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Pharmaceutical Analysis of a Novel Propargyl-linked Antifolate Antibiotic in the Mouse

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Abstract

Antimicrobial resistance to current antibiotics is a significant public health problem and the need for new antibiotics is a compelling one. We have been developing a new series of antibiotics, propargyl-linked diaminopyrimidines, based on the structure of trimethoprim. To date we have discovered compounds that are effective inhibitors of dihydrofolate reductase (the target of trimethoprim), that are potent antibiotics *in vitro* against a range of Gram-positive pathogens including methicillin-resistant *S. aureus*, and that are non-toxic in mammalian cell culture. In this study we report the development of an LC-MS-based protocol for the quantification of our lead antibiotic 37D1-UCP1099 and the application of this assay to follow the concentration of the compound in mouse plasma after intraperitoneal administration. Extraction of 37D1-UCP1099 from mouse plasma was achieved through a liquid-liquid extraction with ethyl acetate. Separation was performed utilizing a reverse-phase C18 column with a ten minute isocratic elution using 47:53 (v/v) 10 mM NH₄HCO₃:acetonitrile. The lower limit of quantitation for 37D1-UCP1099 was 50 ng.mL⁻¹ and the assay showed a dynamic range of 50–4000 ng.mL⁻¹ with good linearity ($r^2 = 0.996$ for all fits). Intra-day and inter-day precision and accuracy were within 11.3% (%RSD) and 6.6% (%RE) respectively. We have demonstrated that the compound is stable under the assay procedures. The compound was shown to have a mean residence time of 26.2 ± 1.0 min and a half-life of 18.2 ± 0.7 min after intraperitoneal delivery at 5 mg.kg⁻¹. These studies now form the foundation of our work to develop additional analogs of 37D1-UCP1099 with improved pharmacokinetic properties.

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Compliance with ethical standards

ACA and DLW declare potential conflicts of interest in that they are named inventors on patents held by the University of Connecticut on the propargyl-linked diaminopyrimidine compound class. These patents have been licensed by Promiliad Biopharma Inc., a company of which NDP is a cofounder and CEO, for preclinical development. Promiliad Biopharma Inc. has sublicensed the patents to Spero Therapeutics LLC.

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Keywords

antibiotic; folate; propargyl-linked; trimethoprim; LC-MS; mouse

Introduction

Antimicrobial resistance to the current available arsenal of antibiotic drugs continues to plague our ability to effectively treat and cure patients who have contracted resistant organisms. According to the Centers for Disease Control and Prevention, over two million people in the United States are infected each year with resistant organisms leading to the death of over 23,000 people.[1] The World Health Organization (WHO) estimates that this costs the United States health care system between \$21 and \$34 billion annually.[2] The increasing emergence of antibiotic resistance in pathogenic microorganisms is an ongoing threat that challenges existing therapeutic options and highlights a need to develop novel treatment strategies.

The creation of novel dihydrofolate reductase (DHFR) inhibitors to target resistant strains is an effective strategy to provide novel antibiotics. DHFR is the enzyme responsible for reducing dihydrofolate to tetrahydrofolate and is essential for the *de novo* synthesis of purines and, therefore, the synthesis of DNA. Inhibition of DHFR leads to a halt in cell growth and proliferation.[3] Trimethoprim (TMP), one of the most widely used DHFR inhibitors, is used to treat a myriad of different microbial infections and is on the WHO Model List of Essential Medicines. TMP is commonly co-administered with the drug sulfamethoxazole (SMX) which is a dihydropteroate synthase inhibitor. Resistance to both TMP and the TMP-SMX combination therapy is increasing which necessitates the search for the discovery of novel DHFR inhibitors that can effectively treat TMP-resistant infections. [4–7]

