European Journal of Cancer (2015) **51**, 2008–2021

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The potential of clofarabine in *MLL*-rearranged infant acute lymphoblastic leukaemia

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Received 9 October 2014; received in revised form 31 May 2015; accepted 7 June 2015
Available online 15 July 2015

KEYWORDS
Infant ALL
MLL
Paediatric leukaemia
Clofarabine
Epigenetic

Abstract *MLL*-rearranged acute lymphoblastic leukaemia (ALL) in infants is the most difficult-to-treat type of childhood ALL, displaying a chemotherapy-resistant phenotype, and unique histone modifications, gene expression signatures and DNA methylation patterns. *MLL*-rearranged infant ALL responds remarkably well to nucleoside analogue drugs *in vitro*, such as cytarabine and cladribine, and to the demethylating agents decitabine and zebularine as measured by cytotoxicity assays. These observations led to the inclusion of cytarabine into the treatment regimens currently used for infants with ALL. However, survival chances for infants with *MLL*-rearranged ALL do still not exceed 30–40%.

Here we explored the *in vitro* potential of the novel nucleoside analogue clofarabine for *MLL*-rearranged infant ALL. Therefore we used both cell line models as well as primary patient cells. Compared with other nucleoside analogues, clofarabine effectively targeted primary *MLL*-rearranged infant ALL cells at the lowest concentrations, with median LC_{50} values of ~25 nM. Interestingly, clofarabine displayed synergistic cytotoxic effects in combination with cytarabine. Furthermore, at concentrations of 5–10 nM clofarabine induced demethylation of the promoter region of the tumour suppressor gene *FHIT* (*Fragile Histidine Triad*), a gene typically hypermethylated in *MLL*-rearranged ALL. Demethylation of the *FHIT* promoter region was accompanied by subtle re-expression of this gene both at the mRNA and protein level. We conclude that clofarabine is an interesting candidate for further studies in *MLL*-rearranged ALL in infants.

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http://dx.doi.org/10.1016/j.ejca.2015.06.117
0959-8049/

1. Introduction

Survival chances for children with acute lymphoblastic leukaemia (ALL) have improved tremendously over the past decades [1]. Nonetheless, the prognosis for infants (<1 year of age) with ALL remains dismal [2,3]. Infant ALL represents a highly aggressive type of leukaemia characterised by chromosomal translocations involving the *MLL* gene (~80% of the cases) [4,5], and typically presents with hepatosplenomegaly, exceedingly high leucocyte counts, and often shows central nervous system involvement [6]. Moreover, infant ALL cells usually are resistant to multiple chemotherapeutic drugs currently used in paediatric ALL treatment regimens, especially to glucocorticoids (such as prednisone and dexamethasone) and L-asparaginase [7,8]. However, infant ALL cells have proven to be highly sensitive to the nucleoside analogue cytarabine (i.e. cytosine arabinoside, or ara-C) [7,8], which appeared to be associated with elevated expression of the human *equilibrative nucleoside transporter 1* (h*ENT1*) on which cytosines are mainly dependent to permeate the cell membrane [9]. Based on these findings a unique infant ALL treatment protocol (Interfant-99) was designed, implementing varying dosages of cytarabine throughout the treatment courses of a standard childhood ALL regimen [3]. The Interfant-99 treatment protocol appeared successful, achieving long-term event-free survival in 47% of the infant ALL cases, realising superior treatment results over earlier attempts exploring therapy intensification [3].

Recently we demonstrated that *MLL*-rearranged infant ALL is characterised by increased levels of DNA methylation at numerous gene promoters, leading to suppressed expression of associated genes [10]. Furthermore this study showed that the degree of promoter methylation is associated with the risk of disease relapse [10]. Interestingly, hypermethylated *MLL*-rearranged ALL cells appeared highly responsive to so-called demethylating agents (e.g. decitabine and zebularine) [10,11]. Like cytarabine, decitabine and zebularine are cytidine analogues, but in contrast to cytarabine, which was originally designed to inhibit DNA synthesis (Fig. 1A), these agents were specifically developed to inhibit DNA methylation. Demethylating cytidine analogues exert their actions by competing with normal cytosines for incorporation into the DNA. Once incorporated, these analogues are able to covalently bind, and thereby trap, DNA methyltransferases (DNMTs) during their donation of methyl groups on receiving cytidines (Fig. 1A). As a consequence, the cell becomes depleted from functional DNMTs and loses its ability to methylate the DNA during subsequent cell cycles [12]. Presumably, the sensitivity of *MLL*-rearranged ALL cells to demethylating cytosine analogues can be ascribed to the aberrant DNA methylation patterns recently found in this type of leukaemia [10,11], but may certainly be enhanced by the elevated expression of h*ENT1* characteristically observed in infant ALL [9]. Thus, demethylating cytosine analogues embody promising candidates for the treatment of *MLL*-rearranged ALL in infants. Unfortunately, despite several clinical trials demonstrating biologic activity and clinical responses for both decitabine and azacitidine in adults diagnosed with myelodysplastic syndromes (MDS) or chronic myelomonocytic leukaemia (CMML) [13,14], clinical results in general remain somewhat disappointing [15,16].

Apart from cytosine analogues, infant ALL cells also appeared to respond remarkably well to another nucleoside analogue, i.e. cladribine [7,8,17]. Although cladribine represents an adenosine analogue, and as such lacks the ability to bind DNMTs, it has been reported to possess methylation-inhibiting properties via an alternative mechanism involving the inhibition of S-Adenosyl Homocysteine Hydrolase (SAHH) [18] (Fig. 1B). As a consequence the amount of intracellular deoxynucleoside triphosphates available for DNA replication becomes impaired, which leads to apoptosis in rapidly dividing cells. Due to its resistance to inactivation by deamination or phosphorolysis, clofarabine is more stable than its predecessors [19]. In addition, clofarabine was shown to inhibit DNA methylation [20], presumably through a mechanism comparable to that observed for cladribine (Fig. 1B). A recent study demonstrated that clofarabine also induces down-regulation of *DNA methyltransferase 1* (*DNMT1*) at the mRNA level in chronic myelogenous leukaemia (CML) cells [21].

While clofarabine has proven activity in the treatment of refractory and relapsed childhood ALL [22–24], we postulate that this agent may be particularly suitable for the treatment of infants with ALL, especially in patients carrying *MLL* translocations and hypermethylated genomes. Therefore we here compared the cytotoxic effects of clofarabine and other nucleoside analogues on primary *MLL*-rearranged infant ALL cells, explored possible synergistic effects between clofarabine and cytarabine, and evaluated the potential of clofarabine to inhibit DNA methylation.

2. Materials and methods

2.1. Patient samples and leukaemic cell isolation

In this study, primary patient samples were used from both infant (<1 year of age) ($n = 10$) and paediatric non-infant (>1 year of age) ($n = 10$) precursor B-ALL patients. All infant ALL cases were enrolled in the international Interfant-99 treatment study [3], and all non-infant paediatric precursor B-ALL samples were derived from the Erasmus MC – Sophia Children's

