

Comparative Pharmacokinetics of HDAC Inhibitors in Pharmacological Species



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Abstract

Histone deacetylase (HDAC) inhibitors are an emerging class of therapeutic agents that induce tumor cell cytostasis, differentiation and apoptosis in various hematologic and solid malignancies [1,2]. They may exert their anti-tumor activity through chromatin remodeling and gene expression modulation that affect cell cycle and survival pathways. Four groups of HDAC inhibitors have been characterized: short chain fatty acids (e.g. sodium butyrate and phenylbutyrate), hydroxamic acids (e.g. suberoylanilide hydroxamic acid (SAHA) and PXD-101), cyclic tetrapeptides (e.g. Depsipeptide and Trapoxin) and benzamides (e.g. CI-994 and MS-275)[3].

In the literature, there is very little information on the pharmacokinetic profiles of these HDAC inhibitors in preclinical species. Our group has attempted to compare the pharmacokinetics of SAHA, PXD-101, and LBH589 to novel HDAC inhibitors synthesized in-house by medicinal chemists. Research grade validation comprising of signal-response, intra and inter-day calibration curve, accuracy and precision of quality control samples and limit of quantification (LOQ) was carried out for the bioanalytical assays (HPLC-tandem mass spectrometry) used in the analysis of mouse plasma samples obtained from pharmacokinetic studies.

A single oral and intravenous administration of this new class of compounds (e.g. EX2) resulted in an oral bioavailability > 50 % in mouse (the pharmacological species) compared to < 10 % observed for SAHA, PXD-101 and LBH589.

Introduction

- There is currently a growing interest and competition in the development of histone deacetylase inhibitors as anticancer agents.
- Inhibition of HDAC activity leads to cell cycle arrest, apoptosis and terminal differentiation of cancer cells and represents a promising approach in the treatment of cell proliferative diseases.
- Several small molecule HDAC inhibitors such as SAHA, PXD-101, and LBH589 are at various stages of clinical trials. However, very little is reported on the preclinical data of these compounds.
- Pharmacokinetic and drug metabolism studies conducted during the preclinical phase provide useful information about the absorption, distribution, elimination and efficacy that can be translated to the clinical setting.
- EX2 has shown antiproliferative activity in various *in vitro* human tumor models, including breast, colon, lung, myeloma, ovarian and prostate cancer cell lines. Furthermore, impressive *in vivo* antitumor activity in xenograft models was observed following oral EX2 administration in nude mice.

Materials and Methods

Chemicals

EX2 was a proprietary, novel, HDAC inhibitor, synthesized by Medicinal Chemistry. HDAC inhibitors, SAHA, PXD-101, and LBH-589, were also synthesized by in-house medicinal chemists or contract laboratories for research purposes. Methanol (HPLC grade) was obtained from JT Baker, methyl-tert-butyl ether (ACS grade) was purchased from Acros, formic acid (ACS grade) was from Merck, and the internal standard, was Cabamazepine (Sigma-Aldrich) or an analogue in the series. Mouse (Balb-c) plasma was harvested from mice purchased from National University of Singapore Centre for Animal Resources (NUS-CARE).

LC/MS/MS Assay

LC/MS/MS system : Waters HT Alliance 2795/MicroMass Quattro Micro MS (Waters Corporation)
HPLC column : Luna, C18, 2x50 mm with 5 µm particle size, set at 40°C (Phenomenex, USA)
Mobile phase : 60% methanol and 40% water with 0.1% formic acid.
Flow rate : 0.3 mL/min
Sample volume injected : 40 µL
Autosampler set at 4°C
Analysis time : 3-5 minutes.
ES+ mode, source temperature set at 120°C.

