SUPPLEMENTARY APPENDIX

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

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Glucocorticoids and selumetinib are highly synergistic in RAS pathway mutated childhood ALL through upregulation of bim

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Additional Methods

Cell lines

The R3F9 cell line is a glucocorticoid resistant subline of PreB697 (t(1;19)) which was a kind gift from Professor Reinhard Kofler and which we have extensively characterised [1-3]. Cells were maintained in RPMI-1640 (Sigma-Aldrich, Dorset, UK) supplemented with 10% foetal bovine serum (FBS) (Gibco, Rugby, UK) and incubated at 37°C with a CO_2 concentration of 5%. Cell cycle analyses and the assessment of annexin V expression were assessed by flow cytometry on a FACSCalibur fitted with a 488 nm laser.

Western blotting

Primagraft cells were washed in PBS and protein extracted using PhoshoSafe extraction reagent (Merck, Nottingham, UK) supplemented with protease inhibitors (Roche, Hertfordshire, UK). Western blotting was carried out using a standard methodology and the resulting blots probed for p-ERK (Santa Cruz), BIM and cleaved Parp and MCL1 (Cell Signaling). Blots were stripped and reprobed for ERK2 (Santa Cruz) and Tubulin (Sigma Aldrich) which acted as loading controls.

Real-time PCR

Total RNA was extracted from cell pellets using the Qiagen RNeasy Mini kit (Qiagen, Crawley, UK) and cDNA synthesis was carried out using the Applied Biosystem High-Capacity cDNA Reverse Transcriptase kit, according to the manufacturer's instructions. To quantify GILZ mRNA levels, the $2^{-\Delta\Delta C_T}$ method was used, using TATA-binding protein (TBP) as the endogenous control, as previously described [3].

Bim Knockdown

Small interfering RNA (siRNA) of BIM was carried out by electroporation using FlexiTube HsBCL2L11 or AllStars negative control (Qiagen) with a concentration of 250nM of siRNA pool and a Gemini twin wave electroporation system (BTX) set to 350 volts and 10 msec. Drug dosing was performed 24 hours after electroporation.

Murine brain histology

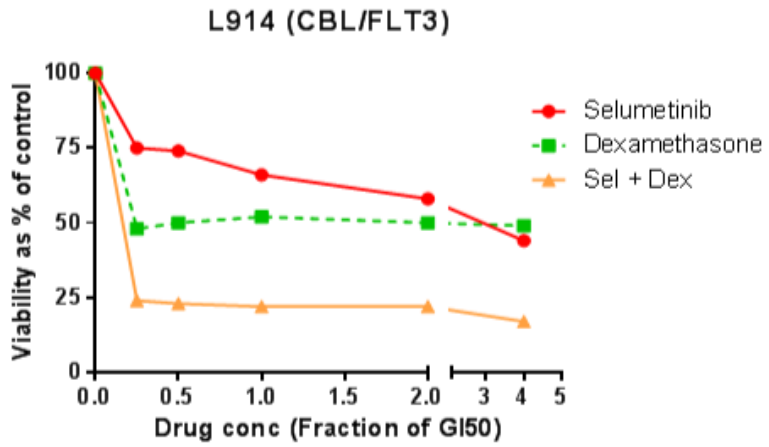
Murine heads were stripped of soft tissues and decalcified in Hilleman and Lee EDTA solution (5·5% EDTA in 10% formalin) for 2-3 weeks, then trimmed and put in fresh EDTA for 3-4 days. Samples were processed on a Tissue-Tek VIP processor using a routine overnight 17.5 hour cycle. Following paraffin wax embedding, 2.5µm sections were cut onto Poly-L-silane coated slides. Sections were then stained with Gills haematoxylin and Putts eosin (both made in house) and viewed on an Axiostar plus (Zeiss) microscope. Quantification of CNS infiltration was performed using a Hamamatsu Nanozoomer Digital Pathology slide scanner with digital slide management/image analysis software from Slidepath (Dublin).

