

## Zebularine-Resistant Human Cytidine Deaminase Mutants for Optimal Chemoprotection of Hematopoietic Stem Cells

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### Introduction

Acute myeloid leukemia (AML) is the most common type of acute leukemia. According to the National Cancer Institute, the new cases and deaths from AML in the United States in 2016 are estimated to be approximately 19,990 and 13,400, respectively, with a 5 year survival rate of only about 26.6% (<http://seer.cancer.gov/statfacts/html/amyl.html>). Thus, there is an urgent need to find more effective ways to improve the clinical treatment of AML.

### Inactivation of chemotherapeutic drugs by cytidine deaminase

AML progresses quickly and the main treatment of AML is chemotherapy and hematopoietic stem cell (HSC)/bone marrow transplant, which puts patients at high risk of relapse or resistance. Cytarabine (cytosine arabinoside or Ara-C) is the most effective single chemotherapeutic agent in the treatment of AML and also shows considerable activity in acute lymphocytic leukemia and non-Hodgkin's lymphoma. However, some patients develop drug resistance and/or profound hematopoietic toxicity after Ara-C treatment, limiting its clinical application. Drug resistance may be mediated by several factors, including low transporter levels and low deoxycytidine kinase (dCK) activity [1]. Furthermore, high human cytidine deaminase (hCDA) activity has been suggested to play an important role not only in drug resistance but also to protect cells from drug-mediated cytotoxicity (myeloprotection) [1,2]. Human CDA is an evolutionarily conserved enzyme involved in the pyrimidine salvage pathway and catalyzes the deamination of cytidine and deoxycytidine to uridine and deoxyuridine, respectively. Importantly, hCDA is also able to deaminate and thereby inactivate a number of clinically used antitumor cytidine nucleoside analogs such Ara-C, decitabine, gemcitabine and azacytidine, resulting in a complete loss of antineoplastic activity. Thus, the cytotoxic efficacy of these cytidine analogs is strongly associated with the level of cellular hCDA.

Human CDA is widely distributed in tumor cells and normal tissues, such as liver, spleen, intestinal mucosa and mature granulocytes, with particularly high levels in liver and spleen, which can rapidly degrade Ara-C and subsequently lead to the development of drug resistance. Ebrahim et al. [3] reported that high hCDA expression in the liver provides protection or sanctuary for cancer cells from decitabine treatment effects. This could explain why some cytidine analogs have greater antitumor efficacy *in vitro* but limited effects *in vivo*, especially in liver cancer cells. Further evidence that high hCDA activity negatively impacts therapeutic outcome was found in leukemic cells, especially in those from patients who have relapsed after Ara-C therapy [1,2]. Mahfouz et al. [4] also found that increased hCDA expression contributes to decreased cytidine analog half-life and likely also to poor outcomes following cytidine analog therapy. In order to neutralize the diminished treatment effect from hCDA activity, one option is to

administer higher doses of Ara-C, which may cause more hematologic toxicity. Another option is to use potent hCDA inhibitors to block hCDA activity. Studies have shown the combined use of potent hCDA inhibitors such as zebularine or tetrahydrouridine with decitabine (a clinically used cytidine analog) can effectively reduce decitabine inactivation by hCDA and significantly enhance antineoplastic action in murine and human leukemic cells [3,5]. Zebularine is among the hCDA inhibitors used clinically and has the additional function as a DNA demethylating agent and exhibits high stability and minimal cytotoxicity *in vitro* and *in vivo*, becoming a highly attractive agent for use with Ara-C [6].

