

IN VITRO AND IN VIVO PK STRATEGIES FOR EVALUATING HDAC INHIBITORS



Pauline L. Yeo*, Liu Xin, Xiaofeng Wu, Tony Ng, Evelyn Goh, New Lee Sun, Lijuan Fang, Khng Hwee Hoon, Noah Tu, Kanda Sangthongpitag, Haishan Wang, Binhui Ni, and Kantharaj Ethirajulu

S*BIO Pte Ltd, 1 Science Park Road, #05-09 The Capricorn, Singapore Science Park II, Singapore 117528 Website: www.sbio.com

* Presenting author

INTRODUCTION

Histone deacetylase (HDAC) inhibitors have been recognised as a promising treatment for cancer [1,2]. Several distinct classes of compounds, such as suberoylanilide hydroxamic acid (SAHA) [3] and depsipeptide [4] have advanced to Phase I/II clinical trials in solid tumors and hematological malignancies.

In our HDAC program, *in silico*, *in vitro* and *in vivo* ADME (Absorption, Distribution, Metabolism and Elimination) studies are incorporated quite early in the flow chart with a view to progress compounds with optimal ADME properties and also to support the medicinal chemistry team during the lead optimisation process.

These ADME results obtained help the medicinal chemists to optimise the properties of the lead compounds.

ADME SCREENING STRATEGY

In silico ADME

Estimation of physicochemical properties like clogP (lipophilicity), HBA (hydrogen bond acceptor), HBD (hydrogen bond donor), PSA (polar surface area), metabolic hotspot, and P450 inhibition are helpful for predicting drug ADME properties.

In vitro ADME

Experimental measure of parameters like solubility, permeability, metabolic stability, and P450 inhibition to prioritise compounds for progression into *in vivo* PK studies.

In vivo ADME

Evaluation of the pharmacokinetics properties of the lead compounds in rat and the pharmacological species to further prioritise and progress compounds in the pharmacodynamic studies.

SCOPE OF WORK

We demonstrate the use of preclinical data such as solubility, permeability, metabolic stability, P450 inhibition, and *in vivo* pharmacokinetics evaluation in rodents to prioritise and advance compounds in the discovery pipeline.

In vitro ADME relevance

Absorption/dissolution	Caco-2/Solubility	Cmax/Tmax
Distribution	Plasma protein binding	Volume Distrib.
Metabolism	T _{1/2} microsomes/hep	Clearance
P450 inhibition	need for Clinical	DDI studies

Suberoylanilide hydroxamic acid (SAHA) as the reference HDAC inhibitor and in-house compound(s) data are presented to emphasise the value of the evaluation of ADME properties and its guidance in further optimisation of the lead compounds to deliver best drug candidates.

METHODS

Solubility Profiling

A high throughput kinetic solubility profiling assay carried out in 96-well format with compounds dissolved in 2.5% DMSO/aqueous buffer at pH 7.0 was developed. 5µL of a 10mM stock solution of the compound to be tested was dispensed into the well and 195µL of phosphate buffer at pH 7.0 was added to the well. The resulting concentration in each well was 250µM. A capmat was placed firmly over the plate, which was then placed on the shaker for 1.5 hours. After shaking, the plate was left standing at room temperature for 2 hours. The supernatant was transferred to another plate and 20µL of DMSO was added to the well. The quantification of the compound is done using a calibration curve on the MicroMass Quattro Micro LC/MS/MS system.

Microsome Stability

The test compound at 1 or 5µM is incubated with microsomal protein at 1 mg/mL (Human/Rat/ Mouse/Dog) for 0, 5, 10, 15, 20 and 30 mins and the % turnover of the parent compound estimated by LC/MS method.

P450 inhibition

A compound being evaluated for its P450 inhibition potential is co-incubated with a known substrate for a specific CYP enzyme using c-DNA expressed isozyme or with human liver microsomes. The effect of the test compound on the metabolism of the substrate is then determined. The concentration of the substrate metabolite has been measured using fluorescence or LC/MS/MS method.

CACO2 PERMEABILITY

Test article at 5µM in HBSSg with maximum DMSO concentration less than 1%. 21 to 28 day confluent monolayers of Caco-2 cells in Transwell® assay plates were used. Apical and basolateral side pH 7.4. Plate shaken. N=2, each direction dosed on apical side for (A→B) assessment Dosed on basolateral side for (B→A) assessment. Both apical and basolateral sides sampled at 120 minutes. Concentration of test article determined using LC/MS with 4 point calibration curve.