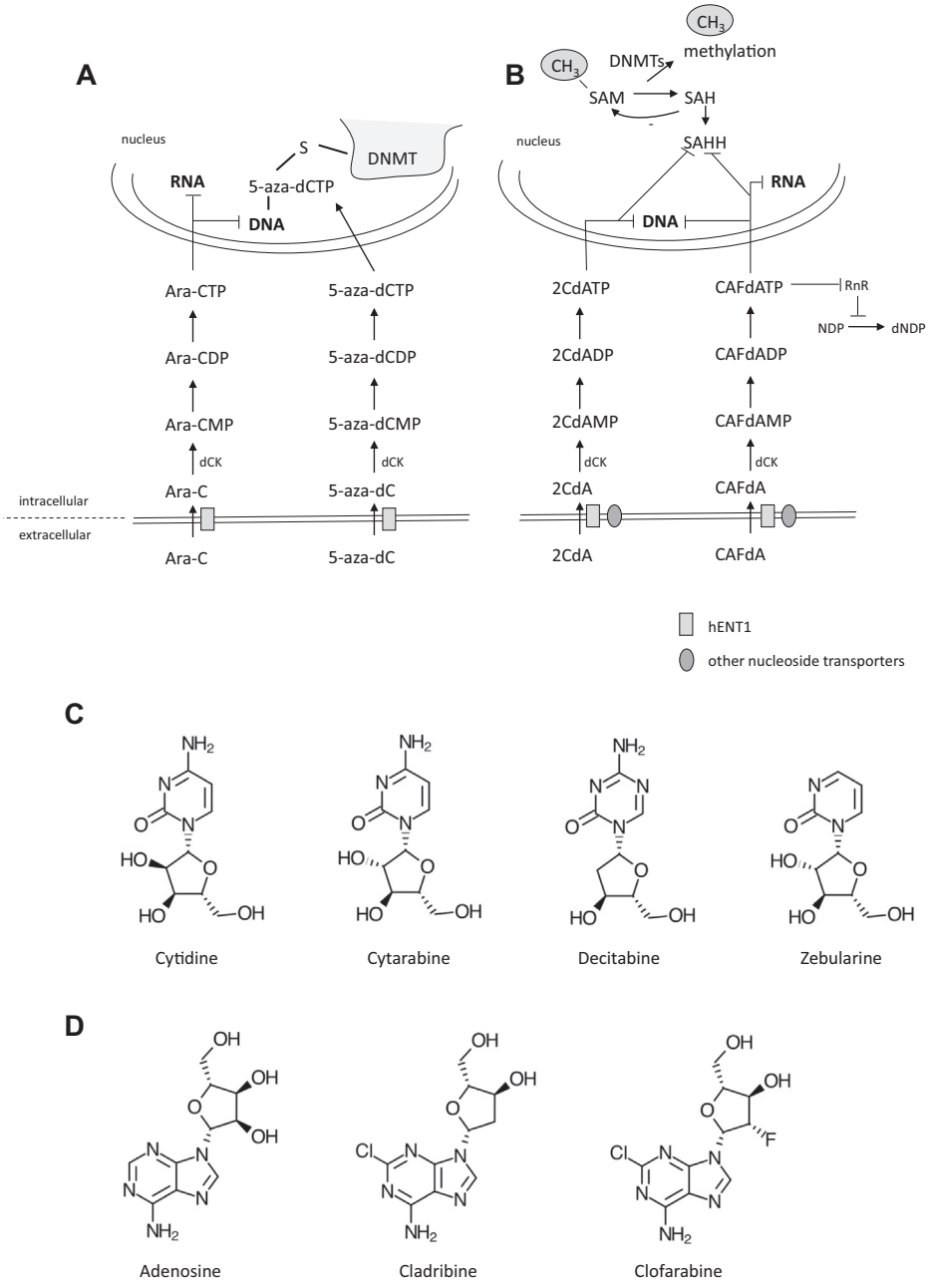

Fig. 1. Mechanisms of action and chemical structures of the different nucleoside analogue drugs. (A) The cytosine analogues cytarabine (Ara-C), as well as the demethylating agents decitabine (5-aza-dC) and zebularine, mainly enter the cell via the human equilibrative nucleoside transporter 1 (hENT1). Inside the cell these pro-drugs are sequentially phosphorylated into active nucleoside tri-phosphates (dNTPs) which compete with normal cytidines for incorporation into the DNA (or RNA) during DNA (and RNA) synthesis. As such, cytarabine blocks RNA and DNA synthesis leading to apoptosis. In contrast, the demethylating agents decitabine and zebularine do not inhibit DNA synthesis, but covalently bind DNA methyltransferases (DNMTs) during methylation events. As a result, DNMTs become trapped onto the DNA, depleting the cell from functional DNMTs and impairing the ability of the cell to distribute methylation in subsequent cell cycles. (B) The adenosine analogues cladribine (2CdA) and clofarabine (CAFdA), comparable to cytosine analogues, require triple phosphorylations to become effective and incorporated into the DNA. Apart from inhibition of DNA synthesis, cladribine, and presumably clofarabine as well, block DNA methylation by inhibition of S-Adenosyl Homocysteine Hydrolase (SAHH), which hydrolyses S-Adenosyl Homocysteine (SAH). The subsequent accumulation of SAH production impairs the formation of the methyl-donor S-Adenosyl Methionine (SAM) (which is used as a methyl-pool by DNMTs), and thereby inhibits DNA methylation. Finally, clofarabine also inhibits ribonucleotide reductase (RnR), blocking the formation of normal dNTPs, and as such eliminating the competition for incorporation into the DNA. (C) Shows the chemical structures of cytidine and the cytidine analogue drugs cytarabine, decitabine and zebularine. The difference between normal cytidines and cytarabine is the inversion of 2′hydroxyl groups from a *trans* position in cytidine to a *cis* configuration in cytarabine. Decitabine lacks the 2′hydroxyl group and carries an additional nitrogen in its base. Zebularine essentially shares its structure with cytarabine, but lacks the amino-group in its base. (D) Shows the chemical structures of adenosine and the adenosine analogues cladribine and clofarabine. Compared with normal adenosine, cladribine carries a chloro-group in its base. Apart from the chloro-group, clofarabine has an additional fluoro group present at the 3′-position of its ribose moiety. *Abbreviations used:* Ara-C = cytarabine, 5-aza-dC = decitabine, 2CdA = cladribine, and CAFdA = clofarabine.

Hospital, Rotterdam, the Netherlands. Infant ALL samples were selected based on the presence of *t*(4;11), the most common *MLL* translocation found among infant ALL patients [3]. Positivity for *t*(4;11) was assessed by split-signal FISH and RT-PCR analysis. None of the paediatric non-infant ALL samples was positive for *t*(4;11). Informed consent was obtained from the parents or legal guardians according to the recommendations of the Helsinki declaration, and approved by the Institutional Review Board of the Erasmus University Medical Center.

Primary bone marrow or peripheral blood samples were obtained before treatment, and mononuclear cells were isolated using sucrose density-gradient centrifugation (density 1.077 g/ml; Lymphoprep, Nycomed Pharma, Oslo, Norway) within 24 h of sampling. Cells were resuspended in RPMI 1640 medium (Invitrogen Life Technologies, Breda, the Netherlands) supplemented with 20% foetal-calf serum (FCS; Integro, Zaandam, the Netherlands), 2 mM L-glutamine, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite (ITS media supplement; Sigma, St Louis, MO, United States of America (USA)), 200 μg/mL gentamycin (Invitrogen Life Technologies, Breda, the Netherlands), 100 IU/mL penicillin, 100 μg/mL streptomycin and 0.125 μg/mL fungizone (Invitrogen Life Technologies, Breda, the Netherlands). When necessary, contaminating non-leukaemic cells were removed using immunomagnetic beads (DynaBeads, Dynal Inc., Oslo, Norway) as previously described [25]. As a result, all leukaemic samples used in this study contained more than 90% of leukaemic blasts.

2.2. Leukaemia cell lines

The cell lines SEM and RS4;11 were used as models for *MLL*-rearranged B-ALL. Both cell lines carry translocation *t*(4;11). SEM was originally derived from a 5-year-old girl at relapse [26] and RS4;11 was established from the bone marrow of a 32-year-old woman in first relapse [27]. The cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), and maintained as suspension cultures in RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen Life Technologies, Breda, the Netherlands) supplemented with 10% FCS (Integro, Zaandam, the Netherlands), 100 IU/mL penicillin, 100 μg/mL streptomycin and 0.125 μg/mL fungizone (Invitrogen Life Technologies, Breda, the Netherlands) at 37 °C in humidified air containing 5% CO_2.

2.3. In vitro cytotoxicity assays and nucleoside analogue exposure

The *in vitro* sensitivity of leukaemia cells to decitabine (Sigma–Aldrich, Zwijndrecht, the Netherlands), zebularine (kindly provided by Dr. Victor E. Marquez, National Cancer Institute of Frederick, Frederick, MD, USA), cladribine (Sigma–Aldrich, Zwijndrecht, the Netherlands), cytarabine (Cytosar, Pharmacia BV, Woerden, the Netherlands) and clofarabine (Genzyme Europe, Naarden, the Netherlands), was determined by 4-day MTT-assays as described previously [28]. Briefly, leukaemic cells were cultured in the absence or presence of varying concentrations of the above mentioned nucleoside analogues for four days, and drug sensitivity was expressed as the LC_{50} value (i.e. the concentration of nucleoside analogue drug lethal to 50% of the leukaemic cells) or, in the case of cell lines as the IC_{50} value (i.e. the concentration of nucleoside analogue drug inhibitory to 50% of the leukaemic cells). Each experiment was performed in duplicate, and carried out at least twice.

The effects of the hENT1 inhibitor NBMPR (*S*-(4-Nitrobenzyl)-6-thioinosine (NBMPR, Sigma–Aldrich, Zwijndrecht, the Netherlands) on clofarabine and cytarabine cytotoxicity were evaluated by performing standard MTT assays in the presence of 3 μM of NMBPR, both in the control cells (untreated cells) and cells exposed to clofarabine or cytarabine.

In order to investigate whether the combination of cytarabine and clofarabine evoked either antagonistic or synergistic cytotoxic effects, cytarabine cytotoxicity (4-day MTT assays) was determined in the *t*(4;11)-positive cell line SEM in the absence and presence of 0.5 or 10 nM clofarabine. For this, cells were pre-incubated with 0.5 or 10 nM clofarabine prior to the actual MTT assay for cytarabine (during which clofarabine remained present throughout the entire experiment). Likewise, clofarabine cytotoxicity was determined in the absence and presence of 25 or 50 nM cytarabine. For all concentrations of cytarabine and clofarabine treatment, a hypothetical maximum additive effect was calculated using the following equation $A \times B/100$, in which A and B indicate survival values with single agents. In case the actual product of cell viability of combined cytarabine/clofarabine treatment reflected the calculated value, the effects were considered additive. When the actual value was lower than the calculated value, the effect was deemed synergistic.

