Research Report
Rhys Axon, Ph.D. Updated 20 November 2020

PROJECT TITLE & AUTHORS

Project Title:	A Comparative Analysis of the Measured Thermodynamic Properties of Azithromycin, Bacitracin and Levofloxacin with In-Silico Methods	
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PROPOSAL CHECKLIST

Completed (Y)	Checklist item
Y	Project title is clear and concise.
Y	Names and emails for project advisor(s) and up to five students per group are provided.
Y	Abstract is no more than 250 words and retains headings
Y	Introduction provides a definition of the topic under study, importance of the topic, and the issue addressed by the study and is no more than one single-spaced page.
Y	There is NO literature review section
Y	Purpose of project is clearly and concisely stated
Y	Methods section uses headings and represents a summary of the methods used. (Actual methods used should be described if they were modified from the proposal.)
Y	Data analysis described is appropriate and responds to the purpose.
Y	Appropriate tables are included in the results section.
Y	Text of results section interprets the findings reported in the tables, not repeating them.
Y	The discussion section includes a description of the most important findings, and relates findings to the literature.
Y	The final section of the discussion is the limitations section.
Y	The conclusions respond to the purpose statement.

Y	Reference list is complete and contains appropriate references, and reference style is applied correctly and consistently.
Y	Data collection/recording form(s) and/or questionnaire(s) are included in the appendix.
Y	Information is placed in the appropriate section—introduction, methods, results, etc.
Y	Template structure is maintained and all required sections are included. Red text instructions/examples are removed. Proposal is written in Times New Roman 12-point font and does not exceed 10 single-spaced pages (excluding appendices). Proposal has been spell-checked and grammar-checked.

ABSTRACT

Specific Aims: Primarily to identify the molecular and physicochemical properties, phase transition temperatures, correlated enthalpies, and confirmation of crystallinity and birefringence of azithromycin, bacitracin, and levofloxacin. Secondarily to highlight the importance of *in-silico* and *in-vitro* experiments in the drug discovery and development process.

Methods: Molecular and physicochemical properties were obtained from molecular modeling software by using SwissADME®, ChemDraw19.1® and Chem3D 19.1®. Phase transition temperatures and correlated enthalpies were obtained by utilizing a technique known as Differential Scanning Calorimetry. The confirmation of crystallinity and birefringence of the three medications were completed via a technique known as Hot Stage Microscopy.

Results: In silico experiments adequately predicted pharmacokinetic and pharmacodynamic parameters for the three antibiotics. DSC experiments confirmed the presence of phase transitions for all three antibiotics. HSM experiments confirmed the presence of crystallinity and birefringence for azithromycin and levofloxacin.

Conclusions: The use of predictive molecular modeling software in conjunction with *in-vitro* thermal experiments remain important and critical experiments in the drug development and discovery process.

INTRODUCTION

Azithromycin is a subclass of a macrolide, binding to the 50S ribosomal subunit.[9] Bacitracin is an inhibitor of cell wall synthesis.[8] Levofloxacin is a third-generation fluoroquinolone that inhibits DNA gyrase which is a requirement for DNA replication.[7] Although differing in class, these medications are equal in their contributions to the medical world. Prior to human use, medications get evaluated via molecular modeling software programs also known as *in-silico* methods. *In-silico* experiments can give insight on how these molecules may behave in the body and why they may make adequate and successful pharmaceuticals. *In-vitro* experiments such as DSC and HSM are important experiments during drug discovery and development because they are used to characterize and identify the stability, purity, and formulation compatibility of a pharmaceutical to others.

DSC is an analytical technique that utilizes small amounts of a drug sample and heat to analyze the properties of the pharmaceutical.[1] Ultimately, DSC will show the difference in heat flow between a sample and a reference. This is shown as a function of time and temperature, providing insight to thermal transitions of the sample. Thermal transitions that can be seen include melting, crystallization, reaction, and phase transitions.[1] DSC can use temperatures ranging from -40 ℃ to 600 ℃.[1] DSC can also be used to predict the extent of miscibility of solid molecular dispersion.[2]

HSM, also known as thermal microscopy, is a combination of thermal and polarized light microscopy techniques used to visualize the sample with temperature.[2] The drug sample is heated on a hot stage while being visualized under an optical light microscope. Using a cross-polarizing lens, the birefringence can be analyzed by either a presence, absence, or change that occurs concurrently with increasing temperature.

The primary purpose of this project is to identify the pharmacokinetic and physicochemical properties of azithromycin, bacitracin and levofloxacin and investigate how they compare to *in vitro* derived findings.

METHODS

Product Selection/Materials

Azithromycin Dihydrate (> 98% purity) ($C_{38}H_{72}N_2O_{12}$; molecular weight (MW): 749), shown in Figure 1, was obtained from Technology Catalysts International (Falls Church, VA, USA). Bacitracin (purity) ($C_{66}H_{103}N_{17}O_{16}S$; MW: 1422.7), shown in Figure 2, was obtained from Sigma-Aldrich (St. Louis, MO, USA). Levofloxacin (> 98% purity), ($C_{18}H_{20}FN_3O_4$; MW: 361.37), shown in Figure 3, was obtained from Technology Catalysts International (Falls Church, VA, USA). The nitrogen gas used was ultra-high purity (UHP) (Cryogenics and gas facility, The University of Arizona, Tucson, AZ, USA).

Software

Data collection will be started by using SwissADME®, ChemDraw19.1® and Chem3D ver 19.1® CambridgeSoft, Cambridge MA, USA for predicted physicochemical properties of Azithromycin, Bacitracin and Levofloxacin. These programs are designed to predict melting points, pKa, boiling point and other factors. Data Collection will be further carried out by utilizing equipment such as a DSC machine and Hot Stage Microscopy can further solidify the predictions obtained by the modeling programs.