The cone voltage and collision energy optimized for the MRM transitions of the compounds are summarized in Table 1:

Table 1: MRM transitions for EX2 and reference HDAC inhibitors

| Compound | Cone Voltage (V) | Collision Energy (eV) | MRM Transition |
|--------------------|------------------|-----------------------|----------------|
| EX2 | 22 | 15 | 294→122 |
| SAHA | 25 | 12 | 265→232 |
| PXD-101 | 20 | 20 | 319→93 |
| LBH-589 | 25 | 18 | 350→158 |
| Carbamazepine (IS) | 25 | 14 | 237→194 |

Extraction Procedure

Samples, QCs and calibration standards were aliquoted to labeled 1.5 mL of Eppendorf tubes using 50 µL aliquots mouse plasma. An aliquot of 10 µL of the internal standard was added to each tube. Methyl-tert-butyl-ether (MTBE) was added to 1.25 mL to each tube. The tubes were placed on a large capacity mixer set at 60% motor speed with regular pulsing intervals for 30 minutes. The solutions were clarified by centrifugation for 10 minutes at 11,000 g on the Eppendorf Centrifuge (Model 5415R) at 4 °C.

The supernatant was transferred to clean labeled Eppendorf tubes and dried in a Speedvac (Thermo Savant) set at medium setting (~35 °C) for about 20 minutes. The resulting residue was reconstituted with 100 µL of 60% methanol/40% water. The reconstituted samples were transferred to HPLC vials for analysis on the Quattro Micro LC/MS/MS system.

Data Analysis

The methods were validated for signal-response of the calibration standards, autosampler stability for ~15 hours (intraday), and interday calibration curve using eight calibration standards excluding the blank plasma. QC samples at three different concentrations in triplicates were prepared to determine the accuracy and precision. The extracted QC samples were compared to unextracted samples to determine the extraction efficiency of the analyte. LLOQ was determined by using triplicate samples of 1 ng/mL and 2 ng/mL to obtain accuracy and precision at the low end.

The Quanlynx software was used for quantification. MRM responses were smoothed twice prior to integration, using a mean smoothing algorithm. The peak area ratios of the analyte to the internal standard versus that of concentration of calibration standards were plotted. The best-fit line was determined by least squares linear regression of the calibration data using a weighting factor of 1/X, where X is the concentration. Concentrations of the analytes were determined using peak area ratios and the regression parameters.

In vivo Pharmacokinetics

Female Balb/c nude mice weighing 18-22 g (8-10 weeks of age) were purchased from Animal Resources Centre (ARC) Western Australia. Animals were quarantined for approximately 1 week prior to the study. They were housed under standard conditions and had *ad libitum* access to water and standard laboratory rodent diet. All studies performed were approved by Institutional Animal Care and Use Committee (IACUC).

All compounds were dissolved in a mixture of DMA/saline (1:9, v/v) for the IV dose (10 mg/kg) and in a mixture of 0.5% methylcellulose, 0.1% Tween 80 and water for the oral dose (50 mg/kg). Mice were randomized according to body weight, grouped three per time point, and administered a single IV dose of compound via the tail vein, or a single oral dose of compound via gavage.

At predefined time points (predose, 5 or 10, 30min, 1, 2, 4, 8, and 24h), mice were sacrificed by overdose CO₂, and blood samples were collected by cardiac puncture. The blood samples were centrifuged for 10 min at 3000 rpm to separate plasma, and the plasma was kept frozen at -80 °C until analysis by LC/MS/MS.

PK Analysis

Pharmacokinetic data analysis was performed by the non-compartmental method using WinNolin 4.0 software (Pharsight, Mountain View, CA, USA). The mean values for the plasma compound concentration-time profiles were used. Oral bioavailability was obtained using the following equation:

$$F(\%) = [AUC_{0-inf}(\text{oral}) \cdot \text{Dose}(\text{IV}) / AUC_{0-inf}(\text{IV}) \cdot \text{Dose}(\text{oral})] \cdot 100$$

In vivo Efficacy Study

Female athymic nude mice, 10 -12 weeks of age, were implanted subcutaneously in the flank with 5 x 10⁶ cells of HCT116 human colon carcinoma. When the tumor reached a size of 100 mm³, the mice were pair matched prior to treatment. Tumor size was measured every second day and the tumor volume calculated as follow:

$$\text{Tumor volume (mm}^3\text{)} = (w^2 \times l) / 2, \text{ where } w = \text{width and } l = \text{length in mm.}$$

SAHA and EX2 were dissolved in an appropriate vehicle (0.5% methylcellulose and 0.1% Tween 80 in water) for oral administration. Drugs were orally administered everyday using a gavage for a period of 21 days.