2.4. Isolation and purification of DNA and RNA

Genomic DNA and total cellular RNA were extracted from a minimum of 5×10^6 cells using the DNeasy Blood and Tissue Kit and the RNeasy mini Kit (Qiagen Benelux BV, Venlo, the Netherlands) according to the manufacturer's protocol. The quality of the extracted DNA was assessed on 1.5% agarose gels, and the RNA integrity was determined using RNA 6000 Nano Assay LabChips on the Agilent 2100 Bio-analyser (Agilent Technologies, Santa Clara, CA, USA).

2.5. Pyrosequencing for quantitative CpG island methylation analysis

Genomic DNA was bisulphite converted using the EZ DNA methylation kit (Zymo Research Corporation, CA, USA) according to the manufacturer's instructions. Bisulphite treatment of the DNA converts non-methylated cytosine bases to uracil (which is replaced by thymine during subsequent PCR cycles), but does not affect 5-methylcytosine residues, allowing discrimination of methylated or unmethylated cytosines [29]. To quantitatively determine the levels of methylation at given loci, we subsequently applied pyrosequencing. In contrast to traditional Sanger sequencing, pyrosequencing is a 'sequencing by synthesis' based method that relies on the detection of DNA polymerase activity (visualised by pyrophosphate release during nucleotide incorporation). Essentially, this technique allows sequencing of a single DNA strand by base per base synthesis of the complementary strand, while monitoring which nucleotide was actually added. As such, pyrosequencing can be used to quantitatively assess the level of methylation in a bisulphite treated DNA strand by comparing the cytosine/thymine ratios incorporated at selected CpGs [29].

Approximately 20 ng of bisulphite converted DNA was amplified using bisulphite-specific primers flanking the CpG island within the promoter of the *Fragile Histidine Triad* (*FHIT*) gene. For this, primers were designed using the Pyrosequencing Assay Design Software (Qiagen Pyrosequencing Inc.), and the sequences were as follows, forward primer: 5′-*GGGGAGGTAAGTTTAAGTGGAATATT*-3′, (biotinylated) reverse primer: 5′-*ATCCCCACCCTAAAACCC TC*-3′. The PCR product amplified using this primer pair includes five separate CpGs within the *FHIT* promoter sequence. Amplification was performed in the presence of 4 mM of $MgCl_2$ using a touchdown PCR with the annealing temperature decreasing from 71 °C to 64 °C over 14 cycles of annealing for 1 min, and subsequent denaturation for 15 s at 95 °C. The PCR was completed by 30 cycles of annealing at 64 °C for 1 min, and denaturation for 15 s at 95 °C. Buffers and hotstart Taq polymerase used for PCR were obtained from the Qiagen Pyromark PCR kit (Qiagen Pyrosequencing, Inc.). Amplified PCR products were initially analysed on 2% agarose gels. Then, PCR products were denatured and antisense strands (containing biotin labels) were bound to Streptavidin Sepharose HP (Amersham Biosciences). Immobilised PCR strands coupled to the Sepharose beads were purified, washed and denatured using a 0.2 M NaOH solution. Next, 0.3 μM of pyrosequencing primer (5′-*AAGTTTAAGTG GAATATTGT*-3′) was annealed to the purified single-stranded PCR product and the sequencing reaction was performed in duplicate and analysed on a PyroMark MD system (Qiagen Pyrosequencing, Inc.). Confirmation of complete bisulphite conversion was assessed by a cytosine/thymine control that was integrated in the *FHIT* CpG island assay. Subsequent quantification of methylation density on selected CpGs was performed using the Pyro Q-CpG software (Qiagen Pyrosequencing, Inc.). The degree of methylation in the *FHIT* CpG sequence was determined from the ratio of thymine and cytosine nucleotides and is presented as the number of methylated cytosine nucleotides divided by the amount of methylated and unmethylated cytosines × 100%.

2.6. Quantitative real-time PCR analysis

Total RNA was reverse transcribed as described previously [9] and the obtained cDNA was used to quantify mRNA expression using quantitative real-time PCR analysis as described elsewhere [30]. All oligonucleotides were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CA, USA). Primer combinations used for transcript amplification of the *FHIT* target gene as well as the housekeeping reference gene *GAPDH* (encoding *glyceraldehyde-3-phosphate dehydrogenase*) have earlier been published [31]. PCR products were amplified using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) according to the manufacturer's recommendations, using SYBR Green as a fluorophore to detect amplified transcripts. Per experiment samples were analysed in duplicate and all experiments were conducted twice.

2.7. Western blotting

Whole cell protein lysates containing 25 μg of protein were resolved on 10% polyacrylamide gels topped with 4% stacking gels, and subsequently transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were then probed with the following antibodies: rabbit polyclonal anti-FHIT (Millipore, Billerica, MA, USA, #07-172), or rabbit polyclonal anti-DNMT1 (New England Biolabs, Ipswich, MA, USA, #M0231S). Anti-beta-Actin mouse monoclonal antibodies (Abcam, Cambridge, USA, #ab6276) were used to detect beta-Actin and confirm equal loading in all lanes. Upon incubation (protected from light) with infrared-labelled secondary antibodies (IRDye 800CW goat-anti-rabbit antibody (#926-32211, LI-COR, Lincoln, NE) and IRDye 680 goat-anti-mouse antibody (#926-32220, LI-COR, Lincoln, NE)) in 5% milk, the membranes were washed in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (Merck Schuchardt OHG, Hohenbrunn, Germany). Finally the membranes were scanned using an Odyssey Infrared Imaging System (LI-COR Inc.,

Lincoln, NE, USA), and protein expression was quantified using the Odyssey software.

2.8. Statistical analysis

Differences in LC_{50} values between *MLL*-rearranged infant precursor B-ALL and non-infant paediatric precursor B-ALL samples were evaluated using two-tailed Mann–Whitney *U* tests (performed in SPSS 17.0 statistical software), and considered statistically significant at *p*-values <0.05. Graphpad Prism graphical software version 5 (La Jolla, CA, USA) was used for data visualisations.

3. Results

3.1. Infant ALL cells are highly sensitive to nucleoside analogues, including clofarabine

Although *MLL*-rearranged infant ALL cells usually are resistant to multiple chemotherapeutic drugs, these cells often are highly sensitive to nucleoside analogue drugs including cytarabine and cladribine [7–9], and the demethylating cytosine analogues zebularine [10] and decitabine [11]. Here we explored the potential of the new-generation nucleoside analogue clofarabine in *MLL*-rearranged infant ALL. Fig. 1C–E shows the chemical structures of the cytosine analogues cytarabine, decitabine and zebularine, and the adenosine analogues cladribine and clofarabine. We compared the effects of these nucleoside analogues on cell death and proliferation in the *MLL*-rearranged ALL cell lines SEM and RS4;11. As shown in Fig. 2A and B, effective concentrations are convincingly lower for both adenosine analogues as compared with the cytosine analogues. The lowest IC_{50} values were observed for clofarabine, with inhibiting concentrations of 3.5 nM and 5 nM for RS4;11 and SEM respectively (Fig. 2A and B).

Next, we compared the LC_{50} values of cytarabine, cladribine and clofarabine in primary samples from *MLL*-rearranged infant ALL patients ($n = 10$), all carrying translocation *t*(4;11), and non-infant paediatric precursor B-ALL patients ($n = 10$). Unfortunately, the demethylating agents zebularine and decitabine could not be tested on primary patient material, as these compounds require several cell divisions to become effective, and primary ALL cells generally stop proliferating once outside the patient's body. Although the other nucleoside analogues also require cell cycles to become incorporated into the genomic DNA, multiple studies have demonstrated that *in vitro* cytarabine and cladribine cytotoxicity can be induced in non-dividing patient cells [9,32], for example by inhibition of RNA synthesis [32]. As expected from earlier studies, *MLL*-rearranged infant ALL cells are significantly more sensitive to cytarabine (~2-fold difference, $p = 0.001$) (Fig. 2C) and cladribine (1.4-fold difference, $p = 0.005$) (Fig. 2D) compared with non-infant paediatric precursor B-ALL cells. Interestingly, *MLL*-rearranged infant ALL cells also appeared marginally (although not significantly) more sensitive to clofarabine (1.7-fold difference, $p = 0.075$) (Fig. 2E). Clofarabine effectively induced leukaemic cell death in primary ALL samples at average LC_{50} values as low as 15–30 nM, while similar effects for cytarabine required ~1–2.5 μM (Fig. 2C–E).

