Novel L-nucleoside analog, 5-fluorotroxacitabine, displays potent efficacy against acute myeloid leukemia

Cytarabine (Ara-C) is a nucleotide analog and a cornerstone of standard chemotherapy in acute myeloid leukemia (AML).\(^1\) Ara-C is phosphorylated to its active metabolite Ara-CTP where it becomes incorporated into replicating DNA strands. Ara-CTP is deaminated to an inactive uridine metabolite by cytidine deaminase (CDA) and CDA overexpression is a clinically relevant mechanism of resistance to Ara-C.\(^2\) Here, we report the preclinical efficacy of a novel L-nucleoside analog 5-fluorotroxacitabine (5FTRX) in AML (Figure 1A).

In a panel of 31 leukemia and lymphoma cell lines, 5FTRX reduced growth and viability with a median IC\(_{50}\) of 200 nM (range 12 nM [EOL-1] to 6 \(\mu\)M [TF-1]) (Figure 1B and Online Supplementary Figure S1). 5FTRX also reduced the clonogenic growth of primary AML cells (Figure 1C; see Online Supplementary Tables S1 and S2 for patients' characteristics), thus demonstrating an ability to target leukemic progenitors. In contrast, normal hematopoietic cells were more resistant to 5FTRX (Figure 1D).

5FTRX is phosphorylated via monophosphate (MP) and diphosphate (DP) to the triphosphate (TP) before being incorporated into nascent DNA strands, leading to subsequent chain termination and cell death. AML cell lines MV4-11 and THP-1 or human peripheral blood mononuclear cells (PBMC) were incubated with 5FTRX and analyzed for the intracellular concentrations of 5FTRX and phosphorylated nucleotides (Online Supplementary Table S3). Detectible concentrations of MP, DP and TP were observed in PBMC and AML cells confirming bioactivation to the active TP in cells.

To test whether 5FTRX damaged DNA in AML cells, we measured changes in phosphorylated H2AX (pH2AX) after 5FTRX treatment. 5FTRX increased pH2AX at concentrations associated with loss of viability (Online Supplementary Figure S2), indicating a likely chain termination during the proliferation of these cells.

CDA overexpression is a mechanism of resistance to Ara-C.\(^3\) We investigated the effects of increased CDA on the anti-neoplastic action of 5FTRX. Wild-type HEK293 and HEK293 cells over-expressing CDA were treated with increasing concentrations of 5FTRX and Ara-C (Figure 2A and Online Supplementary Figure S3A). As expected, overexpression of CDA rendered cells approximately 7-fold more resistant to Ara-C (Figure 2B). Treatment of these cells with the CDA inhibitor tetrahydrouridine (THU) restored sensitivity to Ara-C (Online Supplementary Figure S3B). Interestingly, overexpression of CDA increased sensitivity to 5FTRX by approximately 10-fold (Figure 2C). Further, co-treatment with 5FTRX and THU returned sensitivity of the cells to 5FTRX towards baseline (Online Supplementary Figure S3C). Increased CDA expression did not change the growth rate of these cells (Online Supplementary Figure S4). Potentially, overexpression of CDA increased degradation of endogenous cytidine nucleotide pools enhancing the incorporation of 5FTRX-monophosphate into DNA. Of note, prior studies have reported that increased CDA expression increases the sensitivity of cells to troxacitabine.\(^4\)

5FTRX overcomes drug resistance induced by CDA overexpression, but CDA expression did not fully explain 5FTRX sensitivity in AML cells (Online Supplementary Figure S5A and B), indicating additional mechanisms of resistance and sensitivity. Therefore, we sought to understand potential mechanisms of resistance to 5FTRX. We selected populations of THP-1 cells resistant to 5FTRX (THP1-5FR: 66-fold resistant to 5FTRX) and Ara-C (THP1-ACR; 35-fold resistant to Ara-C) by treating parental THP-1 cells with increasing concentrations of the drugs for up to 4 months (Figure 2D). THP1-5FR and THP1-ACR were cross-resistant to Ara-C and 5FTRX, respectively, suggesting that similar mechanisms were responsible for conferring resistance. Downregulation of dCK is known to confer resistance to Ara-C,\(^5\) so we examined dCK expression in the resistant cell lines. Indeed, dCK levels were reduced in THP1-5FR and THP1-ACR resistant cell populations (Figure 2E). To test whether the reduced dCK was functionally important for resistance to 5FTRX, we analyzed the metabolites of 5FTRX in THP1-5FR cells. THP1-5FR cells treated with 5FTRX showed decreased phosphorylated species, consistent with a reduced ability of dCK to generate the monophosphate species (Figure 2F). Moreover, the reported dCK inhibitor 15C restored THP-1 cells resistant to 5FTRX and Ara-C with IC\(_{50}\) values >50 \(\mu\)M (Figure 2G).

