Protides of 5-Fluorotroxacitabine Display Potent Anti-Proliferative Properties and Circumvent Deoxycytidine Kinase-Mediated Resistance Associated with Cytotoxic Cytidine Analogues; a Novel Approach for Acute Myeloid Leukemia

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INTRODUCTION

The cytotoxic nucleoside cytarabine forms the backbone of acute myeloid leukemia (AML) induction and consolidation therapies, but is associated with severe toxicities that preclude its use in patients unable to tolerate aggressive chemotherapy. Options for patients that do not respond to cytarabine, or relapse post-treatment, are limited. Elderly patients and those with relapsed/refractory AML would particularly benefit from the availability of new agents that provide increased efficacy and tolerability compared to cytarabine, and that have a decreased susceptibility to mechanisms that can limit cytarabine efficacy, such as decreased deoxycytidine kinase (dCK) and/or upregulation of cytidine deaminase (CDA). Our preclinical evaluation of potential new anti-proliferative chemotherapeutics identified 5-fluorotroxacitabine (5FTRX), a chain-terminating cytidine-based L-nucleoside analogue, as having promising anti-proliferative activity against AML cell lines, and resistance to degradation by CDA (Poster # 3939). To improve upon specific properties of 5FTRX, we used protide technology to construct nucleotide monophosphate prodrugs of 5FTRX, including the example characterised by the present study, MV806.

EFFICACY AND TRIPHOSPHATE FORMATION

5FTRX nucleoside | **5FTRX nucleotide prodrug** | **5FTRX nucleotide-triphosphate**

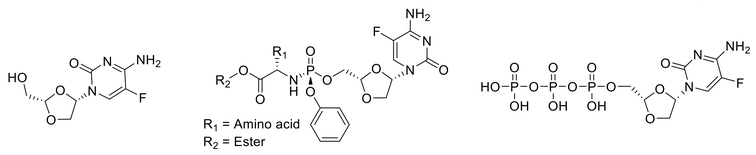

Figure 1. Nucleotide prodrug strategy. 5FTRX forms the scaffold for 5FTRX nucleotide prodrugs consisting of a masked monophosphate moiety (protide). These prodrugs enter cells passively and are processed intracellularly to generate 5FTRX-monophosphate. Subsequent phosphorylation by endogenous kinases generates 5FTRX-triphosphate, which is the active pharmacological species.

Origin	Cell line	CC_{50} (μM) (geomean with n)		
		Cytarabine	5FTRX	MV806
AML	MV411	0.12 (n=8)	0.044 (n=27)	0.0020 (n=4)
AML	THP1	0.48 (n=8)	0.30 (n=40)	0.029 (n=3)
AML	HL60	0.13 (n=4)	0.33 (n=22)	0.19 (n=2)
AML	U937	0.033 (n=4)	0.27 (n=4)	0.034 (n=2)
AML	OCI-AML2	0.11 (n=5)	0.098 (n=5)	0.017 (n=6)
AML	OCI-AML3	0.11 (n=2)	0.087 (n=2)	0.019 (n=7)
AML	EOL-1	0.029 (n=2)	0.019 (n=2)	0.0044 (n=2)
CML	K562	0.24 (n=8)	1.1 (n=17)	0.10 (n=2)
ALL (T cell)	MOLT-4	0.0033 (n=5)	0.027 (n=5)	0.0031 (n=5)
ALL (T-cell)	CEM	0.0036 (n=12)	0.051 (n=11)	0.0096 (n=9)
ALL (T-cell)	Jurkat	0.0042 (n=2)	0.030 (n=2)	0.0037 (n=2)
BL (B cell)	RAJI	0.027 (n=2)	0.26 (n=2)	0.096 (n=2)
Normal blood	PBMC 3d/6d	<3/nd	>100/>100	>100/43
Intracellular triphosphate formation (μM) (geomean with n)				
MV411 cells		6 (n=1)	16 (n=5)	93 (n=3)
THP1 cells		10 (n=1)	26 (n=8)	89 (n=4)

