Targeting Host-Cell Metabolism To Address Multiple Intracellular Pathogens

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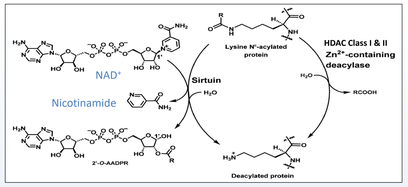
Background

Current anti-infectives and vaccines target the pathogen, whether bacterial, viral, fungal, or protozoan. Unfortunately, pathogens are diverse in their biology requiring a different therapy/vaccine for each pathogen. In the case of biodefense, this ultimately requires the development and stockpiling of a distinct therapy/vaccine for each threat. Given biological diversity, the case scenarios are infinite. Despite improvements in biologic and vaccine technologies, manufacture, and cold chain logistics, the recent Ebola and Zika pandemics are a reminder of the slow response inherent in developing a pathogen-targeted therapy against a newly emergent pathogen. Additionally, pathogens are clever, particularly intracellular pathogens; they have evolved mechanisms to escape detection by the immune system and to develop resistance.

FORGE Life Science is taking a different approach. Intracellular pathogens depend on the host cell's metabolism for energy; metabolic precursors for production of pathogen components and genomic material; as well as the organization of specialized compartments in the cell for replication, maturation, and dissemination. As such, regulation of the host cell's metabolism is a fundamental component of the host-pathogen interaction. FORGE is pursuing a paradigm-shifting anti-infective mechanism-of-action, the modulation of host-encoded sirtuin-proteins, central regulators of host-cell metabolic-homeostasis. Sirtuins are a family of seven enzymes that regulate cellular metabolism and gene activity (including epigenetics) through chemical modification (de-acylation) of other proteins of the cell. Koyuncu et al. (*mBio 5:e02249*) propose sirtuins are evolutionarily-conserved components of the cell's pathogen-defense system (intrinsic immunity); they showed sirtuins modulate the growth of RNA and DNA viruses in mammalian cells, and lytic and lysogenic bacteriophages in bacterial cells. Direct interactions of pathogens with sirtuins actively reprogram the host-cell's metabolism and/or epigenetics to support the pathogen's life-cycle (*e.g.* hepatitis B, *Ren J.Virol. 88:2442*; *Leshmania infantum*, *Moreira PLoS Pathog. 11:e1004684*; *Listeria monocytogenes*, *Eskandarian Science 341: 1238858*). Downstream effects of active disruption of homeostasis can provide a means to circumvent intrinsic immunity such as cellular autophagy that normally eliminate the pathogen (*e.g. Salmonella typhimurium, Ganesan PLoS Pathog. 13:31006227*).



NAD+/NADH — *Nutrients high* — **Sirtuin Activity** — NAD+/NADH — *Nutrients low*

Condition Imposed by Virus Infection

Figure 1. Sirtuins are NAD⁺-consuming protein deacylases expected to be active during viral infection when the NAD⁺/NADH ratio is high.

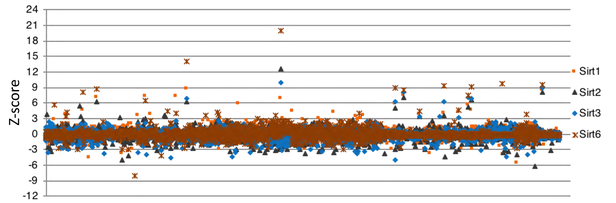
Materials & Methods

1. Develop small-molecule modulators of human sirtuins that are readily synthesized, can be taken as pills, and have properties predicting ambient storage and stability.
2. Test sirtuin-targeted drugs against a prototypical, rapidly-dividing RNA virus, influenza A, and a slowly-replicating, large DNA virus, cytomegalovirus (HCMV).
3. Demonstrate sirtuin-modulators are equivalently effective against RNA and DNA viruses, compared to standard-of-care (SOC), virus-targeted, direct-acting-antivirals (DAA), oseltamivir and ganciclovir.
4. Because sirtuin-modulators target host proteins (as opposed to virus proteins), demonstrate they block drug-resistance and synergize with DAAs.
5. Analyze the mechanism-of-action of sirtuin-targeted drugs through the restoration of metabolic homeostasis and intrinsic immunity.