Statistical analysis

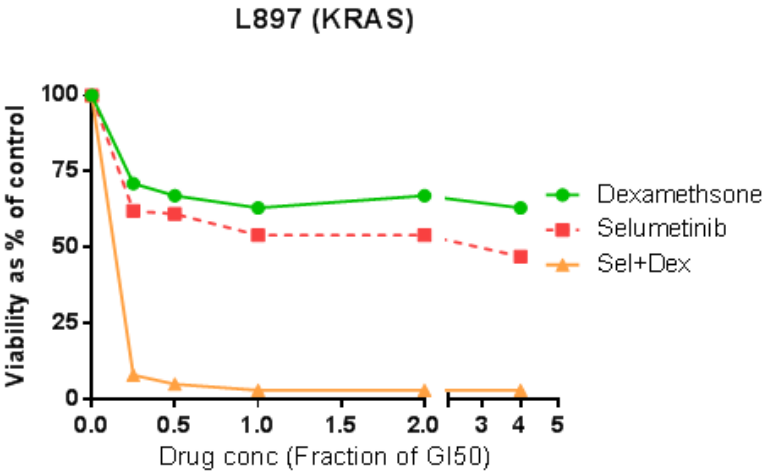
Graph plotting and statistical analyses were performed using GraphPad Prism version 6 (GraphPad software Inc., San Diego, CA, USA).

Figure S1. Selumetinib and dexamethasone show strong synergy *in vitro* in Ras pathway mutated ALL

A



	GI50
DEX	>10 µM
SEL	10 µM
CI	0.15



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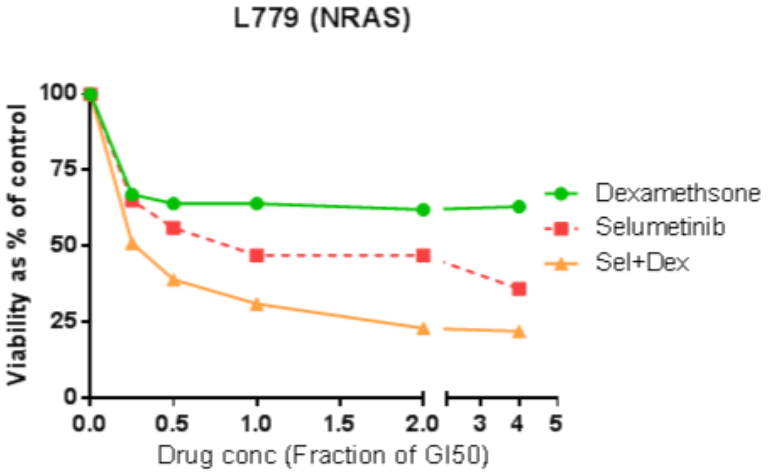
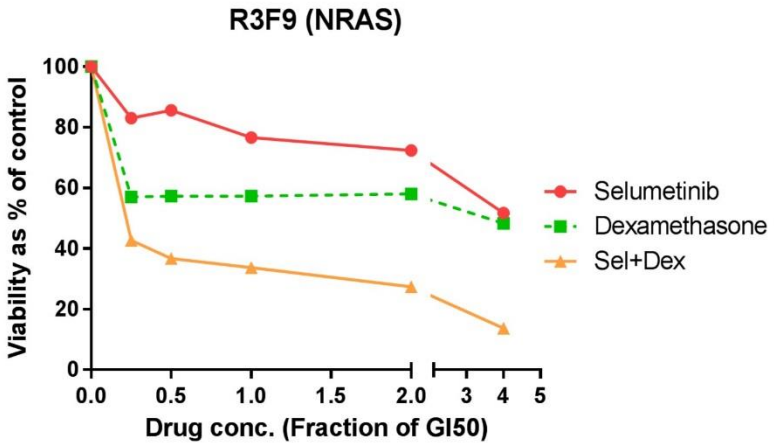
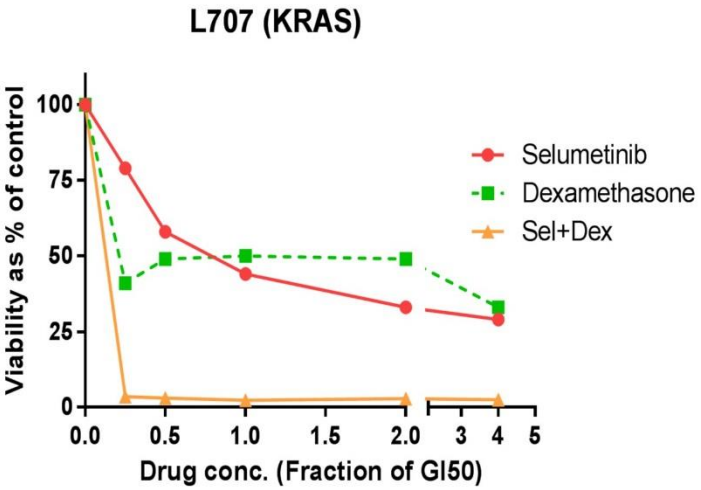
DEX	10 µM
SEL	10 µM
CI	0.09