3.2. Sensitivity to clofarabine is not dependent on the hENT1 transporter

In a previous study we investigated the mechanism underlying cytarabine sensitivity in *MLL*-rearranged infant ALL cells, and found that these cells express significantly higher levels of the human *equilibrative nucleoside transporter 1* (h*ENT1*) [9], on which cytarabine is mainly dependent to permeate the cell membrane [33]. Therefore, we asked whether clofarabine sensitivity may also be dependent on h*ENT1* by measuring the effects of the hENT1 inhibitor NBMPR (*S*-(4-Nitrobenzyl)-6-thioinosine) (which significantly inhibits cytarabine influx and toxicity at 1–3 μM [33]), on clofarabine cytotoxicity. 3 μM of NBMPR itself did not induce any *in vitro* cytotoxicity in *MLL*-rearranged ALL cells. As shown in Fig. 3A, 3 μM of NBMPR markedly inhibited the cytotoxic effects for cytarabine in the *t*(4;11)-positive ALL cell line SEM, whereas the effects on clofarabine cytotoxicity were far less pronounced (Fig. 3B). Next we evaluated the effects of NBMPR on cytarabine and clofarabine cytotoxicity in two *t*(4;11)-positive infant ALL patient samples. These experiments showed that blocking hENT1 using NBMPR reduces the cytotoxic effects of cytarabine (Fig. 3C and D), but does not affect the *in vitro* responses to clofarabine (Fig. 3E and F).

3.3. Additive/synergistic cytotoxic effects for clofarabine and cytarabine in MLL-rearranged ALL

The first observation that infant ALL cells are highly sensitive to cytarabine [7] led to the successful implementation of cytarabine courses in the infant ALL treatment protocol Interfant-99 [3]. However, with a new nucleoside analogue like clofarabine already available for clinical testing, and capable of targeting leukaemic cells at nanomolar concentrations, important questions should be asked: Should cytarabine be replaced by clofarabine, or would *MLL*-rearranged infant ALL patients further benefit from the addition of clofarabine to existing treatment protocols? Interestingly, synergy between clofarabine and cytarabine has been described both *in vitro* and *in vivo* [34,35].

To determine whether these nucleoside analogues also work synergistically in *MLL*-rearranged ALL, we

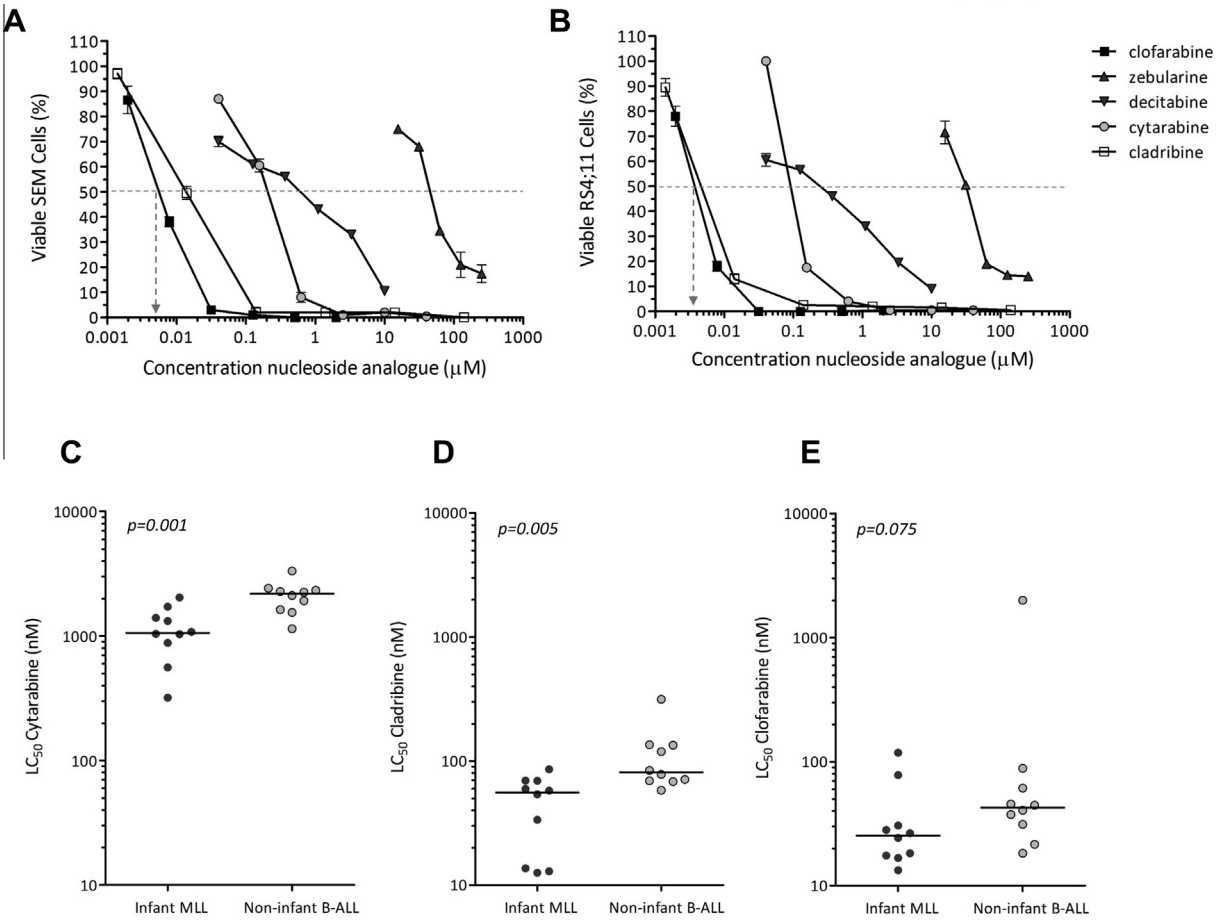

Fig. 2. *In vitro* cytotoxicity of *MLL*-rearranged ALL cells to different nucleoside analogue drugs. (A and B) Dose–response curves showing the *in vitro* cytotoxic response (as determined by 4-day MTT assays) to the nucleoside analogue drugs clofarabine, cladribine, cytarabine, decitabine and zebularine in the cell lines (A) SEM, and (B) RS4;11. Error bars represent standard errors of the mean (SEM). The IC_{50} values (concentrations inhibitory to 50% of the cells) for clofarabine are indicated by grey arrows. (C–E) Comparison of the *in vitro* drug response (as determined by 4-day MTT assays) in primary *MLL*-rearranged infant ALL ($n = 10$) and non-infant germline-*MLL* paediatric precursor B-ALL ($n = 10$) samples for the nucleoside analogue drugs (C) cytarabine, (D) cladribine and (E) clofarabine. Drug sensitivity is expressed as LC_{50} values (concentrations lethal to 50% of the cells). The lines indicate the median LC_{50} values in each patient group, and differences in drug response were statistically evaluated by Mann–Whitney U tests.

tested cytarabine cytotoxicity in the presence of 5 nM or 10 nM of clofarabine, as well as clofarabine cytotoxicity in the presence of 25 or 50 nM of cytarabine in the *t*(4;11)-positive cell line SEM. As shown in Fig. 4A and B, the cytotoxic effects of both drugs are markedly larger in each others' presence, especially at low dosages. However, true synergy exists when the cytotoxic effects of both agents tested simultaneously exceed the combined effect of both agents tested individually. As shown in Fig. 4C, strong synergistic effects were observed at 5 nM and 10 nM of clofarabine in combination with 50 nM of cytarabine, but only in case cells were pre-incubated with cytarabine (also see: material and methods). Four days of culturing SEM cells with a combination of 50 nM cytarabine/5 nM clofarabine and 50 nM cytarabine/10 nM clofarabine resulted in viable cell counts of ~33% and ~25% respectively. The calculated product derived from the effects of both agents when tested separately predicted viable cell counts for the combination of both drugs of ~80% and ~55% respectively. However, the actual combinations of 50 nM cytarabine/5 nM clofarabine and 50 nM cytarabine/10 nM clofarabine resulted in viable cell counts of ~33% and ~25% respectively. Yet, when the same combinations of clofarabine and cytarabine were tested in which clofarabine was added first (1 h pre-incubation), the synergistic effects were either absent or only marginally detectable (Fig. 4C).

