Imatinib and Nilotinib inhibit Bcr–Abl-induced ROS through targeted degradation of the NADPH oxidase subunit p22phox

William D. Landry, John F. Woolley, Thomas G. Cotter*  
Tumour Biology Laboratory, Biochemistry Department, Bioscience Research Institute, University College Cork, College Road, Cork, Ireland

ABSTRACT

Constitutive expression of the Bcr–Abl kinase in Chronic Myelogenous Leukaemia (CML) is known to produce elevated levels of Reactive Oxygen Species (ROS) which can enhance cell survival as well as generate genomic instability. Our laboratory has previously demonstrated that NADPH oxidase (Nox) activity contributes to intracellular-ROS levels in Bcr–Abl-positive cells, while inducing increased pro-survival signalling through the PI3K/Akt pathway. How Bcr–Abl signalling regulates Nox activity still remains to be elucidated. In this study, using the K562 CML cell line we showed that inhibition of Bcr–Abl signalling, by either Imatinib or Nilotinib, led to a significant reduction in ROS levels which was concurrent with the GSK-3β dependent, post-translational down-regulation of the small membrane-bound protein p22phox, an essential component of the Nox complex. Furthermore, siRNA knockdown of p22phox in these cells established its importance in ROS production and proliferation. Taken together we believe our results provide a possible link between Bcr–Abl signalling and ROS production through Nox activity and demonstrate a novel mechanism of action associated with Imatinib and Nilotinib treatment in CML.

1. Introduction

Chronic Myelogenous Leukaemia (CML) is a myeloproliferative disorder characterised by increased proliferation of haematopoietic stem cells in the bone marrow. CML results as a consequence of a reciprocal translocation between chromosomes 9 and 22, producing what is known as the Philadelphia chromosome (Ph1) [1]. This translocation generates the chimeric kinase Bcr–Abl [2], which activates downstream signalling pathways, such as the JAK/STAT, Raf/MEK/ERK and PI3K/Akt pathways, in turn promoting survival and proliferation [3]. It has been shown that cells constitutively expressing Bcr–Abl produce higher levels of Reactive Oxygen Species (ROS) when compared to untransformed cells [4]. This study demonstrated mitochondrial electron transport chain leakage to be one possible source of ROS in Bcr–Abl-positive cells. Work by our laboratory has since demonstrated that NADPH oxidase (Nox) activity, particularly Nox4, is also responsible for producing a significant level of ROS upon Bcr–Abl induction [5]. Furthermore this study and others have shown that Bcr–Abl-induced ROS regulates the PI3K/Akt pathway thereby enhancing survival [5,6]. An additional clinically significant role for Bcr–Abl-induced ROS noted in CML is its ability to contribute to genomic instability [7–9], which along with increased survival and proliferation further contributes to the progression of this myeloproliferative disorder.

In eukaryotic cells ROS are produced by a variety of sources. However, in contrast to the majority of these sources where ROS are produced as by-products, the Nox family of transmembrane proteins primary function is to generate ROS. There are seven members of the Nox family, Nox1, Nox2, Nox3, Nox4, Nox5, DUOX1 and DUOX2, with their activity being involved in various cellular events including survival, growth, differentiation, apoptosis and immune responses [10,11]. Naughton et al. [5] demonstrated that Nox activity was responsible for the increase in ROS production following Bcr–Abl induction, however it is unclear how Bcr–Abl signalling affects Nox activity. In this study we investigated elevated levels of intracellular-ROS associated with Bcr–Abl signalling in the human leukaemic cell line K562. We demonstrate that a significant proportion of ROS in these cells are Nox-derived. Inhibition of Bcr–Abl signalling by either Imatinib or Nilotinib, leads to a significant reduction in ROS levels which is concurrent with the post-translational down-regulation of the small membrane-bound protein p22phox, an essential component of the Nox complex [12,13]. This down-regulation is dependent on GSK-3β activity, which is inhibited downstream of the PI3K/Akt and Raf/MEK/ERK1/2 pathways. Thus, we propose that increased ROS signalling via Bcr–Abl in K562 cells is in part Nox-derived and that inhibition of Bcr–Abl signalling leads to GSK-3β activation which drives down ROS through regulation of p22phox. We believe these
results provide a link between Bcr–Abl signalling and ROS production through Nox activity and demonstrate a novel therapeutic mechanism for both Imatinib andNilotinib.

