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Quantification of OSU-2S, a novel derivative of FTY720, in mouse plasma by liquid chromatography-tandem mass spectrometry

Yicheng Mao^{1,2,*}, Jiang Wang^{1,*}, Yuan Zhao¹, Ribai Yan¹, Hao Li¹, Ching-Shih Chen¹, Robert J. Lee^{1,2}, John C. Byrd⁴, L. James Lee^{2,3}, Natarajan Muthusamy^{4,#}, and Mitch A. Phelps^{1,#}

¹College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA

²Nanoscale Science and Engineering Center for Affordable Nanoengineering of Polymeric Biomedical Devices (CANPBD), The Ohio State University, Columbus, OH43210, USA

³William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus 43210, USA

⁴Division of Hematology, Department of Internal Medicine, College of Medicine, The Ohio State University, Columbus, OH 43210, USA

Abstract

OSU-2S is a novel anti-cancer and immune modulatory agent designed specifically to avert the immunosuppressive effects and related toxicities observed in clinical studies with its predecessor analog, FTY720. To characterize its preclinical pharmacokinetics, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the quantification of OSU-2S in mouse plasma. Ethyl acetate extraction of samples containing OSU-2S and the internal standard, Sph-17, was followed by separation with a 6 minute gradient (water/0.1% formic acid and methanol/0.1% formic acid) on a reverse-phase C18 column at room temperature. Selected reaction monitoring was used for detection on a triple quadrupole mass spectrometer with positive ionization. The assay was linear over the concentration range 3–3000 ng/mL with accuracy ranging from 103 to 111%, and both within- and between-run precision (CV %) 11%. All stability samples were within $\pm 15\%$ of nominal values, and replicates were within 15% CV. The assay was successfully applied to a mouse pharmacokinetic study of OSU-2S with intravenous and intraperitoneal administration. OSU-2S non-compartmental pharmacokinetic parameters, area under the concentration-time curve, clearance, and elimination half-life were estimated at 1522 hr* $\mu\text{g/L}$, 3.06 L/hr/kg and 15.6 hr, respectively, for intravenous injection.

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Correspondence to: Natarajan Muthusamy, DVM., PhD, 455E, OSUCCC, 410, West 12th Avenue, Columbus, OH 43210. raj.muthusamy@osumc.edu, Phone: (614) 292-8135, Fax: (614) 292-3312. Mitch A. Phelps, Ph.D, 230, Parks Hall, 500, West 12th Avenue, Columbus, OH 43210. mitch.phelps@osumc.edu, Phone: (614) 688-4370, Fax: (614) 292-7766.

*These first authors contributed equally

#These senior authors contributed equally

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Systemic availability after intraperitoneal injection was approximately 46%. These data demonstrate the OSU-2S compound displays acceptable pharmacokinetic properties for further *in vivo* pharmacologic evaluation, which can be facilitated by the validated LC-MS/MS assay.

Keywords

OSU-2S; FTY720; liquid chromatography-tandem mass spectrometry; mouse; pharmacokinetics

1. Introduction

OSU-2S was synthesized by Chen and colleagues as a novel derivative of FTY720, an immunosuppressive agent developed from the fungal sphingosine analog ISP-1 [1–3]. Although FTY720 exhibited potent preclinical and clinical activity against diseases including multiple sclerosis [4, 5], various forms of leukemia [6–11], solid tumors [12–16], and organ rejection in kidney transplantation [17], mainly through phosphorylation by sphingosine kinase 2 (SphK2) [3], interaction with the sphingosine-1-phosphate (S1P) receptors [18–21], and apoptosis induction through activation of protein phosphatase 2A (PP2A) [22], its immunosuppressive properties and related side effects have limited its further development for cancer therapy. Our group has investigated various strategies to overcome the immunosuppressive properties of FTY720, including encapsulation of FTY720 in targeted nanoparticles [23] and synthesis of FTY720 derivatives, such as OSU-2S (Figure 1a), which was designed specifically to avoid S1P receptor interaction and has since been demonstrated to lack immunosuppressive activities [24, 25].