Tumor growth inhibition (%TGI) was calculated according to

$$\%TGI = [(C_t - T_t) / (C_t - C_{t1})] \cdot 100$$

Where C_t = the median tumor size of the vehicle control group at time t,
 T_t = the median tumor size of the treatment group at the time t,
 C_{t1} = the median tumor size of the vehicle control group on the first day of treatment.

Results

Table 2: Structures of HDAC Inhibitors

| Structure | HDAC1 IC50 (nM) | Structure | HDAC1 IC50 (nM) |
|-----------|-----------------|-----------|-----------------|
| | 51 | | 64 |
| | 120 | | 7 |

In vitro Pharmacokinetics

Table 3: Liver Microsomal (LM) stability

| | Human LM | Rat LM | Mouse LM |
|--------------------------|----------|--------|----------|
| T _{1/2} (min) | > 60 | > 60 | > 60 |
| Cl _{int} | <13.42 | <22.87 | <45.68 |
| Cl _i (L/h/kg) | <0.49 | <0.97 | <1.82 |

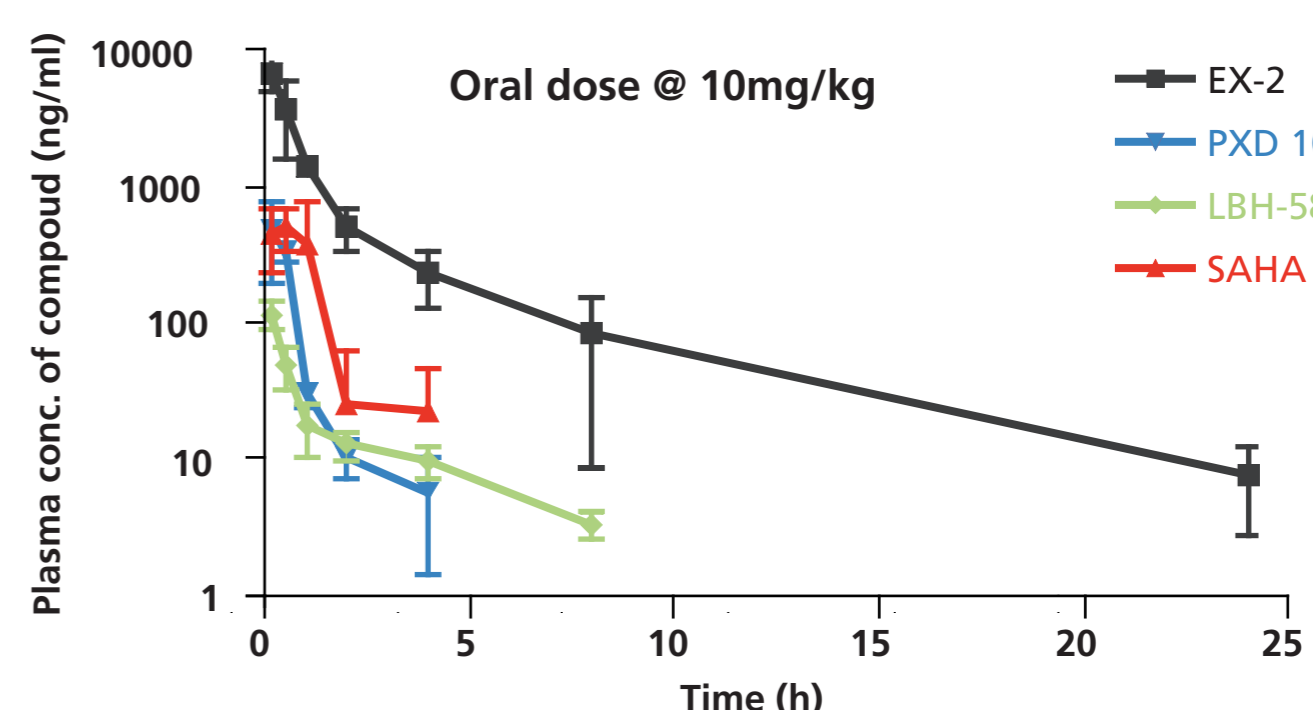
ADME Attributes

- >10 µM for CYP3A4 and CYP2D6 inhibition
- High Caco-2 permeability (pH 7.4)
- Aqueous solubility at 52 µg/mL (pH 7.4)

Table 4: Validation data for LC/MS/MS Assay

