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Targeting oncogenic interleukin-7 receptor signalling with *N*-acetylcysteine in T cell acute lymphoblastic leukaemia

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Summary

Activating mutations of the interleukin-7 receptor (*IL7R*) occur in approximately 10% of patients with T cell acute lymphoblastic leukaemia (T-ALL). Most mutations generate a cysteine at the transmembrane domain leading to receptor homodimerization through disulfide bond formation and ligand-independent activation of STAT5. We hypothesized that the reducing agent *N*-acetylcysteine (NAC), a well-tolerated drug used widely in clinical practice to treat acetaminophen overdose, would reduce disulfide bond formation, and inhibit mutant IL7R-mediated oncogenic signalling. We found that treatment with NAC disrupted IL7R homodimerization in *IL7R*-mutant DND-41 cells as assessed by non-reducing Western blot, as well as in a luciferase complementation assay. NAC led to STAT5 dephosphorylation and cell apoptosis at clinically achievable concentrations in DND-41 cells, and Ba/F3 cells transformed by an *IL7R*-mutant construct containing a cysteine insertion. The apoptotic effects of NAC could be rescued in part by a constitutively active allele of *STAT5*. Despite using doses lower than those tolerated in humans, NAC treatment significantly inhibited the progression of human DND-41 cells engrafted in immunodeficient mice. Thus, targeting leukaemogenic IL7R homodimerization with NAC offers a potentially effective and feasible therapeutic strategy that warrants testing in patients with T-ALL.

Keywords: acute leukaemia, T-cell lymphoma, therapy.

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Coordinated signalling through the JAK and STAT pathways is essential for normal lymphopoiesis (Higuchi *et al*, 1997; Igaz *et al*, 2001). This dependence is exemplified by the development of severe combined immunodeficiency when integral components of this pathway, including interleukin-*7* receptor alpha (IL7R), are inactivated through loss-of-function genetic lesions (Kalman *et al*, 2004). Conversely, constitutive activation of JAK-STAT signalling has been implicated in the development of acute lymphoblastic leukaemia (ALL), with activating mutations in *JAK1, JAK2, JAK3*, and more recently *STAT5B* and *TYK2*, all identified in T-ALL (Flex *et al*, 2008; Zhang *et al*, 2012; Kontro *et al*, 2014; Sanda *et al*, 2013).

Several groups recently reported the presence of somatically acquired activating *IL7R* mutations occurring in approximately 10% of T-ALL cases (Shochat *et al*, 2011; Zenatti *et al*, 2011; Zhang *et al*, 2012). The vast majority of such mutations are short in-frame insertions that result in the introduction of a novel cysteine just extracellular to the transmembrane domain. These unpaired cysteine residues result in inter-molecular disulfide bond formation leading to ligand-independent IL7R homodimerization, and constitutive JAK1 activation with phosphorylation and activation of STAT5 (Shochat *et al*, 2011; Zenatti *et al*, 2011; Zhang *et al*, 2012). Furthermore, inhibition of JAK-STAT signalling results in apoptosis of *IL7R*-mutant cells, suggesting that these tumours depend on this pathway for survival (Shochat *et al*, 2011; Zenatti *et al*, 2011; Porcu *et al*, 2012; Zhang *et al*, 2012).

We hypothesized that leukaemogenic activation of this pathway by cysteine mutations in *IL7R* could be blocked with the reducing agent *N*-acetylcysteine (NAC), which is able to reduce disulfide bond formation *in vitro* and *in vivo* (Cartwright *et al*, 1977; Chen *et al*, 2011). NAC is an approved drug that has been used extensively and safely in clinical practice as an antidote for acetaminophen overdose for over three decades (Peterson & Rumack, 1977; Smilkstein *et al*, 1988). Here we use biochemical, genetic and *in vivo* studies to show that NAC treatment inhibits mutant IL7R-mediated oncogenic signalling by disrupting disulfide bond formation, potentially offering an effective, affordable and well-tolerated therapeutic strategy for T-ALL patients with *IL7R* cysteine insertions.

Materials and methods

Cell culture, IL7R *sequencing and NAC treatment* in vitro

T-ALL cell lines were maintained as previously described (Sanda *et al*, 2013). Exon 6 of *IL7R* was Sanger sequenced from T-ALL cell lines using the published protocol (Shochat *et al*, 2011). For drug assays, cells were grown at a density of 1×10^5/ml in 96-well format and treated with NAC (Sigma-Aldrich, St. Louis, MO, USA). Due to the acidity of NAC in culture, both control and NAC treated wells were additionally buffered with 20 mmol/l HEPES (Invitrogen, Grand Island, NY, USA).

