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Mechanism of Action of the Anti-NASH Effects of Solithromycin in a Predictive NASH HCC Mouse Model

Prabhavathi Fernandes* [1], David Oldach[1], Pierre M. Gholam[2], Taishi Hashiguchi[3], Yuka Shirakata[3], Hiroyuki Yoneyama[3]
[1]Cempra, Inc., Chapel Hill, NC, United States; [2]Liver Center of Excellence, University Hospitals Case Medical Center, Cleveland, OH, United States; [3]Stelic Institute & Co. Inc, Tokyo, Japan

Cempra, Inc.
6320 Quadrangle Drive
Suite 360
Chapel Hill, NC 27517
pfernandes@Cempra.com

Introduction and Rationale

Non-Alcoholic Steatohepatitis (NASH) is a leading cause of liver-related morbidity and mortality worldwide. Finding safe and effective therapies for NASH is a very significant unmet medical need.

Solithromycin (SOLI) is a macrolide antibiotic which is well absorbed when administered orally and has been studied in two large Phase 3 pneumonia trials. A large body of clinical and pre-clinical safety data, including 90-day toxicology in two animal species has been developed. SOLI has demonstrated anti-inflammatory properties in vitro and in the smoking mouse COPD model (Kobayashi Y *et al*., 2013a; Kobayashi Y. *et al*., 2013b). It achieves high concentrations in the liver, the primary organ that is involved with its metabolism and excretion. Hepatic inflammation is thought to play a key role in NASH. We therefore aimed to examine the effect of incremental doses of SOLI in a NASH mouse model.

Methods

Induction of NASH: NASH was induced in male mice by a single subcutaneous injection of 200 µg streptozotocin (STZ, Sigma-Aldrich, USA) solution 2 days after birth and fed with high fat diet (HFD, 57 kcal% fat, cat#: HFD32, CLEA Japan, Inc., Japan) for the duration of the experiment.

Treatment Schedule: Solithromycin was administered orally at doses of 5 mg/kg QD (once daily), 10 mg/kg BID (twice daily), 25 mg/kg BID (twice daily), 50 mg/kg QD (once daily) and 100 mg/kg QD (human equivalent dose (HED) of 400 mg/day) for 4 weeks. Groups of NASH mice were treated (i) between 5-8 weeks of age to examine the effect of early NASH treatment or (ii) between 8-12 weeks of age to examine the effect of treatment after fibrosis had developed.

Measurement of whole blood, plasma and serum biochemistry: Non-fasting blood glucose was measured in whole blood. Plasma insulin levels were quantified by the ultra sensitive Mouse Insulin ELISA kit (Morinaga Institute Biological Science, Inc., Japan). For serum biochemistry, blood was collected without anticoagulant. Serum triglyceride, HDL-, LDL-, VLDL-cholesterol and chylomicron concentrations were quantified by HPLC at Skylight Biotech Inc. (Japan).

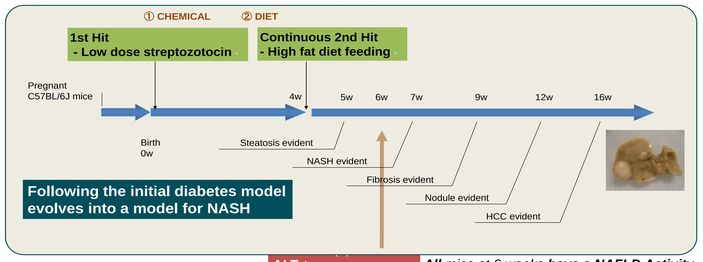
Histological analyses: NAFLD Activity score (NAS) (Kleiner DE. *et al*., *Hepatology*, 2005;41:1313) was determined on H&E stained sections. Collagen was stained using picro-Sirius red solution (Waldeck, Germany). Frozen liver sections were used for immunohistochemistry, using anti-F4/80 antibody (BMA Biomedicals, Switzerland) or a 200-fold dilution of anti-Gr-1 antibody (Abcam, USA). Quantitative analysis of fibrosis and inflammation areas, bright field images of Sirius red-stained and F4/80-immunostained sections were captured around the central vein using a digital camera (DFC280; Leica, Germany) at 200-fold magnification, using ImageJ software (National Institute of Health, USA). Gr-positive cells in 5 fields/section were counted.