Previous *in vitro* studies showed promising cytotoxicity with OSU-2S in cells representing chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), acute lymphoblastic leukemia (ALL) and T cells [25]. OSU-2S also demonstrated high efficiency in suppressing hepatocellular carcinoma tumor growth *in vivo* [3]. Unlike FTY720, OSU-2S did not cause phosphorylation of S1P receptors or SphK2. OSU-2S uniquely targeted protein kinase C, involving caspase 3, 8 and 9 signaling, increased LC3-II levels, and induced phosphorylation of Ser 591 of the SHP1 phosphatase [3, 25, 26]. Though the detailed mechanism of action for OSU-2S remains to be clarified, existing data on OSU-2S is sufficient to confirm that it is a potent anti-tumor and non-immunosuppressive analogue of FTY720. As we and others continue development of this agent for possible future clinical use, thorough characterization and understanding of its pharmacokinetic properties is crucial for its further development.

Herein we present a sensitive and selective liquid chromatography-tandem mass spectroscopy (LC-MS/MS) method for OSU-2S quantification in mouse plasma. We applied this method to characterize pharmacokinetics in mice dosed with OSU-2S through intravenous and intraperitoneal routes of administration. The pharmacokinetic data indicates OSU-2S has favorable disposition with relatively low clearance and long exposures of active drug concentrations. This and additional pharmacokinetic data will support optimization of dose regimens for future preclinical efficacy studies.

2. Experimental

2.1. Materials

OSU-2S was synthesized as previously described [3]. Sphingosine C17 (Sph-17) was purchased from Avanti Polar Lipids (Alabaster, AL). LC-grade ethyl acetate, water and methanol were purchased from Fisher Scientific (Waltham, MA). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO). Blank mouse plasma was obtained from Lampire Biological Laboratories, Inc. (Pipersville, PA).

2.2. Stock and working solutions

Stock solutions (1mg/mL) of OSU-2S and Sph-17 were prepared in methanol and stored at -80°C . Standard solutions of OSU-2S (30 to 30,000 ng/mL) were freshly prepared by series dilution with 50% methanol before each run. Quality control (QC) solutions (100, 300 and 1,000 ng/mL) and a working internal standard (IS) solution (Sph-17 at 10 ug/mL) were prepared separately using the same stock solutions and dilution procedures.

2.3. Sample preparation

Standard and QC sample solutions were prepared by spiking serially diluted OSU-2S (10 μL) into 90 μL blank mouse plasma (total 100 μL volume) to generate calibration curves with plasma concentrations of 3–3,000 ng/mL and QC samples with concentration of 10, 100 and 1,000 ng/mL in plasma. Standards, QCs, and experimental samples of mouse plasma (100 μL) were spiked with 10 μL IS working solution (10 $\mu\text{g/mL}$) and extracted with 1 mL of ethyl acetate by vortex mixing for 1 min. After centrifugation at $11,000\times g$ for 10 min, samples were placed on dry ice for 1 min to solidify the lower plasma layer, and the upper ethyl acetate layer was transferred and collected in a glass tube for evaporation under a gentle stream of nitrogen. The residue was reconstituted with 120 μL of 50% methanol by vortex mixing for 1 min and transferred into microcentrifuge tubes for another 10 min centrifugation at $11,000\times g$. Supernatants were transferred to autosampler vials for LC-MS/MS analysis (20 μL injections).

2.4. LC-MS/MS Analysis

The HPLC system consisted of two LC-20 AD pumps, a SIL-20AC autosampler, DGU-20A5 degasser and a CBM-20A system controller (Shimadzu, Kyoto, Japan). The Chromatographic separation was achieved using an Agilent ZORBAX Extend C18 column (50 $\times$ 2.1mm I.D, 3.5 μm particle size) with a C18 guard cartridge at room temperature. A 6.0 min linear gradient elution was applied with water/0.1% formic acid as mobile phase A and methanol/0.1% formic acid as mobile phase B at a constant flow rate of 200 $\mu\text{L/min}$: 0.0 min (30% A and 70% B), 1.0 min (30% A and 70% B), 3.0 min (100% B), 4.5 min (100% B), 4.6 min (30% A and 70% B) and 6.0 min (30% A and 70% B). The autosampler sample compartment temperature was set to 4°C , and a divert valve was used (0 – 1.7 minutes) to prevent early eluting compounds from entering the ion source.

