Broad *In Vitro* and *In Vivo* Antitumor Activity of TH3424: Preclinical Rationale for a Highly Selective AKR1C3 Prodrug for Treating Cancer

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Abstract

Aldo-keto reductase family 1 member C3 (AKR1C3) catalyzes the reduction of a diverse group of substrates, including prostaglandin (PG) D2 and PGH2. It has been reported that AKR1C3 is overexpressed in the majority of hepatocellular carcinomas (HCC) with 58% of HCC patient surgical tumor samples having strong expression of the enzyme[1]. Tumors overexpressing AKR1C3 can be resistant to radiation therapy[2] and chemotherapies[3]. AKR1C3 is also expressed in normal tissues, but its expression is much lower than in HCC tissue[1].

TH3424 is a prodrug which selectively releases a DNA bis-alkylating agent upon exposure to activated AKR1C3. *In vitro* cell proliferation tests with TH3424 in different HCC cell lines as well as other tumor cell lines showed that it is very potent (IC_{50} ≤ 10nM with 2 hour exposure) in killing cancer cells with high levels of AKR1C3, but less active (IC_{50} ≥ 1 μM) in killing cells with low or no AKR1C3 reductase. The activity of TH3424 correlates with the expression level of AKR1C3 as the potency of TH3424 is inhibited when used with a specific AKR1C3 inhibitor[4] (IC_{50}: 4 nM vs 6.3 μM with SN33638) in a non-small cell lung cancer cell line (H460). *In vivo* orthotopic and patient derived disease (PDX) liver cancer and T-cell leukemia model studies have shown promising efficacy with TH3424 being administered weekly with doses as low as 0.5 mg/kg. TH3424 showed better efficacy than Sorafenib in an orthotopic HepG2 mouse model. Three of eight mice treated with TH3424 at 2.5 mg/kg, Q7Dx3, and 8 of 8 mice treated with TH3424 at 5 mg/kg, Q7Dx3 were tumor free at day 35.

Introduction

Liver cancer is the sixth most common cause of cancer and the second leading cause of cancer death among all cancers with 745,500 deaths occurred worldwide during 2012. Hepatocellular carcinoma (HCC) accounts for majority of liver cancers (70% to 90%). The etiology of HCC is very complex, likely resulting from multiple genetic and epigenetic alterations, chromosomal aberrations, and dysregulated signaling pathways. Many molecular pathways have been well described in the development and progression of HCC, including Wnt-β-catenin pathway, EGFR/RAS/MAPKK pathway, c-MET pathway, IGF signaling, Akt/m TOR pathway, and VEGF/PDGFR signaling pathways. These molecular pathways could theoretically be exploited to slow down, halt or reverse the progression of HCC. Therapeutics targeting these pathways have been undergoing clinical trials aimed at treating advanced HCC[4]. However, currently, the treatment option for late stage HCC is still very limited. Sorafenib, a multi-tyrosine kinase inhibitor that inhibits Raf, VEGFR, PDGFR and other tyrosine kinases, is the only drug that has demonstrated survival benefits for patients with advanced HCC stage disease.

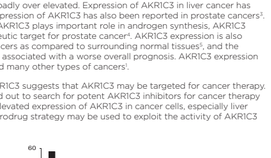
One of the promising targets for treating liver cancer therapy may be AKR1C3 as it was reported that this enzyme is broadly over elevated. Expression of AKR1C3 in liver cancer has been recently reported[1]. Overexpression of AKR1C3 has also been reported in prostate cancers[3]. Related to this observation, as AKR1C3 plays important role in androgen synthesis, AKR1C3 has been proposed as a therapeutic target for prostate cancer[4]. AKR1C3 expression is also increased in some of breast cancers as compared to surrounding normal tissues[5], and the increased AKR1C3 expression is associated with a worse overall prognosis. AKR1C3 expression is also elevated in leukemia[6] and many other types of cancers[1].