Results

A collection of 13,000 novel, drug-like, chemical-compounds was tested for modulation of the deacetylation activity of human sirtuins SIRT1, 2, 3, and 6. 85 hits were validated as significantly modulating the deacetylase activity of SIRT1, 2, 3, and/or 6. Consistent with sirtuin-mediated metabolic homeostasis affecting the replication of an invading pathogen, 61 of the 85 hits significantly inhibited the growth of HCMV.

Results (cont.)



13,000 cpds	screened: SIRT1, 2, 3, and 6 enzyme activity
85 cpds	validated hits modulating SIRT1, 2, 3, or 6
61/85 cpds	active HCMV antiviral in secondary screen

7 structural classes including
FH003 SIRT1,2 inhibitor
FH010 SIRT1,2,6 activator
FH097 SIRT6 activator

Figure 2. Small molecule screen showed sirtuin modulators have antiviral activity. Modulation of sirtuin deacetylase activity measured *in vitro* using an acetylated peptide substrate attached to a fluorophore. Statistical significance in activation or inhibition compared to mean activity is plotted above or below a Z-score = 0 (at the mean) for SIRT1, 2, 3, and 6. *Koyuncu, Kim, MacMillan, Shenk, unpublished.*

Screen Hit	DNA virus				RNA virus		Sirtuin Enzyme			
	cytomegalovirus human MRC5		JC virus monkey Cos7		influenza A canine MDCK		SIRT1	SIRT2	SIRT3	SIRT6
	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	Z-score	Z-score	Z-score	Z-score
FH-003	4.6	>11	3.73	>5	5.0	>10	- -	- -		
FH-010	1.2	>42	0.53	>6.5	1.4	>36	+++	++		+++
FH-097	2.3	>22	0.87	>115	2.9	>9				+++

Table 1. Sirtuin-targeted validated hits are effective against DNA and RNA virus. Virus-type and cell line indicated. Antiviral effectiveness given as 50% viral inhibitory concentration (IC_{50}). Selectivity index (SI) is 50% cytotoxic concentration CC_{50}/IC_{50}. Z-score >1 indicated as the number of "-" or "+" shown for SIRT1, 2, 3, or 6. Blank indicates that the Z-score was <1.

A medicinal chemistry campaign was conducted to improve sirtuin modulation and simultaneous effectiveness against both influenza A and HCMV. Over 300 compounds have been synthesized in the lead program (FH-003 inhibitor series) and effective antivirals demonstrate favorable i*n vitro* pharmaceutical properties (*e.g.* satisfy Lipinski's rules, absence of hERG interaction, absence of drug efflux, weak CYP3A4 inhibition). Importantly, the series demonstrates good pharmacokinetics for administration i*n vivo* (*e.g.* in mice, 30-100% oral bioavailability, half-life up to 5 hours, reasonable clearance, good tolerability; data not shown). Multiple compounds have achieved effectiveness of 200nM for influenza A and HCMV. All effective antivirals are also absent cytotoxic effects on uninfected cells, demonstrating SI of >50-100-fold. In comparison, oseltamivir IC_{50}=50nM against influenza A, and ganciclovir IC_{50}=1400nM against HCMV. Neither SOC DAA works against the other virus.

Drug (μM)	Influenza A		HCMV		MRC-5	SIRT1	SIRT2	SIRT3	SIRT5	SIRT6
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	CC_{50}	dAc IC_{50}	dAc IC_{50}	dAc IC_{50}	dSucc IC_{50}	dMyr IC_{50}
FC-044	7.9	8.4	7.2	10.2	>25	>100	1.8	>200	ND	>100
FC-189	9.1	12.3	0.55	6.2	>25	>100	3.7	11.8	ND	>100
FORGE Lead1	2.4	2.5	0.43	1.8	>100	33	3.3	12.3	>100	>100
FORGE Lead2	0.35		0.66		>25					
ganciclovir	NA	NA	1.4	3.0						
oseltamivir	0.22	0.9	NA	NA		*Note: FORGE Lead1 shown below*				