3.4. Inhibition of DNA methylation by clofarabine

Clofarabine is thought to inhibit DNA methylation through either inhibition of S-Adenosyl Homocysteine Hydrolase (SAHH) as shown for cladribine [18], or suppression of *DNA methyltransferase 1* (*DNMT1*) expression [21]. The demethylating cytosine analogues zebularine and decitabine inhibit DNA methylation by replacing normal cytosines in the genomic DNA where

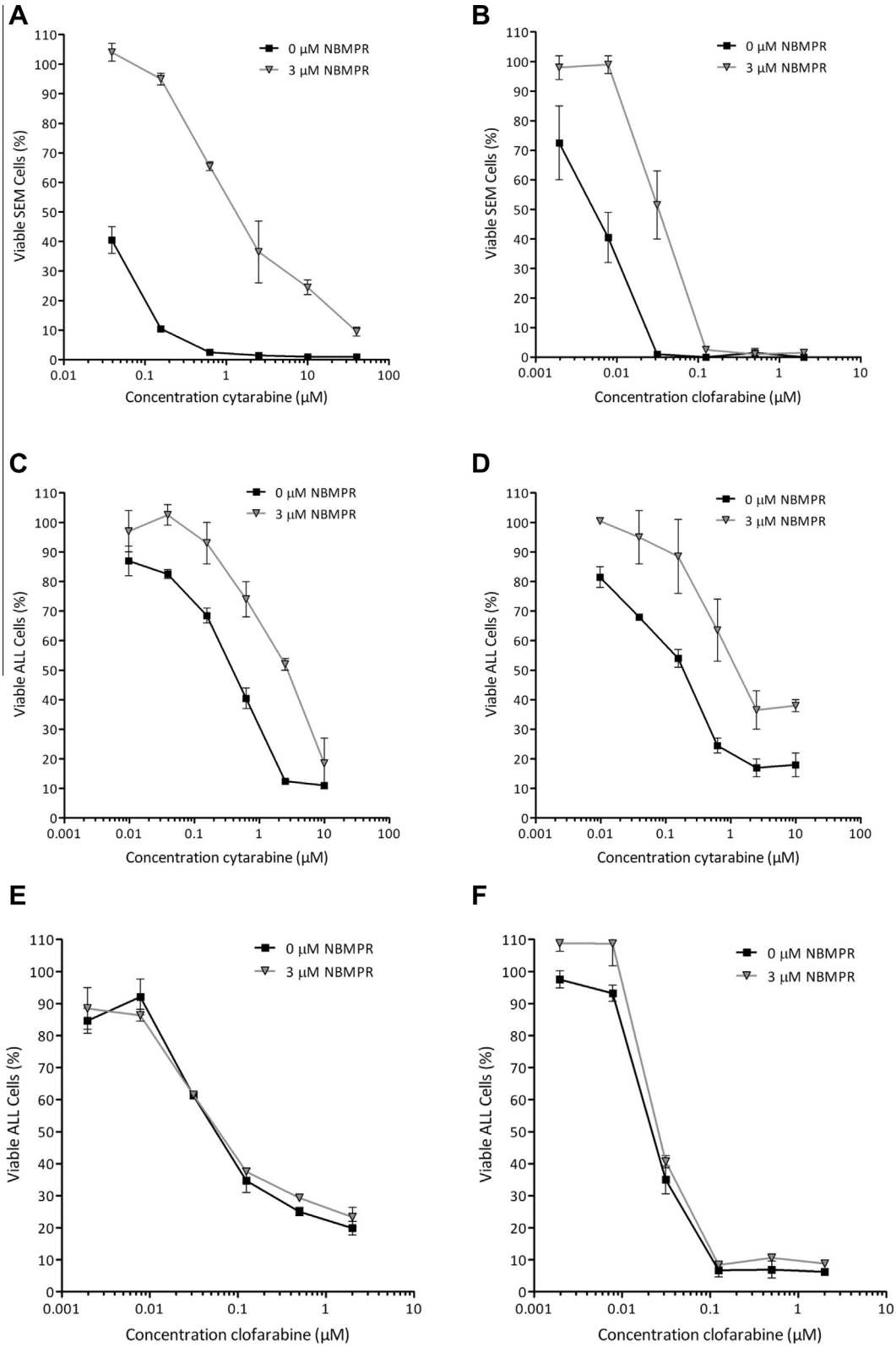

Fig. 3. Effects of NBMPR on cytarabine and clofarabine cytotoxicity in *MLL*-rearranged ALL cells. The *MLL*-rearranged ALL cell line SEM was pre-incubated for 1 h with or without 3 μM of the pharmacological hENT1-inhibitor NBMPR prior to determining (A) cytarabine, and (B) clofarabine cytotoxicity using 4-day MTT assays. (NBMPR remained present throughout the experiment). Similarly, (C and D) cytarabine and (E and F) clofarabine cytotoxicity was determined in two primary *t*(4;11)-positive infant ALL patient samples in the absence and presence of NBMPR (3 μM).

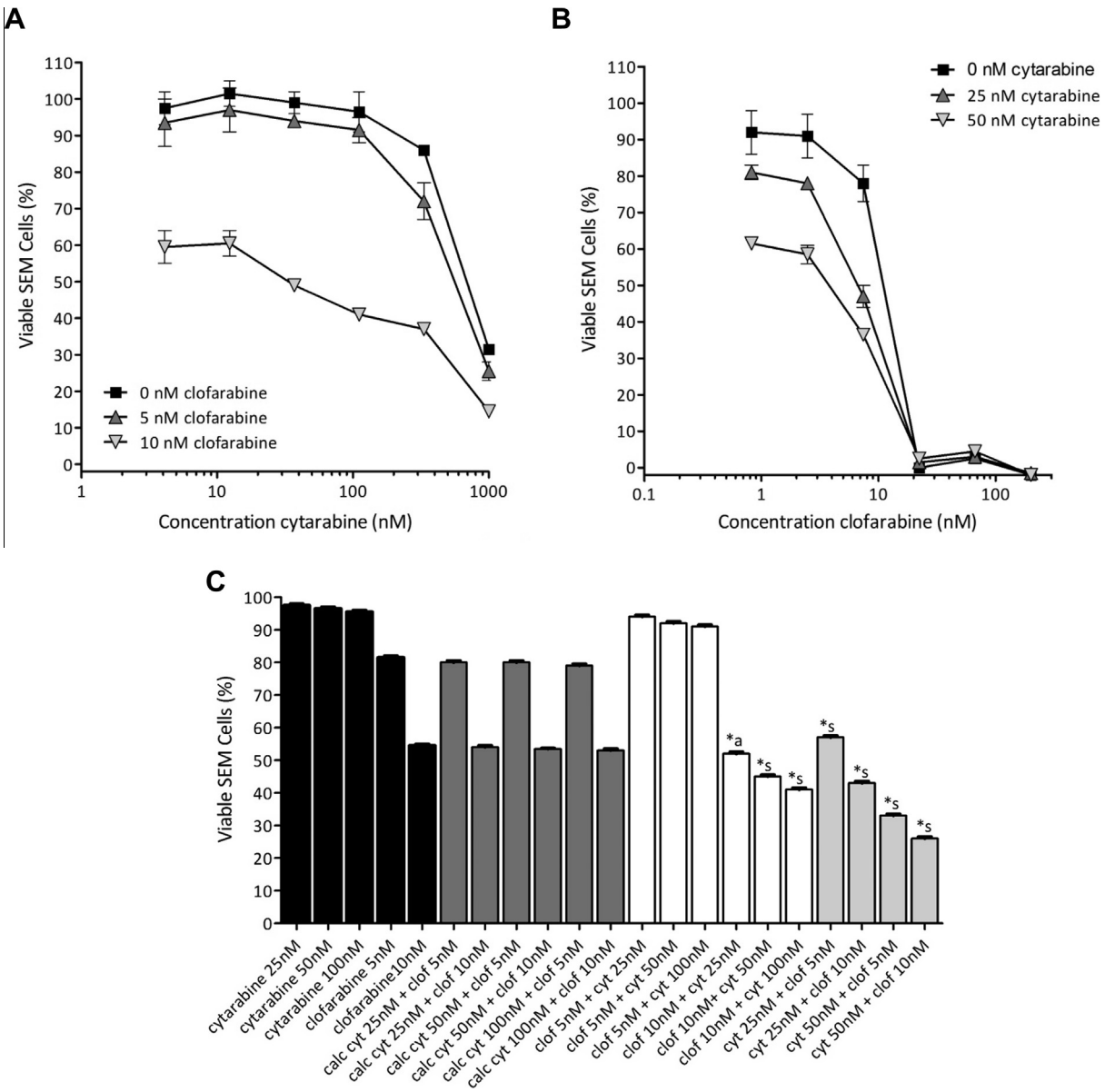

Fig. 4. Evaluation of synergy between cytarabine and clofarabine cytotoxicity in the cell line SEM. (A) The *MLL*-rearranged ALL cell line SEM was pre-incubated for 1 h in the absence or presence of either 5 nM or 10 nM clofarabine before initiating 4-day MTT assays to determine cytarabine cytotoxicity (clofarabine remained present throughout the entire experiment). (B) Likewise, SEM cells were pre-incubated for 1 h in the absence or presence of either 25 nM or 50 nM cytarabine before 4-day MTT assays were performed using low concentrations of clofarabine (cytarabine remained present throughout the entire experiment). (C) Shows the leukaemic cell viability after 4-day exposures to cytarabine and/or clofarabine. Black bars indicate SEM cell viabilities in response to cytarabine or clofarabine alone at indicated concentrations. Dark grey bars represent the hypothetical additive effects of the cytarabine and clofarabine combined as predicted from the actual exposures of these agents individually (see: black bars). White and light grey bars show the cell viabilities of the actual combined exposures of cytarabine and clofarabine tested simultaneously. Cytarabine is abbreviated as 'cyt', and clofarabine as 'clof'. Calculated is abbreviated as 'calc'. Additive effects are indicated by: *a, and synergistic effects are marked by: *s.

they covalently bind and trap DNMTs, thereby impairing the ability of the cell to methylate CpGs in consecutive cell cycles [12].

