Simvastatin hydroxy acid fails to attain sufficient CNS tumor exposure to achieve cytotoxic effect: Result of a preclinical cerebral microdialysis study

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Supplemental material

Materials and methods

Animals

CD1 nude mice were purchased from Charles River (Wilmington, MA). Animals were kept under controlled temperature and humidity conditions, and were exposed to 12 hour day and night cycles. All animal studies performed were approved by the Institutional Animal Care and Usage Committee (IACUC) of St. Jude Children's Research Hospital.

Chemicals and reagents

SV dosing suspension was prepared in 0.5% carboxymethylcellulose at a concentration of 10 mg/mL. SV (analytical standard) and fluvastatin (FV) were purchased from Sigma-Aldrich (St. Louis, MO). Simvastatin hydroxy acid ammonium salt (SVA, 98%) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Lovastatin (LV, 99.8%) was purchased from EMD Millipore Chemicals (Billerica, MA). Acetonitrile, methyl t-butyl ether (TBME), ammonium acetate, and acetic

acid purchased from Fisher Scientific (Fair Lawn, NJ). All solvents used were HPLC grade. Water was purified using Milli-Q Advantage A10 system (Millipore, Billerica, MA). CD1 mouse plasma (Na heparin) was purchased from BioChemed (Winchester, VA). Artificial cerebrospinal fluid (aCSF) consisting of NaCl (148 mM), KCl (4 mM), $MgCl_2$ (0.8 mM), $CaCl_2$ (1.4 mM), Na_2HPO_4 (1.2 mM), NaH_2PO_4 (0.3 mM), and dextrose (5 mM), was prepared in-house and pH was adjusted to 7.4 using 1N NaOH (Benveniste and Huttemeier, 1990).

Use of in-silico approach to prioritize compounds

We used two different published *in-silico* models to predict brain to plasma partition coefficient of a drug using its physico-chemical properties to prioritize statins for preclinical PK studies. The first *in-silico* model was a mathematical model (**Equation A**) designed by Feher et al that correlates physico-chemical descriptors to logBB (logarithm of ratio between brain and plasma exposure) (Feher et al., 2000),

$$\log BB = 0.4275 - 0.0017 \times PSA + 0.1092 \times clogP - 0.3873 \times HBA \qquad \textbf{Equation A}$$

The second model was designed based on several molecular discreptors identified by Mensch et al and Pajouhesh et al as having influence on CNS penetration (Pajouhesh and Lenz, 2005; Mensch et al., 2009). We have assigned binary code (1 or 0) to these molecular descriptors based on their favorable and unfavorable value and calculated a “rule of thumb” score. Favorable values of molecular descriptors were: molecular weight (MW) <450 gram/mole, logarithm of hydrophobicity (clogP) <5, number of hydrogen (H) bond donor (HBD) <3, number of H-bond acceptor (HBA) <7, number of rotatable bond (NRB) <8, number of H-bond (NHB) <8, polar surface area (PSA) <90 $Å^2$, sum of nitrogen and oxygen atom (N + O) <6, and clogP - (N + O) > 0

(Pajouhesh and Lenz, 2005; Mensch et al., 2009). Molecular descriptors required for these *in-silico* models were obtained from the PubChem database (http://www.ncbi.nlm.nih.gov/pccompound).

Plasma pharmacokinetic study

A plasma PK study of SV was performed in CD1 nude mice bearing cortical implants of mouse ependymoma (Mohankumar et al., 2015). Simvastatin (10 mg/mL prepared in 0.5% carboxymethylcellulose) was administered at dosage of 100 mg/kg via oral gavage. A serial sacrifice design was used to allow for collection of adequate plasma volume for analysis of SV and its active metabolite SVA. Blood samples from three mice were collected at each of 0.25, 1.5, 3.5, 6, and 8 hr after the SV dose using heparinized cardiac punctures. Immediately after blood sample collection, plasma was separated and samples were stored at -80 °C until analysis.