PHARMACOKINETICS AND BIOANALYSIS

Pharmacokinetic profiling was carried out using oral and intravenous (iv) dosing in rodents. The compounds were dissolved in saline for iv dosing and a vehicle containing 0.5% methylcellulose, 0.1% Tween 80 in water was used for oral dosing. The plasma samples collected as a function of time were frozen at -80°C until ready for bioanalysis. Research grade method validation was carried out for the bioanalytical assays. The components of validation included specificity, limit of quantification, extraction efficiency, accuracy of the calibration standards, inter-day calibration curve, and accuracy and precision of quality control (QC) samples. Liquid-liquid extraction procedure using methyl-tert-butyl-ether (MTBE) was used in the sample preparation for bioanalysis. PK samples and spiked

calibration standards in plasma and QC samples were extracted using MTBE, dried in a speedvac and reconstituted with the mobile phase. A MicroMass Quattro Micro LC/MS/MS system was used in the quantification of the samples in the multiple reaction monitoring (MRM) mode.

LC-MS METHOD

Liquid Chromatography (Waters HT Alliance 2795)

- Column: Luna, C18, 2x50mm, 5µm
- Column temperature: 60°C
- Mobile phase: Water + 0.05%TFA (A)
Acetonitrile + 0.05% TFA (B)
- Injection volume: 50µL
- Flow rate: 1.1 mL/min [1(MS):5(UV) split]
- Run time: 3minutes (5-95%B)
- Autosampler temp.: 20°C

Mass Spectrometry (MicroMass ZQ)

- Electro-spray (ESI) ionisation in the positive mode
- Cone voltage: 20-35V
- SIR mode

LC-MS/MS METHOD (BIOANALYSIS)

Liquid Chromatography (Waters HT Alliance 2795)

- Column: Luna, C18, 2x50mm, 5µm
- Mobile phase: Methanol: 0.1% formic acid in water (60:40) (v/v)
- Injection volume: 40µL
- Flow rate: 0.3mL/min
- Column temperature: 40°C
- Run time: 3-5minutes
- IS: Carbamazepine
- Autosampler temp.: 4°C

Mass Spectrometry (MicroMass Quattro Micro)

- Electro-spray (ESI) ionisation in the positive mode
- Cone voltage: 20-35V
- Collision energy: 12-20 eV
- MRM mode for quantification

RESULTS AND DISCUSSION

S*BIO HDAC Inhibitors Possess Drug-like Properties

Typical range of physicochemical properties

- MW: 350-400 (free base)
- Solubility: ≥50 mg/ml (salt form, aqueous solution); >250µM (pH 7)
- Log P: 3.0-5.0 (MOE ClogP)
- # HB Donor: 2-4 (MOE)
- # HB acceptor: 4-5 (MOE)
- PSA (polar surface area): 80-110
- Rule of 5 violations: NIL

PERMEABILITY

Compound ID	%Recovery		Papp (X10 ⁻⁶ cm/s)		Efflux Ratio	Permeability Classification	Significant Efflux
	A-B	B-A	A-B	B-A			
A	68	74	19.4	14.9	0.8	High	No
B	98	119	3.12	14.4	4.6	High	Yes
C	83	109	0.97	10.5	11	Low	Yes
D	78	103	7.04	19.4	2.8	High	No
E	73	99	9.59	26.0	2.7	High	No
F	17	20	7.11	6.91	1.0	High	No

Permeability Classification: A-to-B Papp < 1.0 X 10⁻⁶ cm/s: Low
A-to-B Papp > 1.0 X 10⁻⁶ cm/s: High
Significant Efflux: Efflux Ratio > 3.0 and B-A Papp > 1.0 X 10⁻⁶ cm/s

Compounds with good aqueous solubility (> 50 mg/mL) and classified as highly permeable in the Caco2 assay are taken forward for *in vivo* PK evaluation.

Compounds identified as potential substrates for P-gp require additional mechanistic experiments before further progression.

P450 DRUG-DRUG INTERACTION POTENTIAL

Compound ID	CYP1A Inhibition	CYP2C9 Inhibition	CYP2C19 Inhibition	CYP2D6 Inhibition	CYP3A4 Inhibition
	IC ₅₀ (µM)	IC ₅₀ (µM)	IC ₅₀ (µM)	IC ₅₀ (µM)	IC ₅₀ (µM)
G	>25	24.1	11.5	>25	>25
H	>25	>25	8.10	13.9	>25
I	>25	>25	5.78	>25	>25
J	>25	10.4	10.3	>25	>25
K	1.24	>5	>5	>25	>25

Weak inhibitors (> 10 µM) – probability for DDI low.

Moderate (1-5µM) and potent inhibitors (<1µM) – inhibition data only indicate, give a signal/flag, there is the likelihood for a clinical drug interaction.