For several years, we have been working on the synthesis and biological evaluation of a series of novel DHFR inhibitors including compound 37D1-UCP1099. Compound 37D1-UCP1099 showed antibiotic activity against several strains of *S. aureus* as well as significant activity against several other bacterial and fungal strains (Table 1). Previous publications on 37D1-UCP1099 have reported the results of enzymatic inhibition studies using DHFR from multiple organisms as well as structure-activity relationships and crystallography data for this class of compound.[8, 9] In addition, we have studied the rates of penetration across different bacterial cell walls and identified molecular changes that occur inside bacteria when subjected to molecules of this class.[10] The goal of this current study was to develop a rapid and sensitive method, based on LC/MS quantitation, for the pharmacokinetic evaluation of 37D1-UCP1099 in a mouse model. The goal was not only to elucidate the pharmacokinetic profile of 37D1-UCP1099 but also to create a method that can serve as a blueprint for future pharmacokinetic analysis of other compounds in this class.

Materials and Methods

Materials

Acetonitrile (HPLC grade) was purchased from Fisher Scientific. Ethyl acetate (HPLC grade) was from EMD Millipore. Nanopure water was obtained from an in-house Millipore ion-exchange filtration system. All mobile phases were filtered using 2.0 μm filtration discs (Millipore). Trimethoprim was from Sigma-Aldrich and ammonium bicarbonate (LC/MS grade) was from Fluka. Compound 37D1-UCP1099 was synthesized as described in a previous publication.[8]

Instrumentation

Analysis was performed on an Agilent HPLC system equipped with an autosampler (Agilent 1260 Infinity, Model G1329B), a degasser (Agilent 1200, Model G1379B) and a binary pump (Agilent 1200, Model G1312B) coupled to a Bruker Amazon SL ion trap mass spectrometer. Data was analyzed using the Bruker Compass DataAnalysis™ software suite (Version 4.2, Build 383.1). A Phenomenex Gemini (3.0mm $\times$ 150 mm, 100 $\text{\AA}$, 5 μm) column with an attached Phenomenex SecurityGuard™ (C18, 4 $\times$ 2.0 mm, Part No. AJ0-4286) guard column was used for analysis. The analysis was performed under isocratic conditions at ambient temperature. The mobile phase was premixed at a ratio of 47:53 (v/v) 10 mM NH_4HCO_3 :acetonitrile. The mobile phase was filtered through 2.0 μm filtration discs (Millipore, Cat. No. AP2504700). The flow rate used was 0.320 mL/min with a resultant backpressure of approximately 50 bar. The total run time was 10 minutes with an injection volume was 10 μL .

Detection was performed with positive mode electrospray ionization (ESI) and selected ion monitoring (SIM). The $[\text{M}+\text{H}]^+$ ions at m/z 388.1 and 291.1 were chosen for SIM data collection for quantitation of 37D1-UCP1099 and the internal standard (IS) trimethoprim, respectively. The following MS parameters were used during data collection: electrospray voltage +4,500 V, source temperature 250 $^\circ\text{C}$, nebulizer gas (nitrogen) 29.0 psi, and dry gas flow rate (nitrogen) 12.0 L/min.

Sample extraction procedure

For each plasma sample a small volume (20 μL), held in an Eppendorf tube, was mixed briefly by vortexing with IS spiking solution (10 μL); the IS spiking solution consisted of 500 ng.mL^{-1} trimethoprim. The samples were vortexed for 3 minutes. Ethyl acetate (500 μL) was added to each sample, mixed by vortexing, shaken for 10 minutes, vortexed briefly again and then the phases separated by centrifugation at 10,000 rpm for 10 minutes at room temperature. The ethyl acetate supernatant (450 μL) was transferred to a new Eppendorf tube and concentrated to dryness in vacuo using a Savant SpeedVac. The dried samples were reconstituted in HPLC mobile phase (50 μL) for analysis.