To assess the future potential for 5FTRX to be included in standard treatment regimens for AML treatment, the anti-proliferative effects of 5FTRX were examined in combination with Ara-C, azacytidine or doxorubicin in MV4-11 and THP-1 cells. In both cell types, 5FTRX exhibited strong synergy in combination with azacytidine or doxorubicin. The combination of 5FTRX and Ara-C was additive in MV4-11 cells, whereas in THP-1 cells, a moderately synergistic interaction was observed (Online Supplementary Table S5 and Online Supplementary Figure S6A-F).

Finally, we examined the preclinical efficacy and toxicity of 5FTRX in mouse models of leukemia. In mice xenografted with MV4-11 cells, 5FTRX produced dramatic and sustained tumor regressions and showed a marked superiority over Ara-C at its maximum tolerated dose (Figure 3A) without evidence of toxicity or body weight loss (Online Supplementary Figure S7). Furthermore, we assessed the activity of 5FTRX at a reduced frequency of treatment (qd x5 instead of BID x5) in vivo using MV4-11 cells. Sustained tumor regressions were observed at all doses (Figure 3C) without loss of body weight (Online Supplementary Figure S8). The OCI-AML2 model displayed even more sensitivity to 5FTRX, with tumor regression observed at both 100 mg/kg and 80 mg/kg doses. Mice remained tumor free for 45 days after termination of treatment (Figure 3B) and did not display any signs of toxicity (Online Supplementary Figures S9-S11).