Cerebral microdialysis study

Cerebral microdialysis studies were performed in CD1 nude mice (20 - 30 g) bearing cortical implants of mouse ependymoma. Tumor cells (*Ink4a/Arf-null* + *RTBDN* + *Luci*, 2000 cells) and microdialysis guide cannula (MD-2255, BASi) were stereotactically implanted into the cerebral cortex using a previously reported method (Carcaboso et al., 2010; Atkinson et al., 2011). Once *in vivo* tumor bioluminescence measured to be minimum of 10^7 photons/sec (~ 14 days), the microdialysis study was performed by removing the stylet in the guide cannula, and replacing it with a semipermeable 1 mm microdialysis probe with molecular weight cut-off of 38 KDa (MD-2211, BASi). The microdialysis probe was continuously perfused with aCSF at a flow rate of 0.5 µL/min using a perfusion pump. To improve microdialysis recovery of SVA,

β-cyclodextrin (BCD) was added to the microdialysis perfusate (aCSF) at a concentration of 10% w/v. After probe equilibration for an hour, the microdialysis study was initiated by dosing animals with SV suspension (100 mg/kg, orally) prepared in 0.5% carboxymethylcellulose at a concentration of 10 mg/mL. Over the next six hours, the microdialysis fractions were collected at flow rate of 0.5 µL/min for 1 hr interval using a fraction collector. Dialysate samples were collected in a tube containing 20 µL of 100 mM ammonium acetate (pH 4.5) to maintain an acidic pH of the dialysate. During the microdialysis experiment, three blood samples were collected by retroorbital bleed at 0.083, 1.5, and 4.75 hr after the dose to measure plasma concentration of SVA. Plasma and dialysate samples collected during the microdialysis study were stored at -80 °C until analysis.

Recovery of each microdialysis probe was determined using in vitro recovery experiments (de Lange et al., 1997), where microdialysis probes were submerged into the SVA solution prepared in aCSF at a concentration of 750 ng/mL (C_{bulk}) and perfused with aCSF solution containing 10% w/v BCD at a flow rate of 0.5 µL/min. After probe equilibration for 1 hour, subsequent dialysate fractions of 1 hr interval were collected for next three hours. Microdialysis probe recovery was calculated using **Equation B**.

$$\%\,\text{Recovery Ratio (RR)} = \frac{C_{dialysate}}{C_{bulk}} \times 100 \qquad \textbf{Equation B}$$

Sample analysis

SV and SVA concentrations in mouse plasma samples and SVA concentration in dialysate samples were analyzed using a validated liquid chromatography tandem mass spectrometry (LC MS/MS) method. Calibration standards were prepared at a range of 1 - 500 ng/mL for SV in mouse plasma, and at 0.5 - 250 ng/mL for SVA in mouse plasma

and aCSF. Initial sample preparation was done on wet ice. Protein precipitation with acetonitrile and liquid-liquid extraction with TBME was used for SV and SVA assays, respectively.

For the SV assay, an aliquot of 25 µL of mouse plasma was mixed with 10 µL of IS working solution (LV, 250 ng/mL) and 20 µL of 100 mM ammonium acetate buffer (pH 4.5 ± 0.05) in a glass tube. The resulting mixture was vortexed with 80 µL of acetonitrile for 1 min, followed by centrifugation at 3000 rpm for 5 min. A 5 µL of organic supernatant was injected into the chromatographic system.