Preparation of calibration standards and quality control (QC) standards

The primary, standard stock solution of 37D1-UCP1099 was prepared at a concentration of 6.4 mg/mL in dimethyl sulfoxide (DMSO). The standard spiking solutions were prepared in 50:50 v/v acetonitrile:water at concentrations of 100, 250, 500, 750, 1000, 1250, 2500, 5000,

10000, 15000, 20000, and 40000 ng.mL⁻¹. The IS stock solution was prepared at a concentration of 2.5 mg/mL in 50:50 v/v acetonitrile:water from which the IS spiking solution was prepared at a concentration of 500 ng/mL in 50:50 v/v acetonitrile:water. Standard spiking solutions and IS spiking solution were stored at -20 °C or 4 °C until use. Plasma standards were prepared by adding 11 µL of respective standard spiking solution to 99 µL of drug-free mouse plasma. The working standard concentrations for plasma standards were thus 10.0, 25.0, 50.0, 75.0, 100.0, 125.0, 250.0, 500.0, 750.0, 1000.0, 1500.0, 2000.0, and 4000.0 ng.mL⁻¹.

Method Validation

Linearity was evaluated through the preparation and analysis of replicates of eight calibration standards prepared at concentrations of 50, 100, 125, 250, 500, 1000, 2000, and 4000 ng.mL⁻¹ 37D1-UCP1099 in mouse plasma. In addition, quality control standards were prepared at concentrations of 75, 750, and 1500 ng.mL⁻¹. Accuracy and precision (intra-day and inter-day) were assessed by replicate analysis at the LLOQ (lower limit of quantitation), ULOQ (upper limit of quantitation), and the three QC standard concentrations. Accuracy values are reported as percent relative error (RE%) and precision values are reported as relative standard deviation (%RSD). Recovery and matrix effects were also evaluated at the LLOQ, ULOQ, and the three QC standard concentrations. Evaluation of recovery and matrix effects necessitated the preparation of two additional sets of standards. The “neat” standard set was prepared by spiking 1.8 µL of the respective stock standard into 450 µL of ethyl acetate. The “unextracted” standard set was prepared by taking mouse plasma samples with no drug present and processing them through the standard work up procedure. After this known amounts of drug were added to the ethyl acetate extractions of these blank samples (1.8 µL of the respective stock standard into the 450 µL of ethyl acetate from the blank extraction). The ethyl acetate was then evaporated from each sample and the residue was reconstituted in 50 µL of mobile phase in the same fashion as all of the other standards. Recovery percent was calculated by dividing the average peak area from each extracted standard set by the average peak area from the “unextracted” standard set and multiplying the result by 100%. Matrix effects were calculated by dividing the average peak area from each “unextracted” standard set by the average peak area from the “neat” standard set and multiplying the result by 100%. Stability of 37D1-UCP1099 plasma samples was evaluated under several conditions. Benchtop and 0 °C stability were each measured at 5 hours. Post-preparative (autosampler) stability was measured at 12 hours. Freeze-thaw stability at -80 °C was measured after three 24 hour cycles. Short-term (4 days) storage stability at -80 °C was also evaluated. Acceptance criteria for all measurements were based on guidelines set forth in the United States Food and Drug Administration document entitled “Guidance for Industry: Bioanalytical Method Validation.” [11]

Animals

Experimental procedures were performed according to IACUC protocol AUP022-14 which was approved by The University of Montana and its Institutional Animal Care and Use Committee (IACUC). NIH Guidelines for the Care and Use of Laboratory Animals were followed for all experiments. Thirteen to fifteen-week-old female, CD1 mice (Harlan-Envigo) were housed in static micro isolated cages under pathogen free, HEPA-filtered

conditions. Other housing conditions were as follows: 12-hour light/dark cycles, controlled temperature (20.5–22.5 °C) and humidity (25–45 %), with weekly cage changes of bedding. Food and water were provided *ad libitum*. After animal delivery from Harlan-Envigo, animals were given at least three days to acclimate to their new environment prior to the start of any experiments.