Retroviral transductions

The pMSCV-IL7R-243insPPCL-IRES-GFP and pMSCV-IL7R-V253G-IRES-GFP were created by site directed mutagenesis from pMSCV-IL7R-IRES-GFP wild-type vector (Stratagene/Agilent, Santa Clara, CA, USA) (Shochat *et al*, 2011). pMSCV-BCR-ABL-puro has been previously described (Yoda *et al*, 2010). The pMX-Stat5b1*6-IRES-GFP constitutively active murine STAT5B mutant (aStat5b) and control pMX-IRES-GFP retroviral vectors were kind gifts from Professor Toshio Kitamura (University of Tokyo, Tokyo, Japan), and were used in DND-41 rescue experiments (Onishi *et al*, 1998). The pMSCV-cS5^F-IRES-eGFP encoding a constitutively active murine STAT5A mutant (S710F=cS5) and control pMSCV-IRES-eGFP were cloned and validated previously (Moriggl *et al*, 2005). These constructs were used in the Ba/F3 rescue experiments because their high GFP expression allowed sorting of a Ba/F3-IL7R-PPCL population co-expressing cS5. The generation of retroviral supernatants, viral transductions and cell selection have been described previously (Sanda *et al*, 2013).

Western blots

Immunoblotting was carried out with the following antibodies: anti-IL7Rα (clone-40131; R&D Systems, Minneapolis, MN, USA) and anti-pY-STAT5 (Y694) (Cell Signaling Technology, Danvers, MA, USA), both diluted 1 in 1000; anti-β-actin (ACTB); Sigma-Aldrich) diluted 1 in 5000 and secondary horseradish peroxidase (HRP)-linked antibody to mouse or rabbit (Cell Signaling Technology) diluted 1 in 10 000.

Cell viability and apoptosis assays

Cell viability *in vitro* was determined at 48 h after the initiation of treatment with NAC using the Cell Titer Glo assay (Promega, Madison, WI, USA). For Annexin V staining, cells were washed twice in phosphate-buffered saline (PBS) at 24 h after drug treatment, labelled with Annexin V fluorescein isothiocyanate (FITC) antibody (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's recommendations and assessed by flow cytometry.

Luciferase complementation assay

The luciferase complementation assay was performed according to the protocol of Cassonnet *et al* (2011). Wild-type and mutant (p.L242_L243ins PPCL) IL7R sequences were amplified from MSCV-IL7R-IRES-GFP plasmid templates (Shochat *et al*, 2011) using primers IL7R-GW-F 5′-GGGGACAACTT TGTACAAAAAAGTTGGCATGACAATTCTAGGTACA and IL7R-GW-R 5′-GGGGACAACTTTGTACAAGAAAGTTGAG AGACTGGGCCATACGATAGG, containing *att*B1 and *att*B2 Gateway cloning sites. Polymerase chain reaction (PCR)

products were purified and transferred by BP recombination into pDONR223 vector. Generated entry clones were used in a LR recombination reaction with destination vectors pSPICA-C1 and pSPICA-C2 [kindly provided by Dr. Yves Jacob, Unité de Génétique, Papillomavirus et Cancer Humain (GPCH), Institut Pasteur, Paris, France] allowing C-terminal fusion of IL7R sequences with the amino acids 18–109 or amino acids 110–185 of the humanized *Gaussia princeps* luciferase, respectively. All clones were verified by Sanger sequencing.

HEK-293T cells were seeded at 5×10^4 cells per well in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) without antibiotics in a 24-well plate format, and transfected at 24 h using Fugene reagent and 250 ng of total DNA/well. Cells were cultured at 37°C for 48 h, after which the media was changed with fresh DMEM containing 10% FCS buffered with 20 μmol/l HEPES (to maintain pH after the addition of NAC) and then treated with or without NAC or β-mercaptoethanol (BME 2 mmol/l) for 90 min. *Gaussia princeps* luciferase activity was measured using the Promega kit (E2810) according to the manufacturer's instructions. Luciferase activity was measured on a FLUOstar Omega plate reader.

Xenograft model

1×10^6 luciferase-expressing DND-41 cells were injected into the tail vein of 7-week-old female NOD-SCID-IL2Rcg^{null} (NSG) mice (The Jackson Laboratory, Bar Harbor, ME, USA). Tumour burden was assessed by bioluminescence imaging (BLI) using an IVIS Spectrum system (Caliper Life Sciences, Santa Clara, CA, USA), every 3–5 d until leukaemia was established, generally 2–3 weeks after intravenous injection, at which time NAC therapy was initiated. For each BLI time point, cancer bioluminescence was visualized by intraperitoneal (IP) injection of d-luciferin (Promega) in PBS at 75 mg/kg. In the pilot study, NAC-treated mice received the drug (Sigma-Aldrich) both in drinking water (10 mg/ml, supplemented with 45 mg/ml dextrose for palatability) and by IP injection 1 g/kg bid (twice daily) for 7 d, because continuous intravenous (IV) administration is not feasible in mice and osmotic administration using implanted pumps is precluded by the need to achieve micromolar concentrations.