A Thermo Finnigan (Waltham, MA) TSQ Quantum Ultra EMR mass spectrometer equipped both with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources was operated in positive ionization mode. Ion transition channels for

selected reaction monitoring mode (SRM) were m/z 322→268 for OSU-2S and 286→238 for Sph-17 with both Q1 and Q3 set to 0.7 m/z resolution. MS parameters were optimized by a direct infusion of 10 $\mu\text{g/mL}$ OSU-2S and Sph-17 at a flow rate of 10 $\mu\text{L/min}$ via a direct syringe pump into a stream of mobile phases at 200 $\mu\text{L/min}$. The ion spray voltage, skimmer offset and tube lens offset were set to 5000V, 10V and 100V, respectively. Argon was used as the collision gas at 1.5 mTorr, and nitrogen was used as sheath and auxiliary gas set at 30 and 15 (arbitrary units), respectively. The collision energies were 13eV for OSU-2S and 16eV for Sph-17. The ion transfer tube was maintained at 325 °C. The scan time for both the ion transition channels was set to 30 ms.

2.5. Assay validation

The validation experiments performed included selectivity, linearity, precision and accuracy, matrix effect, carryover, and stability tests in accordance with FDA guidance for bioanalytical methods. Standard and QC sample solutions were prepared as described above to generate calibration curves with plasma concentrations of 3–3,000 ng/mL and QC samples with concentration of 10, 100 and 1,000 ng/mL in plasma. Within-run precision and accuracy (%CV) were determined using $n=6$ replicate samples at each QC concentration level. Between-run precision and accuracy were determined by evaluating QC data from three runs. The lower limit of quantification (LLOQ) was defined as the lowest concentration that could be quantified with precision less than or equal to 20% and accuracy between 80% and 120%.

Assay selectivity was determined by comparing signals from six different lots of extracted blank mouse plasma to those of LLOQ samples. Carry-over was evaluated by blank sample injected after injection of the highest concentration standard samples (3,000 ng/mL). Recovery was determined by comparison of peak areas of plasma QC samples to those of neat solution QC samples (OSU-2S in 50% methanol) that were used to reconstitute extracted blank plasma samples. Matrix effects were evaluated by comparing peak areas of neat solution QCs to those of neat solution QCs containing extract (matrix) from blank plasma. The stability of OSU-2S was also evaluated with triplicates of QC samples at three concentrations for all experimental conditions in mouse plasma/50% MeOH at 1:9 (v:v).

2.6. Mouse pharmacokinetic study

All work performed on animals was approved by the Institutional Animal Care and Use Committee at The Ohio State University. Sterile dosing solutions of OSU-2S were prepared as follows: OSU-2S was first dissolved in DMSO with vortex mixing followed by addition of Cremophor EL and phosphate buffered saline (PBS, pH 7.4) to produce 1.0 mg/mL OSU-2S in 10% DMSO, 18% Cremophor EL and 72% PBS (v:v:v). Female ICR mice 6 weeks of age (Harlan Laboratories, Madison, WI) were dosed with OSU-2S at 5 mg/kg body weight (ranging from 25–33g) via intravenous tail vein or intraperitoneal injection. At each of 15 different time points of 5, 10, 20, 30, 45 min, 1, 1.5, 2, 4, 8, 12 and 24h post dosing, 5 mice were sacrificed by CO₂ asphyxiation, and blood was immediately collected via cardiac puncture then transferred into heparinized tubes. After centrifugation, plasma was separated and collected for storage at –80°C till processing and analysis. No signs of toxicity in the mice were observed throughout the studies. Pharmacokinetic analyses were performed with

non-compartmental analysis using WinNonlin Professional software (v. 5.2, Pharsight, Mountain View, CA).

3. Results and discussion

3.1. Method development

Mass spectrometer detector signal intensities were compared using APCI and ESI ionization probes. Both OSU-2S and Sph-17 exhibited higher signal using ESI with protonated ions as the most abundant parent ions. Full-scan product ion spectra of OSU-2S and Sph-17 are shown in Figure 1. The predominant product ions at m/z 268 for OSU-2S and m/z 238 for Sph-17 were chosen to establish SRM transition channels. Both collision energy and tube lense were optimized at m/z 322→268 for OSU-2S and m/z 286→238 for Sph-17 while the remaining mass spectrometer parameter settings were established to optimize the OSU-2S signal.

