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.[2-4] 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 12nM [EOL-1] to 6 μM [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.[2-5] 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.[6,7]

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,[6] 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[7] rendered THP-1 cells resistant to 5FTRX and Ara-C with IC_{50} values >50 μM (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*). Furthemore, we assessed the activity of 5FTRX at a reduced frequency of treatment (qD x5 instead of BIDx5) 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 30 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.

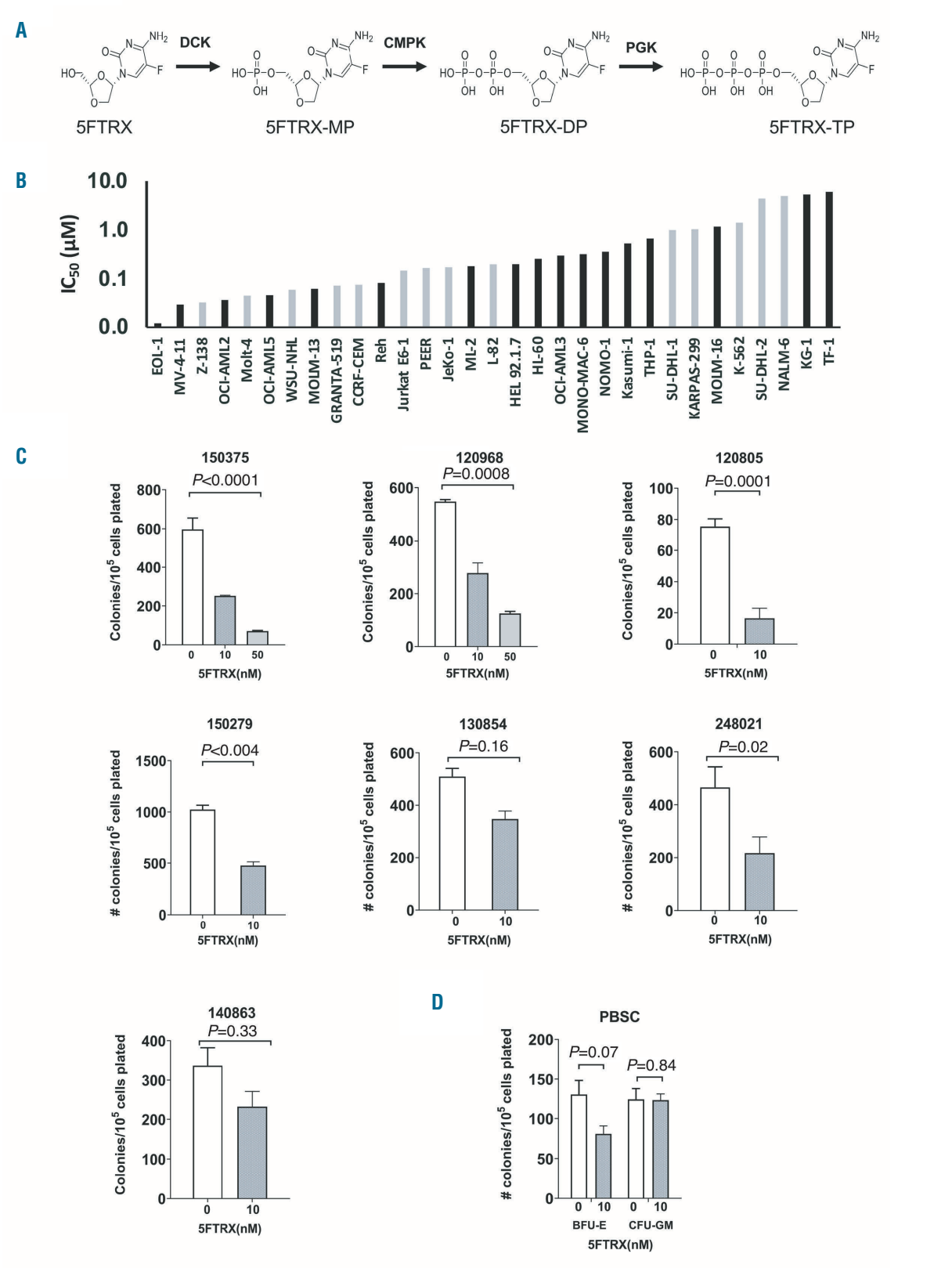
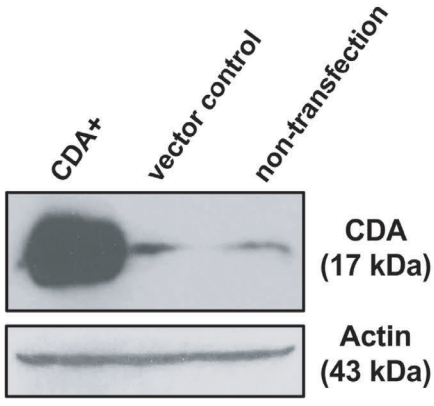
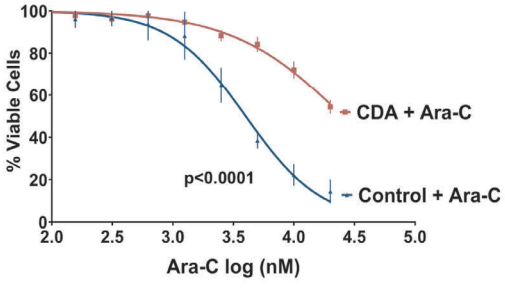