In the follow-up experiment, mice either received NAC by IP injection (1 g/kg) bid, or IP injections of PBS. For IP injections, NAC was made up with water and brought to pH = 5 with NaOH and filter-sterilized. Mice treated with this approach for 19 d displayed no detectable toxicity or weight loss upon daily monitoring. Femurs were fixed in 10% formalin, sectioned, paraffin-embedded and stained with anti-human-CD45 antibody (Clone 2B11 + PD7/26, diluted 1 in 50; Dako/Agilent, Santa Clara, CA, USA). Stained slides were photographed using an Olympus BX41 microscope and Q-colour5 digital camera (Olympus, Center Valley, PA, USA) and staining scores were calculated using APERIO software (Leica, Newcastle, UK).

Statistical analyses

Dose-response curves were fitted using least squares (ordinary) fit as log (inhibitor) versus response using GRAPHPAD PRISM software (GraphPad Software, La Jolla, CA, USA). Differential sensitivities were calculated by non-linear regression. Two-tailed Student's *t*-test was used to calculate statistical differences between continuous variables, with $P < 0{\cdot}05$ considered as statistically significant.

Results

To identify a cell model of mutant IL7R signalling, we first sequenced exon 6 of *IL7R* in 21 T-ALL cell lines. We identified one somatic mutation; a 12 bp insertion in DND-41 cells resulting in a 4-amino-acid insertion (p.L242_L243insLSRC), as described previously (Table I) (Porcu *et al*, 2012). Modelling the insertion on the crystal structure of IL7R using the PYMOL software (Schrodiner, New York, NY, USA) predicted that it would reside just extracellular to the transmembrane domain and form homodimers through disulfide bond formation with the unpaired cysteine from the other mutant IL7R molecules (Fig 1A) (McElroy *et al*, 2009, 2012). Of note, we had previously detected particularly high levels of phospho-STAT5 (pSTAT5) in this cell line by Western blotting (see fig S3 in Sanda *et al*, 2013]) consistent with its activation by mutant IL7R signalling.

We next tested the effects of NAC on the viability of cells from a panel of ten T-ALL cell lines over a broad dose range. Cell lines could be broadly categorized as resistant [MOLT-16, RPMI-8402; 50% inhibitory concentration (IC50) >300 μmol/l], moderately sensitive (for example, KOPT-K1 and SUP-T13; IC50 257 μmol/l and 141 μmol/l respectively) and sensitive (for example, DND-41 and CCRF-CEM; IC50 74 μmol/l and 62 μmol/l respectively; Fig 1B and Table I). IL7R-mutant DND-41 cells were among the most sensitive of the cell lines tested. We hypothesized that the efficacy of NAC in DND-41 cells was mediated, at least in part, through disruption of mutant IL7R homodimers, while the sensitivity identified in cell lines such as CCRF-CEM was probably mediated through disruption of disulfide bonds in other cell surface receptors required for cell survival. The reduction in cell viability in DND-41 cells occurred through apoptosis as determined by Annexin V staining (Fig 1C).

Given that we were able to find only a single T-ALL cell line harbouring an IL7R mutation, we recapitulated oncogenic IL7R signalling in Ba/F3 cells using two different IL7R mutations previously described in patients: IL7R-ins243PPCL and IL7R-V253G (Shochat *et al*, 2011). The IL7R-ins243PPCL mutation is similar to the DND-41 IL7R-243insLSRC mutation in both its insertion position and size, and is predicted to lead to receptor homodimerization through disulfide bond

Table I. Sanger sequencing results for *IL7R* exon 6 from 21 T-ALL cell lines together with STAT5 phosphorylation status.

T-ALL cell line	*IL7R* exon 6	Phospho-STAT5 status* (Y964)	IC_{50} to NAC (μmol/l)
ALL-SIL	WT	+	
Be13	WT		91·2
CCRF-CEM	WT	−	61·7
DND-41	L242_L243 ins LSRC	++	74·1
HPB-ALL	WT	−	>300
H-SB2	WT	+	
JURKAT	WT	−	177·8
K3P	WT		
KOPT-K1	WT	++	257·0
LOUCY	WT	−	
MOLT-3	WT		
MOLT-4	WT	−	
MOLT-16	WT	−	>300
P12-ICHIKAWA	WT	−	
PEER	WT	+	
PF382	WT	−	
RPMI-8402	WT	−	>300
SKW-3/KE-37	WT	−	
SUP-T1	WT		
SUP-T11	WT	−	
SUP-T13	WT	+	141·3
T-ALL1	WT		162·2

−, no pYSTAT5 detectable; +, detectable pYSTAT5; ++, strong pYSTAT5; IC_{50}, 50% inhibitory concentration; WT, wild-type.
*As determined by Western blot. Data from (Sanda *et al*, 2013).

formation from the novel cysteine. By contrast, the IL7R-V253G resides deep within the membrane, and mediates constitutive STAT5 activation through an alternative allosteric mechanism that does not involve the formation of disulfide bonds (Shochat *et al*, 2014). Both mutations transformed Ba/F3 cells to IL3-independence. In support of our hypothesis, Ba/F3 cells transformed by IL7R-ins243PPCL were significantly more sensitive to NAC than Ba/F3 cells transformed by IL7R-V253G or BCR-ABL1 ($P < 0{\cdot}001$, Fig 1D).