Depletion of cellular DNMT is therefore commonly used as a reliable read-out for demethylation [36]. Depletion of functional DNMT1 usually is already observed after 24 h of cell exposure to 0.5 μM of decitabine or 100 μM of zebularine (Fig. 5A and B). In contrast, the DNMT1 binding capacity of both agents appeared to be largely diminished at nanomolar concentrations (Fig. 5A and B). As clofarabine appeared effective at 5–10 nM, we assessed its ability to deplete DNMT1 at these concentrations. As shown in Fig. 5C, DNMT1 protein expression seems very

moderately reduced after 7–10 days, but certainly not to the extent that inhibition of methylation as a result of DNMT1 depletion may be expected.

Although direct DNMT1 depletion by clofarabine appears an unlikely mechanism by which this agent inhibits DNA methylation, clofarabine-induced demethylation remains possible through alternative actions. Therefore, we further investigated demethylation as a measure of re-expression of the *FHIT* gene, known to be silenced by promoter methylation in *MLL*-rearranged ALL cells [31], in response to clofarabine exposure. Using a quantitative bisulphite pyrosequencing assay specifically designed for the *FHIT* promoter CpG island, we quantitatively analysed the level of *FHIT* promoter CpG methylation in SEM cells in the absence and presence of decitabine (0.5 μM), zebularine (100 μM) and clofarabine (5 or 10 nM). As expected, both the demethylating agents decitabine and zebularine induced pronounced demethylation of three out of the five interrogated CpG loci within the *FHIT* promoter at concentrations of 0.5 μM and 100 μM respectively (Fig. 6A). At a concentration of 10 nM neither decitabine nor zebularine appeared capable of demethylating the *FHIT* promoter, although a modest and temporary response was observed for 10 nM of decitabine after 24 h of exposure (Fig. 6A). Interestingly, 5 nM or 10 nM of clofarabine induced demethylation of the same CpGs to a comparable extent as 0.5 μM decitabine and 100 μM zebularine, demonstrating that clofarabine indeed is capable of CpG demethylation at nanomolar concentrations (Fig. 6A).

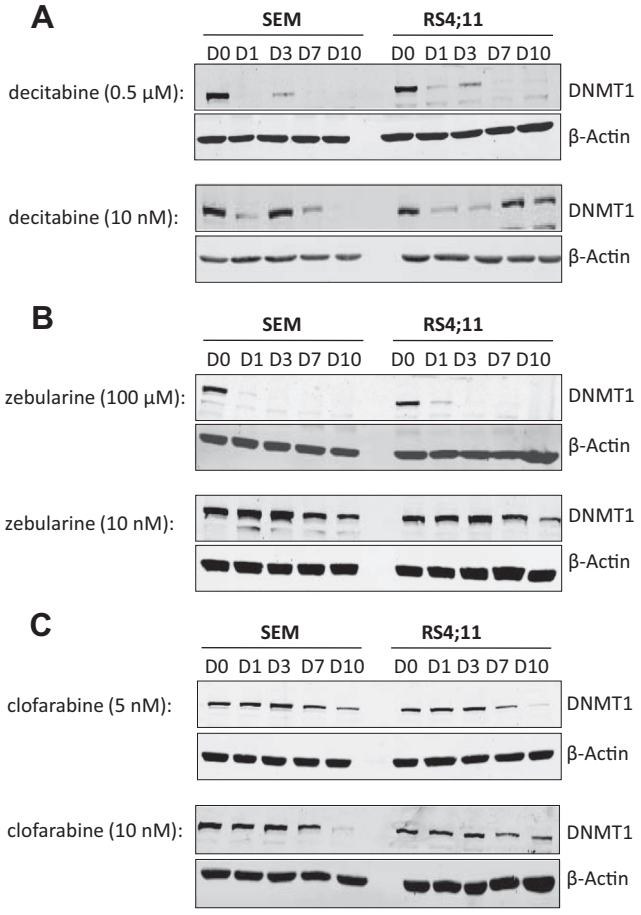

Fig. 5. Cellular DNMT1 depletion induced by nucleoside analogues. Western blot analysis demonstrating protein expression of DNMT1 in the cell lines SEM and RS4;11 in the absence or presence of the demethylating cytidine analogues (A) decitabine and (B) zebularine, and for the adenosine analogue (C) clofarabine at indicated concentrations and progressing exposure times (expressed in days). Beta-actin was used as a control for equal loading.

Next, we investigated whether demethylation of these *FHIT* promoter CpGs as induced by decitabine, zebularine and clofarabine also led to the re-activation of mRNA and protein expression. As shown in Fig. 6B, SEM cells exposed to 0.5 μM decitabine or 100 μM zebularine showed 3 to 4-fold increases in *FHIT* mRNA expression at day 7. Clofarabine (5 nM or 10 nM) showed a >2-fold increase in *FHIT* expression at the mRNA level (Fig. 6B). At the protein level, FHIT was most convincingly re-expressed in response to 0.5 μM decitabine and 100 μM zebularine. At nanomolar concentrations these agents did not show re-activation of FHIT protein expression. In contrast, 5 or 10 nM of clofarabine appeared induced subtle but detectable re-expression of FHIT protein (Fig. 6C).

4. Discussion

While the pathophysiology underlying *MLL*-rearranged infant ALL is slowly being unraveled, it remains the genetic subtype of ALL with the worst clinical outcome. We here showed that clofarabine, a next generation adenosine analogue, is capable of demethylating aberrant gene promoter methylation in *MLL*-rearranged infant ALL cells. This confirms earlier observations by Zhang et al. who demonstrated that clofarabine displays demethylating properties in lymphoma cells [22]. Inhibition of aberrant DNA methylation may become very important in the treatment for *MLL*-rearranged infant ALL, as we recently showed that the majority of these patients display abnormal genome-wide DNA methylation patterns [10]. While the clinical effectiveness of the more conventional demethylating cytosine analogues like decitabine and zebularine is not fully convincing [15,16], clofarabine may provide an alternative. Especially since we here show that primary *MLL*-rearranged infant ALL cells are highly sensitive to clofarabine *in vitro*, which evokes cell death in 50% of the leukaemic cells at concentrations as low as ~25 nM. At the same time, our present study showed that 5–10 nM of clofarabine seems sufficient to trigger gene promoter demethylation and re-expression of the affected gene. Although the demethylating effects of clofarabine appear modest in comparison with the demethylating agents decitabine and zebularine, it must