The increased expression of AKR1C3 suggests that AKR1C3 may be targeted for cancer therapy. Extensive work has been carried out to search for potent AKR1C3 inhibitors for cancer therapy without success, however, the elevated expression of AKR1C3 in cancer cells, especially liver cancer cells, suggested that a prodrug strategy may be used to exploit the activity of AKR1C3 to kill cancer cells.

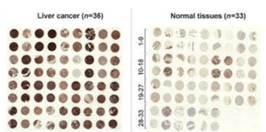
AKR1C3 Expression Across Tumor Types

Frequency of STRONG (6+) AKR1C3 positivity across 19 human cancers (2700 samples)

Guise et al., Cancer Res 2010

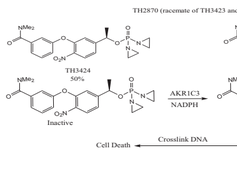


AKR1C3 Expression is Much Higher in Liver Cancer Than in Normal Tissue

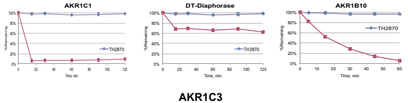


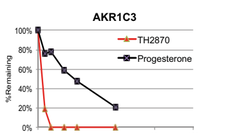
TH3424 and Its Activation

Structure of TH3424 and Its Activation



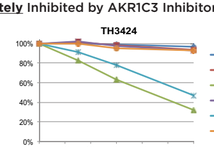
TH2870 (racemate of TH3424) is selectively activated by AKR1C3





Incubate 1 μg/mL (protein concentration) of enzyme (AKR/DT Diaphorase) in 100 mM phosphate buffer, pH 7.4 at 37°C for at least 5 minutes; add substrate (TH2870) at a final conc of 0.5 μM and add NADPH to a final concentration of 100 μM and BSA at the final concentration of 0.5 mg/mL to initiate reaction. Take samples at pre-dose, 10, 20, 40, 60 and 120 min. Add acetonitrile containing internal standard to stop reaction and centrifuge at 3000 g for 10 min and inject 5 μL of supernatant on LC/MS.

Activation of TH3424 in Monkey and Human Cytosol Can Be Completely Inhibited by AKR1C3 Inhibitor (TH3021)

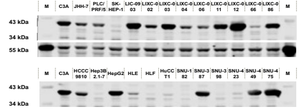


TH3021=SN33638#: Flanagan J. U. et al; Bioorg. Med. Chem.; 22(2014); 967

Incubate 1 mg/mL cytosol in 100 mM phosphate buffer, pH 7.4 at 37°C for at least 5 min; add substrate to a final conc of 5 μM and add NADPH to a final conc. of 2 mM to initiate reaction. Take samples at pre-dose, 15 and 30 min. Add acetonitrile containing internal standard to stop reaction and centrifuge at 3000 g for 10 min and inject 5 μL of supernatant on LC/MS.

TH3424 Showed Broad AKR1C3 Dependent Cytotoxicity Against Different Cell Lines

AKR1C3 Expression is Frequently Overexpressed in Liver Cancer Cell Lines



For cancer cell lines, cells were cultured in complete medium with 10% FBS at 2.5 X 10^5 cells/well, and incubated at 37°C, 5% CO_2 overnight. Cells were then washed with cold 1 ml PBS once, and lysed with lysis buffer (cell signaling technology, Cat. No. 9803) containing protease inhibitor cocktails (Sigma, Cat. No.P8340), the protein samples (30 ug each) were then used for western blot analysis. Different primary antibodies were diluted in 5% slim milk. The goat anti-mouse or goat anti-rabbit IgG HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) were used for detection. The western blots were developed with chemiluminescent HRP substrate (Millipore Corporation, Billerica, USA) and detected using Bio-Rad imager (ChemiDocTM XRS+). Antibodies used in this study include mouse monoclonal AKR1C3 antibody (clone NP6.G6.A6; Sigma-Aldrich), rabbit polyclonal antibody (Abcam), GADPH (14C10) Rb mAb (Cell Signaling), and α-tubulin antibody (clone B-5-1-2; Sigma-Aldrich).