2. Materials and methods

2.1. Cell lines, culture conditions and treatment

The human leukaemic cell line K562 (purchased from DSMZ) were maintained in RPMI-1640 medium supplemented with 10% foetal calf serum, 2 mM L-glutamine and 1% penicillin/streptomycin in a humidified incubator at 37 °C with 5% CO₂. Cell counts were obtained using a haemocytometer (trypan blue exclusion) under a light microscope, 0, 24, 48 and 72 h following treatments. Inhibition of Bcr–Abl signalling was achieved using Imatinib Mesylate (Imatinib; 1.0 µM) or Nilotinib (0.1 µM) for up to 16 h (both from Selleck Chemicals [Strategic Scientific]). PKC412 (from Tocris) was used at 1.0 µM for up to 16 h. Nox inhibition was via flavoprotein inhibitor diphenyleneiodonium chloride (10 µM DPI; Sigma) or 3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo[4,5-b]pyrimidine (10 µM VAS2870; Enzo Life Sciences) for up to 1 h. Inhibition of the 205 proteins was via lactacystin (5 µM Lact; Sigma) for up to 16 h. GSK-3β inhibition was via SB216763 (5 µM SB; Tocris) for 16 h. P38 kinase and MEK inhibition was achieved via LY294002 (10 µM LY) and U0126 (10 µM U0), respectively, for up to 16 h (both from Cell Signaling Technology). All reagents were from Sigma-Aldrich unless otherwise stated.

2.2. Measurement of intracellular ROS by flow cytometry

Following treatments, ROS levels were determined using the cell-permeable fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (H₂DCF-DA; Invitrogen, Bio Sciences Ltd.) as previously described [5]. Briefly, 50 µM H₂DCF-DA was added to cells in suspension for 15 min, and incubated at 37 °C in the dark. 10,000 cells were then analysed in the FL-1 channel on a FACSCalibur (BD Biosciences, Europe) using CellQuest Pro software. H₂O₂ and O²⁻ production was calculated by the increase in mean fluorescence.

2.3. Antibodies

Primary antibodies used for immunoblotting or immunoprecipitation were Akt (#9272), phospho-Akt (ser473; #9275), ERK (#4692), phospho-ERK (Thr202/Tyr204; #9275), GSK-3β (#9315), phospho-GSK-3β (Ser9; #93365), phospho-CrkL (Tyr207; #3181), p47phox (#4312; all from Cell Signaling Technology), p67phox (#SC15342), DUOX1 (#548853), p22phox (#SC2078; all from Santa Cruz Biotechnology), DUOX2 (#AB5831; Abcam), GAPDH (#RCM2-500; Advanced Immunochecrs), β-Actin (#A5441), Nox2 (#HPA019362 all from Sigma), ubiquitin (#MB1510), Nox1 (#50; all from Millipore), Nox4 was a kind gift from Dr JD Lambeth (Emory University School of Medicine, Atlanta, GA, USA). All secondary antibodies for western blotting were peroxidase conjugated (Dako).

2.4. Small interfering RNA (siRNA)

RNA interference mediated by duplexes of 21-nucleotide RNA was performed in K562 cells. Different Ambion Silencer Select predesigned siRNA (Applied Biosystems) were used for silencing. For p22phox, two different siRNA were used siRNA ID #3786 (siRNA1) and ID #140372 (siRNA2). For the negative control, the siRNA used were Silencer Select Negative Control #1 siRNA (Negative; Ambion). The transfection of siRNA used the Amazax Nucleofactor technology with the Amazax cell optimisation kit V (Amazax) and followed the Amazax guidelines using programme X-001.

2.5. Live cell imaging

Cells were electroporated with either negative siRNA or p22phox siRNA (as described) and plated in poly-L-lysine coated glass bottomed dishes (15 mm Petri dishes with 14 mm microwells; MatTek Corporation) for 24 h. Cells were incubated in 500 µM H₂DCF-DA for 1 h at 37 °C in the dark. Following this incubation cells were rinsed and imaged live in growth medium using the Multiphoton Laser scanning microscope Flouview1000 MPE (Mason Technology) as previously described [14]. Images are represented as a single slice from a Z stack projection. During acquisitions, saturation levels were kept constant for H₂DCF-DA to allow direct comparison of ROS levels between negative siRNA treated cells and p22phox siRNA treated cells.