For the SVA assay, an aliquot of 25 µL mouse plasma or aCSF was mixed with 10 µL of IS (FV, 25 ng/mL) and 20 µL of 100 mM ammonium acetate buffer (pH 4.5 ± 0.05) in a glass tube. The resulting mixture was vortexed with TBME (1 mL) for 10 min, followed by centrifugation at 3000 rpm for 10 min. The organic supernatant was then transferred to a glass vial, dried, and reconstituted with 80 µL of mobile phase. An aliquot of 5 µL was injected onto the chromatographic column. All separations were performed using a Phenomenex Luna C18 column (3µL, 100 Å 50 x 2.00 mm) maintained at 25°C. The mobile phase consisted of 1 mM ammonium acetate (pH 4.5 ± 0.05) and acetonitrile with 0.01% acetic acid (20:80, v/v) for the SV assay, and 5 mM ammonium acetate (pH 4.5 ± 0.05) and acetonitrile (25:75 v/v) for the SVA assay and were pumped at a flow rate of 0.25 mL/min. Mass spectrometric analysis was performed using AB SCIEX API-4000 mass spectrometer system (Framingham, MA). The MRM transitions of m/z 436.30 > 285.20 and m/z 422.30 > 199.20 were chosen for SV and LV, respectively for SV assay, and m/z 435.25 > 319.25 and m/z 410.10 > 348.25 were chosen for SVA and FV, respectively for SVA assay. All the methods were found to be

linear and reproducible with typical r values > 0.99. The intra- and inter- day assay coefficients of variation were < 10% with accuracies ranging from 91.4 to 108%.

Pharmacokinetic data analysis

A population based pharmacokinetic model was used to derive pharmacokinetic parameters for the plasma disposition of SV and SVA. A drug metabolism pharmacokinetic (DMPK) model (**Figure 1 in the manuscript**) consisting of a gut and a plasma compartment was fitted to the plasma concentration time data obtained from plasma PK study. Mathematically K_a represents first order absorption of simvastatin after oral drug administration, CL_m/F represents metabolic clearance of simvastatin lactone to simvastatin hydroxy acid, CL_{sva}/F represents first order systemic clearance of simvastatin hydroxy acid from the central compartment, whereas V_{sv}/F and V_{sva}/F represent the plasma volumes of distribution for simvastatin and simvastatin hydroxy acid. The apparent mean pharmacokinetic parameters along with their standard error of estimates (SEE) and inter-individual variability (IIV) were estimated using nonlinear mixed effect modeling (NONMEM 7.2, ICON development solutions). First order conditional estimation (FOCE) method with interaction was used derive population mean parameter estimates and variance terms, whereas SEE were derived using importance sampling method (IMP) with interaction by performing only the expectation step (EONLY=1) (Bauer, 2011). To characterize the extent of SVA penetration in ependymoma tumor, it is necessary to derive extensive plasma as well as tumor extracellular fluid (tECF) profile in each mouse used in microdialysis study. However, we were limited to three plasma samples per animal because of volume of plasma that could be withdraw and required for bioanalysis of SVA. Therefore, we derived a limited-

sampling model (LSM) to collect plasma samples during microdialyisis study using D-optimality implemented in ADAPT 5 (D'Argenio, 1981; D'Argenio et al., 2009). Plasma PK parameters obtained previously were used as priors to derive LSM.

SVA plasma concentrations obtained during microdialysis study were modeled together with SA and SVA data obtained during plasma PK study to derive full SVA plasma concentration time profile for each mouse enrolled in microdialysis study. Estimation method and modeling techniques were similar to that mentioned above. SVA area under plasma concentration time curve ($AUC_{plasma}^{0\rightarrow t\,hr}$) was estimated by integration of concentration time profile using modeling, whereas area under tECF concentration time curve ($AUC_{tECF}^{0\rightarrow t\,hr}$) was estimated using the trapezoidal method by replacing below LLOQ data with LLOQ as depicted in **Equation C**.

$$AUC_{tECF}^{0\rightarrow t\,hr} = \sum_{i=1}^{t} C_i \times \tau \qquad \textbf{Equation C}$$

Where C_i is the SVA concentration observed in i^{th} dialysate sample collected over 1 hour interval (τ). The extent of SVA distribution in tECF ($K_{p,u}$, tumor to plasma partition coefficient of SVA) was calculated as a ratio of area under unbound tECF to total plasma concentration time profile ($AUC_{tECF}^{0\rightarrow 6hr} / AUC_{plasma}^{0\rightarrow 6hr}$).

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