TH3424 Shows AKR1C3 Dependent *In Vitro* Cytotoxicity in Liver Cancer Cell Lines

Cell Line	Expression	IC_{50} (uM)	Max Inhibition%
C3A	Strong	0.0021	98.1
Hep G2	Strong	0.0055	98.9
SNU-387	Strong	0.042	102.8
SNU-449	Strong	0.04	99.6
SNU-475	Strong	0.008	100.4
LIC-0903	Strong	0.082	96.3
LIXC-003	Strong	0.0054	44.6
LIXC-012	Strong	0.027	87.1
LIXC-086	Strong	0.041	92.8
LIXC-011	Strong	0.041	97.4
HCCC-9810	Strong	0.029	95.4
JHH-7	Medium	0.11	69.8
PLC/PRF/5	Medium	0.47	53.3
LIXC-002	Medium	0.056	99.1
LIXC-004	Medium	0.031	83.6
LIXC-006	Medium	0.12	71.7
HLE	Medium	0.084	80.5
LIXC-066	Medium	0.094	75.4
HuCCT1	Low	0.13	66.7
SNU-423	Low	>1	43.3
Hep 3B2.1-7	No	>1	8.1
HLF	No	>1	22.5
SNU-182	No	>1	19.4
SNU-398	No	>1	15.3
SK-HEP-1	No	>1	44.3
Normal Hepatocytes	?	>1	No Inhibition

TH3424 Showed Excellent Activity in AKR1C3 Over Expressed Lung Cancer Cell Lines

Cell lines	IC_{50} (μM)	AKR1C3 Expression
NCI-H460	0.0006	Strong
NCI-H322M	0.0023	Strong
NCI-H1651	0.0079	Strong
HCC44	0.0028	Strong
NCI-H2110	0.0039	Strong
NCI-H2342	0.014	Strong
NCI-H1563	0.0099	Strong
NCI-H1793	0.015	Strong
NCI-H28	0.047	Strong
NCI-H1435	0.18	Strong
NCI-H1869	0.18	Strong
NCI-H522	>1	Strong
NCI-HCC366 (Slow growth)	>1	Strong

Cell lines	IC_{50} (μM)	AKR1C3 Expression
NCI-H2052 (slow growth)	#intersect	Medium
NCI-H2347	0.12	Medium
NCI-H2073	>1	Medium
HCC2444	>1	Medium
NCI-H2085	>1	Medium
NCI-H155	0.91	None
NCI-H838	>1	None
95D	>1	None
HCC15	>1	None
NCI-H23	>1	None
NCI-H661	>1	None

TH3424 Showed Excellent Activity in AKR1C3 Over Expressed Esophageal Cancer Cell Lines

Cell Lines	IC_{50} (uM)	AKR1C3
TE-11	0.0027	Strong
TE-14	0.0047	Strong
OE 21	0.0052	Strong
TT	0.006	Strong
TE-6	0.021	Strong
TE-9	0.011	Strong
ECa-109	0.017	Strong
KYSE 410	0.011	Strong
TE-4	0.0099	Medium
TE-8	0.019	Medium
TE-15	0.0029	Medium
COLO680N	0.066	Medium

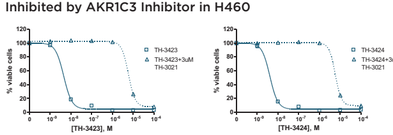
Cell Lines	IC_{50} (uM)	AKR1C3
EC-GI-10	0.013	Medium
KYSE 70	0.033	Medium
TE-5	0.033	Medium
OE 33	0.084	Medium
KYSE 510	0.18	Medium
KYSE 270	0.27	Medium
KYSE 180	0.45	Medium
KYSE 140	>1	Low
KYSE 150	>1	Low
TE-10	>1	No
KYSE 30	>1	No
TE-1	>1	No