2.6. Immunoblotting

Immunoblotting was carried out under conditions previously described by us [5]. Densitometric analysis was carried out on immunoblots using ImageJ software (http://rsb.info.nih.gov/ij/index.html) and data are presented in bar charts, calculated as the ratio of the intensity of target bands quantified by densitometry factored by the densitometric measurements of loading control bands.

2.7. Immunoprecipitation

Immunoprecipitation (IP) was carried out using the p22phox antibody (as above) and the Pierce Crosslink IP kit (#26147; Thermo Scientific). Manufacturer’s protocol was followed. Immunoprecipitate was resolved using SDS-polyacrylamide gel electrophoresis, as described above.

2.8. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed on oligo-dt generated cDNA using the MJ Research Opticon 2 detection system in combination with the Quantitect SYBR Green PCR Master Mix (Qiagen). The primers for p22phox and β-Actin were purchased as Quantitect Primer Assays (Qiagen). PCR parameters and data analysis was as described previously [14].

2.9. Statistical analysis

In all cases data are expressed as percentage of control, where the control was defined as 100% ±1. Values are the mean ± standard deviation (SD) and are representative of three independent experiments. Statistical significance was evaluated by Student’s t-test for comparisons between groups. P-values of <0.05 were considered significant.

3. Results

3.1. Inhibition of Bcr–Abl signalling reduces Nox-derived intracellular ROS

The human leukaemic cell line K562 is a Ph1-positive cell line which expresses the p210 isoform of Bcr–Abl. This cell line was originally isolated in 1975 [15] and is a well established model used in the study of Bcr–Abl signalling. The main aim of this work was to elucidate the mechanism by which Bcr–Abl signalling induces Nox activity and ROS production. Initial experiments were carried out to demonstrate that an inhibition of Bcr–Abl signalling resulted in decreased ROS production as well as to demonstrate that a significant proportion of endogenous ROS produced by K562 are Nox-derived. Treatment of K562 cells with the tyrosine kinase inhibitor (TKI) Imatinib resulted in decreased endogenous ROS of 51%, which was measured using the ROS-sensitive probe H₂DCF-DA (Fig. 1a). This result corresponded with previous studies [4]. To ensure this reduction was due to specific inhibition of Bcr–Abl signalling, cells were treated with Nilotinib, another small molecule TKI of Bcr–Abl and a derivative of Imatinib with a lower IC₅₀. Similarly, this treatment gave an average reduction in ROS of 61% (Fig. 1a). The FLT3 inhibitor, PKC412, was used as a control and demonstrated no reduction in ROS levels (Fig. 1a). PKC412 was used as a control TKI as it does not affect Bcr–Abl signalling, but is known to inhibit similar non-specific tyrosine kinases as Imatinib [16,17]. To confirm that Bcr–Abl activity was indeed inhibited after TKI treatments, the phosphorylation status of Crkl was examined (Fig. 1b). A reduction in p-CrkL was noted after treatments, which taken together with the previous results confirmed that the reductions in ROS levels were due to the inhibition of Bcr–Abl signalling. We showed that treatment with DPI, a flavoprotein inhibitor commonly used as a Nox inhibitor, significantly reduced the levels of ROS in K562 cells (Fig. 1c). This result was confirmed with the Nox-specific inhibitor VAS2870, which was shown to also reduce ROS levels following treatment (Fig. 1c). Taken together these results suggest that Nox proteins are involved in the production of ROS downstream of Bcr–Abl signalling in K562 cells. It should be noted that treatments with Imatinib, Nilotinib, PKC412, VAS2870 and DPI at these concentrations and time-points were chosen as they showed maximal reduction in ROS levels with no significant effect on cell viability (data not shown).
2.1. Bcr–Abl inhibition results in the post-translational down-regulation of p22phox