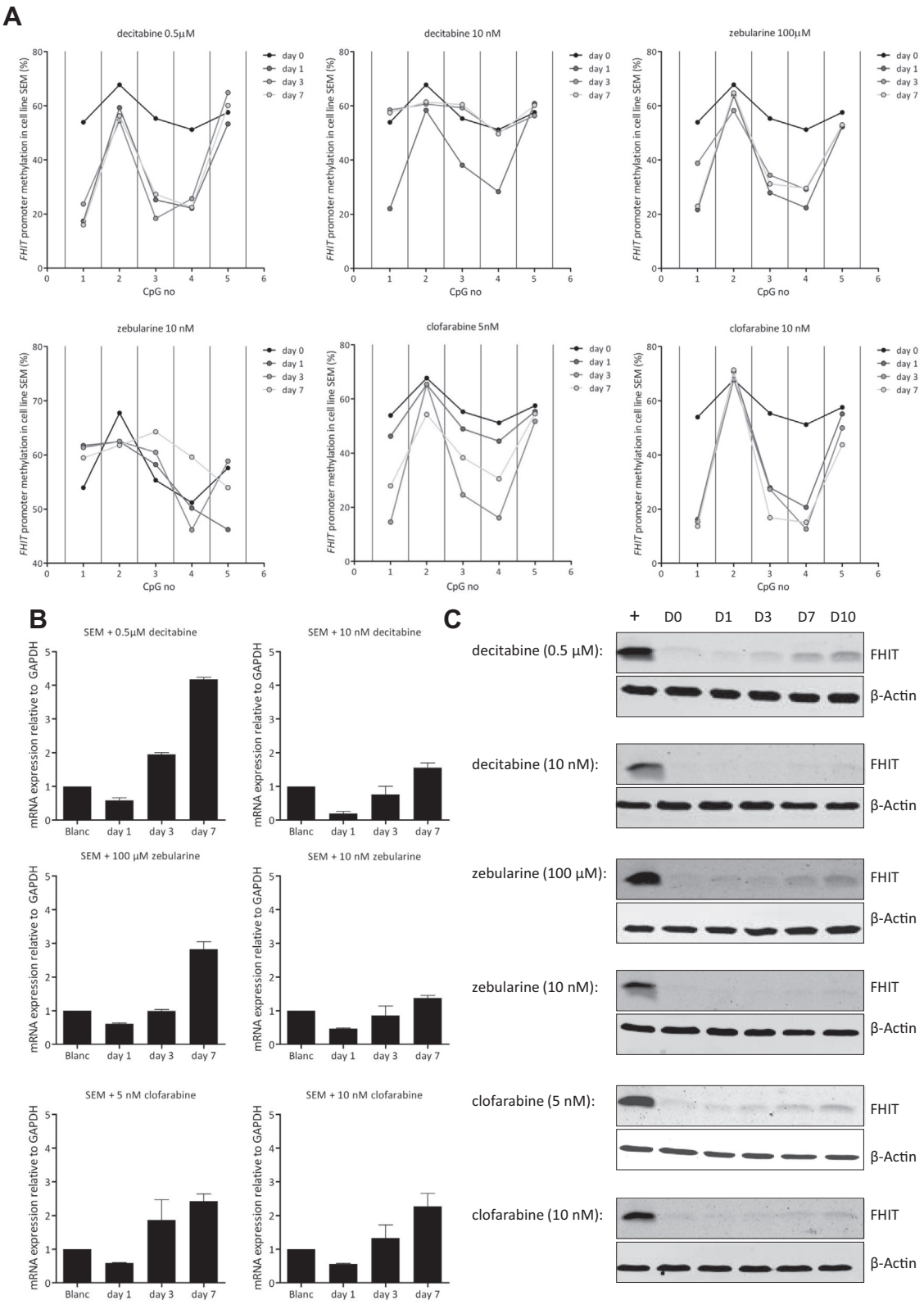

Fig. 6. Demethylation and re-expression of the *FHIT* gene and protein by nucleoside analogues. (A) Sensitivity of five individual CpGs within the *FHIT* gene promoter to demethylation by indicated nucleoside analogues at depicted concentrations as determined in the *MLL*-rearranged ALL cell line SEM. The progression of CpG demethylation was assessed at day 0 (untreated; black lines), at day 3 (dark grey lines) and day 7 (light grey lines). (B) Quantitative Real-time PCR analysis showing the relative mRNA expression levels of the *FHIT* gene in the cell line SEM before and after exposure to the different nucleoside analogues decitabine, zebularine and clofarabine at indicated concentrations and time points. (C) FHIT protein expression levels in the *MLL*-rearranged ALL cell line SEM in the absence or presence of decitabine, zebularine, or clofarabine at indicated concentrations and exposure periods. Beta-actin was used as a control for equal loading.

be noted that the performed experiments involved a single dosage of each agent. In order to be clinically effective, demethylating agents generally require the administration of multiple dosages over relatively long periods of time. Therefore, the demethylating properties of clofarabine may well be more pronounced at repeated or continued administrations. Nonetheless, whether demethylation by clofarabine would actually contribute to the remarkably good cytotoxic response as here observed in primary *MLL*-rearranged infant ALL cells *in vitro* remains to be confirmed. Yet, the sensitivity of this aggressive type of leukaemia to clofarabine is promising and encouraging in terms of clinical implementation of this drug in *MLL*-rearranged infant ALL treatment regimes. In fact, a recent study using clofarabine in heavily pretreated paediatric ALL patients, included five *MLL*-rearranged infant ALL cases of which three patients achieved complete remission on clofarabine treatment [23].

Another important finding in our study is the observation that clofarabine and cytarabine have a synergistic cytotoxic effect on *MLL*-rearranged ALL cells. These data are in concordance with previously published data on myeloid leukaemia cells [34] although the concentrations used in our study were significantly lower. From this point of view, addition of clofarabine to cytarabine-based treatment protocols may seem a highly attractive treatment option, especially since cytarabine has been successfully included in the treatment of infant ALL [3]. Addition of clofarabine, effectively targeting *MLL*-rearranged ALL cells as a single agent at nanomolar concentrations, may not only be very beneficial, but it may also allow the decrease of cytarabine dosages without losing its required cytotoxic effects. Clofarabine may also kill a portion of cells non-responsive to cytarabine [37]. In a clinical setting, the combined use of clofarabine and low-dose cytarabine has already proven effective in adult and paediatric leukaemias without *MLL* translocations [35,38].

Studies have revealed that, besides abnormal DNA methylation, *MLL*-rearranged leukaemias are linked to and sustained by aberrant activity of the histone methyltransferase DOT1L [39]. Preclinical studies using potent and selective inhibitors of DOT1L have demonstrated successful killing of the leukaemic clone in animal models [39]. The first clinical trial using a DOT1L inhibitor has been initiated. Interestingly, DOT1L inhibitors have shown to work synergistically with demethylating agents and with cytarabine in the killing of *MLL*-rearranged leukaemia cells [40]. No data exist on the combination of a DOT1L inhibitor and clofarabine. Most likely, a combination of drugs is needed to tackle the erroneous epigenetic landscape of *MLL*-rearranged infant ALL. We here demonstrate that clofarabine has significant cytotoxic effects on *MLL*-rearranged ALL cells from infants. In addition, it has demethylating effects on these cells. Pre-incubation and co-incubation with cytarabine induced synergistic cytotoxicity with clofarabine. We conclude that clofarabine, either as a single agent, or in combination with other promising drugs, is an interesting candidate for further studies in *MLL*-rearranged infant ALL.

Authorship contributions

D.J.P.M.S.: designed and performed research, and wrote the paper; P.S.: performed research; R.W.S. and R.P.: designed and guided research, and wrote the paper.

Financial disclosure

This study was financially supported by grants from the Sophia Foundation for Medical Research (SSWO grants 495 and 600). RWS was financially supported by the Dutch Cancer Society (EMCR 2005-2662). The institutions financially supporting this study had no role in study design, data collection, data analysis, data interpretation or writing of the report. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Conflict of interest statement

None declared.

Acknowledgments

The authors wish to express gratitude to the members and participating hospitals of the INTERFANT-99 study for supporting our research by providing leukaemic samples. Members of INTERFANT-99 include M. Campbell (Programa Infantil Nacional de Drogas Atineoplasicas), M. Felice (Argentina), A. Ferster (Children's Leukemia Group), I. Hann, and A. Vora (UK Children's Cancer Study Group); L. Hovi (Nordic Society of Paediatric Haematology and Oncology), G. Janka-Schaub (Cooperative Study Group for Treatment of ALL), C.K. Li (Hong Kong), G. Mann (Berlin-Frankfurt-Münster Group-Austria), T. LeBlanc (French ALL Group), R. Pieters (Dutch Childhood Oncology Group), G. de Rossi, and A. Biondi (Associazione Italiana Ematologia Oncologia Pediatrica); and J. Rubnitz (St Jude Children's Research Hospital), M. Schrappe (Berlin-Frankfurt-Münster Group-Germany), L. Silverman (Dana-Farber Cancer Institute), J. Stary (Czech Paediatric Haematology), R. Suppiah (Australian and New Zealand Children's Haematology/Oncology Group), T. Szczepanski (Polish Paediatric Leukemia and Lymphoma Study Group), M. Valsecchi, and P. de Lorenzo (Trial Operating Center).

References

[1] Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. Lancet 2008;371(9617):1030–43.

[2] Pieters R. Infant acute lymphoblastic leukemia: lessons learned and future directions. Curr Hematol Malig Rep 2009;4(3):167–74.

[3] Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, et al. A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. Lancet 2007;370(9583):240–50.

[4] Greaves MF. Infant leukaemia biology, aetiology and treatment. Leukemia 1996;10(2):372–7.

[5] Biondi A, Cimino G, Pieters R, Pui CH. Biological and therapeutic aspects of infant leukemia. Blood 2000;96(1):24–33.

[6] Pui CH, Kane JR, Crist WM. Biology and treatment of infant leukemias. Leukemia 1995;9(5):762–9.

[7] Pieters R, den Boer ML, Durian M, Janka G, Schmiegelow K, Kaspers GJ, et al. Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia–implications for treatment of infants. Leukemia 1998;12(9):1344–8.