	GI50
DEX	>10uM
SEL	5 nM
CI	0.14

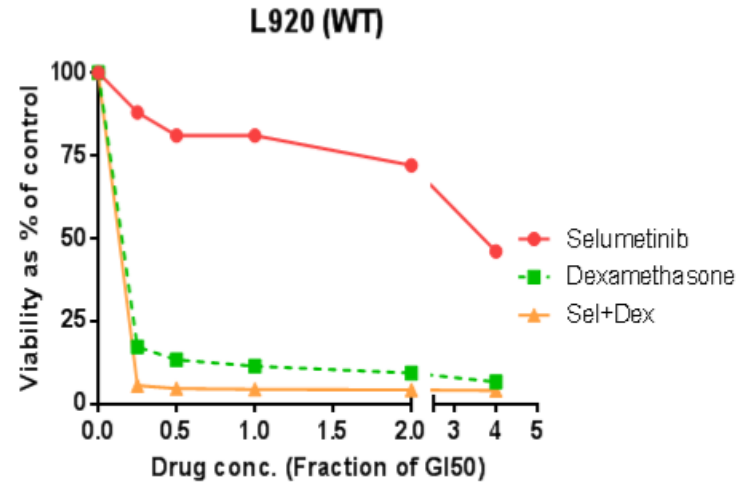


	GI50
DEX	10 µM
SEL	20 µM
CI	0.1

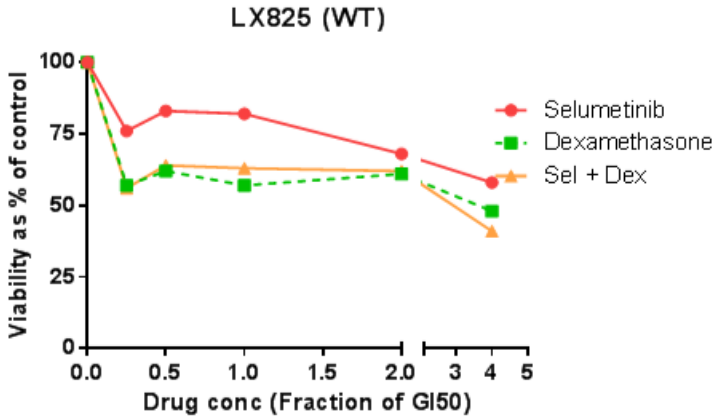


	GI50
DEX	50 nM
SEL	200 nM
CI	<0.1

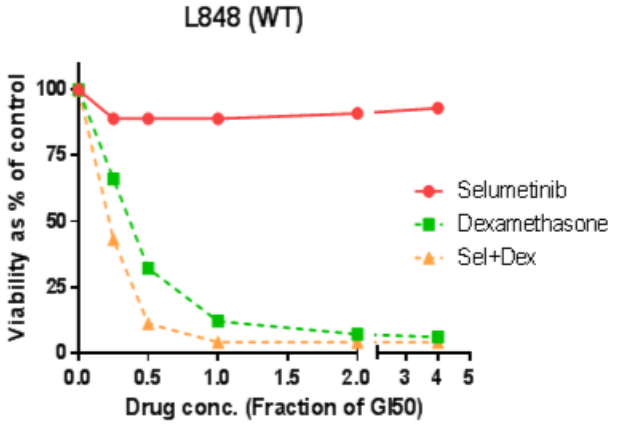
B



	GI50
DEX	50 nM
SEL	>20 µM
CI	>1.2



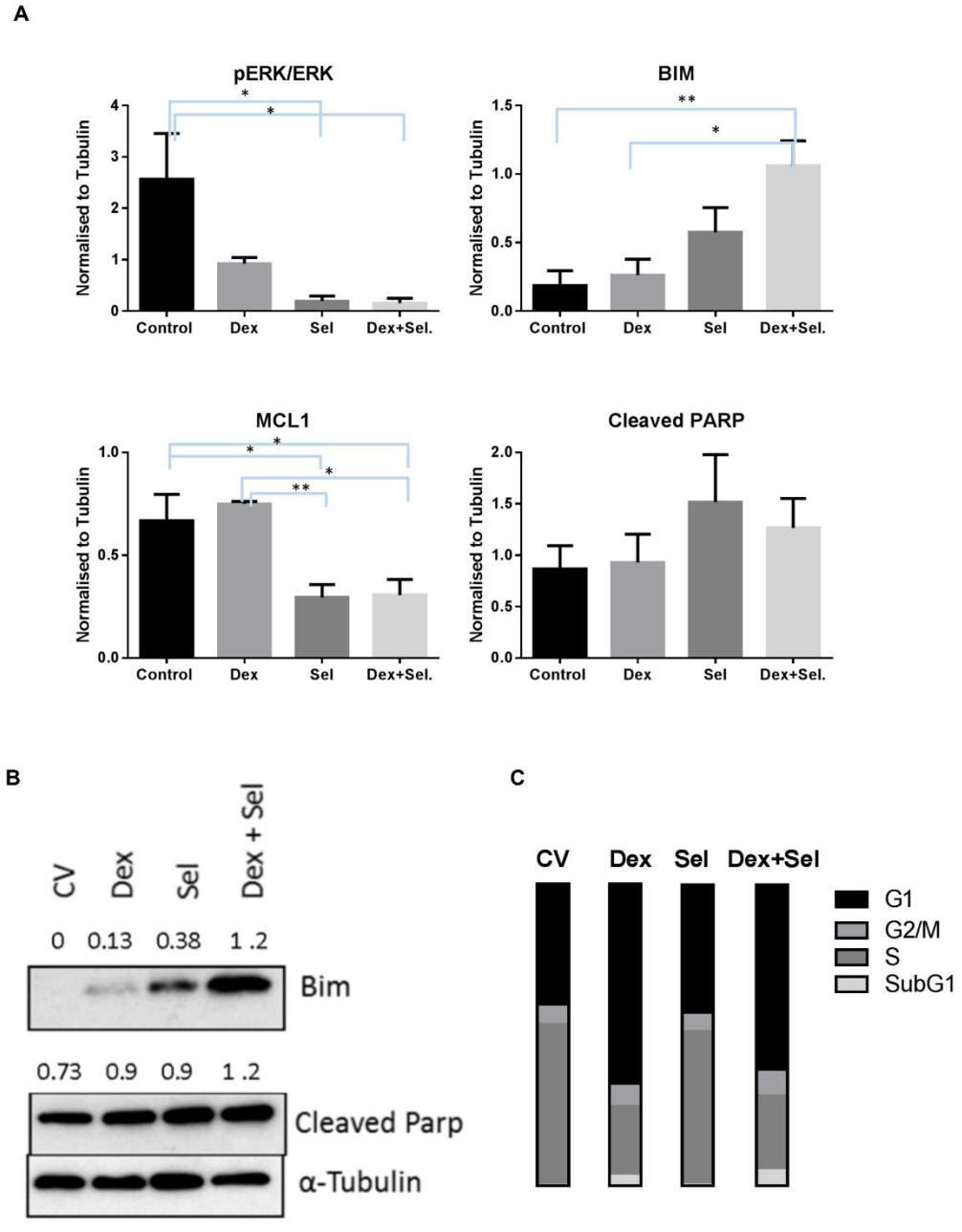
	GI50
DEX	5 µM
SEL	>20 µM