Compound 37D1-UCP1099 was formulated for intraperitoneal (i.p.) delivery at a concentration of 0.2 mg.mL⁻¹ as follows: 2.8 mg 37D1-UCP1099 was dissolved in 0.280 mL of *N*-methylpyrrolidone (NMP), 3.111 mL of 45% w/v (2-hydroxypropyl)- β -cyclodextrin in phosphate buffered saline (PBS), and 10.609 mL of PBS. Five mice were used in the study. The average mouse weight was 33 g and the mice were dosed at 5 mg.kg⁻¹. Blood was collected at 7, 14, 32, 47, 60, 77, 90, 120, 181, 239, 300, and 358 minutes following drug delivery. Blood samples were collected via the saphenous vein into heparinized capillary tubes followed by transfer to 1.5 mL Eppendorf tubes with EDTA (30 μ L of 100 mM EDTA at pH 8) and mixed. Blood was not taken more than three times from any one mouse. Samples were stored at 0 °C until centrifuging later that same day. Following the last time point, all blood samples were centrifuged at 13,500 rpm for 15 minutes at 4 °C. The supernatant was then transferred to a new 1.5 mL Eppendorf tubes and stored at –80 °C until the sample extraction step.

Results

Specificity

Chromatographic selectivity was assessed by comparing chromatograms from blank plasma extracts with chromatograms spiked with 37D1-UCP1099 at the LLOQ (50 ng.mL⁻¹). Compounds 37D1-UCP1099 and TMP both elute in the retention time window between 2 and 5 minutes and there are no observed interferences in the blank plasma extract (Fig. 2). There are peaks in the blank outside this retention time window which were seen across all samples with consistent retention times.

Internal standard

Having access to our library of novel DHFR inhibitors with structural similarity to 37D1-UCP1099, we had ready access to numerous compounds that could be used as internal standards. We also had ready access to known DHFR inhibitors (such as trimethoprim) which feature the 2,4-diaminopyrimidine moiety that could also be used as the internal standard. Through screening many candidates, we found that trimethoprim could be used as the most effective internal standard for analysis of 37D1-UCP1099. Trimethoprim and 37D1-UCP1099 could both be eluted and readily resolved in our isocratic LC-MS method which negated any issues associated with gradient profiles. Trimethoprim also showed adequate repeatability in the sample extraction process.

Linearity

Linearity in the concentration-response curves was observed from 50–4000 ng.mL⁻¹ when using a 1/x² weighting factor for the eight calibration concentrations (50, 100, 125, 250, 500, 1000, 2000, 4000 ng.mL⁻¹ 37D1-UCP1099). Curve fitting was done using the regression

capabilities (Solver) of Microsoft Excel 2013 with the relative response ratio (37D1-UCP1099 peak area/IS peak area) and known standard concentrations. The weighting factor of $1/x^2$ was used to assure linearity at the lowest of the standard concentrations; and the R^2 value was 0.996 or greater for all curve fits. The dynamic, linear range of the quantitation method was found to be 50–4000 ng.mL⁻¹. The LLOQ and ULOQ were 50 ng.mL⁻¹ and 4000 ng.mL⁻¹ respectively.

Accuracy and precision

Intra-day and inter-day accuracy and precision were evaluated at the LLOQ, the ULOQ, and three additional concentrations (75, 750, and 1500 ng.mL⁻¹) between the LLOQ and ULOQ. For these concentrations, the accuracy results (%RE) ranged from -5.2% to 1.9% and from -6.6% to 3.9% for intra-day and inter-day respectively. The precision results (%RSD) ranged from 4.7% to 9.4% and from 4.1% to 11.3% for intra-day and inter-day respectively (Table 2). Based on these results, our method was deemed both accurate and precise. Standard concentrations (10 and 25 ng.mL⁻¹) less than the LLOQ were also evaluated. The analyte was readily detected at these lower concentrations and precision was acceptable but quantitation accuracy did not meet acceptance criteria.

Matrix effects and recovery

The observed matrix effect in this study was minimal with an average of 95.1% at five concentrations (50, 75, 750, 2000, and 4000 ng.mL⁻¹) spanning the dynamic range of the method (Table 3). The %RSD for the matrix effect is 3.7% indicating the matrix effect was relatively consistent across the concentrations tested. Recovery was excellent with an average of 99.2% across the five concentrations tested. Recovery was also consistent across the concentrations tested as shown by the %RSD value of 4.8%.