Having established that DPI and VAS2870 treatments as well as Imatinib and Nilotinib treatments resulted in a significant reduction in ROS, we investigated whether the levels of any of the Nox proteins or regulators were altered. A significant reduction in p22phox protein levels was observed following 16h of Imatinib treatment (Fig. 2a). DPI had no effect on p22phox protein levels (Fig. 2a). Again to ensure this was a specific effect of the small-molecule inhibitor on Bcr–Abl signalling we treated the cells with Nilotinib and PKC412. Nilotinib treatment produced a comparable reduction in p22phox to Imatinib treatment (Fig. 2b). However, PKC412 treatment had no effect on p22phox protein levels (Fig. 2b). These results indicated that specific inhibition of Bcr–Abl signalling affects p22phox protein levels. Interestingly it was noted that the reduction in p22phox protein levels was proportional to the level of CrkL dephosphorylation after TKI treatment (data not shown). In order to elucidate how inhibition of Bcr–Abl signalling affects p22phox protein levels, we investigated if the reduction was mediated at a transcriptional level. Following treatment with Imatinib we observed through quantitative PCR that p22phox mRNA levels did not change significantly upon inhibition of Bcr–Abl suggesting p22phox was post-translationally regulated (Fig. 2c). To establish this, Bcr–Abl signalling was inhibited as before using Imatinib, which was then followed by the immunoprecipitation of p22phox protein and probing for ubiquitination. We demonstrated that p22phox ubiquitination increased following Imatinib treatment (Fig. 2d).

Furthermore, Imatinib treatment along with the presence of lactacystin, an inhibitor of the proteasome, causes an accumulation of ubiquitinated p22phox in the cell (Fig. 2d). This result indicated that p22phox is first ubiquitinated and then degraded by the proteasome. Taken together these data suggest that p22phox is regulated post-translationally following Bcr–Abl inhibition.

2.2. Bcr–Abl inhibition results in the post-translational down-regulation of p22phox

There are three major signalling pathways activated by Bcr–Abl, namely the JAK/STAT, PI3k/Akt and Raf/MEK/ERK1/2 pathways [3]. As the regulation of p22phox was post-translationally focused was placed on the PI3k/Akt and Raf/MEK/ERK1/2 pathways. To determine if either of these pathways had an involvement in p22phox regulation, K562 cells were treated with the PI3k inhibitor LY294002 and the MEK inhibitor U0126. Protein levels of p22phox were down-regulated following 16h inhibition of both of these pathways, with PI3k/Akt inhibition showing the greatest decrease (Fig. 3a). However, decreases in p22phox protein levels were minimal when compared to the decrease previously observed following Imatinib treatment (Fig. 3a). As inhibition of both pathways individually had a minor effect on p22phox levels the possibility was proposed that both pathways may collectively be involved in its regulation. In order to establish this hypothesis, both pathways were inhibited simultaneously. This resulted in a significant reduction in p22phox protein levels and demonstrated that the downstream signalling of both the PI3k/Akt and
Raf/MEK/ERK1/2 pathways was required to regulate p22phox levels (Fig. 3a). Given its frequently cited role in the regulation of proteasomal degradation and its noted presence downstream of both the PI3k/Akt and Raf/MEK/ERK1/2 pathways we investigated whether the Serine/Threonine Kinase GSK-3β had a role in p22phox degradation [18–20]. Utilising SB216763, a known GSK-3β inhibitor, down-regulation of p22phox following Imatinib treatment was completely reversed (Fig. 3b). Additionally the use of SB216763 inhibited the p22phox degradation noted following simultaneous inhibition of the PI3k/Akt and Raf/MEK/ERK1/2 pathways (Fig. 3b). These results indicated that the post-translational regulation of p22phox is assisted by the activation of GSK-3β following Bcr–Abl inhibition and the subsequent inactivation of Akt and Erk1/2.

3.4. Knockdown of p22phox by means of siRNA results in a marked reduction in intracellular ROS levels and a decreased rate of proliferation