Quantitative RT-PCR: Total RNA was extracted from liver samples. One µg of RNA was reverse-transcribed using a reaction mixture and MMLV-RT (Invitrogen) and real-time PCR was performed. Relative mRNA expression level was calculated using the expression of each gene normalized to that of the reference gene 36B4.

Statistical tests: Statistical analyses were performed using Bonferroni Multiple Comparison Test or Student t-test on GraphPad Prism 4 (GraphPad Software, USA). *P* values < 0.05 were considered statistically significant. Results were expressed as mean ± SD.

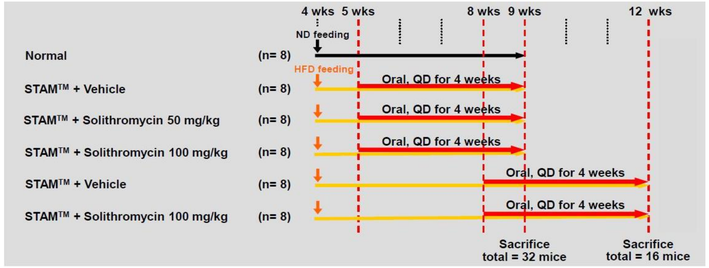
STAM™: In Vivo Predictive Pharmacology Model for NASH[1]

The STAM™ NASH model in diabetic C57BL/6J mice fed a high fat diet (Fujii M. *et al*. 2013), is reproducible and follows a predictable path of steatosis, hepatic inflammation, hepatocellular ballooning degeneration, fibrosis and HCC (hepatocellular carcinoma).



*All mice at 6 weeks **have a NAFLD Activity Score (NAS) ≥5, similar to human NASH***

Study Plan for Assessing the Anti-NASH/fibrosis Effects of SOLI in STAM™ Model of NASH



Results

Dose-response for the anti-NASH and glucose lowering effect

N = 16	Blood Glucose (mg/dL)	NAFLD Score	G6pc	FBPase
SOLI 50 mg/kg QD	380 ± 170**	3.0 ± 0.9****	Not done	Not done
Vehicle (Expt.1)	628 ± 85	5.4 ± 0.5	Not done	Not done
N = 32	4 weeks of treatment as above except 3 dose levels lower than Expt. 1 were tested			
SOLI 25 mg/kg BID	497± 259*	2.9 ± 1.0***	1.9 ± 0.7***	1.0 ± 0.2***
SOLI 10 mg/kg BID	713± 172	3.3 ± 0.9**	2.4 ± 0.5***	1.2 ± 0.2*
SOLI 5 mg/kg QD	726 ± 101	4.8 ± 0.9	3.0 ± 0.7**	1.2 ± 0.2*
Vehicle (Expt. 2)	749± 125	4.9 ± 0.8	4.2 ± 1.1	1.5 ± 0.3
Normal	227± 38	0.0	1.0 ± 0.3	1.0 ± 0.1
N = 48	Treatment at a higher daily dose			
SOLI 100 mg/kg QD[a] (4 weeks of treatment)	338 ± 160**	4.1 ± 0.4	0.81 ± 0.34***	0.7 ± 0.13 ***
Vehicle (Expt. 3)	640 ± 146	5.6 ± 0.7*	2.44 ± 0.82	1.63 ± 0.27
Normal	187 ± 24	0.0	1.0 ± 0.56	1.0 ± 0.16

Values are means ±SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001
a = 100 mg/kg QD for 4 weeks treatment did not provide additional benefit over the 50 mg/kg QD dose

Relative Gene Expression in the Liver (qRT-PCR)