Figure 1. 5-fluorotroxacitabine (5FTRX) reduces cell growth and viability in hematologic cell lines and primary acute myeloid leukemia (AML) samples. (A) The structure of 5FTRX and its bio-activation by phosphorylation to the monophosphate (MP), diphosphate (DP), and triphosphate (TP). The enzymes known to catalyse 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.

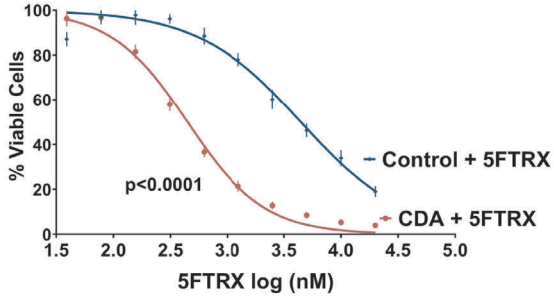
A



B



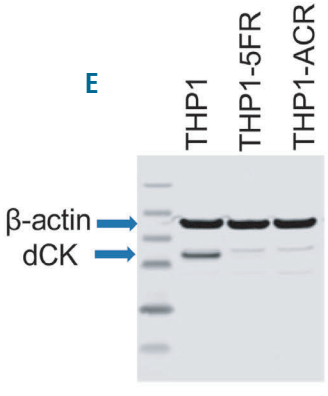
C



D

Compound	THP1 Parental	THP1-ACR	IC_{50} fold shift	THP1-5FR	IC_{50} fold shift
Ara-C	1.45 μM	>50 μM	>35	>50 μM	>35
5FTRX	0.76 μM	>50 μM	>66	>50 μM	>66

E



F

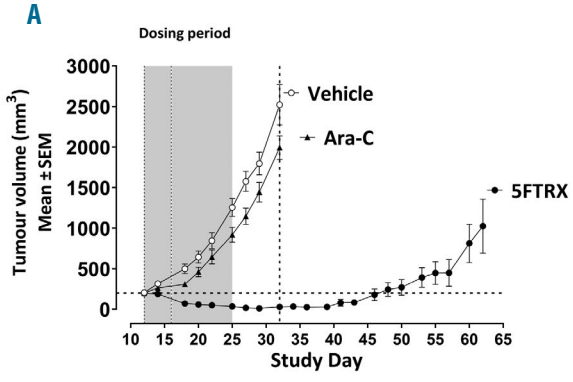
	5FTRX	MP	DP	TP
THP1	11	3	19	16
THP1-5FR	12	<1	2	<1

