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

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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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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