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Short Communication

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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Running Title:

Limited CNS tumor penetration of simvastatin hydroxy acid

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Abbreviations:

tECF, tumor extracellular fluid; SVA, simvastatin acid; SV, simvastatin lactone; $K_{p,u}$, tumor to plasma partition coefficient; HTS, high throughput screening; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; BBB, blood brain barrier; AUC, area under concentration time curve; DMPK, drug metabolism pharmacokinetic; LLOQ, lower limit of quantification; MTD, maximum tolerated dose

Abstract

HMG-CoA reductase inhibitors were potent hits against a mouse ependymoma cell line, but their effectiveness against CNS tumors will depend on their ability to cross the blood-brain barrier and attain a sufficient exposure at the tumor. Among HMG-CoA inhibitors that had activity in vitro, we prioritized simvastatin as the lead compound for preclinical pharmacokinetic studies based on its potential for CNS penetration as determined from *in-silico* models. Further we performed systemic plasma disposition and cerebral microdialysis studies of simvastatin (100 mg/kg, PO) in a murine model of ependymoma to characterize plasma and tumor extracellular fluid (tECF) pharmacokinetic properties. The murine dosage of simvastatin (100 mg/kg, oral) was equivalent to the MTD in patients (7.5 mg/kg, PO) based on equivalent plasma exposure of simvastatin acid (SVA) between the two species. Simvastatin (SV) is rapidly metabolized in murine plasma with 15 times lower exposure compared to human plasma. SVA exposure in tECF was <33.8 ± 11.9 µg/L*hr, whereas tumor to plasma partition coefficient of SVA ($K_{p,u}$) was <0.084 ± 0.008. Compared to *in vitro* washout IC_{50}s, we did not achieve sufficient exposure of SVA in tECF to suggest tumor growth inhibition, therefore simvastatin was not carried forward in subsequent preclinical efficacy studies.

Introduction

Ependymomas are rare tumors arising from ependymal tissue of the brain and spinal cord (McGuire et al., 2009). Although the current treatment options for ependymoma of aggressive surgical resection and conformal radiotherapy are effective in selected patients, approximately 40% of patients either fail to respond or relapse from their disease. Moreover, in children less than 3 years of age, the use of radiotherapy is limited by the occurrence of neurocognitive deficits (Merchant et al., 2009; Netson et al., 2012). Thus, effective chemotherapies for ependymoma are sorely needed, and represent an unmet medical need. We have applied a high throughput screening (HTS) approach using various chemical libraries including FDA-approved compounds to find effective chemotherapeutic agents against ependymoma (Mohankumar et al., 2015). In our HTS, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) inhibitors (e.g., simvastatin, lovastatin, fluvastatin and pitavastatin) were identified as potent and selective cytotoxic agents against a mouse ependymoma cell line.

HMG CoA reductase is an essential enzyme for synthesis of cholesterol via the mevalonate pathway. Interest in the mevalonate pathway as a target for cancer therapy was initially raised when its end product farnesyl isoprenoid was found to be involved in anchoring small GTPase (e.g., RAS and RHO protein) to the cell membrane. This anchorage drives intracellular signal transduction that regulates vital cellular processes such as growth, proliferation, and survival (Casey et al., 1989; Konstantinopoulos et al., 2007). Therefore inhibition of mevalonate pathway is considered a reasonable strategy to hamper tumor growth. Statins, which are inhibitors of the HMG CoA reductase enzyme and sterol synthesis showed tumor inhibitory effect against various human cell

lines including human glioma cell lines (Kikuchi et al., 1997). However, when evaluated in an *in vivo* mouse model of glioblastoma multiforme, simvastatin failed to show tumor inhibitory effects (Bababeygy et al., 2009). Multiple factors could be responsible for failure of chemotherapies against central nervous system (CNS) tumors, but often a primary factor is limited blood-brain barrier permeability (Parrish et al., 2015). Very limited data are available to provide insight into the CNS penetration of statins, and the data available typically provide only a point estimate of total plasma and brain homogenate concentrations (Thelen et al., 2006).

To gain confidence in translating *in vitro* activity results to *in vivo* efficacy studies, it is crucial that distribution of the compound in the target tissue be characterized. We have utilized cerebral microdialysis to assess local drug distribution as part of our standard preclinical drug developmental strategy (Jacus et al., 2014). Objectives of the current study were to prioritize statins for preclinical pharmacokinetic (PK) studies using a physico-chemical property-based *in-silico* brain-to-plasma partition coefficient prediction model, to characterize the plasma PK of simvastatin (SV) and its active metabolite simvastatin acid (SVA), and to determine tumor disposition of SVA using a cerebral microdialysis technique in our murine model.