[8] Ramakers-van Woerden NL, Beverloo HB, Veerman AJ, Camitta BM, Loonen AH, van Wering ER, et al. In vitro drug-resistance profile in infant acute lymphoblastic leukemia in relation to age, MLL rearrangements and immunophenotype. Leukemia 2004;18(3):521–9.

[9] Stam RW, den Boer ML, Meijerink JP, Ebus ME, Peters GJ, Noordhuis P, et al. Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. Blood 2003;101(4):1270–6.

[10] Stumpel DJ, Schneider P, van Roon EH, Boer JM, de Lorenzo P, Valsecchi MG, et al. Specific promoter methylation identifies different subgroups of MLL-rearranged infant acute lymphoblastic leukemia, influences clinical outcome, and provides therapeutic options. Blood 2009;114(27):5490–8.

[11] Schafer E, Irizarry R, Negi S, McIntyre E, Small D, Figueroa ME, et al. Promoter hypermethylation in MLL-r infant acute lymphoblastic leukemia: biology and therapeutic targeting. Blood 2010;115(23):4798–809.

[12] Lyko F, Brown R. DNA methyltransferase inhibitors and the development of epigenetic cancer therapies. J Natl Cancer Inst 2005;97(20):1498–506.

[13] Garcia-Manero G, Gore SD, Cogle C, Ward R, Shi T, Macbeth KJ, et al. Phase I study of oral azacitidine in myelodysplastic syndromes, chronic myelomonocytic leukemia, and acute myeloid leukemia. J Clin Oncol 2011;29(18):2521–7.

[14] Lubbert M, Suciu S, Baila L, Ruter BH, Platzbecker U, Giagounidis A, et al. Low-dose decitabine versus best supportive care in elderly patients with intermediate- or high-risk myelodysplastic syndrome (MDS) ineligible for intensive chemotherapy: final results of the randomized phase III study of the European Organisation for Research and Treatment of Cancer Leukemia Group and the German MDS Study Group. J Clin Oncol 2011;29(15):1987–96.

[15] Kadia TM, Jabbour E, Kantarjian H. Failure of hypomethylating agent-based therapy in myelodysplastic syndromes. Semin Oncol 2011;38(5):682–92.

[16] Blum KA, Liu Z, Lucas DM, Chen P, Xie Z, Baiocchi R, et al. Phase I trial of low dose decitabine targeting DNA hypermethylation in patients with chronic lymphocytic leukaemia and non-Hodgkin lymphoma: dose-limiting myelosuppression without evidence of DNA hypomethylation. Br J Haematol 2010;150(2):189–95.

[17] Hoeksema KA, Jayanthan A, Cooper T, Gore L, Trippett T, Boklan J, et al. Systematic in-vitro evaluation of the NCI/NIH Developmental Therapeutics Program Approved Oncology Drug Set for the identification of a candidate drug repertoire for MLL-rearranged leukemia. Onco Targets Ther 2011;4:149–68.

[18] Wyczechowska D, Fabianowska-Majewska K. The effects of cladribine and fludarabine on DNA methylation in K562 cells. Biochem Pharmacol 2003;65(2):219–25.

[19] Bonate PL, Arthaud L, Cantrell Jr WR, Stephenson K, Secrist 3rd JA, Weitman S. Discovery and development of clofarabine: a nucleoside analogue for treating cancer. Nat Rev Drug Discov 2006;5(10):855–63.

[20] Lubecka-Pietruszewska K, Kaufman-Szymczyk A, Stefanska B, Cebula-Obrzut B, Smolewski P, Fabianowska-Majewska K. Clofarabine, a novel adenosine analogue, reactivates DNA methylation-silenced tumour suppressor genes and inhibits cell growth in breast cancer cells. Eur J Pharmacol 2014;15(723):276–87.

[21] Majda K, Kaufman-Szymczyk A, Lubecka-Pietruszewska K, Bednarek A, Fabianowska-Majewska K. Influence of clofarabine on transcriptional activity of PTEN, APC, RARB2, ZAP70 genes in K562 cells. Anticancer Res 2010;30(11):4601–6.

[22] Jeha S, Gaynon PS, Razzouk BI, Franklin J, Kadota R, Shen V, et al. Phase II study of clofarabine in pediatric patients with refractory or relapsed acute lymphoblastic leukemia. J Clin Oncol 2006;24(12):1917–23.

[23] O'Connor D, Sibson K, Caswell M, Connor P, Cummins M, Mitchell C, et al. Early UK experience in the use of clofarabine in the treatment of relapsed and refractory paediatric acute lymphoblastic leukaemia. Br J Haematol 2011;154(4):482–5.

[24] Shukla N, Kobos R, Renaud T, Steinherz LJ, Steinherz PG. Phase II trial of clofarabine with topotecan, vinorelbine, and thiotepa in pediatric patients with relapsed or refractory acute leukemia. Pediatr Blood Cancer 2014;61(3):431–5.

[25] Kaspers GJ, Veerman AJ, Pieters R, Broekema GJ, Huismans DR, Kazemier KM, et al. Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. Br J Cancer 1994;70(6):1047–52.

[26] Greil J, Gramatzki M, Burger R, Marschalek R, Peltner M, Trautmann U, et al. The acute lymphoblastic leukaemia cell line SEM with *t*(4;11) chromosomal rearrangement is biphenotypic and responsive to interleukin-7. Br J Haematol 1994;86(2):275–83.

[27] Stong RC, Korsmeyer SJ, Parkin JL, Arthur DC, Kersey JH. Human acute leukemia cell line with the *t*(4;11) chromosomal rearrangement exhibits B lineage and monocytic characteristics. Blood 1985;65(1):21–31.

[28] Pieters R, Loonen AH, Huismans DR, Broekema GJ, Dirven MW, Heyenbrok MW, et al. In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. Blood 1990;76(11):2327–36.

[29] Tost J, Gut IG. DNA methylation analysis by pyrosequencing. Nat Protoc 2007;2(9):2265–75.

[30] Stam RW, Schneider P, Hagelstein JA, van der Linden MH, Stumpel DJ, de Menezes RX, et al. Gene expression profiling-based dissection of MLL translocated and MLL germline acute lymphoblastic leukemia in infants. Blood 2010;115(14):2835–44.

[31] Stam RW, den Boer ML, Passier MM, Janka-Schaub GE, Sallan SE, Armstrong SA, et al. Silencing of the tumor suppressor gene FHIT is highly characteristic for MLL gene rearranged infant acute lymphoblastic leukemia. Leukemia 2006;20(2):264–71.

[32] de Vries JF, Falkenburg JH, Willemze R, Barge RM. The mechanisms of Ara-C-induced apoptosis of resting B-chronic lymphocytic leukemia cells. Haematologica 2006;91(7):912–9.

[33] Gati WP, Paterson AR, Larratt LM, Turner AR, Belch AR. Sensitivity of acute leukemia cells to cytarabine is a correlate of

cellular es nucleoside transporter site content measured by flow cytometry with SAENTA-fluorescein. Blood 1997;90(1):346–53.

[34] Cooper T, Ayres M, Nowak B, Gandhi V. Biochemical modulation of cytarabine triphosphate by clofarabine. Cancer Chemother Pharmacol 2005;55(4):361–8.

[35] Faderl S, Ravandi F, Huang X, Garcia-Manero G, Ferrajoli A, Estrov Z, et al. A randomized study of clofarabine versus clofarabine plus low-dose cytarabine as front-line therapy for patients aged 60 years and older with acute myeloid leukemia and high-risk myelodysplastic syndrome. Blood 2008;112(5):1638–45.

[36] Marquez VE, Barchi Jr JJ, Kelley JA, Rao KV, Agbaria R, Ben-Kasus T, et al. Zebularine: a unique molecule for an epigenetically based strategy in cancer chemotherapy. The magic of its chemistry and biology. Nucleosides Nucleotides Nucleic Acids 2005;24(5–7):305–18.

[37] Yamauchi T, Uzui K, Nishi R, Shigemi H, Ueda T. Cytarabine-resistant leukemia cells are moderately sensitive to clofarabine *in vitro*. Anticancer Res 2014;34(4):1657–62.

[38] Moreno L, Fernandez-Navarro JM, Del Mar Andres M, Bautista F, Tasso M, Verdeguer A. Institutional experience with clofarabine and cytarabine in relapsed pediatric acute myeloid leukemia. J Pediatr Hematol Oncol 2012;34(1):e17–21.

[39] Stein EM, Tallman MS. Mixed lineage rearranged leukaemia: pathogenesis and targeting DOT1L. Curr Opin Hematol 2015;22(2):92–6.

[40] Klaus CR, Iwanowicz D, Johnston D, Campbell CA, Smith JJ, Moyer MP, et al. DOT1L inhibitor EPZ-5676 displays synergistic antiproliferative activity in combination with standard of care drugs and hypomethylating agents in MLL-rearranged leukemia cells. J Pharmacol Exp Ther 2014;350(3):646–56.