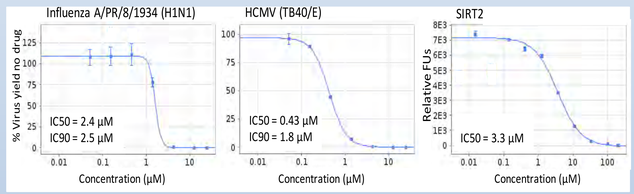
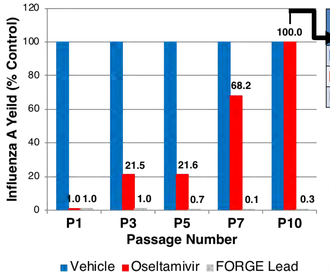


Table 2 & Figure 3. SIRT2 inhibitors are potent broad-spectrum antivirals.

Results (cont.)

Passage experiments, wherein virus is grown in sub-lethal doses of drug, find that oseltamivir-resistant virus can be selected within 2-4 passages, and resistance is absent after 10 passages of administering FORGE Lead1. Virus selected in the presence of oseltamivir is resistant but remains susceptible to FORGE Lead1. Virus selected in the presence of FORGE Lead1 remains susceptible to both FORGE Lead1 and oseltamivir. Combination experiments confirm synergy in co-administration of oseltamivir and a FC-044 across a broad range of relative concentrations.



Virus from P10	FORGE IC_{50}	oseltamivir IC_{50}
vehicle passaged	2.75μM	0.081μM
oseltamivir passaged	1.27μM	40.6μM
Lead1 passaged	3.05μM	0.058μM

Figure 4. Absence of acquired drug resistance targeting SIRT2. Influenza A in the presence of FORGE Lead1 or oseltamivir at a concentration permitting 1% break-through: yield was determined and the virus was passaged into new cells. P10 virus was tested against oseltamivir and FORGE Lead1.

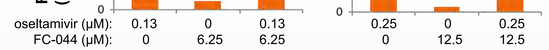
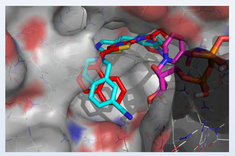


Figure 5. SIRT2-targeted antiviral synergizes with oseltamivir. Shown are two combinations of oseltamivir and FC-044 demonstrating greater than additive effects of the combination. Proof of synergy was demonstrated by the Chou-Talalay model demonstrating a combination index less than one, 0.68 < CI < 0.71, across the full spectrum of drug concentration combinations and degree of viral inhibition.



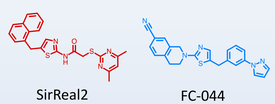


Figure 6. The FH-003 series acts through SIRT2 inhibition. High resolution crystal structures have been solved for SIRT2 with binding to multiple inhibitory ligands. FH-003 series compounds including FC-044 dock with high predicted stability into a hydrophobic binding pocket discovered for SIRT2 ligand SirReal2 (*Rumpf Nature Communications 6:6263*). Kinetic enzyme data (not shown) confirms uncompetitive inhibition by FH-003 series compounds.

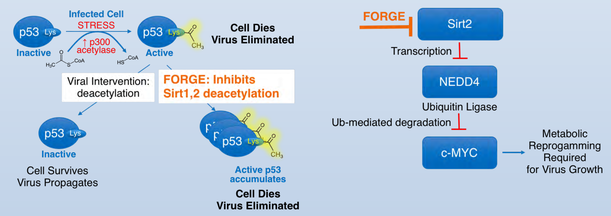


Figure 7. SIRT2 inhibition can activate p53 (*Hoffmann J.Biol.Chem. 289:5208*) and down-regulate c-myc (*Jing Cancer Cell 29:297*).

Results (cont.)

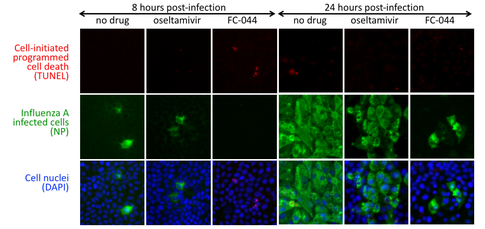


Figure 8. SIRT2 inhibition restores intrinsic apoptosis. p53 is a tumor-suppressor, that initiates selective suicide of cells undergoing genotoxic stress (tumor-inducing condition) or viral replication. FC-044 eliminates infected cells by programmed-cell-death and protects uninfected cells as assayed by TUNEL and DAPI stain, respectively. In addition, FC-044 is significantly better than oseltamivir in preventing virus spread in culture, predicting a wide, effective window-of-administration. Oseltamivir must be administered within 48 h. of infection to be effective in patients.