AKR1C3 Overexpressed Leukemia Cell Lines are Very Sensitive to TH3424 Treatment

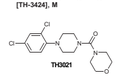
Cell Lines	AKR1C3 Expression	IC_{50} (nM)	Leukemia
CCRF-CEM	Strong	3.7	T-ALL
MOLT-4	Strong	10	T-ALL (19ys)
PF-382	Strong	15	T-ALL (child)
SUP-T1	Strong	3	T-ALL (child)
TALL-1	Strong	30	T-ALL (child)
Jurkat	Medium	40	T-ALL? (child)
Jurkat, Clone E6-1	Medium	24	T-ALL
NOMO-1	Medium	11	T-ALL (adult)
PER	Medium	84	Jurkat mutant
P30/OHK	Medium	>3μM	T-ALL(child)
GR-ST (not T ?)	Strong	9.9	B- ALL (transfected with G-CSF.)

Cell Lines	AKR1C3 Expression	IC_{50} (nM)	Leukemia
KG-1	Strong	15.3	Erythroleukemia (59 years old)
TF-1	Strong	2.8	Erythroleukemia (35 years old)
HEL	Medium	224	Erythroleukemia (30 years old)
Reh	Medium	3	ALL (non-T, non-B)
HL-60	Medium	52.6	APL (36ys)
HL-60 Clone 15	Medium	87	APL (36ys)
K-562	Medium	>1uM	CML(53ys)
ATN-1	Medium	>1uM	T-ALL (adult)
Mono-Mac-6	Medium	29.8	AML-MS (64ys)
THP-1	Medium	>1uM	AML(child)
Kasumi-1	None	>1uM	AML
P31/FUJ	None	>1uM	AML

The Cytotoxicity of the Two Enantiomers is Dramatically Inhibited by AKR1C3 Inhibitor in H460



Compound	IC_{50} (nM) -3uM TH-3021	IC_{50} (nM) +3uM TH-3021	Fold Difference
TH-2870	0.005	>5	>1000
TH-3423	0.005	6.3	1400
TH-3424	0.004	6.3	1500

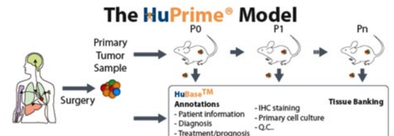


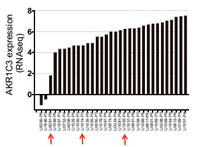
SN33638=TH3021 Flanagan J. U. et al; Bioorg. Med. Chem.; 22(2014); 967

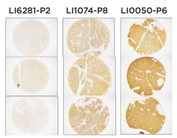
Procedure for proliferation assay: cells were plated into 96-well plates supported with 50 ul complete medium at 1500-8500 cells/well, incubated overnight at 37°C (5% CO_2). Each well was then added with 50 ul complete medium supplemented with indicated concentrations of compounds dissolved in 0.1% DMSO, and incubated at 37°C (5% CO_2). After 72 hrs unless indicated otherwise, Cell Counting Kit-8 reagent (CCK8, Dojindo), or CellTiter Glo reagent (CTG, Promega) was added into each well, and read on TECAN microplate reader (Infinite F50). The data were then analyzed either by Excel or Graphics. Alternatively, after incubating for indicated times, the medium with compounds is aspirated and cells were washed off once with PBS, and then incubated with complete medium without compounds. Live cells were then measured either by CCK8 or CTG assay.