Table 1. MV806 demonstrated broad and high potencies with elevated triphosphate levels compared to 5FTRX in cells derived from haematological malignancies. Inhibitor efficacies were assessed in haematological cell lines using a 5 day cytotoxicity assay. Intracellular triphosphate levels were quantified following 24h incubation with 10μM of compounds. Improved efficacies of MV806 compared to 5FTRX correlated with increased intracellular triphosphate formation. The half-life of 5FTRX-triphosphate in THP-1 cells was shown to be 14h ± 1.4h.

dCK INHIBITION

Compound	CC_{50} in THP-1 cells (μM)		CC_{50} fold shift
	-dCKi	+ 50μM dCK inhibitor	
Cytarabine	0.553	>50	>90
5FTRX	0.275	>50	>181
MV806	0.031	0.13	4.2

Table 2. MV806 retained potency in THP-1 cells treated with a dCK inhibitor. Compound efficacies were assessed in the absence/presence of a dCK inhibitor (Murphy *et al* 2013) using a 5 day cytotoxicity assay. Substantial potency decreases were observed for both nucleosides, but not for MV806, following dCK inhibition.

GENERATION AND CHARACTERISATION OF RESISTANT THP-1 CELLS

Gene Symbol	Description	Fold change (n=3) in mRNA levels compared to control cells	
		Cytarabine selected	5FTRX selected
dCK	Deoxycytidine kinase	-16.2	-3.3
NT5E	5'-Nucleotidase	5.3	4.4
CDA	Cytidine deaminase	3.1	1.9

Table 3. dCK mRNA downregulation in resistant THP-1 cells. THP-1 cells were serially passaged in increasing concentrations of cytarabine or 5FTRX. Resistant cells (growing in >10x CC_{50} compound concentrations) were submitted for Affymetix gene expression analysis and gene expression profiles were compared to those from parental THP-1 cells.

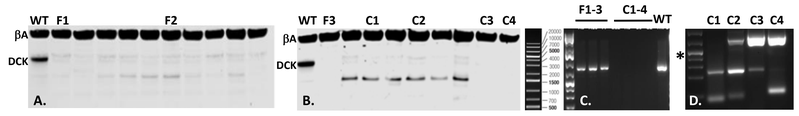

Figure 2. Aberrant dCK expression in 5FTRX/cytarabine resistant THP-1 clones. A) and B); Western blot analysis of dCK (βA; β-actin) levels in THP-1 clones from 5FTRX (F) and cytarabine (C) resistant cells. C) RT-PCR for dCK in THP-1 clones. D) Nested PCR for dCK in cytarabine resistant clones (* 500bp marker). Sequence analysis of dCK from 5FTRX-resistant clones revealed premature stop codons that correlated with absent dCK protein. In cytarabine resistant clones, both truncated dCK protein and mRNA were detected.

Compound	CC_{50} in THP-1 cells (μM)				
	Parental	Cytarabine selected	CC_{50} fold shift	5FTRX selected	CC_{50} fold shift
Cytarabine	1.45	>50	>35	>50	>35
5FTRX	0.761	>50	>66	>50	>66
MV806	0.117	0.149	1.3	0.403	3.4

Table 4. MV806 retained potency in cytarabine/5FTRX resistant THP-1 cells. Downregulation of dCK in resistant cells correlated with a marked decrease in sensitivity to cytarabine and 5FTRX nucleosides, but not to MV806.

INCREASED CDA DOES NOT DECREASE SUSCEPTIBILITY TO MV806

CDA overexpression in HEK cells | **Cytarabine and MV806 efficacies in CDA overexpressing cells**

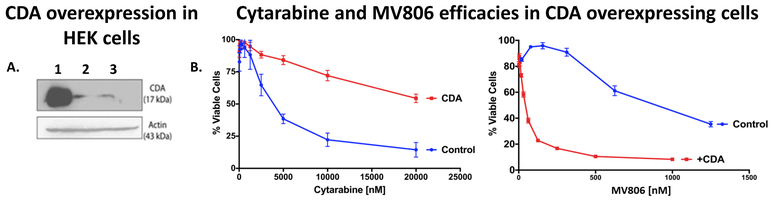

Figure 3. MV806 was not a substrate for CDA. A) Western blot of CDA protein in 1: CDA transfected; 2: vector control; 3: non-transfected HEK cells. B) Efficacy of cytarabine was reduced 7-fold in CDA overexpressing cells. Conversely, efficacy of MV806 was increased 23-fold in CDA overexpressing cells.