Stability

The stability of 37D1-UCP1099 in mouse plasma was evaluated under various conditions used in the extraction and quantification procedures (Table 4). Freeze-thaw, post-preparative, and 4-day stability all passed acceptance criteria. Benchtop stability at ambient temperature for five hours failed acceptance criteria for both the LLOQ and ULOQ with accuracy %RE values of -20.8 and -18.7 respectively. When stored at 0°C (in vial rack placed in cooler of ice) until warming to room temperature immediately before the extraction procedure, the samples passed acceptance criteria for both the LLOQ and ULOQ with accuracy %RE values of -6.6 and 2.0 respectively. A long-term storage stability study has not yet been completed.

Pharmacokinetics for IP delivery in CD1 mice

Our developed method was used to evaluate the pharmacokinetic profile of 37D1-UCP1099 following intraperitoneal (i.p.) delivery of 37D1-UCP1099 at 5 mg.kg⁻¹. The 37D1-UCP1099 plasma concentration vs. time profile (Fig. 3) shows a rapid increase in plasma concentration followed by an exponential decay.

To derive standard parameters describing the plasma concentration-time dependence we used a non-compartmental analysis. First, we directly calculated the PK parameters using

standard equations, the time-concentration data and the estimates of accuracy derived from the standard curve fitting parameters (Table 5). Second we used Microsoft Excel 2013 together with the PKSolver add-in function provided by Y. Zhang *et al.*[12] Both the manual calculation and the Excel tool gave the same results.

Discussion

Antimicrobial resistance to current antibiotics is a significant public health problem and the need for new antibiotics is compelling. Over several years we have been developing second generation TMP analogs, a series of propargyl-linked diaminopyrimidines such as 37D1-UCP1099. Over several rounds of iterative chemical synthesis and biological evaluation we have obtained compounds that are potent inhibitors of the DHFR enzyme and that are also potent and selective against standard pathogens as well as clinical pathogen isolates and pathogens resistant to TMP. At this point in compound development it is crucial to understand the pharmacokinetics of this series of compounds so that the data can be used to develop a new antibiotic clinical candidate. In this report we describe two major advances in our program. First, we have developed a sensitive, accurate and precise LC-MS-based method for the quantification of 37D1-UCP1099, our first promising compound to undergo DMPK evaluation. Second we have determined the plasma concentrations of 37D1-UCP1099 after i.p. administration in the mouse. For extraction and quantification of 37D1-UCP1099 we have shown that recoveries are repeatable and high and that matrix effects are negligible. The method allows for quantification of 37D1-UCP1099 with a dynamic range of 50–4000 ng.mL⁻¹. We have confirmed that 37D1-UCP1099 is stable and that no degradative effects are confounding our quantification. Overall, when administered i.p. to the mouse, the compound rapidly enters the bloodstream where metabolic breakdown and/or excretion of the compound occurs in a manner best described by a first-order exponential decay. A non-compartmental analysis of the data shows a mean residence time of 26.2 minutes and a half-life of 18.2 minutes.

Conclusion

The method developed here and the data obtained are important. We now have a reliable quantification method that we can adapt to other propargyl-linked diaminopyrimidine analogs, a method that will be of use to other groups studying such analogs. The data can be used to inform choices of dosing regimens for efficacy studies as well as serve as the basis of dose profiles for in vitro pharmacokinetic modeling experiments. We also have baseline data for our first compound to which later data can be compared in order to develop structure-activity relationships and to understand how structural modifications to our new class of antibiotic affect the pharmacokinetics of these compounds. These are important steps toward the clinical development of our propargyl-linked diaminopyrimidine antibiotics.

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Figure 1.
Structures of the antifolate antibiotics trimethoprim and 37D1-UCP1099

Figure 2.

LC-MS selectivity. **A.** MS for TMP, $[M+H]^+$ 291.1; **B.** MS for 37D1-UCP1099, $[M+H]^+$ 388.1; **C.** Total ion chromatogram for extracted mouse plasma; **D.** Total ion chromatogram for mouse plasma spiked with TMP and 37D1-UCP1099 at the LLOQ (50 ng.mL⁻¹).

Figure 3.
Plasma concentration of 37D1-UCP1099 in the mouse after an i.p. dose of 5 mg.kg^{-1} .