| Assay Requirement | Acceptance Criteria | *EX2 | SAHA | PXD-101 | LBH-589 |
|--|--|--|--|--|--|
| Selectivity | Free of interference | Yes | Yes | Yes | Yes |
| Extraction efficiency | ≥ 50% | 50-56% | 60-67% | 59-75% | 59-65% |
| LLOQ | Accuracy and precision ≤30% | 2ng/mL 97±5% | 2ng/mL 96±14% | 2ng/mL 111±14% | 2ng/mL 92±8% |
| Calibration curve | Accuracy ≤ 20% at all concentrations but ≤ 30% at the LLOQ. R ² ≥ 0.95 | Accuracy satisfied. R ² ≥ 0.999 | Accuracy satisfied. R ² ≥ 0.995 | Accuracy satisfied. R ² ≥ 0.995 | Accuracy satisfied. R ² ≥ 0.987 |
| Calibration curve (interday) | Slope of standard curve must not change significantly (≤ 15%) | 15% | 7% | 4% | 10% |
| Stability of extracted samples (intra-day stability of standard curve) | Slope of standard curve must not change significantly (≤ 15%) | 10% | 8% | 9% | 8% |
| QC samples: LQC | Accuracy ≤ 20% at mid and high QCs but ≤ 30% at low QCs. Precision of triplicates ≤ 20%. | 101±6% | 98±6% | 97±11% | 97±14% |
| QC samples: MQC | | 93±2% | 98±8% | 93±5% | 92±6% |
| QC samples: HQC | | 92±2% | 96±14% | 97±5% | 89±6% |