CI	>1.2



	GI50
DEX	5 nM
SEL	10 µM
CI	>1.2

Viability curves of Ras pathway mutant ALL cells (A) and wildtype/pERK negative ALL cells (B) after 96 hours incubation with individual drugs and the selumetinib/dexamethasone drug combination.

Figure S2. The selumetinib and Dexamethasone drug combination is associated with enhanced induction of BIM and apoptosis.



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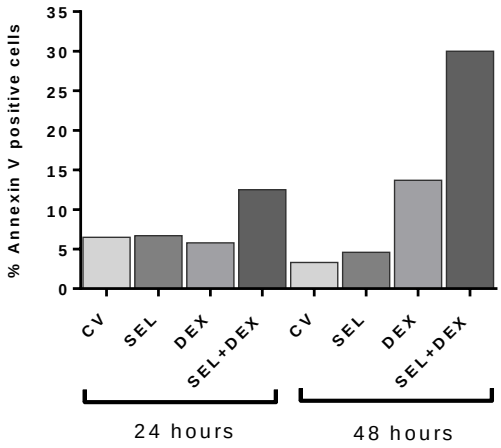

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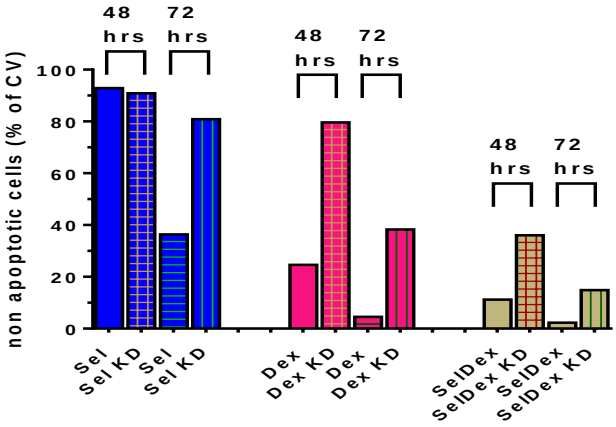

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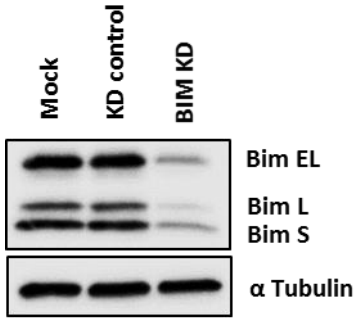
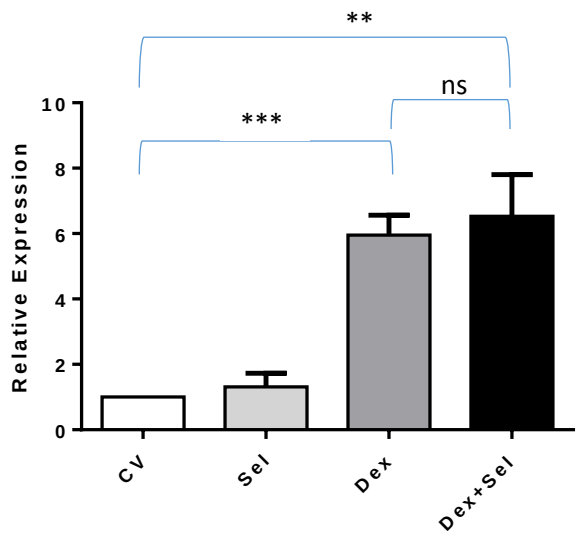