Western blotting of endogenous IL7R in DND-41 cells under non-reducing conditions demonstrated disruption of the IL7R homodimer at the same doses as those required to induce apoptosis *in vitro*, with associated loss of STAT5 phosphorylation (Fig 2A). We also tested IL7R homodimerization quantitatively in a luciferase complementation assay in HEK-293T cells using constructs encoding the N- or C-terminus of *Gaussia princeps* luciferase fused to the intracellular domain of IL7R, such that functional luciferase activity occurs on IL7R protein-protein interaction. When expressed individually, the N- or C-terminal luciferase constructs alone were unable to generate a luciferase signal (Fig 2B). However, a robust luciferase signal was obtained when the N- and C-terminal IL7R constructs were expressed concurrently, which was significantly stronger ($P < 0{\cdot}001$) with IL7R-PPCL than the wild-type (WT) IL7R constructs (notably, WT-IL7R has previously been shown to homodimerize in an inactive configuration through an N-terminal hydrogen bond) (McElroy *et al*, 2012). NAC had no effect on WT IL7R constructs but, consistent with what we had observed through Western blotting, treatment with NAC significantly inhibited homodimerization of IL7R-PPCL ($P < 0{\cdot}001$, Fig 2B). In this assay, NAC had the same effect as β-mercaptoethanol (BME), a reducing agent commonly used *in vitro*, which has been previously shown to inhibit mutant IL7R signalling *in vitro* (Zenatti *et al*, 2011). However, given the reported toxicity of BME *in vivo* compared to the excellent tolerability of NAC, we chose not to pursue BME as a therapeutic agent for T-ALL (White *et al*, 1973).

To determine whether loss of viability in DND-41 cells from NAC treatment was mediated predominantly through inhibition of STAT5 signalling, we retrovirally transduced DND-41 cells with a constitutively active STAT5 construct (termed aStat5b) (Onishi *et al*, 1998), such that signalling downstream of STAT5 was no longer dependent on mutant IL7R homodimerization. Consistent with our proposed mechanism, DND-41-aStat5 cells were significantly more resistant to NAC treatment than control transduced DND-41 cells ($P < 0{\cdot}001$, Fig 2C). Similarly, when IL7R-PPCL transformed Ba/F3 cells were engineered to co-express constitutively active STAT5 (aStat5a) (Moriggl *et al*, 2005), they demonstrated increasing resistance to NAC therapy ($P < 0{\cdot}05$, Fig 2D), suggesting the effects of NAC on cell viability were mediated at least in part through STAT5 signalling. The lack of a complete rescue may be attributable to the fact that the IL7R pathway also activates PI3K-AKT signalling in a STAT5 independent fashion (Dadi & Roifman, 1993; Sharfe *et al*, 1995; Barata *et al*, 2004; Silva *et al*, 2011).

Given that NAC concentrations of 150–300 μmol/l are required to kill DND-41 cells *in vitro*, we examined published pharmacokinetic studies to determine if therapeutic levels of NAC are potentially achievable in human subjects. A single oral dose of 600 mg of NAC results in plasma levels of approximately 30–60 μmol/l (De Caro *et al*, 1989; Holdiness, 1991; Chen *et al*, 2007), with pharmacokinetics influenced by poor bioavailability, extensive first-pass metabolism and short half-life (Table II) (Borgstrom *et al*, 1986; Hong *et al*, 2005). Consequently, a widely adopted approach of treating acetaminophen overdose is continuous IV infusion of NAC (Smilkstein *et al*, 1991), which produces steady-state NAC plasma levels up to 930 μmol/l (Table II), well within the therapeutic range required to induce apoptosis of DND-41 cells (Borgstrom *et al*, 1986; Brown *et al*, 2004; Chen *et al*, 2007). This suggests that the therapeutic concentrations of NAC required to treat IL7R-mutant leukaemias are readily achievable in humans.