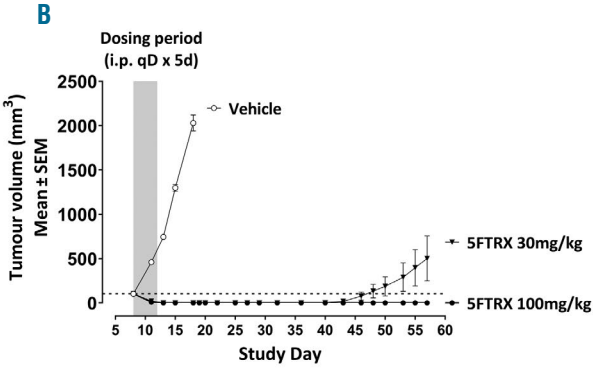
G

	IC_{50} in THP1 cells (μM)		
Compound	- dCKi	+ 50 μM dCKi	IC_{50} fold shift
Gemcitabine	0.0128	7.27	568
Ara-C	0.553	>50	>90
5FTRX	0.275	>50	>181

Figure 2. Resistance mechanisms to 5-fluorotroxacitabine (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 IC_{50} values for control and CDA^+ cell line was calculated using Student's *t*-test. (D) THP-1 cells resistant to 5FTRX (THP1-5FR) and Ara-C (THP1-ACR) were selected. Data represent the IC_{50} 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) IC_{50} (μM) of gemcitabine, Ara-C and 5FTRX alone or in combination with 50 μM dCK inhibitor (dCKi 15c7) in THP-1 cells.

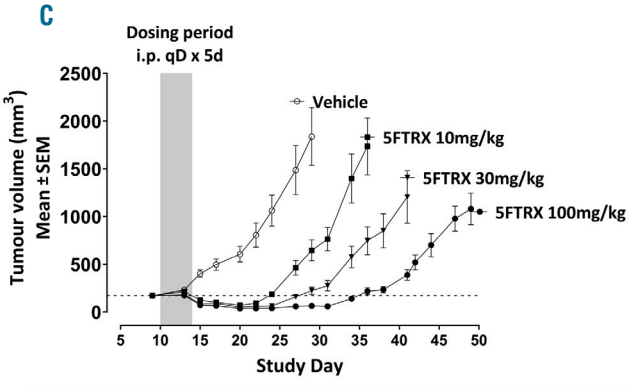
In order to understand the strong anti-tumor activity and mechanism of action of 5FTRX *in vivo*, pharmacokinetic and pharmacodynamic studies were undertaken in the MV4-11 model. Mice bearing subcutaneous tumor xenografts were treated with control or 5FTRX twice daily for 5 days and were sacrificed 2 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-

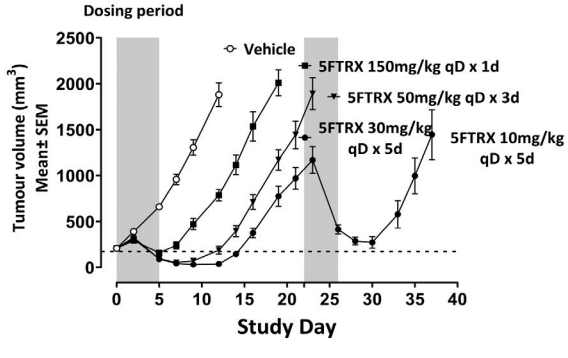




Group	Dose (mg/kg) i.p.	Dosing schedule	TGI d32	Regression d32	TGD (at RTV4)
5FTRX	100	BID x 5 d	108%	85%	38d
Ara-C	60	qDx5/7d x 2wks	20%	-	2d