Histograms of combined densitometry values (mean and SD) normalised to tubulin for Ras pathway mutant ALL PDX samples (n=4, L829R, L897, L779 and L914) treated with control vehicle or GI50 values of selumetinib and dexamethasone for 24 hours, singly and in combination. (One way ANOVA with Tukeys multiple comparison test, * $p<0.05$, **$p<0.01$) (A). Western analyses showing densitometry values relative to α-tubulin for PDX, L897 showing clear induction of BIM and cleaved PARP (B). Cell cycle analyses showing the percentage of cells in each stage of the cell cycle after 48 hours dosing of the R3F9 cell line with CV or GI50 values of selumetinib (20uM) and dexamethasone (10uM) as single agents or as a drug combination (C). Histogram showing the percentage of R3F9 cells positive for Annexin V under the same treatment conditions at 24 and 48 hours (D). Histogram showing the percentage reduction in apoptotic cells in R3F9 cells with BIM knockdown compared to scrambled control, for selumetinib, dexamethasone and the drug combination (E). Western blot of Bim and the loading control, α tubulin, 24 hours after electroporation with mock (no siRNA), a negative control (scrambled siRNA) or Bim siRNA (F).

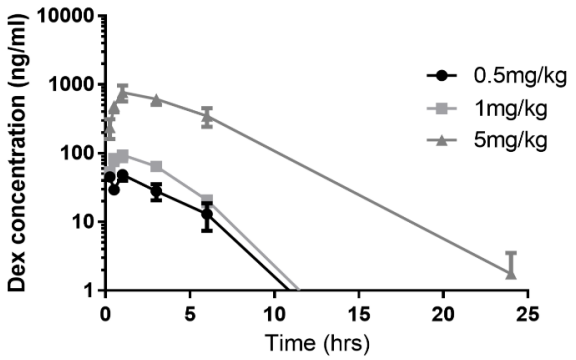
Figure S3. GILZ induction in response to dexamethasone is similar with and without selumetinib



Relative expression of GILZ mRNA (mean and SEM) compared to CV after 24 hours dosing with GI50 concentrations of selumetinib and dexamethasone, as quantified by RQPCR in RAS pathway mutant PDX ALL cells (n=3) (**p<0.01, ***p<0.001; ns, not significant).

Figure S4. Dexamethasone and Selumetinib pharmacokinetic analyses define optimal oral dexamethasone dosage and exclude drug-drug interaction