Parameter (mean ± SD)	Normal (n=8)	Vehicle (n=8)	Solithromycin 5 mg/kg (n=8)	Solithromycin 10 mg/kg (n=8)	Solithromycin 25 mg/kg (n=8)
TNF-α/36B4	1.1 ± 0.6*	2.8 ± 1.3	3.1 ± 1.2	3.0 ± 0.7	3.4 ± 1.3
MCP-1/36B4	1.0 ± 0.4***	7.4 ± 3.4	7.2 ± 4.0	5.2 ± 1.9	4.9 ± 1.9
MMP-9/36B4	1.0 ± 0.2**	2.8 ± 1.5	2.9 ± 0.8	3.0 ± 1.1	2.6 ± 0.5
Col1a2/36B4	1.0 ± 0.2**	3.1 ± 1.2	3.6 ± 1.7	2.8 ± 1.0	2.9 ± 1.2
α-SMA/36B4	1.1 ± 0.3*	2.7 ± 1.2	3.8 ± 1.9	3.7 ± 1.0	3.3 ± 1.2
Timp-1/36B4	1.0 ± 0.5	9.7 ± 4.0	16.0 ± 15.7	11.9 ± 6.8	11.0 ± 8.6
TGF-β/36B4	1.0 ± 0.2***	2.1 ± 0.6	2.1 ± 0.4	1.8 ± 0.4	1.8 ± 0.5
Gck/36B4	1.0 ± 0.3**	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.6 ± 0.4
G6pc/36B4	1.0 ± 0.3***	4.2 ± 1.1	3.0 ± 0.7**	2.4 ± 0.5***	1.9 ± 0.7***
Pck1/36B4	1.0 ± 0.4*	1.6 ± 0.4	1.5 ± 0.1	1.5 ± 0.4	1.3 ± 0.6
FBPase/36B4	1.0 ± 0.1***	1.5 ± 0.3	1.2 ± 0.2*	1.2 ± 0.2*	1.0 ± 0.2***
Glut 2/36B4	1.0 ± 0.2	1.0 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1

vs Vehicle group, *P<0.05, **P<0.01, ***P<0.001

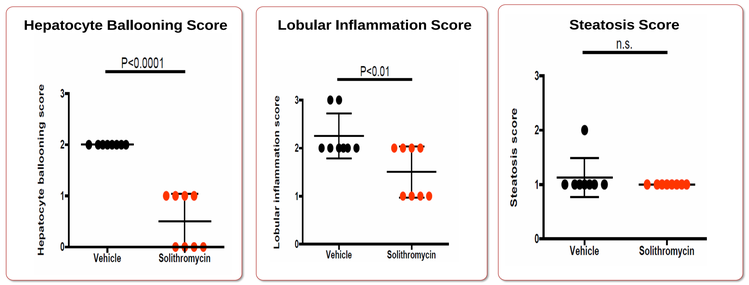
Effect on Blood Lipids

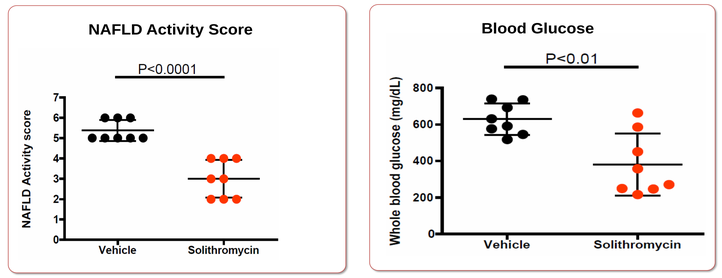
Parameter (mean ± SD)	Normal 8W (n= 5)	Vehicle control 4-8W (n=8)	Soli 4-8W (n=8)	Vehicle control 8-12W (n=8)	Soli 8-12W (n=8)
Serum total-cholesterol (mg/dL)	80.8 ± 8.0	108.2 ± 19.4	107.2 ± 16.2	127.5 ± 23.9	146.9 ± 18.1
Serum chylomicron (mg/dL)	1.1 ± 0.4	1.9 ±1.5	1.3 ± 0.2	2.9 ± 2.1	0.8 ± 0.5
Serum HDL-cholesterol (mg/dL)	53.8 ± 5.6	86.8 ± 15.5	77.2 ± 8.8	96.7 ± 16.9	111.9 ± 12.4
Serum LDL-cholesterol (mg/dL)	11.2 ± 1.6	11.6 ± 2.2	20.0 ± 6.6	13.4 ± 2.4	25.2 ± 6.3
Serum VLDL cholesterol (mg/dL)	14.7 ± 3.1	7.8 ± 4.5	8.7 ± 2.8	14.5 ± 9.8	8.9 ± 2.5
Serum triglyceride (mg/dL)	111.7 ± 54.1	103.7 ± 83.6	36.0 ± 9.5	96.7 ± 16.9	111.9 ± 12.4
Liver triglyceride (mg/g liver)	3.9 ± 2.0	29.3 ± 9.4	12.1 ± 4.8	49.4 ±15.5	19.7 ± 7.0
Liver diacylglycerol (mg/g liver)	3.3 ± 0.9	4.2 ± 0.7	3.6 ± 0.6	5.1 ± 1.1	5.1 ± 0.9