5FTRX Dose (mg/kg)	T/C (d18)	TGI (d18)	Mean TGI (d13-18)	Regression d13	Regression d18	TGD (at RTV4)
30	0.003%	108%	108%	99.8%	100%	Not reached at day 61
100	0%	108%	108%	99.8%	100%	





5FTRX Dose (mg/kg)	T/C (d29)	TGI (d29)	Mean TGI (d13-29)	Regression d20	Regression d29	TGD (at RTV4)
10	35%	72%	94%	58%	-	9d
30	12%	97%	112%	70%	-	14d
100	3%	106%	119%	79%	63%	23d

Group (n)	Dosing period in first cycle (day) 1	2	3	4	5	Total dose
	Dose in mg/kg					
1 (10)	0	0	0	0	0	0
2 (10)	30	30	30	30	30	150
3 (10)	50	50	50	-	-	150
4 (10)	150	-	-	-	-	150

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 mm^3, 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 ΔT/ΔC where ΔT = T1-T0 and ΔC = 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 mm^3, 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 mm^3, 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 mm^3, 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.

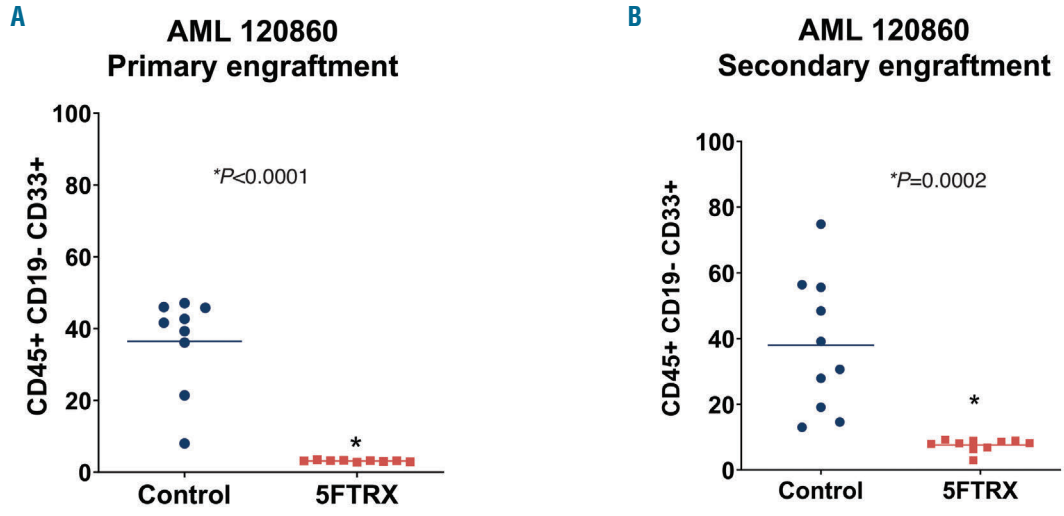


Figure 4. 5-fluorotroxacitabine (5FTRX) targets acute myeloid leukemia (AML) stem cells *in vivo*. (A) Primary AML cells were injected intra-femorally into irradiated female NOD/SCID mice preconditioned with CD122. Mice were treated with 100 mg/kg per day of 5FTRX by intraperitoneal (i.p.) injection or control from days 12-14 for 5 days (n=10 per group). Six weeks after injection, cell engraftment was assessed by flow cytometry. Data represent mean±standard deviation (SD). (B) Secondary engraftment was assessed by injecting viable leukemia cells from the bone marrow of 5FTRX treated and control mice and injected into the secondary recipient mice, which remained untreated. Six weeks later, cell engraftment was measured by flow cytometry. Data represent mean±SD. For all experiments, *P*-value was calculated using Student's *t*-test; $P<0.05$ was considered significant.