A



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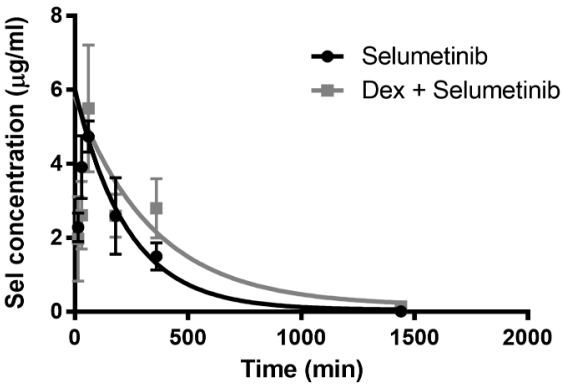
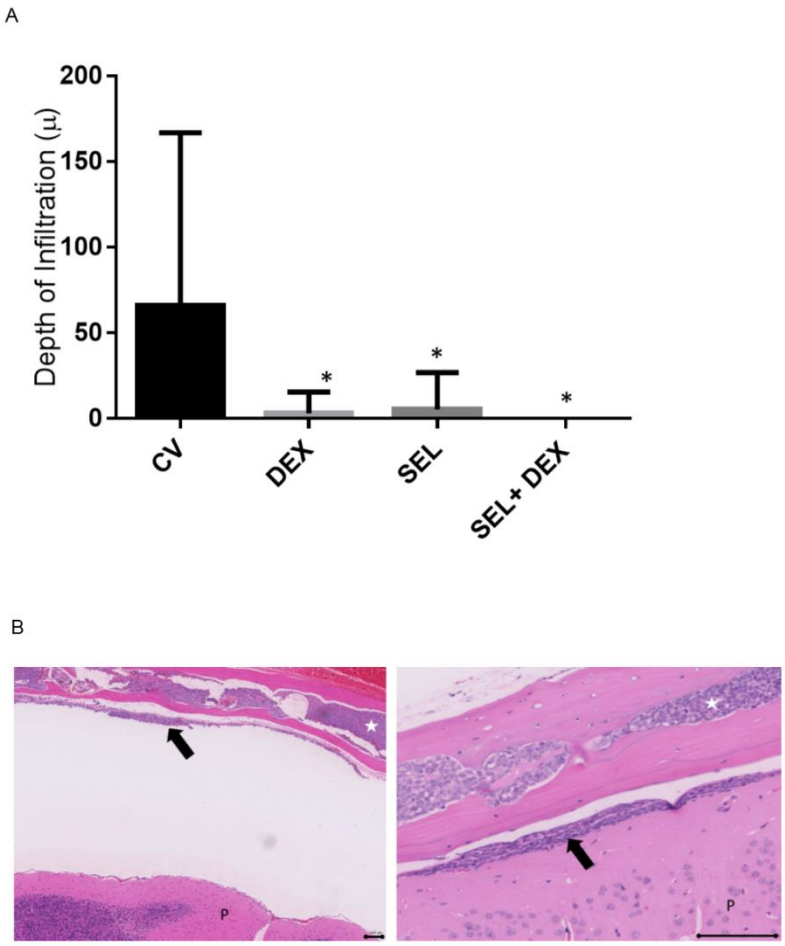


C

	Sel	Sel + Dex
AUC last (µg/ml.min)	1814	2728
AUC inf (µg/ml.min)	1817	2785
C Max (µg/ml)	4.74	5.49
T Max (min)	60	60
Half Life (min)	158.6	272.9
$Clearance_{app}$ (ml/min/kg)	13.8	9.0
$Vdss_{app}$ (L/kg)	3351.0	2715.53

Pharmacokinetic analyses in CD-1 mice dosed with 0.5, 1 and 5mg/kg dexamethasone, showing mean and SD of triplicate values at each time point (A). Selumetinib pharmacokinetic analyses, alone (25mg/kg) and after co-administration of 1mg/kg dexamethasone, showing mean and SD of triplicate values at each time point (B). Table of key pharmacokinetic parameters of selumetinib, alone and when co-administered with dexamethasone ($p>0.05$ for all) (C).

Figure S5. The selumetinib/dexamethasone combination clears CNS leukaemia



Histogram showing the depth of leukaemic infiltrate in the leptomeninges of mice treated with CV versus selumetinib, dexamethasone and drug combination for 2-4 weeks (n=4 mice per group) (A). Brains were cut into 5 equal sections prior to embedding and the maximal depth of infiltration was measured for each section and then summed per mouse. Mean and SD are shown, *p <0.05 by student t test when compared to CV.

Photomicrograph of a brain section stained with Haematoxylin and Eosin from a mouse engrafted with ALL cells (L779) with a peripheral blood level of ~ 1% (B, left panel X6 magnification of posterior section (with some shrinkage artefact widening the leptomeningeal space), right X20 magnification of middle section). Thick arrow indicates the leukaemic infiltrate within the leptomeninges, white star indicates calvarial bone marrow, and P indicates the brain parenchyma. Scale bar = 100µm.

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3. Nicholson L, Evans CA, Matheson E, et al. Quantitative proteomic analysis reveals maturation as a mechanism underlying glucocorticoid resistance in B lineage ALL and re-sensitization by JNK inhibition. Br J Haematol. 2015;171(4):595-605.