered on-treatment CNS relapse

<sup>b</sup>L829 at diagnosis was *NRAS* G12D

<sup>c</sup> patient relapsed with the same *KRAS* mutation

B-other group:-

L897 is negative by FISH for ETV6-RUNX1, BCR-ABL1, MLL and TCF3-PBX1/HLF

LX825 is negative by FISH for ETV6-RUNX1, BCR-ABL1, MLL, CRLF2, IKZF1, PAX5, IGH and PDGFRB

## Figure Legends

### **Figure 1. Selumetinib and dexamethasone show synergy *in vitro* in RAS pathway mutated ALL and is associated with enhanced levels of BIM**

Viability curves of Ras pathway mutant ALL cells (L829R) with individual drugs and the selumetinib/dexamethasone drug combination (A). Histogram of combination indices (CI) for the selumetinib/dexamethasone combination in wild type and Ras pathway mutant ALL cells; mutated genes are shown in brackets (B). Western analyses of ALL cells (L829R) treated with CV or GI50 values of selumetinib (10  $\mu$ M) and dexamethasone (10  $\mu$ M), singly and in combination (C). A representative median effect curve (data shown are from L897) after simultaneous drug dosing and with each drug added 24 hours prior to the partner drug, followed by a further 72 hours incubation (D).

### **Figure 2. Selumetinib and dexamethasone show synergy *in vivo* in RAS pathway mutated ALL**

*In vivo* drug efficacy studies of single drug and combination in RAS pathway mutated ALL showing dose scheduling and peripheral blood monitoring before and during dosing and spleen weights at the end of dosing for L779-*NRAS* (A and B, respectively), L897-*KRAS* (C and D) and L829 relapse- *KRAS* (E and F). For L779, mice were dosed with selumetinib at 25mg/kg and dex at 1mg/kg twice daily and then once daily after a recovery period. For L897, selumetinib was 25mg/kg and dex at 0.5mg/kg (BID), with the dex being increased to 1mg/kg (SID) following a recovery period and for L829R, selumetinib was dosed at 25mg/kg (BID) and dex at 0.25mg/kg (SID). Mean and SD are shown for combined spleen weight data for all 3 efficacy experiments (G) (One way ANOVA with Tukeys multiple

comparison test, \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ;  $n=17$  for CV,  $n=17$  for Sel,  $n=15$  for Dex and  $n=14$  for Sel and Dex treated mice).

**Figure 3. Pharmacodynamic analyses in ALL cells after drug dosing *in vivo*, supports *in vitro* data.**

Western analyses of spleen cells from mice engrafted with RAS pathway mutant ALL cells after 72 hours of dosing (A, L779; NRAS; 25mg/kg selumetinib and 1mg/kg dexamethasone bid and B, L897; KRAS; 25mg/kg selumetinib and 0.5mg/kg dexamethasone bid).

Histograms of densitometry from western analyses, showing mean  $\pm$  SEM (3-4 mice per treatment) (One way ANOVA with Tukeys multiple comparison test, \*  $p < 0.05$ , \*\* $p < 0.01$ , (C). Relative expression of GILZ mRNA (mean and SEM) compared to CV as quantified by RQPCR expression in all 3 PDX experiments, again after 72 hours dosing (ANOVA as before \*\* $p < 0.01$ ; ns, not significant (D). Histograms of annexin V positive ALL cells (mean  $\pm$  SEM) determined by flow cytometric analyses of peripheral blood at 24 and 48 hours after dosing (2 mice per group)(E).

Figure 1

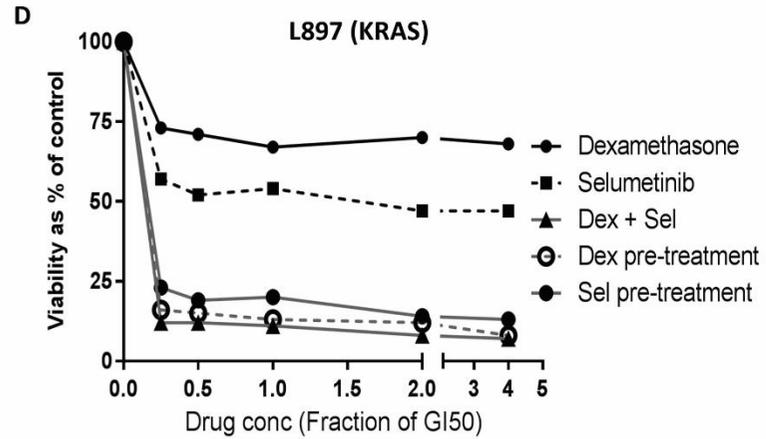
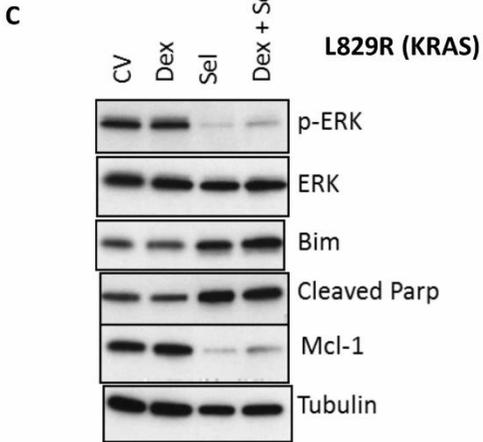
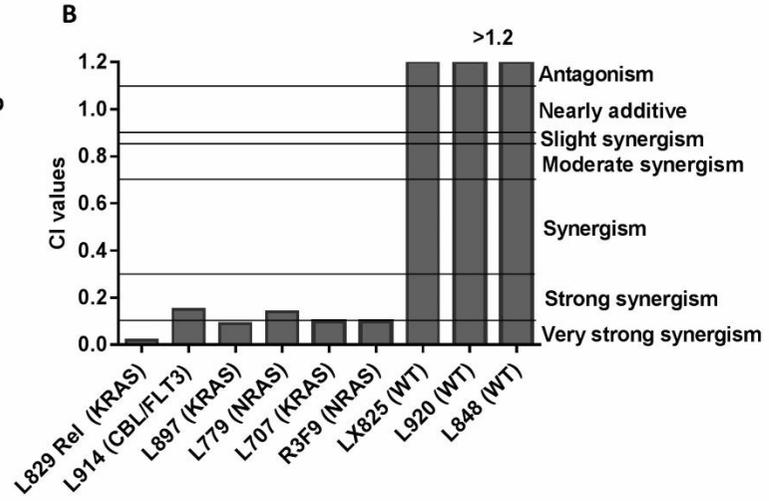
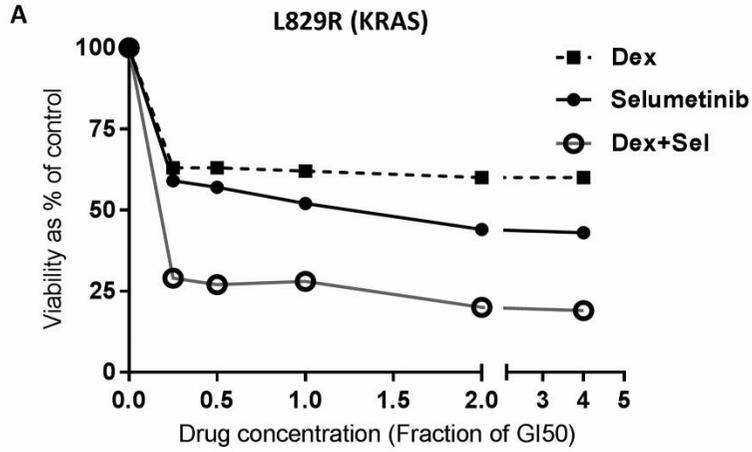