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Materials and methods

Use of in-silico approach to prioritize compounds

Two different published *in-silico* models were used to prioritize the statins for preclinical studies based on their predicted ability to cross the blood-brain barrier (BBB) (see **Supplement Data** for additional details). The first *in-silico* model predicted logBB (logarithm of ratio between brain and plasma exposure) using compound specific physico-chemical descriptors (Feher et al., 2000). In the second *in-silico* model, we calculated a 'rule of thumb' score using favorable and unfavorable values of compound specific molecular descriptors for CNS penetration. (Pajouhesh and Lenz, 2005; Mensch et al., 2009).

Preclinical pharmacokinetic study

Plasma PK and cerebral microdialysis studies of SV and SVA were 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 100 mg/kg via oral gavage. For the plasma PK study, a serial sacrifice design was used and plasma samples were collected at 0.25, 1.5, 3.5, 6, and 8 hr postdose (n=3 mice per time point) via cardiac puncture for measurement of SV and SVA (see **Supplemental Data** for additional details). For the microdialysis study, a microdialysis probe, precalibrated for recovery, was implanted in the intracerebral murine ependymoma tumor through a preimplanted guide cannula. The microdialysis probe was continuously perfused with aCSF containing 10% w/v β-cyclodextrin (BCD) at a flow rate of 0.5 µL/min. After the probe was allowed to equilibrate for 1 hr, mice were dosed with SV (100 mg/kg, orally) and plasma samples were collected at 0.083,

1.5, and 4.75 hr postdose whereas 1-hr microdialysis fractions were collected up to 6 hr for analysis of SVA (see **Supplemental Data** for additional details).

Pharmacokinetic data analysis

A population PK model was used to derive PK parameters for the plasma disposition of SV and SVA. A drug-metabolite pharmacokinetic (DMPK) model (**Figure 1a**) consisting of a gut and a plasma compartment was fitted to the plasma concentration time data. The apparent mean PK 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 to derive population mean parameter estimates and variance terms, whereas SEE were derived using importance sampling method (IMP) with interaction by running the expectation step only (EONLY=1). 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 I**.

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

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 ($AUC_{tECF}^{0\rightarrow t\ hr} / AUC_{plasma}^{0\rightarrow t\ hr}$). Additional details of the PK data analysis are provided in **Supplemental Data**.

Results and discussion

In-silico approach to prioritize compound

From *in vitro* HTS simvastatin, fluvastatin, lovastatin, and pitavastatin were identified as drugs with antitumor activity against murine ependymoma tumors with *in vitro* 72 hr IC_{50} values below 0.1 µM. To optimize resources and time required for preclinical efficacy studies, it was necessary to establish a method to prioritize which of these drugs would be carried forward into these studies. We chose to prioritize compounds based upon their likelihood for CNS penetration, however, for the statins scarce reliable data on brain distribution is available. Thus, we adapted an alternative approach to assess CNS penetration using *in-silico* models. We used the Feher model and calculated a logBB for simvastatin, lovastatin, fluvastatin, and pitavastatin of -1.120, -1.164, -1.268, and -1.669, respectively. This is compared to our second *in-silico* model which calculated "rule of thumb" scores for the same compounds of 8, 8, 5, and 4, respectively. Based on the results of our *in-silico* model calculations, we ranked the priority of the statins in order of high to low: simvastatin, lovastatin, fluvastatin, and pitavastatin. When Sierra and colleagues measured *in vitro* passive permeability using parallel artificial membrane permeation assay, a similar rank order of the statins was determined (Sierra et al., 2011). Although the results of our *in-silico* approach did not clearly distinguish between the top two statins (i.e., simvastatin and lovastatin), we used other characteristics (i.e., previously published antitumor activity, dosing data in pediatrics) to make the decision to carry simvastatin forward as our lead statin for further preclinical PK studies (Hindler et al., 2006).

Plasma pharmacokinetic study

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After oral absorption, the prodrug SV converts to the active SVA by enzymatic and non-enzymatic means (Vickers et al., 1990). To describe the plasma disposition and quantitate systemic exposures of SV and SVA in CD1 nude mice for comparison with tolerable exposures in human, and to derive a plasma limited sampling model (LSM) for use during cerebral microdialysis studies, we first studied plasma PK of SV (100 mg/kg, oral) and resultant SVA in the murine EPY model using a serial sacrifice design. As shown in **Figure 1b**, plasma SV and SVA concentration-time data were well described using a population based DMPK model (**Figure 1a**). Model predicted population mean parameter estimates ± standard errors were 0.67 ± 0.45 hr^{-1} for absorption constant of SV, 2812 ± 591 L/hr/kg and 281 ± 9.8 L/hr/kg for metabolic clearance of SV (CL_m/F) and systemic clearance of SVA (CL_{SVA}/F), respectively, and 159 ± 1.0 L/kg and 51.4 ± 0.47 L/kg for plasma volume of distribution of SV (V_{SV}/F) and SVA (V_{SVA}/F), respectively. The IIV for K_a, CL_m/F, and CL_{SVA}/F were estimated to be 31%, 84%, and 26%, respectively.