Table 1

Minimum inhibitory concentrations (MIC) of 37D1-UCP1099 against select Gram-positive and Gram-negative bacteria and fungi. The MIC is the lowest concentration of antibiotic that reduces growth by more than 99.9%.

Organism	MIC/ $\mu\text{g.mL}^{-1}$	Organism	MIC/ $\mu\text{g.mL}^{-1}$
<i>Bacillus subtilis</i>	0.10	<i>Candida albicans</i>	6.2
<i>Enterococcus faecalis</i>	0.002	<i>Candida glabrata</i>	0.4
<i>Staphylococcus aureus</i>	0.19	<i>Escherichia coli</i>	6.2
<i>Streptococcus pyogenes</i>	0.20	<i>Klebsiella pneumoniae</i>	0.2
		<i>Pseudomonas aeruginosa</i>	> 390

Table 2

Accuracy and precision data for the quantitation of 33D7-UCP-1099.

Concentration ng.mL ⁻¹	Intra-day (n=5)			Inter-day (n=10)		
	Measured (mean ± SD)	Accuracy (RE%)	Precision (%RSD)	Measured (mean ± SD)	Accuracy (RE%)	Precision (%RSD)
50	50.9 ± 4.8	1.9	9.4	50.1 ± 4.3	0.2	8.7
75	72.1 ± 6.3	-3.9	8.7	77.9 ± 8.8	3.9	11.3
750	754.2 ± 42.0	0.6	5.6	744.5 ± 36.1	-0.7	4.8
1500	1449.6 ± 67.6	-3.4	4.7	1432.0 ± 59.3	-4.5	4.1
4000	3791.1 ± 192.6	-5.2	5.1	3736.6 ± 161.4	-6.6	4.3

Matrix and recovery effects. Numbers in the top three rows are average peak area ($n=5$) for each concentration $\times 10^{-9}$. Matrix % is unextracted peak area/ neat peak area $\times 100$ %. Recovery % is extracted peak area/unextracted peak area $\times 100$ %.

Table 3

Concentration ng.mL ⁻¹	50	75	750	2000	4000	Average	%RSD
Neat	0.096	0.130	1.019	2.567	5.018		
Unextracted	0.090	0.126	0.918	2.530	4.909		
Extracted	0.094	0.123	0.933	2.333	4.885		
Matrix (%)	92.8	96.4	90.1	98.5	97.8	95.1	3.7
Recovery (%)	104.9	97.6	101.7	92.2	99.5	99.2	4.8

Table 4

Stability of 37D1-UCP1099 under various conditions used in the extraction and quantification procedure.

Conditions	Concentration ng.mL ⁻¹	Accuracy %RE	Precision %RSD
Benchtop (5 h)	50.0	-20.8	3.5
	4000.0	-18.7	0.9
0 °C	50.0	-6.6	5.0
	4000.0	2.0	NA
Post-preparative (12 h)	50.0	-7.5	4.4
	4000.0	0.4	4.3
Freeze-thaw, 3 cycles (24 h)	50.0	-6.5	15.5
	4000.0	6.5	1.2
Stability (4 d)	50.0	-13.6	3.1
	4000.0	6.3	10.6

Table 5

Derived PK parameters for i.p. delivery of 37D1-UCP1099 in the mouse at a dose of 5 mg.kg⁻¹. AUC, area under the curve; AUMC, area under the moment curve; C_{max}, maximum plasma concentration; MRT, mean residence time; k_{elim}, apparent rate constant for first-order exponential decrease in plasma concentration brought about by elimination of compound; t_{1/2}, plasma half life.

Parameter	37D1-UCP1099
AUC _{0-∞}	63.0 ± 1.0 mg.min.mL ⁻¹
AUMC _{0-∞}	1651 ± 58 µg.min ² .mL ⁻¹
C _{max}	1.6 µg.mL ⁻¹
MRT	26.2 ± 1.0 min
k _{elim}	0.0382 ± 0.0015 min ⁻¹
t _{1/2}	18.2 ± 0.7 min