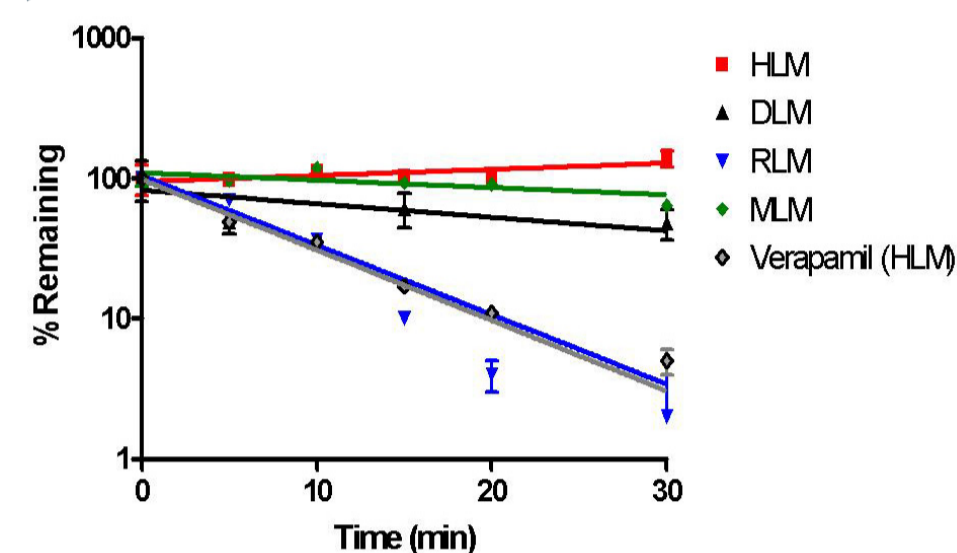
Compounds with no P450 inhibition potential with major drug metabolising isozymes are prioritised and taken forward.

METABOLIC STABILITY

- Compounds that exhibit T_{1/2} > 30 mins (CL_{int}<30 ml/min/kg) [5] in HLM (human liver microsomes) are further screened in the rodents and non-rodent liver microsomes.

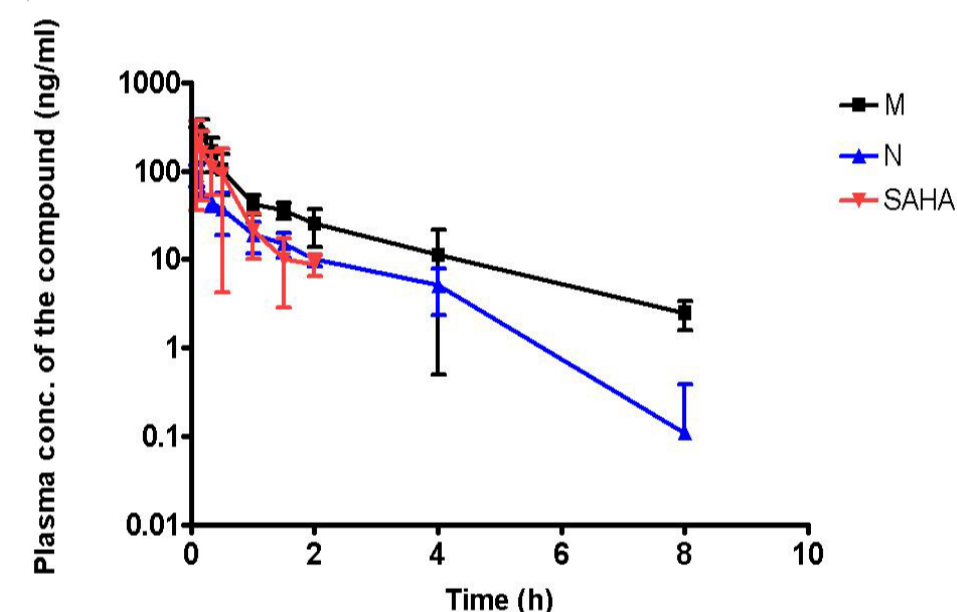
- Compounds that exhibit T_{1/2} > 30 mins (CL_{int}<100 ml/min/kg) in the mice and T_{1/2} > 30 mins (CL_{int}<50 ml/min/kg) in the rat are further prioritised for *in vivo* PK evaluation in these species.

STABILITY OF COMPOUND L IN MICROSOMES



	HLM	DLM	RLM	MLM
T _{1/2} (min)	>30	>30	7	>30
Cl _{int} (ml/min/kg)	<30	<35	196	<100

PK PROFILES OF A SINGLE ORAL DOSE OF COMPOUND AT 5 MG/KG IN RAT



PO dosing exposure levels for compounds M and N were better than SAHA.

CONCLUSIONS

Compounds comply with Lipinski rule of 5

Caco2 Permeability classification-Highly permeable

Aqueous Solubility > 50 µg/mL

Compounds exhibit metabolic stability in the human, dog and mouse liver microsomes

Metabolic instability observed in Rat – Class effect?

Short half-life *in vivo* could be due to glucuronidation-class effect observed with most HDAC inhibitors

No P450 inhibition potential with major drug metabolising isozymes

Compound(s) with most optimal ADME properties are progressed further in *in vivo* for pharmacodynamic evaluation

The *in vitro* and *in vivo* strategies outlined in this work enabled the medicinal chemists to progress towards the goal of obtaining a drug candidate with favourable PK properties for further preclinical development.

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