As shown in **Table 1**, we compared model predicted $AUC_{plasma}^{0\rightarrow 12\,hr}$ in our murine model with that in patients studied with high-dosage simvastatin (maximum tolerated dosage - 7.5 mg/kg simvastatin given orally, twice a day) to determine the human equivalent dosage of SV for our murine model (Ahmed et al., 2013). The SVA AUC values observed in our murine model were comparable to those in patients. However, SV AUC values in our murine model were ~15 times lower than patients. This suggests that the metabolism of SV to SVA occurs more rapidly in mouse plasma than in human plasma, which may be due to presence of caboxylesterase enzyme (responsible for metabolism conversion of SV to SVA) in mouse plasma (Bahar et al., 2012). The active

metabolite SVA has been shown to inhibit HMG CoA reductase enzyme of the mevalonate pathway and reduce cholesterol synthesis. Therefore it was suggested that exposure to SVA is associated with its tumor inhibitory activity in several cancers (Gazzerro et al., 2012). In fact, SVA was twice as potent as SV, with respect to cytotoxicity, when applied to our murine EPY cell line *in vitro* (data not shown). Considering the similar exposures of SVA between mice and human patients, 100 mg/kg SV in mice was considered equivalent to the human MTD (7.5 mg/kg) and was used in the subsequent microdialysis study. The optimal plasma sampling time points derived using LSM for SVA for microdialysis experiments were found to be 0.08, 1.5, and 4.75 hr postdose.

Cerebral microdialysis study in tECF

We have used cerebral microdialysis to measure unbound SVA concentration in the tECF of mice with ependymoma tumors. The design of our cerebral microdialysis experiments allows us to collect small volumes of plasma and dialysate; therefore, we were only able to evaluate disposition of SVA during this study. Mean ± standard deviation of plasma PK parameter post hoc estimates from individual microdialysis experiments (n=4) were 0.52 ± 0.30 1/hr for K_a, 2936 ± 313 L/hr/kg for CL_m/F, 12.3 L/kg for V_{sv}/F, 157 L/kg for V_{sva}/F and 238 ± 133 L/hr/kg for CL_{sva}/F. Variance parameters for V_{sv}/F and V_{sva}/F were fixed to zero. The *in vitro* percent recovery of microdialysis probe for SVA was 10.9 ± 4.0%. SVA concentrations in dialysate were corrected for probe recovery to calculate actual SVA concentration in the tECF. Although we used a sensitive (LLOQ = 0.5 ng/mL) LC-MS/MS method to quantify SVA in dialysate samples, over half the samples were below the LLOQ. To depict the SVA tECF disposition, we

used all tECF concentration data obtained during our microdialysis experiments. **Figure 2** shows total plasma and unbound tECF concentration time profile of SVA in four microdialysis experiments. The mean tECF to plasma partition coefficient of SVA ($K_{p,u}$) of SVA estimated by replacing below LLOQ data with LLOQ was 0.084 ± 0.008. Unbound tECF concentration of SVA was below the lowest *in vitro* IC_{50} (0.04 µM ~ 17 µg/L for 72 hr exposure, data not shown) against our mouse ependymoma cell line. Assuming similar *in vitro* and *in vivo* tumor inhibitor potency of SVA against ependymoma and considering the *in vitro* IC_{50} as a minimum concentration cut-off for promoting a compound to further preclinical efficacy studies, simvastatin was unsuccessful in achieving sufficient SVA tECF concentrations at clinically tolerable plasma exposures. Based on these data, simvastatin was not carried forward in our preclinical pipeline. In the literature, simvastatin had a tumor inhibitory effect against several glioma cell lines (Gliemroth et al., 2003). However, when tested *in vivo* at 10 mg/kg oral SV using mouse model of glioblastoma multiforme, simvastatin failed to show tumor growth inhibition (Bababeygy et al., 2009). The disconnect between these two studies could be due to the low concentration of SVA in tECF.