TH3424 Showed Excellent *In Vivo* Efficacy in HCC and T-Cell Leukemia Animal Models

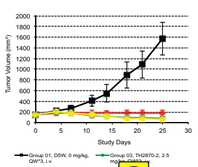
Liver Cancer PDX Models

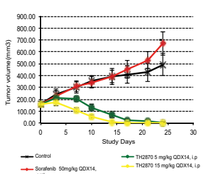






TH3424 Showed Excellent Activity in Liver Cancer PDX Models, Even Sorafenib Resistant Model





Patient derived tumor xenograft studies were performed using HuPrime® model LI1005 (Crownbio, Taizhou, China), which were from a female liver cancer patient (HBV, HCV negative). Tumor xenograft slices were transplanted subcutaneously into the right flank of 6-8 weeks old female BALB/c-nu mice. When tumor volumes reached about 100-200 mm^3, the animals were randomly grouped (n=5 each). Treatment was started as indicated. Tumor volume was measured every four days and calculated based on the formula: V=length X (width)2/2. All animals were sacrificed at the end of experiments.

For HepG2 orthotopic xenograft efficacy studies, HepG2-GFP cell line was first subcutaneously transplanted. Then HepG2-GFP tumor tissue were taken and excised under a dissecting microscope to remove necrotic tissue. The tumor tissue was then cut into a size of 1 mm^3. The sliced tumor tissues were then surgically transplanted to the liver of 6-week-old female BALB / c-nu. The liver was then released back into the abdominal cavity with 6-0 surgical suture (ETHICON,INC). After three days, based on whole-body imaging (FluorVivo Model-100 fluorescence imager), tumor-bearing mice were randomly divided into different groups for treatment. Whole-body imaging was then taken every 3-4 days to record tumor growth.

Expression of AKR1C3 In HepG2 Xenografts was in the Range Seen in 21 Human HCC Specimens

Pathology Review of AKR1C3 Immunohistochemical Staining in HCC Xenografts and HCC Surgical Specimens

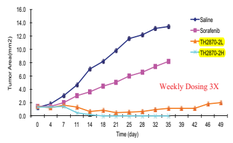
Sample	% Tumor Cells Staining at Each Intensity							Percent Positive	MAX SI
	4+		3+		2+		1+		
	Cells	%	Cells	%	Cells	%	Cells	%	
SNU-398	1	CN	2	CN	10	C	87	13	3+
PLC/PRF/5	0	CN	0	CN	15	C	85	15	3+
Hep3B	2	CN	1	CN	7	C	90	10	3+
HepG2	0	CN	0	CN	0	C	100	0	0
Surgical Samples ID									
RPB00079	10	NC	50	NC	30	CN	10	100	3+
RPB00098	10	NC	20	NC	60	CN	10	90	3+
RPB00062	50	NC	30	NC	10	CN	10	90	3+
RPB00075	40	NC	30	NC	30	CN	0	100	3+
RPB00063	60	NC	20	NC	20	CN	0	100	3+
RPB00064	50	NC	30	NC	20	CN	0	100	3+
RPB00067	60	NC	20	NC	20	CN	0	100	3+
RPB00072	50	NC	30	NC	20	CN	0	100	3+
RPB00068	0	C	0	C	20	CN	80	20	3+
PLC00008	0	NC	0	NC	0	CN	0	100	3+
PLC00049	85	NC	5	NC	5	CN	5	90	3+
PLC00044	80	NC	5	NC	5	CN	5	90	3+
PLC00045	60	NC	5	NC	5	CN	5	90	3+
PLC00028	50	NC	30	NC	10	CN	10	100	3+
PLC00055	2	NC	3	NC	10	CN	85	15	3+
PLC00060	95	NC	5	NC	0	C	0	100	3+
PLC00027	60	NC	10	NC	30	CN	0	100	3+
RPB00069	80	NC	10	NC	10	CN	0	100	3+
PLC00035	95	NC	5	NC	0	C	0	100	3+
PLC00073	50	NC	50	CN	0	C	0	100	3+
RPB00070	90	NC	10	CN	0	C	0	100	3+

Abbreviations: SCL - Subcellular Location; N –Nuclear, C - Cytoplasmic, NC –Nuclear < Cytosolic; CN –Cytosolic < Nuclear; Max SI –Maximum Staining Intensity