We then tested the efficacy of NAC *in vivo* using a murine xenograft model. NOD-SCID-IL2Rcg^{null} (NSG) mice were injected with 1×10^6 luciferase-expressing *IL7R*-mutant DND-41 cells and were treated with NAC once tumour

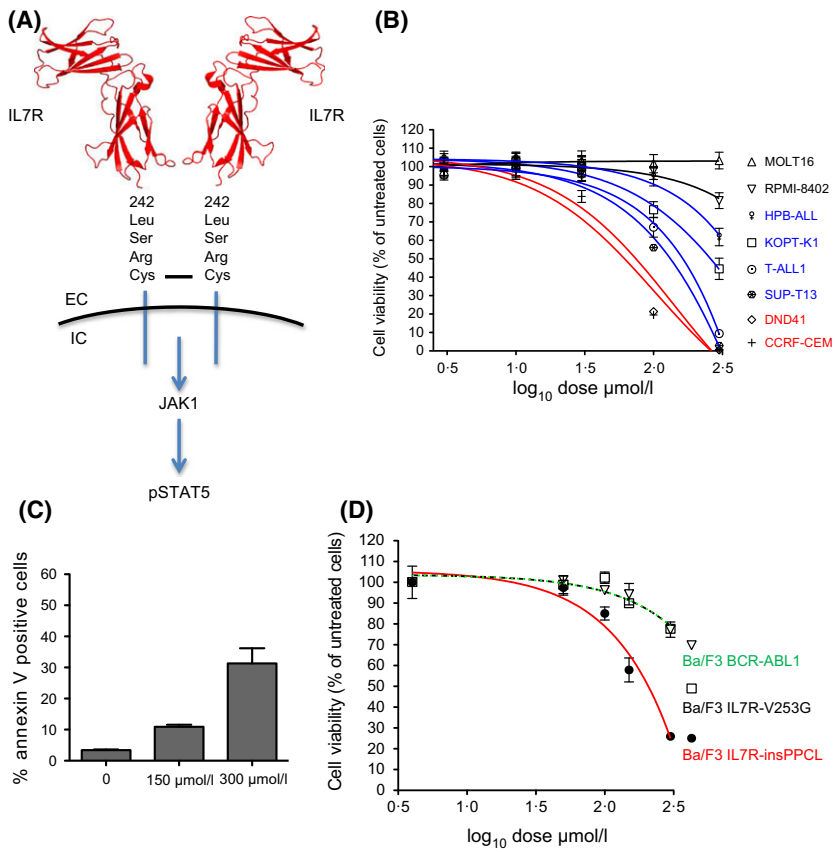

Fig 1. NAC induces apoptosis in *IL7R* mutant DND-41 T-ALL cells and Ba/F3 cells transformed by a cysteine-containing *IL7R* mutant allele. (A) Structural model of a mutant IL7R homodimer and schematic of downstream signalling events. The disulfide bond between unpaired cysteines of the LSRC insertion of *IL7R*-mutant DND-41 cells is shown as a black bar. Images were created with PYMOL software based on the published crystal structure (Protein Databank ID 3DI2) (McElroy *et al*, 2009, 2012). EC, extracellular; IC, intracellular. (B) Viability of T-ALL cell lines determined by the Cell Titer Glo assay after 48 h of treatment with NAC. (C) Apoptosis measured as the percentage of Annexin V-FITC positive DND-41 cells 24 h after treatment with NAC, as determined by flow cytometry. (D) Ba/F3 cells were transformed to IL3 independence with retroviral constructs encoding BCR-ABL, IL7R-insPPCL or IL7R-V253G. Cell viability was tested using the Cell Titer Glo assay after 48 h of NAC treatment.

engraftment was established by BLI. In an attempt to achieve therapeutic levels of NAC, and limited by the difficulty in administering continuous IV infusions to NSG mice due to their small size, we opted to administer NAC both in drinking water (10 mg/ml) and by IP injection (1 g/kg bid) in our initial pilot study ($n = 4$ per group), starting after leukaemia was established, generally 2–3 weeks after intravenous injection of leukaemic cells. After 7 d of treatment, we observed striking responses in two of four mice, with few if any detectable leukaemic cells by BLI, as compared to widespread disease in all four control treated animals (Fig 3A). However, one of the mice died of dehydration because of refusal to drink the NAC-containing drinking water, which has the foul odor of rotten eggs.

To avoid the difficulty with oral administration, we gave mice free access to drinking water and administered NAC by IP injection 1 g/kg twice per day (Fig 3B). Despite the suboptimal pharmacokinetics of IP NAC (*in vivo* half-life in mice, 9–11 min) (Neuwelt *et al*, 2004), NAC treatment nonetheless significantly delayed tumour progression in this model (Fig 3C,D): mean bioluminescence on day 19 was $13{\cdot}9 \times 10^9$ for control versus $7{\cdot}75 \times 10^9$/photons (ph)/s/cm^2/steradian (sr) for NAC-treated animals ($P < 0{\cdot}001$; $n = 8$ in each group). We confirmed these findings by analysing bone marrow biopsies of mice for the presence of human leukaemia cells, as measured by immunohistochemistry against human CD45 (Fig 3E). We consistently found a marked reduction of human T-ALL burden in mice treated with NAC as compared to control animals (Fig 3F, $P < 0{\cdot}005$).