Figure 2

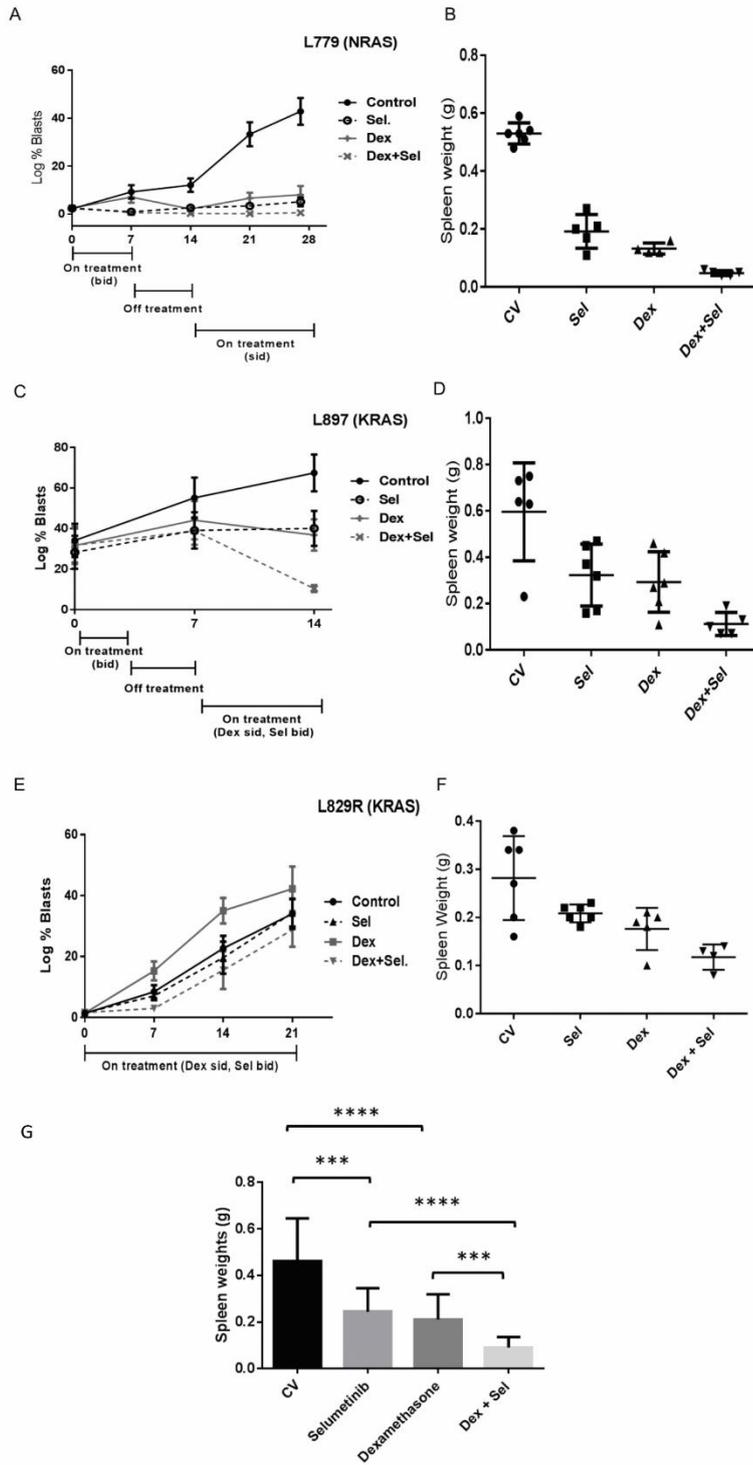


Figure 3