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.[1] 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.[2] 5FTRX targets patient-derived AML blasts and stem cells in physiologically relevant intra-femoral engraftment models *in vivo*.[3] 5FTRX overcomes CDA overexpression which is a known mechanism of Ara-C resistance.[4] 5FTRX has a strong anti-tumor activity *in vivo* that can lead to durable tumor regressions at well-tolerated doses.[5] 5FTRX is synergistic in combination with doxorubicin and azacytidine 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.

Aniket Bankar,[1] Thirushi Piyumika Siriwardena,[1*] Biljana Rizoska,[2] Christina Rydergård,[2] Helen Kylefjord,[2] Vilma Rraklli,[2] Anders Eneroth,[2] Pedro Pinho,[2] Stefan Norin,[2] Johan Bylund,[2] Sara Moses,[2] Richard Bethell,[2] Simon Kavanagh,[1] Neil Maclean,[1] Marcela Gronda,[1] Xiaoming Wang,[1] Rose Hurren,[1] Mark D. Minden,[1,3,4] Paul Targett-Adams,[2] Aaron D. Schimmer[1,4] and Mark Albertella[2]*

[1]*Princess Margaret Cancer Center, University Health Network, Ontario, Canada;* [2]*Medivir AB, Huddinge, Sweden;* [3]*Department of Medical Biophysics, Faculty of Medicine, University of Toronto, Ontario, Canada and* [4]*Division of Hematology, Faculty of Medicine, University of Toronto, Ontario, Canada*

**AB and TPS contributed equally as co-first authors.*

Correspondence:
AARON D. SCHIMMER - aaron.schimmer@uhn.ca

doi:10.3324/haematol.2019.226795

Disclosures: BR was an employee of Medivir when the work was performed; MA and PTA were employees of Medivir at the time the research was performed and have an equity share in the company; ADS received research funding from Medivir and has received consulting fees from Novartis, Jazz, Takeda and Otsuka Pharmaceuticals; ADS holds shares in AbbVie.

Contributions: AB, TPS, NM, MG, RH, XW, BR, SR, PTA and MA, performed research and analyzed data; BR, MA and PTA analyzed data, supervised research and provided critical reagents; ADS

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.

Acknowledgments: we thank Jill Flewelling (Princess Margaret Cancer Center) for administrative assistance. We would like to thank Andrew Minchinton and Alistair Kyle for the immunofluorescence analyses.

Funding: this work was supported by Medivir AB, the Canadian Institutes of Health Research, the Princess Margaret Cancer Centre Foundation, and the Ministry of Long-Term Health and Planning in the Province of Ontario. ADS holds the Barbara Baker Chair in Leukemia and Related Diseases.

References

1. Döhner H, Estey EH, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115(3):453-474.
2. Dumontet C, Fabianowska-Majewska K, Mantincic D, et al. Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. Br J Haematol. 1999;106(1):78-85.
3. Galmarini CM, Thomas X, Calvo F, et al. Potential mechanisms of resistance to cytarabine in AML patients. Leuk Res. 2002;26(7):621-629.
4. Hyo Kim L, Sub Cheong H, Koh Y, et al. Cytidine deaminase polymorphisms and worse treatment response in normal karyotype AML. J Hum Genet. 2015;60(12):749-754.
5. Schroder JK, Kirch C, Seeber S, Schutte J. Structural and functional analysis of the cytidine deaminase gene in patients with acute myeloid leukaemia. Br J Haematol. 1998;103(4):1096-1103.
6. Veuger MJ, Honders MW, Landegent JE, Willemze R, Barge RM. High incidence of alternatively spliced forms of deoxycytidine kinase in patients with resistant acute myeloid leukemia. Blood. 2000;96(4):1517-1524.
7. Murphy JM, Armijo AL, Nomme J, et al. Development of new deoxycytidine kinase inhibitors and noninvasive in vivo evaluation using positron emission tomography. J Med Chem. 2013; 56(17):6696-6708.