Discussion

Peterson and Rumack (1997) initially reported the clinical use of NAC to reverse the severe hepatic toxicity associated with acetaminophen overdose. Since then, this antidote has saved countless lives and to this day remains the mainstay of effective treatment for this indication (Ferner *et al*, 2011). Given that acetaminophen overdose is the commonest form of poisoning worldwide, with over 50 000 cases per annum in the USA alone, the experience of using NAC in the clinic is extensive (Nourjah *et al*, 2006). Despite the remarkably high steady-state plasma concentrations achievable on standard treatment protocols, it has an exceptionally favourable tolerability profile.

As predicted based on the known reducing properties of NAC, it was able to disrupt cysteine-bond-mediated homodimers of mutant IL7R, resulting in reduced pSTAT5 expression and the induction of apoptosis in IL7R mutant DND-41 human T-ALL cells. Unfortunately, we found only a single human T-ALL cell line that harbours an IL7R mutation. For this reason, we also analysed Ba/F3 cells immortalized with a cysteine-mutated activated IL7R, and these cells also responded to treatment with NAC. We also observed a

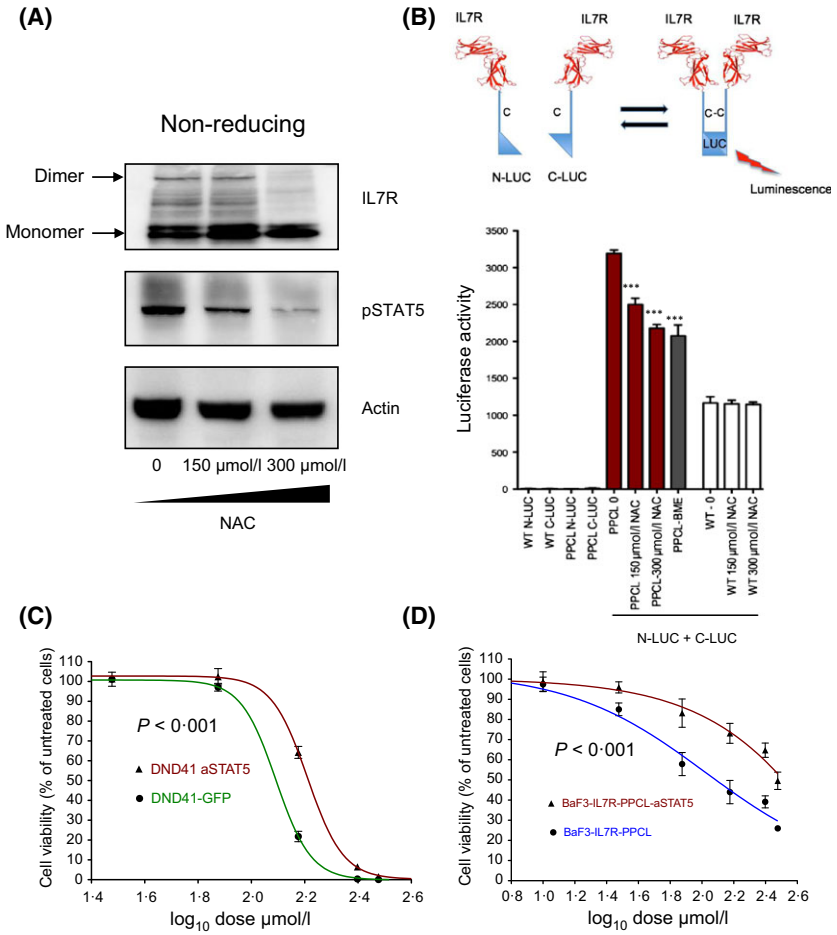

Fig 2. NAC disrupts mutant IL7R homodimerization, inhibits STAT5 signalling and its apoptotic effects can be partially rescued by activated STAT5. (A) Western blotting under non-reducing conditions of lysates extracted from DND-41 cells 24 h after treatment with NAC or control medium. (B) A luciferase complementation assay to quantify IL7R homodimerization. The assay is based on the principle that expression of two separate inactive fragments of luciferase will produce a functionally active luciferase protein only after significant protein–protein interaction (Cassonnet *et al*, 2011). Either the N- or C-terminal fragments of luciferase (LUC) were fused to the intracellular domain of wild-type and mutant IL7R (ins PPCL) constructs and expressed in HEK-293T cells for 48 h, treated with or without NAC or β-mercaptoethanol (BME 2 mmol/l) for 90 min, before measurement of *Gaussia princeps* luciferase activity. Of note, homodimerization of wild-type IL7R has been previously described by crystallography, where the IL7R homodimers are held together by an intermolecular hydrogen bond at the N-terminus, maintaining the dimer in a non-active conformation (McElroy *et al*, 2012). (C) Expression of a constitutively active mutant of Stat5b partially rescues DND-41 cells from apoptosis induced by NAC. DND-41 cells were transduced with either a control pMX-IRES-GFP or pMX-Stat5B*-IRES-GFP retroviral vector encoding a constitutively active Stat5b construct, selected by fluorescence-activated cell sorting (FACS) and then treated with NAC for 48 h before cell viability was measured by the Cell Titer Glo assay. The *P* value was calculated using non-linear regression. (D) Ba/F3 cells transformed by IL7R-insPPCL were infected with retroviral vectors expressing a constitutively active murine Stat5a mutant (S710F) or control pMSCV-IRES-eGFP. Cells were sorted by FACS for high GFP expression, then treated with NAC for 48 h before cell viability was measured by the Cell Titer Glo assay. The *P* value was calculated using non-linear regression.