Original gradient conditions evaluated yielded matrix effects ranging from 125% to 150%, but this was resolved by adjusting the gradient to achieve later retention times using the conditions described above. A methanol mobile phase yielded improved signal intensity compared to acetonitrile, and the described gradient conditions yielded elution times of approximately 2.7 and 4.3 minutes for OSU-2S and IS, respectively. Formic acid was added to mobile phases to further enhance signal and to decrease peak tailing. Since both OSU-2S and Sph-17 are relatively lipophilic, liquid–liquid extraction using ethyl acetate was evaluated and found to provide high recoveries.

3.2. Assay validation

Representative chromatograms of blank mouse plasma samples, plasma spiked with LLOQ, and a low concentration sample from the PK study are shown in Figure 2. No peaks above background were observed at the retention times of OSU-2S and IS, which yielded high selectivity for the assay. The calibration curve was linear in the range 3–3,000 ng/mL with an LLOQ of 3 ng/mL and correlation coefficients (r^2) of 0.99 or better using $1/x^2$ weighting, which provided a consistently superior fit to the standard curve data relative to equal or $1/x$ weighting. There was no significant carry-over found for either OSU-2S or IS in this assay using the described conditions. Data for linearity, precision and accuracy are displayed in Tables I and II.

Matrix effect was evaluated in triplicate QC samples at 10, 100, 1000 ng/mL OSU-2S with 100ng/mL Sph-17 as described above. The matrix effects at three QC levels ranged from 75.78 % to 82.16%, which suggested matrix components slightly suppress the OSU-2S ion signal. Recovery of OSU-2S in this method ranged from 83.12 to 94.52 %. Sph-17, as the internal standard, had an average matrix effect of 75.02 % and recovery of 84.10 % in this developed method.

Measured concentrations for all stability samples were within $\pm 15\%$ of nominal values and replicates were within 15% CV. OSU-2S was therefore determined to be stable at -80°C in mouse plasma for three weeks and in methanol for 3 months. Results also demonstrated good stability of OSU-2S in 3 cycles of freeze-thaw and at 8 hrs room temperature. The post

extracted OSU-2S was stable up to 12 hrs under autosampler conditions. Notably, earlier versions of this method utilized a refrigerated speed vacuum system for sample dry-down. However, we observed high variability and an apparently low recovery for both OSU-2S and IS. We ultimately utilized nitrogen evaporation as it produced a lower sample temperature and shorter time for drying, which ultimately produced acceptable recovery.

3.3. Pharmacokinetic data analysis

Mice injected with 5mg/kg OSU-2S were euthanized at time points between 5 min and 24 hrs post administration, and blood was collected through cardiac puncture. Separated plasma was further extracted and processed for analysis, and OSU-2S concentrations were determined using the validated assay. Resulting concentration versus time curves after IV and IP injection are presented in Figure 3. Non-compartmental pharmacokinetic parameter estimates were determined using average data at each time point; parameter estimates are presented in Table III. Mean maximum observed concentrations for IV and IP administration routes were 2803 ± 285 ng/mL and 305 ± 55 ng/mL, respectively. Areas under the concentration vs. time curves indicated that the IP administration route systemic availability was approximately 46%. Pharmacokinetic data for OSU-2S has not been previously reported. As per our current results, OSU-2S is adequately absorbed from the peritoneal cavity with bioavailability of 46%. The mean observed $C_{\max}$ of 0.305 $\mu\text{g/mL}$ ($\sim 1\mu\text{M}$) after 5 mg/kg IP injection occurs at 10 min post dosing, and drug levels remain high for several hours given the long terminal phase half-life of approximately 16 hours.

4. Conclusion

OSU-2S is a potent derivative of FTY720 and has shown *in vitro* and *in vivo* efficacy in hepatocellular carcinoma [3] as well as promising cytotoxicity in leukemia [24, 25]. Herein we present the development and validation of an LC-MS/MS assay for quantification of OSU-2S, a novel anti-tumor agent derived from FTY720. In this study, a LC-MS/MS method was developed and validated for the quantification of OSU-2S in mouse plasma. The assay was linear over the concentration range 3–3000 ng/mL and demonstrates within- and between-run precision $\leq 11\%$, and accuracy ranging from 103% to 111%. At selected concentrations, all stability samples were within $\pm 15\%$ of nominal values with replicates within 15% CV. The assay has been successfully applied to a pharmacokinetic study of OSU-2S in mice, thus demonstrating its practical use for future studies related to OSU-2S measurement, such as pharmacokinetic and *in vivo* pharmacodynamic studies of dose proportionality, tissue distribution and dose-response relationships.