In conclusion, among the four statins with in vitro antitumor activity against mouse ependymoma, we have used an *in-silico* approach to prioritize simvastatin for plasma and tumor microdialysis studies. We have systematically characterized the plasma disposition of simvastatin (SV and SVA) and tECF disposition of SVA, and conclude that the SVA tECF concentration in our mouse model was not sufficient to achieve ependymoma tumor growth inhibition, therefore simvastatin was not pursued further in our preclinical pipeline.

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Authors Contributions:

Participated in research design: Patel, Jacus, Boulos, Freeman, Gilbertson, Stewart

Conducted experiments: Patel, Jacus, Davis, Boulos, Vuppala

Contributed new reagents or analytic tools: Vuppala, Gilbertson, Stewart

Performed data analysis: Patel, Freeman, Stewart

Wrote or contributed to the writing of the manuscript: Patel, Turner, Vuppala, Freeman, Stewart

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Footnotes

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Legends for figures

Figure 1: **(a)** Schematic diagram of drug metabolism pharmacokinetic (DMPK) model (K_a - first order absorption constant; V_{sv} and V_{sva} - plasma volume of distribution for SV and SVA, respectively; CL_m - metabolic clearance for SV to SVA metabolism; CL_{sva} - systemic clearance of SVA from plasma compartment) **(b)** Simvastatin (SV) and simvastatin acid (SVA) plasma concentration time profile for full plasma pharmacokinetic study (open circle and open triangle represents observed SV and SVA concentrations, respectively; solid and dotted line represents population mean predicted SV and SVA concentrations, respectively)

Figure 2: Simvastatin acid (SVA) concentration time profile in plasma and tumor ECF for individual microdialysis experiment (open circle represents observed SVA concentrations in plasma, Open triangle and diamond represents SVA concentrations in tumor ECF that are above and below LLOQ, respectively; solid and dashed line represents model predicted concentration in plasma and fractional mean concentration profile in tumor ECF for SVA, respectively; horizontal dotted line represents recovery corrected LLOQ for individual mouse for SVA method for tumor ECF)

Table

Table 1: Comparison of plasma pharmacokinetic parameters for SV and SVA between mouse ependymoma model (100 mg/kg, oral simvastatin) and human patients (Ahmed et al., 2013) (7.5 mg/kg, oral simvastatin)

Parameters	Mice (n=15)* (Mean ± SD)		Human (n=3) (Ahmed et al., 2013) (Mean ± SD)	
	SV	SVA	SV	SVA
AUC_{0-12} (ug/L*hr)	51.9 ± 39.5	362.8 ± 54.2	795.3 ± 753.4	349.3 ± 218.3
C_{max} (ug/L)	28.6 ± 19.0	178.3 ± 36.3	376.7 ± 460.4	109 ± 131.0
T_{max} (hr)	0.23 ± 0.13	0.55 ± 0.14	1.3 ± 0.5	3.3 ± 2.5