### Cytidine deaminase mediated HSC myeloprotection strategies

In bone marrow transplantations, because HSCs contain low levels of hCDA, long-term or intensive doses of Ara-C may cause severe hematopoietic toxicity and life-threatening myelosuppression. Overexpression of drug resistance genes in HSCs has become a promising approach to overcome this hematologic toxicity. Several chemotherapy drug resistance genes such as human O<sup>6</sup>-methylguanine-DNA-methyltransferase, multidrug resistance 1 (MDR-1) and hCDA have demonstrated myeloprotective potential *in vitro* and *in vivo* [2,7,8]. Human CDA represents the most relevant drug inactivating gene for myeloprotection in the context of acute leukemia or myelodysplasia therapy. The hCDA overexpression approach has several advantages including non-immunogenicity, small size (438 bp) to facilitate genetic manipulation and delivery, and the ability to confer high levels of resistance to cytidine nucleoside analogs with low toxic and mutagenic potential. The significant protection from toxicity of several cytidine analogs of cells overexpressing hCDA has been demonstrated in murine and human HSCs *in vitro* and *in vivo* in murine transplant models [2,8]. Overexpression of hCDA has the added benefit of supporting *in vivo* enrichment of transduced HSCs under prolonged Ara-C application [9].

Tandem transgenic HSCs transplantation and combination therapy of zebularine and Ara-C may provide robust antileukemic potency due to the increased production of Ara-CTP, the cytotoxic agent, however, transplantation failure during combination therapy can occur because zebularine also inhibits hCDA activity in the transgenic HSCs overexpressing hCDA and prevents inactivation of Ara-C, thereby

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restoring sensitivity to Ara-C. However, if HSCs express a modified zebularine-resistant hCDA that restricts the ability of zebularine to block hCDA activity, Ara-C can be rapidly deaminated to its inactive form (Ara-U), and protect transfected stem cells from the cytotoxic action of Ara-C. In a recent paper, we and colleagues described the creation of unique zebularine-resistant and Ara-C active hCDA mutants [10]. Our approach used a combination of protein engineering strategies (random mutagenesis strategy of error-prone PCR and DNA shuffling) and an *E. coli* genetic complementation system to select for zebularine resistant, functional hCDA mutants. Nine variant hCDAs were identified from a library of over a million transformants and further characterized. CDA deficient *E. coli* harboring hCDA-mutants in the presence of cytidine containing selection medium cultures grew at rates indistinguishable from *E. coli* expressing wild-type hCDA, however, in the presence of zebularine, the nine mutant hCDA expressing *E. coli* strains grew robustly whereas growth of the wild-type hCDA expressing strain was significantly impaired. Results from enzyme assays using purified wild-type and mutant enzymes with either deoxycytidine or Ara-C as substrate revealed that all mutants retained activity towards these substrates albeit reduced to varying degrees from wild-type hCDA activities. Further kinetic parameter determinations were made on three variant and the wild-type hCDA enzymes. Those assays revealed the mutants displayed two log orders of decreased sensitivity to zebularine in the presence of Ara-C as compared to wild-type hCDA further supporting the idea that they will confer resistance to zebularine in the context of gene modified HSC transplantations when Ara-C is used and thus provide a level myeloprotection that is currently not achievable.

## Conclusion

Acute myeloid leukemia has a 5 year survival rate of only 26.6% (<http://seer.cancer.gov/statfacts/html/amyl.html>). One key problem associated with the use of Ara-C (alone or in combination with HSC transplantations) is its inactivation by cytidine deaminase, which leads to myelosuppression and diminished therapeutic outcome. Various strategies are being explored to enhance myeloprotection during prolonged Ara-C treatment including overexpression of hCDA to enrich HSCs and to reduce Ara-C toxicity of HSCs. Our approach was to use protein engineering to create hCDA variants that retain activity towards Ara-C (needed to inactivate the drug) but are resistant to the hCDA inhibitor zebularine. Such variants should allow Ara-C mediated cytotoxicity of leukemic cells while providing transgenic HSCs protection from the toxic effects of Ara -C and with the additional

benefit of selective pressure to enhance engraftment. From over a million transformants, nine mutants were identified with desirable characteristics. Further biochemical analyses of three select mutants revealed that, compared to wild-type hCDA, these novel mutants retain hCDA deaminase activity towards Ara-C and confer resistance to zebularine at clinically relevant doses. As such, these mutants may be highly beneficial in clinical applications using gene-modified HSCs transplantation to enhance chemoprotection of HSCs during intensive Ara-C and zebularine combination chemotherapy. Our results indicate that these Ara-C active/zebularine resistant mutant hCDAs warrant further exploration *in vitro* and *in vivo* in appropriate cells and animal models for AML and other diseases states where HSC transplantations are beneficial.

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