partial rescue in cell viability when Ba/F3 cells were transduced with an activated STAT5 construct, suggesting that a significant proportion of the effect of NAC was mediated through loss of IL7R-mediated STAT5 activation.

NAC also showed effects on cell viability in IL7R-WT T-ALL cell lines, such as SUP-T13, when the cells were treated at high concentrations. We are not sure of the mechanism of activity, although one could surmize that intramolecular cysteine bonds in key cellular proteins might also be required for T-ALL cell viability. For example, many cell surface receptors contain reactive cysteines involved in the formation of intra- and inter-molecular disulfide bond formation (Metcalfe *et al*, 2011), the reduction of which are likely to have consequences on receptor configuration and affect 'outside-in' signalling; increased dependency on such signals in leukaemic cells compared to normal cells may offer the therapeutic window predicted from our data. Further studies will be required to investigate the cytotoxic effects of NAC in T-ALLs lacking IL7R mutations. The fact that patients can tolerate continuous NAC infusions for over 24 h, where steady state plasma levels are >300 µmol/l, without undergoing catastrophic organ failure, suggests that this leukaemia-

Table II. Summary of reported pharmacokinetic studies assessing steady state *N*-acetylcysteine concentrations in humans.

Reference	Patient group	Route of administration	Steady state plasma concentration (μmol/l)
Ahola *et al* (1999)	Pre-term newborns	IV	510
Borgstrom *et al* (1986)	Pediatric	IV	400
	Adult	IV	200
Brown *et al* (2004)	Adult	IV	930
Jones *et al* (1997)	Adult	IV	210
Olsson *et al* (1988)	Adult	IV	37
Prescott *et al* (1989)	Adult	IV	230
De Caro *et al* (1989)	Adult	PO (single 600 mg dose)	33
Holdiness (1991)	Adult	PO	65

IV, intravenous; PO, oral.Adapted from Chen *et al* (2007), with permission.

specific cytotoxicity may prove clinically useful in a subset of T-ALL cases that lack IL7R mutations. Other mutations leading to aberrant disulfide bond formation have been shown to activate different oncogenic receptors in several other cancers – F232C mutation of *CRLF2* in B-ALL (Hertzberg *et al*, 2010; Yoda *et al*, 2010), *RET* mutations in thyroid cancers (Asai *et al*, 1995; Santoro *et al*, 1995) and *HER2* R896C mutation in breast cancer (Bose *et al*, 2013) – also suggesting that NAC may have application beyond IL7R mutant T-ALL.

One limitation of our study was the inability to adequately dose NSG mice to levels that are comparable to those that are achievable in humans. The most striking responses occurred in mice that were treated with both oral and IP NAC in our pilot study, suggesting that efficacy was dose-dependent, but oral NAC was repulsive to mice and several mice became dehydrated. With an *in vivo* half-life of NAC in mice of approximately 10 min (Neuwelt *et al*, 2004), a continuous IV infusion schedule would have been optimal, but we were technically unable to deliver the drug by this route in such small animals. Nonetheless, we observed significant responses, particularly with respect to leukaemic infiltration of the bone marrow.