Cancer Biol Ther. 2015;14(4):610-22

Compelling Efficacy in HepG2 Orthotopic Xenograft Model

Tumor Growth Curve

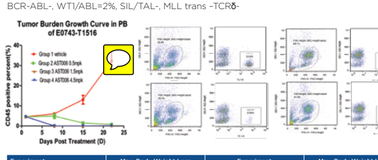


Quick and Excellent Efficacy in T Cell Leukemia PDX Model

AL7473 **Model:** T cell leukemia

Abnormal Cell: 78.5%, express CD10, CD3, CD7, CD2, CD5, CD38, TDT, CD34, CD45

BCR-ABL-, WT1/ABL1=2%, SIL/TAL-, MLL trans -TCRδ-



Experiment	Max. Body Weight Loss	Experiment	Max. Body Weight Loss
Orthotopic HepG-2		PDX LI0752	
Vehicle	0%	Vehicle	2.00%
Sorafenib	0%	TH3424 (1.25 mg/kg)	6.10%
TH3424 (1.25 mg/kg)	0%	TH3424 (2.50 mg/kg)	7.10%
TH3424(2.5 mg/kg)	0%	TH3424 (5.0 mg/kg)	3.90%
T-cell leukemia		PDX LI1501	
Vehicle	0%	Vehicle	2.90%
TH3424 (0.5 mg/kg)	0%	Sorafenib (50 mg/kg)	4.90%
TH3424 (1.5 mg/kg)	0%	TH2870 (5mg/kg)	0%
TH3424 (4.5 mg/kg)	0%	TH2870 (15 mg/kg)	1.20%

Conclusion

- AKR1C3 is over expressed in many human tumors particularly liver cancer
- TH2870 (racemate) and its enantiomers are selectively activated by AKR1C3
- *In vitro* cytotoxicity of TH3424 is AKR1C3 dependent
- TH3424 shows excellent *in vivo* efficacy in HCC and T-cell leukemia animal models

References 1) Guise C. P.; Abbattista M. R.; Singleton R. S.; Holford S. D.; Connolly J.; Dachs G. U.; Fox S. B.; Pollock R.; Harvey J.; Guiford P.; Da[[unable to display character: ň]]ate F.; Wilson W. R.; and Patterson A. V.; Cancer Res.70(4), 2010, 1573. **2)** Xiong W.; Zhao J.; Yu H.; Li X.; Sun S.; Li Y.; Xia Q.; Zhang C.; He Q.; Gao X.; Zhang L.; Zhou D.; Plos One, V. 9 (11), 2014, e111911. **3)** Liu C.; Lou W.; Zhu Y.; Yang J.; Nadiminty N.; Gaikwad N.; Evans C.; Gao A.; Cancer Res.; 2015, 75(7), 1413. **4)** Flanagan J. U.; Atwell G. J.; Heinrich D. M.; Brooke D. G.; Silva S.; Rigoreau L. J. M.; Trivier E.; Turnbull A. P.; Raynham T.; Jamieson S. M. F.; Denny W. A.; Bioorg. Med. Chem.; 22(2014); 967. **5)** Linb H.-K, Steckelbroecka S., Fungc K.-M., A. N. Jonesb, Penningа, T. M., Steroid, V69, 2004, 795. **6)** Konopleva M, Thall PF, Yi CA, Borthakur G, Coveler A, Bueso-Ramos C, Benito J, Konopev S, Gu Y, Ravandi F, Jabbour E, Faderl S, Thomas D, Cortes J, Kadia T3, Kornblau S, Daver N, Pemmaraju N, Nguyen HQ, Feliu J, Lu H, Wei C, Wilson WR, Melink TJ, Gutheil JC, Andreeff M, Estey EH, Kantarjian H., Haematologica. 2015, 100(7):927-34.