We determined the optimal treatment schedule by delivering a total dose of 150 mg/kg at different time points: a single dose of 150 mg/kg; three daily doses of 50 mg/kg; or five daily doses of 30 mg/kg. All regimens led to tumor regressions, although tumor regrowth varied between doses (Figure 3D). The maximal response was observed at five daily doses of 30 mg/kg. To test whether the tumors remained sensitive to the drug, tumors were allowed to regrow to approximately 1,000 mm\(^3\) from the 5x30 mg/kg dose group, and then challenged with the same schedule of 5FTRX treatment. Despite the larger size of these tumors at this re-treatment stage, they still showed pronounced regression (Figure 3D), confirming they remained sensitive to the agent.
Figure 1. 5-fluorotroxacitabine (5FTRX) reduces cell growth and viability in hematologic cell lines and primary acute myeloid leukemia (AML) samples. (A) The structure of 5FTRX and its bio-activation by phosphorylation to the monophosphate (MP), diphosphate (DP), and triphosphate (TP). The enzymes known to catalyze these phosphorylation reactions are indicated. DCK: deoxycytidine kinase; CMPK: cytidine monophosphate (CMP) kinase; PGK: phosphoglycerate kinase. (B) Hematologic cell lines were treated with increasing concentrations of 5FTRX for 96 hours (h). After incubation, cell growth and viability were measured by Cell Titer-Glo assay. Black bars indicate the myeloid cell lines; gray bars represent lymphoid cell lines. Data represent IC50, mean±standard deviation (SD). (C) Primary AML cells were treated with increasing concentrations of 5FTRX for 24 h and then plated into clonogenic growth assays. Data represent the number of colonies: mean±SD. (D) Peripheral blood stem cell (PBSC) were treated with increasing concentrations of 5FTRX or control for 24 h and then plated into clonogenic growth assays. Data represent the number of colonies: mean±SD. For all experiments, P-value was calculated using Student’s t-test; P<0.05 was considered significant.
Figure 2. Resistance mechanisms to 5-fluorotrexacitabine (5FTRX). (A) HEK293 cells were transduced with cDNA encoding cytidine deaminase (CDA) or vector control. Levels of CDA were measured by immunoblotting. (B and C) HEK293 cells over-expressing CDA or vector control were treated with increasing concentrations of cytarabine (Ara-C) (B) or 5FTRX (C) for 72 hours (h). After incubation, cell growth and viability were measured by MTS assay. Significance of difference in mean IC₅₀ values for control and CDA⁺ cell line was calculated using Student’s t-test. (D) THP-1 cells resistant to 5FTRX (THP-1-5FR) and Ara-C (THP-1-ACR) were selected. Data represent the IC₅₀ in μM for Ara-C and 5FTRX in the parental and resistant cell populations. (E) Levels of deoxycytidine kinase (DCK) were measured by immunoblotting in the parental and resistant THP-1 cells. (F) THP-1 parental and resistant cells (THP1-5FR) were treated with 10 μM 5FTRX for 24 h. After incubation, levels of intracellular metabolites (5FTRX monophosphate [MP], 5FTRX diphosphate [DP], 5FTRX triphosphate [TP]) were measured by liquid chromatography-mass spectrometry (LC-MS). (G) IC₅₀ (μM) of gemcitabine, Ara-C and 5FTRX alone or in combination with 50 μM dCK inhibitor (dCKi 15c7) in THP-1 cells.
In order to understand the strong anti-tumor activity and mechanism of action of 5FTRX in vivo, pharmacokinetic and pharmacodynamic studies were undertaken in the MV4-11 model. Mice bearing subcutaneous tumor xenografts were treated with control or 5FTRX twice daily for 5 days and were sacrificed 2 hours after the last dose. Two hours prior to analysis the mice received BrdU and pimonidazole (i.p.) to label S-phase cells and hypoxic tumor regions. Proliferating BrdU positive cells were significantly decreased in a dose-dependent manner upon treatment with 5FTRX (Online Supplementary Figure S12A). A concomitant increase in necrosis as assessed by the reduction in nuclear density was observed (Online Supplementary Figure S12B). Widespread elevation of DNA damage in tumor nuclei throughout the tumors was observed by measuring pH2AX levels at all doses (Online Supplementary Figure S12C), indicating double-strand breaks as a result of incorporation of chain terminating 5FTRX nucleotide. A reduction in %pH2AX-positive nuclei were observed in the 100 mg/kg group compared to the lower doses of 5FTRX, but this finding may reflect the extensive necro-

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**Table 1.**

| Group  | Dose (mg/kg) | Dosing schedule | TG1 (d32) | Regression d32 | TGD (at RTV4) |
|--------|--------------|-----------------|-----------|----------------|----------------|
| 5FTRX  | 100          | BID x 5 d       | 108%      | 85%            | 38d            |
| Ara-C  | 60           | qDx5/7dx2 wks   | 20%       | -              | 2d             |

**Figure S12A.** A concomitant increase in necrosis as assessed by the reduction in nuclear density was observed (Online Supplementary Figure S12B). Widespread elevation of DNA damage in tumor nuclei throughout the tumors was observed by measuring pH2AX levels at all doses (Online Supplementary Figure S12C), indicating double-strand breaks as a result of incorporation of chain terminating 5FTRX nucleotide. A reduction in %pH2AX-positive nuclei were observed in the 100 mg/kg group compared to the lower doses of 5FTRX, but this finding may reflect the extensive necro-