Figure 1: Plasma concentration-time profiles for EX2, PXD-101, LBH589 and SAHA



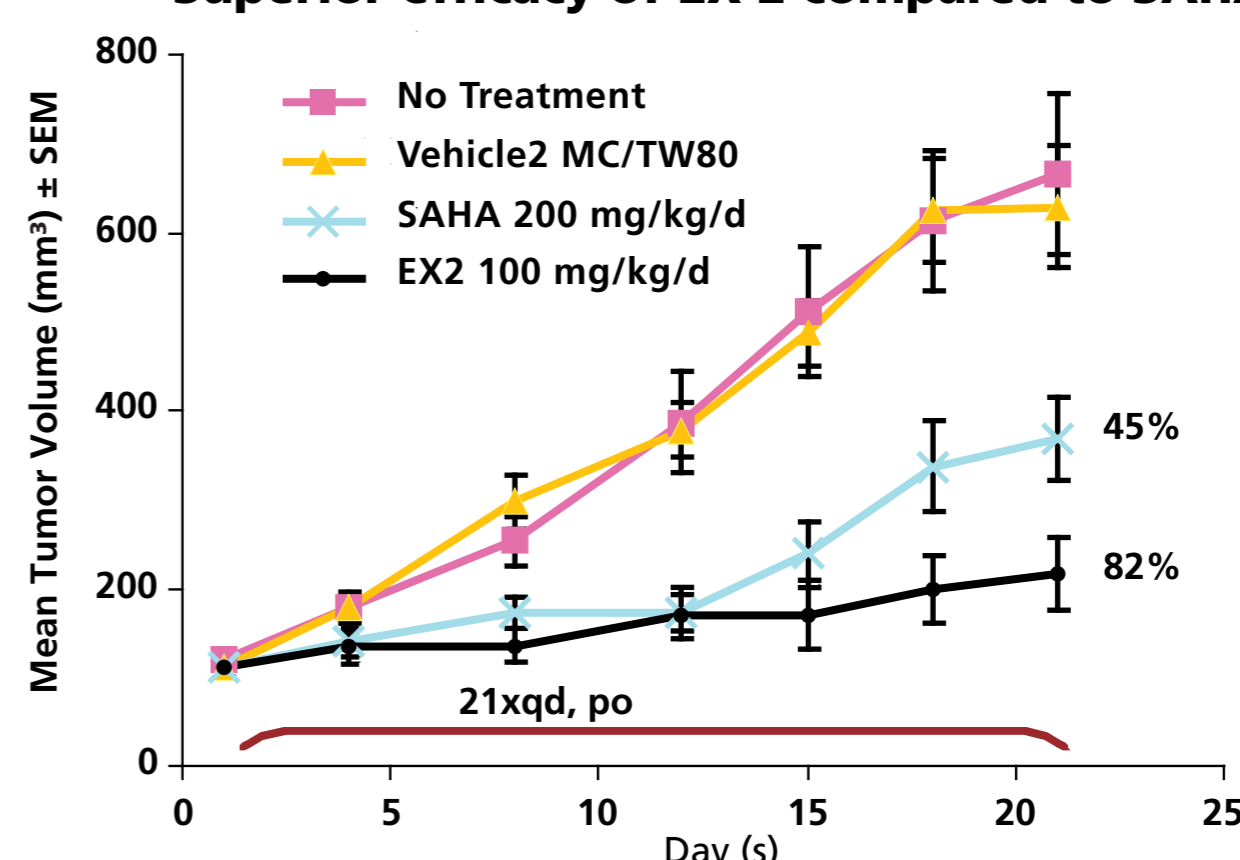
EX2 has longer half life compared to PXD-101, LBH589 and SAHA in mice

Table 5: Pharmacokinetic parameters of EX2, PXD-101, LBH589 and SAHA in mice

| Parameter | IV at 10 mg/kg | | | | PO at 50 mg/kg | | | | |
|------------------------------|----------------|---------|---------|------|------------------------------|---------|---------|------|------|
| | EX-2 | PXD 101 | LBH-589 | SAHA | EX-2 | PXD 101 | LBH-589 | SAHA | |
| T _{1/2} (h) | 1.54 | 1.21 | 1.37 | 0.38 | C _{max} (ng/ml) | 6565 | 489 | 116 | 501 |
| CL (L/h/kg) | 5.03 | 11.6 | 18.3 | 6.73 | T _{1/2} (h) | 4.21 | 1.34 | 2.90 | 0.75 |
| V _d (L/kg) | 11.2 | 20.2 | 36.1 | 3.70 | AUC _{0-∞} (ng.h/ml) | 6118 | 287 | 126 | 619 |
| V _d (L/kg) | 2.16 | 5.03 | 15.1 | 0.81 | F (%) | 61.6 | 6.66 | 4.62 | 8.33 |
| AUC _{0-∞} (ng.h/ml) | 1987 | 862 | 546 | 1486 | | | | | |

Excellent oral bioavailability for EX-2 in the pharmacological species compared to competitors' compounds

Figure 2: EX2 and SAHA in HCT-116 Xenograft Model Superior efficacy of EX-2 compared to SAHA



Conclusions

- Superior oral bioavailability in mice compared to PXD-101, LBH589 and SAHA.
- Potent *in vivo* anti-tumor activity.
- Drug-like physicochemical properties.
- Potential drug development candidate.

References

- 1 Johnstone, RW, Nat. Rev. Drug Discov. 1:287-99, 2002.
- 2 Marks PA, Richon VM, Rifkind RA, J Natl Cancer Inst (Bethesda) 92:1210-6, 2000.
- 3 Yoo CB and Jones PA, Nat. Rev. Drug Discov. 5:37-50, 2006.