Given that optimal and safe dosing schedules to achieve high micromolar plasma concentrations are well-established in humans, our findings suggest that NAC use in IL7R-

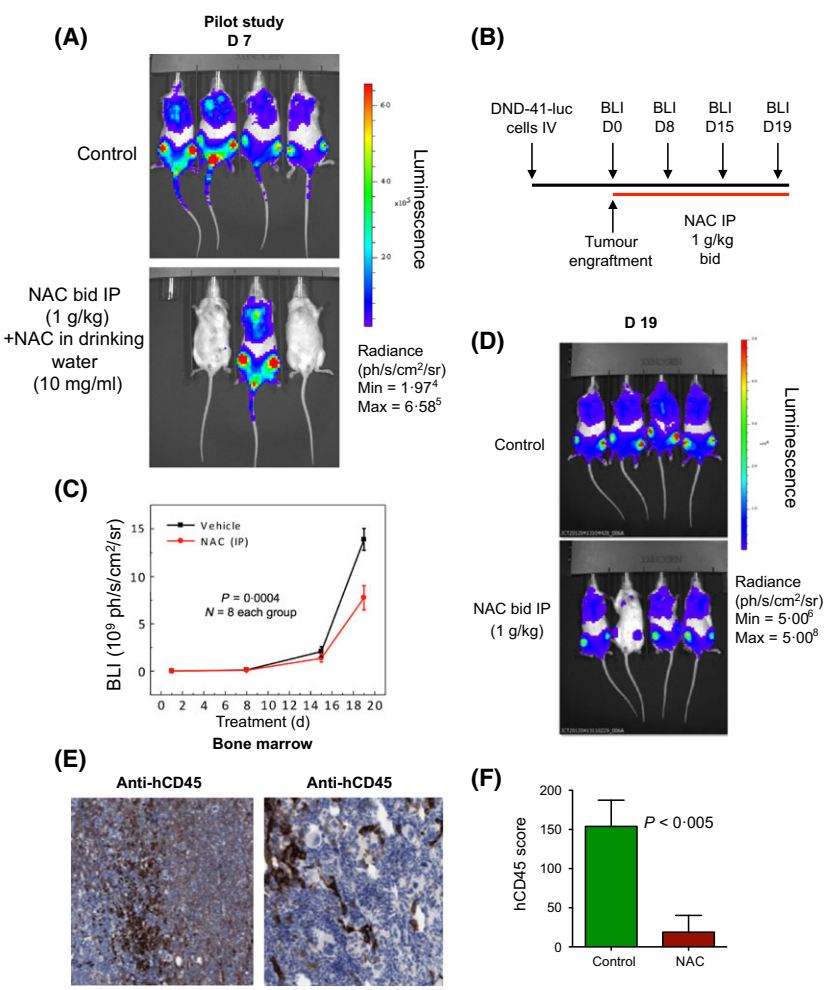

Fig 3. NAC inhibits the growth of DND-41 leukaemic cells *in vivo* in NSG mice. (A) Bioluminescent images of NSG mice in the pilot study when treated with a combination of oral (10 mg/ml in drinking water) and IP injected NAC (1 g/kg bid) compared to vehicle for 7 d (n = 4 per group). (B) Schematic showing the timeline for the follow-up *in vivo* study. 1×10^6 luciferase-expressing DND-41 cells were injected into the tail vein of 7-week-old female NOD-SCID-IL2Rcg^{null} mice. After determination of tumour engraftment by bioluminescent imaging (BLI), mice received treatment with either IP injected NAC 1 g/kg bid or vehicle (n = 8 per group). (C) *In vivo* monitoring of tumour burden of DND-41 cells as assessed by BLI. The P value at day 19 was calculated using two-tailed Student's t test. (D) Bioluminescent images of NSG mice after 19 d of treatment with IP NAC (1 g/kg bid) compared to vehicle. Four representative animals from each group are shown. (E) Immunohistochemistry of human CD45 from representative paraffin-embedded femur sections from NSG mice treated with either control or NAC, photographed at 40× magnification. (F) Score for human CD45 staining of femurs from NSG mice treated with either control or NAC, as calculated with Aperio software, based on the abundance of positively staining cells over a set area of magnification (n = 3 per group). The P value was calculated using two-tailed Student's t test.

mutant T-ALL could afford a safe and effective new therapeutic strategy warranting testing in clinical trials. Lastly, it is worth noting that 11 of the 12 US Food and Drug Administration (FDA)-approved cancer drugs in 2012 cost over $100 000 per annum (Experts in Chronic Myeloid Leukemia, 2013). In the UK, a 2-g ampoule of NAC for IV use costs £1·96 (equivalent to approximately $3·20), meaning that a 24-h- infusion on a typical NAC protocol for a 70 kg adult for acetaminophen overdose costs £21·56 (*c*. $34·50). Given the fact that NAC is well tolerated in clinical use by continuous infusion, we recommend that it be evaluated in clinical trials as an approach to targeted therapy for a subset of T-ALL patients, the economical implications of which may be particularly relevant to healthcare in the developing world.

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Authorship contributions

M.R.M. and A.K. designed, performed and analysed research and wrote the paper; C.R., A.E., J-C.T., S.D., A.Y., N.T., R.M., A.B., and J.T. performed experiments and/or provided crucial reagents; A.L.K., D.J.D., D.M.W., S.I. and S.E.S. designed and analysed research; S.J.R. performed and analysed research; and A.T.L. supervised research, analysed data and co-wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests.

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