*Parameters obtained by simulation

Figure 1

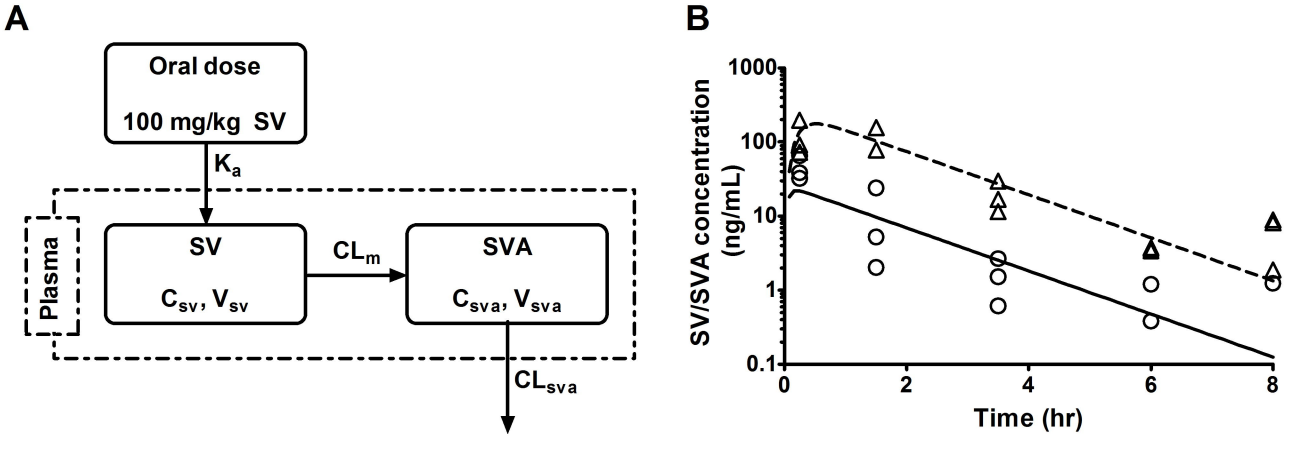
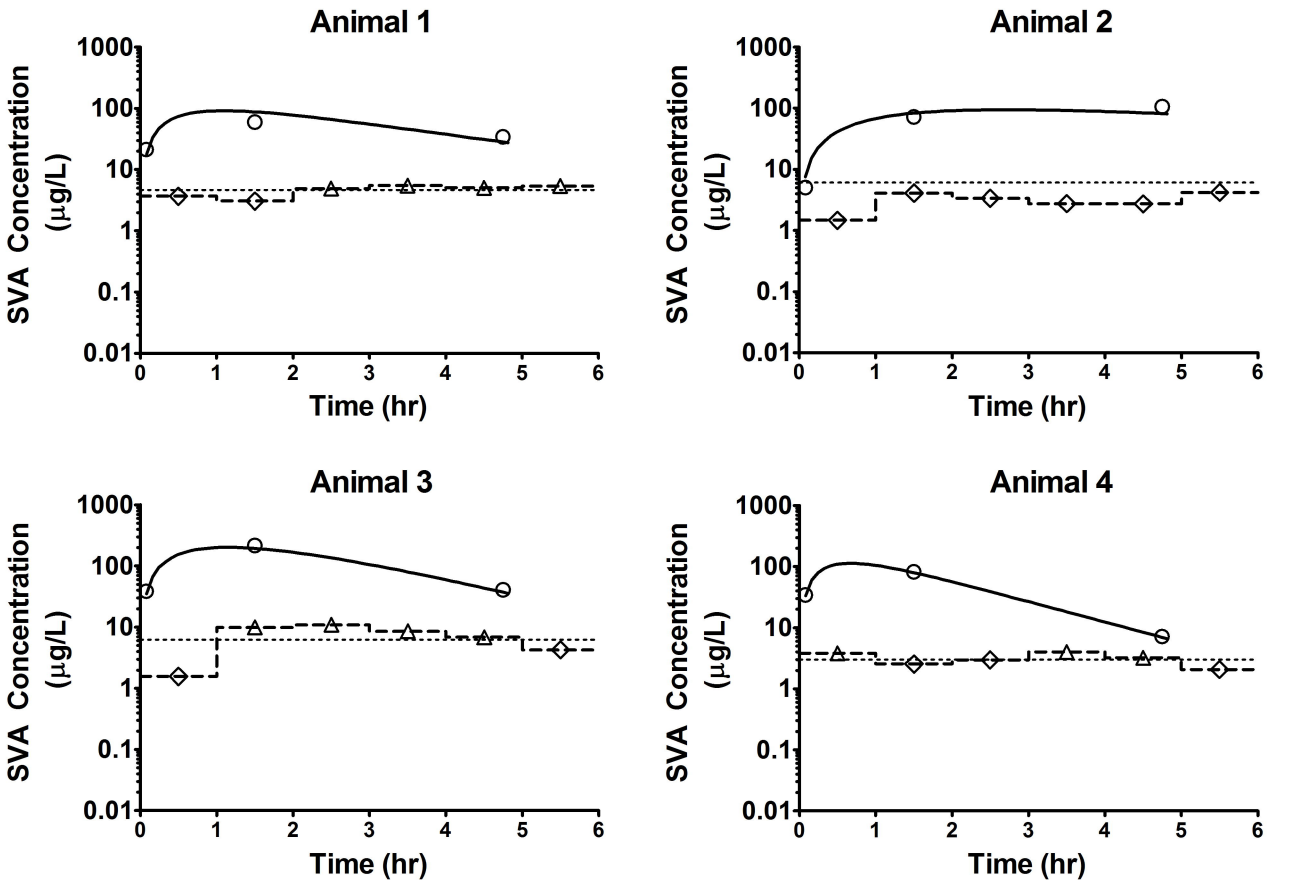

A
Oral dose
100 mg/kg SV
K_a
Plasma
SV
C_{sv}, V_{sv}
CL_m
SVA
C_{sva}, V_{sva}
CL_{sva}
B
SV/SVA concentration (ng/mL)
1000
100
10
1
0.1
0
2
4
6
8
Time (hr)

Figure 2


Animal 1
Animal 2
Animal 3
Animal 4
SVA Concentration (μg/L)
Time (hr)
1000
100
10
1
0.1
0.01
0
1
2
3
4
5
6

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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Journal: Drug Metabolism and Disposition

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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