IN VITRO COMBINATION ANALYSIS

MV806 with azacytidine | **MV806 with doxorubicin** | **MV806 with midostaurin**

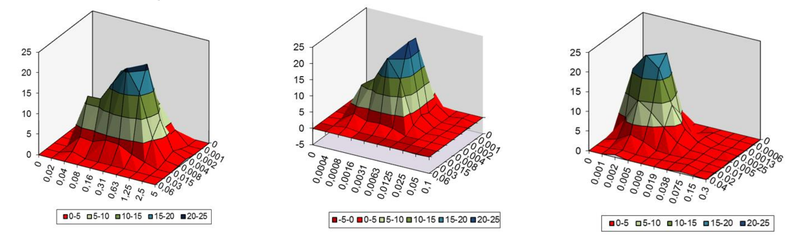

Figure 4. Bliss Independence analysis of MV806 in combination with AML drugs in MV411 cells. 3D synergy plots at 99.9% confidence for MV806 in combination with azacytidine, doxorubicin, or midostaurin indicated substantial synergy in inhibiting proliferation of MV411 cells.

MV806 in combination with	Synergy volumes at 95% CI (μMol²%)		Conclusion
	Synergy	Antagonism	
Azacytidine	273.30	-0.07	Strong synergy with insignificant antagonism
Doxorubicin	278.05	-0.41	Strong synergy with insignificant antagonism
Midostaurin	178.43	0	Strong synergy with insignificant antagonism

Table 5. Quantification of synergy for MV806-containing combinations in MV411 cells. Quantification of data presented in Figure 4 using MacSynergy software revealed MV806 exhibited strong synergy with no/insignificant antagonism in perturbing proliferation of MV411 cells when combined azacytidine, doxorubicin, or midostaurin. The profile of MV806 was superior to that of cytarabine, which demonstrated moderate synergy with insignificant antagonism in combination with doxorubicin or midostaurin, and insignificant synergy with strong antagonism in combination with azacytidine

IN VITRO DMPK PROPERTIES

Compound	Human whole blood Clint (μl/min/ml)/t ½ (h)	Dog / cyno whole blood Clint (μl/min/ml)	Human / dog / cyno hepatocyte metstab Clint (μl/min/million cells)	Solubility (thermodynamic pH7.4, μM)	CYP IC_{50} (2A6, 2C9, 2D6, 3A4)
MV806	8.9/1.3h	<2/2.2	36/50/81	1310	>1μM

Table 6. MV806 demonstrated favourable *in vitro* DMPK properties. MV806 exhibited promising *in vitro* blood and metabolic stabilities with high levels of aqueous solubility. MV806 did not inhibit major CYP enzymes and no time-dependent CYP inhibition was observed with 10μM of the compound.

SUMMARY

Using protide technology to directly deliver the active monophosphate species of 5FTRX intracellularly; MV806 exhibited broad, high potencies in haematological cancer cell lines together with increased triphosphate formation compared to 5FTRX. Importantly, MV806 could overcome resistance to cytarabine due to down-regulation of dCK and increased CDA expression. *In vitro* data indicated that protides such as MV806 could be suitable for combination with a broad range of AML drugs, as evidenced by synergistic pharmacology and low risk for CYP-mediated drug-drug interactions. Taken together, our findings support the further development of protides of 5FTRX for the treatment of AML, including AML patients with reduced sensitivity to cytarabine through high CDA expression and/or low dCK expression.

DISCLOSURES

PP, HK, VR, CR, BR, AE, JB, SM, SN, RB, MA, and PTA are/were employed by Medivir AB own equity in the company. AS receives research funding from Medivir AB, and consultancy fees from Jazz Pharmaceuticals, Otsuka Pharmaceuticals, and Novartis.