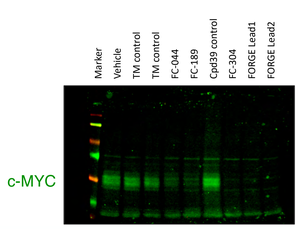


Figure 9. SIRT2 inhibition restores host metabolism. c-myc is an oncogene that can improve virus growth by activating glutamine metabolism and nucleoside biosynthesis needed for optimal viral nucleic-acid replication (*Smallwood Cell Rep. 19:1640*). FORGE SIRT2-inhibiting antivirals reduce c-MYC protein expression by Western blot and inhibit the growth of c-MYC-addicted tumor cells (data not shown).

Summary

FORGE Life Science has developed broad-spectrum antivirals that are well-tolerated and orally bioavailable, comparable to SOC in culture, and work by inhibiting a host protein (SIRT2), thereby significantly reducing virally-acquired drug-resistance. As part of their productive lifecycle, many pathogens actively inhibit p53 or upregulate c-MYC (*e.g.* adenovirus, *Thai Nature Comm. 6:8873; liver-stage malaria, Kaushansky Cell Rep. 3:630*). Nucleotide biosynthesis inhibitors have shown broad-spectrum efficacy against flaviviruses like Dengue (*Boldescu Nature Rev. 16:565*). By optimizing target-organ delivery and effectiveness against diverse pathogens, the vision is to develop a panel of sirtuin-based drugs that are field ready in event of future pandemic or biodefense threat. Because each sirtuin-modulator including SIRT2 inhibitors is broad-spectrum, the path to clinical readiness would be through preclinical/clinical studies for a traditional pathogen such as seasonal influenza. These studies would validate the dosing regimen required for safe, sufficient drug-exposure in humans to achieve an anti-infective effect against the traditional pathogen. In the future, a newly emerging pathogen would be tested in culture against a panel of clinical-stage sirtuin-based drugs to select the most effective one for rapid roll-out and field trials.

Likelihood and expected robustness of anti-infective sirtuin moduation.
Prediction based on known sirtuin substrates, pathogen biology, literature reports, and FORGE genetic & pharmacology data

Activation	Putative Sirtuin Target(s) Affecting Virus Growth	Adenovirus	Flavivirus HCV, Dengue	Hepadnavirus HBV	Herpes Virus HCMV, KSHV	Orthomyxovirus Influenza	Polyomavirus BKV, JCV	Retrovirus HIV, HTLV-1	Rhabdovirdae Rabies	Picornavirus HAV	Papillomavirus HPV	Intracellular parasite literature report or demonstrated experimental effect	Intracellular bacteria
SIRT1	NF-KB, dsDNA break repair, glycolysis, fatty acid oxidation, histones, rRNA transcription, autophagy	***	***	?	***	***	**	?	**	**	**	M. tuberculosis S.typhimurium	
SIRT2	alpha-tubulin	***	*	**	**	***	*	*	*	*	*		
SIRT3	TCA cycle, fatty acid oxidation	***	***	*	***	*	*	*	*	**	**		
SIRT4	TCA cycle, fatty acid oxidation	***	**	*	***	*	*	*	*	**	**		
SIRT5	TCA cycle, fatty acid oxidation	***	***	*	***	*	*	*	*	**	**		
SIRT6	NF-KB, TNF-alpha release, dsDNA break repair, glycolysis, histones	***	**	***	**	***	***	***	*	**	**		
SIRT7	ribosome biogenesis, histones	***	*	*	*	*	*	*	*	*	*		
Inhibition													
SIRT1,2	p53, c-myc	**	***	***	***	***	***	***	*	***	***	Leishmania infantum	Listeria Chlamydia

*** direct tie to sirtuins in literature or demonstrated experimental effect
** literature support tying a target of sirtuin deacylation to pathogen
* expected from general viral restriction effect
? unpredictable (conflicting reports)

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