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

Marc R. Mansour,1,2 Casie Reed,1 Amy R. Eisenberg,1,2 Jen-Chieh Tseng,4 Jean-Claude Twizere,5 Sarah Daakour,5 Akinori Yoda,6 Scott J. Rodig,7 Noa Tal,8 Chen Shochat,8,9 Alla Berezovskaya,1 Daniel J. DeAngelo,1 Stephen E. Sallan,1 David M. Weinstock,6 Shai Izraeli,8 Andrew L. Kung,10 Alex Kentsis1,3 and A. Thomas Look1,11

1Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA, 2Department of Haematology, UCL Cancer Institute, University College London, London, UK, 3Molecular Pharmacology and Chemistry Program, Sloan-Kettering Institute, Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, New York, NY, 4Laboratory of Protein Signalling and Interactions, Interdisciplinary Cluster for Applied Genoproteomics (GIGA-R), University of Liège, Sart-Tilman, Belgium, 5Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA, USA, 6Department of Pediatric Oncology, Dana-Farber Cancer Institute, 7Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA, USA, 8Cancer Research Centre, Sheba Medical Centre, Tel Hashomer and Tel Aviv University Medical School, Tel Aviv, 9Migal-Galilee Bio-Technology Centre and Tel Hai Academic College, Tel Hai, Israel, 10Department of Pediatrics, Columbia University, New York, NY, and 11Division of Hematology/Oncology, Children’s Hospital, Boston, MA, USA

Received 17 April 2014; accepted for publication 9 July 2014
Correspondence: A. Thomas Look, Department of Pediatric Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, M tyer 630, Boston, MA 02115, USA.
E-mail: thomas_look@dfci.harvard.edu
Or
Alex Kentsis, Molecular Pharmacology & Chemistry Program, Memorial Sloan Kettering Cancer Center, 1275 York Ave., New York, NY 10065, USA.
E-mail: kentsisresearchgroup@gmail.com

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.
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 leukemogenic 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-cSS^2-IRES-eGFP encoding a constitutively active murine STAT5A mutant (S710F=cSS) 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 cSS. 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’-GGGGAACACTTTGTACAAAGCAAATTTGGTGA GGACTGGCCATAGTACG, containing attB1 and attB2 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 × 10⁶ 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 × 10⁶ luciferase-expressing DND-41 cells were injected into the tail vein of 7-week-old female NOD-SCID-IL2Rcgnull (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 < 0.05 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_L243insPPCL or 12 bp insertion), 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 formation.
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 < 0.001, 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 *Gausia 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 < 0.001) 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 < 0.001, 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 < 0.001, Fig 2C). Similarly, when IL7R-PPCL transduced Ba/F3 cells were engineered to co-express constitutively active STAT5 (aStat5a) (Moriggl et al, 2005), they demonstrated increasing resistance to NAC therapy (P < 0.05, 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 steadystate 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-IL2R<sup>γc</sup><sup>null</sup> (NSG) mice were injected with 1 × 10<sup>6</sup> luciferase-expressing IL7R-mutant DND-41 cells and were treated with NAC once tumour

---

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

<table>
<thead>
<tr>
<th>T-ALL cell line</th>
<th>IL7R exon 6</th>
<th>Phospho-STAT5 status* (Y964)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; to NAC (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL-SIL</td>
<td>WT</td>
<td>+</td>
<td>91-2</td>
</tr>
<tr>
<td>Be13</td>
<td>WT</td>
<td>−</td>
<td>61-7</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>WT</td>
<td>++</td>
<td>74-1</td>
</tr>
<tr>
<td>DND-41</td>
<td>L242_L243</td>
<td>ins LSRC</td>
<td>&gt;300</td>
</tr>
<tr>
<td>HPB-ALL</td>
<td>WT</td>
<td>−</td>
<td>&gt;300</td>
</tr>
<tr>
<td>H-SB2</td>
<td>WT</td>
<td>+</td>
<td>257-8</td>
</tr>
<tr>
<td>JURKAT</td>
<td>WT</td>
<td>−</td>
<td>177-8</td>
</tr>
<tr>
<td>K3P</td>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KOPT-K1</td>
<td>WT</td>
<td>++</td>
<td>230-2</td>
</tr>
<tr>
<td>LOUCY</td>
<td>WT</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>MOLT-3</td>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOLT-4</td>
<td>WT</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>MOLT-16</td>
<td>WT</td>
<td>&gt;300</td>
<td></td>
</tr>
<tr>
<td>P12-ICHIKAWA</td>
<td>WT</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>PEER</td>
<td>WT</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PF382</td>
<td>WT</td>
<td>−</td>
<td>&gt;300</td>
</tr>
<tr>
<td>RPMI-8402</td>
<td>WT</td>
<td>−</td>
<td>&gt;300</td>
</tr>
<tr>
<td>SKW-3/KE-37</td>
<td>WT</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>SUP-T1</td>
<td>WT</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>SUP-T11</td>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUP-T13</td>
<td>WT</td>
<td>+</td>
<td>141-3</td>
</tr>
<tr>
<td>T-ALL</td>
<td>WT</td>
<td></td>
<td>162-2</td>
</tr>
</tbody>
</table>

*, no pYSTAT5 detectable; +, detectable pYSTAT5; ++, strong pYSTAT5; IC<sub>50</sub> 50% inhibitory concentration; WT, wild-type.

*As determined by Western blot. Data from (Sanda et al, 2013).
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 LSR_C 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-ABL1, 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 \(13.9 \times 10^9\) photons (ph)/cm\(^2\)/sr for control versus \(7.75 \times 10^7\) ph/s/cm\(^2\)/sr for NAC-treated animals (\(P < 0.001\); 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, \(P < 0.005\)).

**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
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 surmise 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-

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-IRE5-GFP or pMX-Stat5b*-IRE5-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-IRE5-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.
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- Table II. Summary of reported pharmacokinetic studies assessing steady state N-acetylcysteine concentrations in humans.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Patient group</th>
<th>Route of administration</th>
<th>Steady state plasma concentration (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borgstrom et al (1986)</td>
<td>Pediatric</td>
<td>IV</td>
<td>400</td>
</tr>
<tr>
<td>Jones et al (1997)</td>
<td>Adult</td>
<td>IV</td>
<td>210</td>
</tr>
<tr>
<td>De Caro et al (1989)</td>
<td>Adult</td>
<td>PO (single 600 mg dose)</td>
<td>33</td>
</tr>
</tbody>
</table>

Holdiness (1991) Adult PO 65

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

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 x 10⁶ luciferase-expressing DND-41 cells were injected into the tail vein of 7-week-old female NOD-SCID-IL2Rcg-/-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 40x 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.
References