Bcr–Abl inhibition by Imatinib or Nilotinib, led to a reduction in ROS in parallel with the post-translational down-regulation of p22phox (Figs. 1 and 2). Expression of p22phox is essential for the activity of Nox1, Nox2, Nox3 and Nox4 as it is integral in stabilising these proteins at the membrane which is a necessary process for ROS production [12,13]. Therefore, having established the mechanism by which p22phox is regulated we sought to determine if variations in p22phox protein levels affected ROS levels in K562 cells which may in turn account for the reductions seen upon Bcr–Abl inhibition. Selective knockdown of p22phox mediated by siRNA was carried out in K562 cells, these cells were then compared to cells transfected with negative control siRNA. Knockdown via siRNA resulted in an almost complete loss of p22phox protein in the cell for up to 72 h (Fig. 4a) and was accompanied by a significant decrease in endogenous ROS when compared to cells transfected with the negative control siRNA (Fig. 4b). This decrease in ROS was visualised in live cells using the H$_2$DCF-DA probe (Fig. 4c). From this result it is evident that p22phox expression contributes to ROS production in K562 cells. Furthermore, 72 h after transfection it was observed that cell number of p22phox knockdown cells remained lower than that of cells transfected with negative control siRNA (Fig. 4d). Interestingly at 72 h cell number of both untreated and negative control siRNA transfected cells were the same; however cells transfected with siRNA(i) and siRNA(ii) showed an average decrease of 34% and 17%, respectively, when compared to control cells (Fig. 4d). At each time-point, cells transfected with siRNA(ii) were demonstrated to have a higher level of p22phox expression when compared to siRNA(i) transfected cells (Fig. 4e). This may have accounted for the higher cell count recorded at 72 h in siRNA(ii) transfected cells and show that the proliferation rates of these cells are dependent on p22phox protein levels. This set of data demonstrates a possible role for p22phox in the proliferation of K562 cells.
4. Discussion

Several previous studies have shown that induction of Bcr–Abl and subsequent signalling events increase ROS production in cells [4–6]. Naughton et al. [5] demonstrated that Nox activity significantly contributed to intracellular-ROS levels in Bcr–Abl-positive cells, while inducing increased pro-survival signalling through the PI3K/Akt pathway. Nox-derived ROS have been demonstrated to be involved not only in survival but also the migration, proliferation and differentiation of leukaemia cells as well as other cell types [10,11,21–23]. One effect of ROS generation, which is of clinical significance in CML is the link between ROS production and genomic instability [7–9], which has recently been linked to Nox activity in oncogenic cells [24,25]. Furthermore, genomic instability in CML is known to be associated with disease progression and development of resistance to key drugs such as Imatinib [26]. Here, K562 cells, a CML cell line with constitutive Bcr–Abl expression, were used as a model to elucidate a possible novel mechanism of regulation of Nox-dependent ROS production downstream of Bcr–Abl signalling.

We have shown that K562 ROS generation is inhibited by both Bcr–Abl inhibitors (Imatinib and Nilotinib) and Nox protein inhibitors (DPI and VAS2870), indicating that ROS is both Bcr–Abl and Nox-dependent (Fig. 1). Reduction in ROS levels following Bcr–Abl inhibition coincided with the down-regulation of p22phox, but did not affect any other Nox protein (Fig. 2). p22phox is membrane-bound protein essential for full activity of Nox proteins 1, 2, 3 and 4 [12,13], consequently endogenous-ROS production is very likely to be significantly affected by a reduction in p22phox protein levels. Knockdown of p22phox using siRNA verified this and demonstrated a decrease in ROS levels (Fig. 4b) establishing a link between p22phox and ROS production in these cells. Nox-1 and Nox-3 proteins were undetectable in K562 cells (Fig. 2a). Nox-5, DUOX-1 and DUOX-2 are not regulated by p22phox [27]; therefore Nox-2 and Nox-4 are the only potentially p22phox-regulated Nox proteins in this model. From these results we can say that p22phox down-regulation due to Bcr–Abl inhibition mediates a decrease in ROS levels through deactivation of one if not both of these Nox proteins. Interestingly, ROS generated from both of these Nox proteins have been linked to cell survival and proliferation [5,28–30] and suggests a possible role in K562 survival signalling.

Following Bcr–Abl inhibition by Imatinib we demonstrated that p22phox mRNA levels were unaffected (Fig. 2c) but p22phox protein was shown to be extensively ubiquitinated and subsequently directed to the proteasome for degradation (Fig. 2d). This post-translational reduction of p22phox protein levels mediated by both Imatinib and Nilotinib is a novel mechanism of action of the drugs, not previously described. Interestingly, this method of p22phox degradation is not just specific to CML and has also been demonstrated on the reintroduction of von Hippel-Lindau tumour suppressor gene (VHL) into VHL-deficient carcinoma cells [31,32]. Furthermore, a recent study in Acute Myelogenous Leukaemia (AML) from our laboratory demonstrated a similar method of p22phox regulation upon inhibition of the FLT3-ITD oncogene [14]. In both these studies the decrease in p22phox protein levels resulted in a significant ROS reduction and effected survival signalling. Taken together, this study and the previous work mentioned here raise the possibility of an involvement for p22phox in the development of these cancers and further compound the significance of this result in CML.