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Highlights

- OSU-2S is a derivative of FTY-720, which is FDA approved for multiple sclerosis
- OSU-2S retains anti-tumor activity but lacks FTY-720 immunosuppressive properties
- Validation of an LC-MS/MS assay for OSU-2S quantification in mouse plasma
- First report of OSU-2S PK, including full mouse plasma PK after IP and IV delivery

Figure 1. Chemical structures and mass spectra of OSU-2S and internal standard, Sph-17
Spectra were obtained from direct infusion of 10 μ g/mL of each compound in positive electrospray ionization mode. Mass spectra and chemical structures of A) OSU-2S and B) Sph-17 displaying proposed fragmentation pathways for each compound.

Figure 2.

Total ion chromatograms of OSU-2S and relative separation of OSU-2S and Sph-17 in mouse plasma. Chromatograms from A) blank plasma and B) plasma spiked with 3 ng/mL OSU-2S (retention time = 2.70 min), and relative separation of C) 100ng/mL Sph-17 (4.26 min) and D) 100ng/mL OSU-2S (2.72 min).

Figure 3.

Plasma concentration vs. time curve of OSU-2S. Data points are means $\pm$ SD of OSU-2S in mouse plasma after intravenous and intraperitoneal administration. N = 5 mice per time point. The inset figure displays the data up to 2 hours.

Table I

OSU-2S within- and between-run precision and accuracy

QC (ng/mL)	Mean calculated concentrations			
	Within-run (n=6)		Between-run (n=18)	
	ng/mL (% accuracy)	% CV	ng/mL (% accuracy)	% CV
10	10.46 (105)	4.55	10.08 (101)	8.27
100	100.93 (101)	7.86	104.27 (104)	8.75
1000	971.58 (97)	3.10	945.29 (95)	5.05

Accuracy values are mean concentrations; % accuracy was determined as mean concentration/nominal concentration $\times$ 100%; CV% was determined as standard deviation/mean $\times$ 100%.

Table II

OSU-2S standard curve linearity, precision and accuracy

Levels (ng/ml)	Measured concentrations (ng/ml)					Mean (ng/ml)	% accuracy	CV%
	Run 1	Run 2	Run 3	Run 4	Run 5			
3	3.00	2.99	2.91	2.95	2.96	2.96	98.70	1.15
10	10.18	10.03	10.78	10.28	10.41	10.33	103.35	2.84
30	28.74	30.25	32.60	33.09	30.93	31.12	103.73	5.89
100	100.08	105.80	91.26	99.22	88.46	96.96	96.96	7.03
300	320.07	309.86	320.36	279.65	295.75	305.14	101.71	5.81
1000	894.30	1007.65	1030.33	901.60	1034.75	973.73	97.37	7.00
3000	3190.38	2682.82	2564.85	3182.11	3104.94	2945.02	98.17	9.93

Linearity, precision and accuracy were determined in the standard curves of OSU-2S from five separate runs. Percent accuracy was determined as mean concentration/nominal concentration $\times 100\%$; CV% was determined as standard deviation/mean $\times 100\%$.

Table III

OSU-2S non-compartmental PK parameters

Parameter	Units	IV	IP
Dose	mg/kg	5	5
λ_z	1/hr	0.0445	0.0443
$T_{1/2} \lambda_z$	hr	15.59	15.64
T_{max}	min	5	10
C_{max}	$\mu\text{g/mL}$	2.803	0.305
V_z/F_{-obs}	L/kg	68.89	144.05
$AUC_{0-\infty_{obs}}$	hr* $\mu\text{g/L}$	1522.13	698.25
CL/F_{-obs}	L/hr/kg	3.06	6.38
F	%	100	45.87

Table values are non-compartmental parameter estimates. λ_z , elimination rate constant; $T_{1/2}\lambda_z$, elimination half-life; T_{max} , time of C_{max} ; C_{max} , observed maximum concentration; $AUC_{0-\infty_{obs}}$, area under the observed concentration-time curve extrapolated to infinity; V_z/F_{-obs} , estimated volume of distribution divided by F ; CL/F_{-obs} , clearance divided by F ; F , bioavailability.