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**Figure 3.** 5-fluorotroxacitabine (5FTRX) targets primary acute myeloid leukemia (AML) cells. (A) MV4-11 cells were xenografted into NOD/SCID mice. Once tumors were approximately 200 mm³, mice were treated with 5FTRX (100 mg/kg BID x 5 days [d]) or cytarabine (Ara-C) (60 mg/kg qD x 5/7 x 2 weeks). Tumor volume was measured over time. Tumor growth inhibition (TGI%) defined as (C1-T1)/(C0-T0) x 100 where C1 and T1 are the mean tumor volumes of control and 5FTRX or Ara-C treated groups at time of tumor extraction, while C0 and T0 are the mean tumor volumes at the start of treatment. Percent regression calculated using: 100 x DT/DC where DT = T1-T0 and DC = C1-C0. Tumor growth delay (TGD) represents anti-tumor efficacy defined as the time taken for tumors to quadruple in size (RTV4). (B) OCI-AML2 cells were xenografted into SCID mice. Once tumors were approximately 100 mm³, mice were treated with 5FTRX (30 or 100 mg/kg x 5 days). Tumor volume was measured over time. T/C represents the ratio of mean tumor volume for 5FTRX treated group versus the mean tumor volume for the control group. (C) MV4-11 cells were xenografted into NOD/SCID mice. Once tumors were approximately 200 mm³, mice were treated with increasing amounts of 5FTRX. Tumor volume was measured over time. (D) MV4-11 cells were xenografted into NOD/SCID mice. Once tumors were approximately 200 mm³, mice were treated with increasing amounts of 5FTRX on days 1-4. Mice treated with 30 mg/kg received a second treatment with 5FTRX 10 mg/kg on days 22 to 26. Tumor volume was measured over time.
sis and profound anti-tumor effect induced by this dose.

To assess changes in 5FTRX metabolites in vivo, mice bearing MV4-11 tumors were treated with 5FTRX and levels of 5FTRX and metabolites were measured in plasma and tumor samples over time. Approximately linear increases in plasma and tumor exposures were observed with increasing doses (Online Supplementary Figure S13A and B). 5FTRX MP, DP and TP exposures were observed in the tumor samples, indicating phosphorylation of the nucleoside within the tumor. High levels of TP were maintained within the tumor for a longer duration than plasma, consistent with a long half-life of this metabolite, and retention within the tumor, as expected from a charged metabolite (Online Supplementary Figure S13C).

Finally, we assessed whether 5FTRX can target primary AML cells and stem cells in vivo. NOD/SCID mice were injected intra-femorally with primary AML cells (Online Supplementary Table S1). Eight days after injection of the primary cells, mice were treated with 5FTRX 100 mg/kg daily for 5 days. Four to six weeks after the initial injection of cells, mice were sacrificed, and the percentage of human myeloid cells defined as CD45+CD19–CD33+ was quantified by flow cytometry in the mouse marrow. 5FTRX reduced primary AML engraftment >95% without toxicity (P<0.0001, Student’s t-test) (Figure 4A and Online Supplementary Table S4). Moreover, we assessed the effects of 5FTRX on the leukemic stem cells (LSC) by evaluating secondary engraftment. Primary AML cells harvested from bone marrow of 5FTRX treated mice were engrafted into secondary untreated mice and human leukemic cell engraftment was assessed after 5 weeks. 5FTRX significantly reduced leukemic engraftment (P<0.001, Student’s t-test) in secondary transplants, demonstrating an effect of the drug on AML stem cells (Figure 4B).

Thus, in summary, this study demonstrated that the L-nucleoside analog, 5FTRX, has a number of favorable properties for a potential new treatment for AML. 5FTRX has a potent and broad-ranging anti-tumor activity against a range of AML and other leukemic cell lines, which is maintained against primary patient AML cells. 5FTRX targets patient-derived AML blasts and stem cells in physiologically relevant intra-femoral engraftment models in vivo. 5FTRX overcomes CDA overexpression which is a known mechanism of Ara-C resistance. 5FTRX has a strong anti-tumor activity in vivo that can lead to durable tumor regressions at well-tolerated doses. 5FTRX is synergistic in combination with doxorubicin and azacitidine which could be good combination agents for clinical evaluation. Thus, 5FTRX warrants further preclinical study as a novel agent for patients with refractory AML and increased CDA expression.
analyzed data and supervised research; AB, ADS, PTA and MA wrote the paper; all authors reviewed and edited the paper; 5FTRX was developed by Medivir AB, Sweden, which has interests in nucleotide/nucleoside science to develop pharmaceuticals that meet unmet medical needs.

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