Two major survival signalling pathways activated downstream of Bcr–Abl are the PI3K/Akt and Raf/MEK/ERK1/2 pathways [3]. As shown, inhibition of both these pathways individually had minimal effect on p22phox levels, yet simultaneous inhibition resulted in a reduction comparable to that observed on Bcr–Abl inhibition (Fig. 3a). This result suggests a possible synergy or compensatory effect between the pathways with deactivation of both necessary for p22phox down-regulation. Such signalling crosstalk between these two pathways is not unusual has been noted before [33,34].

Fig. 3. Imatinib-mediated degradation of p22phox requires GSK-3β activation. (a) Western blot analysis of p22phox protein expression in K562 cells following treatment with LY294002 (LY; 10 μM) and UO126 (UO; 10 μM) for 8 and 16 h. (b) Western blot analysis of p22phox protein expression in K562 cells following treatment with Imatinib (10 μM), SB216763 (SB; 5 μM), LY (10 μM) and UO (10 μM) for 16 h. Bar charts in (a) and (b) represent densitometric analysis of p22phox protein levels following treatments. GAPDH is shown as a loading control. Vehicle control is DMSO. All values are expressed as mean ± SD and are representative of three independent experiments.
Using inhibitors we demonstrated that GSK-3β activity is essential for the reduction of p22phox levels (Fig. 3b). The activity of GSK-3β is well known to target proteins for proteasomal degradation and its effect on β-catenin ubiquitination and degradation is extensively studied in the Wnt-signalling pathway [19]. Like many proteins GSK-3β activity is regulated by phosphorylation. Interestingly phosphorylation at Serine 9 inactivates GSK-3β inducing a pro-survival effect by inhibiting its proapoptotic functions. It is already known that Bcr–Abl signalling induces the phosphorylation of GSK-3β at this residue [5]. Here we believe that following Bcr–Abl inhibition there is an increase in activated GSK3–β through dephosphorylation of Serine 9 which was observed following imatinib treatment as well as simultaneous inhibition of both the PI3K/Akt and Raf/MEK/ERK1/2 pathways (Fig. 3b). These data demonstrate that inhibition of Bcr–Abl deactivates both these pathways, in turn making them unable to inhibit GSK-3β via Serine 9 phosphorylation, resulting in GSK-3β activation and promotion of p22phox degradation. This result demonstrated for the first time that GSK-3β is involved in p22phox regulation and therefore ROS production in CML. While we have demonstrated that p22phox can be directed to the proteasome via ubiquitination it remains unclear how GSK-3β may be mediating this effect.

Targeted knockdown of p22phox resulted in a notable reduction in the proliferation rates of these cells which was very dependent on p22phox protein levels (Fig. 4d and e). Such a role for p22phox and Nox-derived ROS in proliferation has been noted before [23,28,29,35]. Enhanced proliferation is a major characteristic of CML blast crisis cells defining disease phenotype [36]. K562 cells were originally isolated from a CML patient in blast crisis [15], demonstrating a role for Nox-derived ROS in proliferation in these cells is significant and provides a possible role for ROS production in the increased proliferation of CML cells. In this study we have demonstrated a link between Bcr–Abl signalling and ROS production through Nox activity. These results also describe a possible role for Nox-derived ROS in proliferation of CML cells. Taken together we believe these results demonstrate a novel mechanism of action associated with imatinib and nilotinib treatment, thus providing a better understanding of the actions of these drugs which currently play an important role in patient treatment.

Conflict of interest statement

The authors declare no conflict of interest.
Acknowledgements

The authors thank Dr. Lavina Bhatt for technical assistance in live cell microscopy and are grateful to Miss Catherine Harrington for providing helpful discussion and support throughout this work.

Contributors: WDL, JPW and TGC provided conception and design of the study; WDL provided data; WDL, JPW and TGC provided analysis and interpretation of data; WDL and TGC drafted and critically reviewed the manuscript for important intellectual content; TGC gave final approval of the submitted version.

Role of funding source. This work was supported by an Irish Cancer Society Research Scholarship Award, CRS10LAN, and a grant from The Children’s Leukaemia Research Project.

References