Results Continued

NAFLD Activity Score

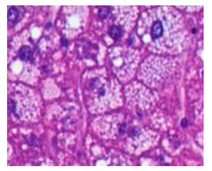
Mice treated orally for 4 weeks at 50 mg/kg QD (once daily)



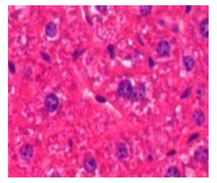


Mean ± SD
Student's T-test

H & E stained liver sections



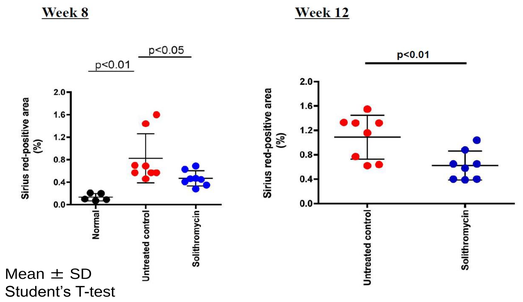
Control untreated



Treated

The 50 mg/kg QD dose showed the greatest effect, while the 5 mg/kg/day (QD) dose had no effect. There was no increased benefit at the 100 mg/kg QD dose. In order to determine the effect on fibrosis and HCC that reproducibly occurs in the mouse model, the experiment was repeated with treatment initiated from 4-8 weeks after birth (4 weeks of treatment) and 8-12 weeks after birth (4 weeks of treatment). mRNA expression levels for glucose-6-phosphatase and fructose-1,6-bisphosphatase, two enzymes involved in gluconeogenesis, were suppressed.

Effect on Fibrosis



Mean ± SD
Student's T-test

Size and Number of Visible Tumor Nodules

Weeks 4-8 and Weeks 8-12: (total of 4 weeks of treatment for each group)
The fibrosis area and the tumor nodules (both size and number) were significantly decreased in the SOLI treated group.

Conclusions

- SOLI was effective in reducing NASH and significantly reduced NAFLD scores in a diabetes and high fat diet induced NASH mouse model.
- SOLI produced statistically significant reduction in inflammation, hepatocellular ballooning degeneration, fibrosis, and HCC at a daily dose that is equivalent or lower than the HED that has been well tolerated and effective in two global Phase 3 trials to treat moderate to moderately severe CABP.
- SOLI was effective in reducing blood glucose, without significant improvement in insulin levels in the NASH mice.
- SOLI does not have activity against anaerobic Gram-negative intestinal microflora. Therefore, the anti-NASH activity is not believed to occur as a result of SOLI's antibacterial activity.
- The anti-NASH activity is proposed to be through SOLI's anti-inflammatory properties and through the suppression of gluconeogenesis. Specifically, SOLI was shown to suppress the key enzymes, fructose-1,6-bisphosphatase and glucose-6-phosphatase in the liver.
- The availability of a large safety database and the data in the NASH mouse model support exploratory development of SOLI for the treatment of NAFLD and NASH in humans.
- An exploratory trial in patients with biopsy-proven NASH is currently underway.

Disclosures

P. Fernandes and D. Oldach are employees of Cempra and receive compensation in salary and option share. P. Gholam is a paid consultant for Cempra. T. Hashiguchi, Y. Shirakata, and H. Yoneyama work at the Stelic Institute in Tokyo, Japan and this work was paid for under contract to Stelic.