Differential Scanning Calorimetry (DSC)

Thermal analysis and phase transition measurements will be performed on a TA Q1000 differential scanning calorimeter (DSC) (TA Instruments, New Castle, DE, USA) equipped with T-Zero® technology, RSC90 automated cooling system, auto sampler and calibrated with indium. Approximately 1-2 mg of sample will be placed into an anodized aluminum hermetic DSC pan. The T-Zero® DSC pans were hermetically sealed with the T-Zero hermetic press (TA Instruments). Using an empty hermetically sealed aluminum pan as a reference pan for all the experiments. UHP nitrogen will be used as the purging gas at a rate of 40 mL/min. The samples will be heated from -5.00ºC to 350.00ºC at a scanning rate of 5.00ºC/min. All measurements will be carried out in triplicate ($n = 3$).

Hot Stage Microscopy (HSM) under Cross-Polarizer

Hot-stage microscopy (HSM) studies used a Leica DMLP cross-polarized microscope (Wetzlar, Germany) equipped with a Mettler FP 80 central processor heating unit and Mettler FP82 hot stage (Columbus, OH, USA). Samples were mounted on glass slides and heated from 25.0ºC to 200.0ºC at a heating rate of 5.00ºC/min. The images were digitally captured using a Nikon Coolpix 8800 digital camera (Nikon, Tokyo, Japan) under 10x optical objective and 10x digital zoom.

Design

The phase transition temperatures and corresponding enthalpies of azithromycin, bacitracin, and levofloxacin will be tested by triplicate (n=3) (Table specifics can be found in Appendix C). The results obtained were expressed as mean ± SD.

Subjects

Human subjects are not involved in this research. Documentation of the type of IRB approval required is in Appendix B.

Measures

The data collection forms are shown in Appendix C. Data was collected on the date of lab test, DSC protocol, reference mass Tzero alodined pan (g), mass of each analyte (g), final mass of analyte and Tzero alodined pan (g), DSC results for each analyte and analysis of DSC results. All data was recorded in a standard laboratory notebook.

Data Collection

Data collection was started by using SwissADME®, ChemDraw19.1® and Chem3D ver 19.1® CambridgeSoft, Cambridge MA, USA for predicted physicochemical properties of Azithromycin, Bacitracin and Levofloxacin.

Thermal analysis and phase transition measurements were performed on a TA Q1000 differential scanning calorimeter (DSC) (TA Instruments, New Castle, DE, USA) equipped with T-Zero® technology, RSC90 automated cooling system, auto sampler and calibrated with indium. Hot-stage microscopy (HSM) studies used a Leica DMLP cross-polarized microscope (Wetzlar, Germany) equipped with a Mettler FP 80 central processor heating unit and Mettler FP82 hot stage (Columbus, OH, USA). Data was kept in Skaggs Lab 460 in a laboratory notebook property of Dr. Heidi Mansour. Experimental data was accessed through the University of Arizona's College of Pharmacy VPN under Dr. Mansour's shared workgroup drive. Employees, research students, and post graduate medical students under the supervision of Dr. Heidi Mansour had access to the shared workgroup drive. Access to these folders required access to the University of Arizona College of Pharmacy virtual private network (VPN), as well two-step username and password verification.

Data Analysis

The molecular weight, tPSA, LogP, LogS, the amount of H bond donors, GI absorption, Blood Brain Permeability, the Lipinski's Rule of 5 were retrieved for each of the drugs using two separate *in-silico* methods SwissADME® and ChemDraw®. The boiling point and melting point were obtained from Thermo Fisher Scientific Inc® and ChemSpider®. All predicted values were analyzed and reviewed.

DSC experiments were conducted in triplicate n=3 for each of the antibiotics. From the DSC thermograms, the Max Peak temperature (phase transition temperature) and corresponding enthalpies were recorded. Mean and SD of Max Peak temperatures and correlated enthalpies were obtained via Standard Deviation Calculator derived from Calculator Soup.

HSM experiments were conducted in one single run for each drug. Thermal changes were digitally recorded via Nikon camera. Starting from room temperature, images were captured approximately every five degrees up to 300°C. The birefringence of each drug was visually analyzed, and each revealed the ability or inability of a substance to exhibit crystallinity.

This data analysis differs from the proposed data analysis because comparisons were difficult to formulate amongst the three drugs due to their different mechanisms of actions and some data was unobtainable via the *in-silico* methods.

RESULTS

Results from PK and Physicochemical properties can be found under Table 1. in the Tables and Figures section of this report. The DSC thermogram analysis can be found under Table 2. in the Tables and figures section.

Azithromycin (Zithromax®)

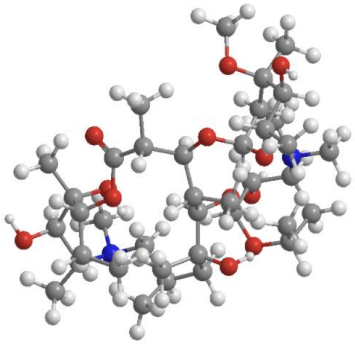

Figure 1. Ball and Stick model of azithromycin via Chem3D 19.1®

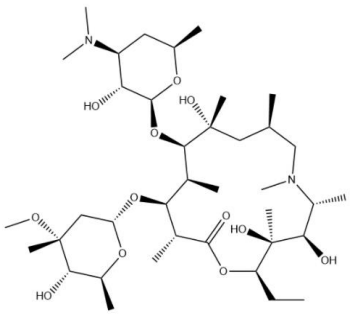

Figure 2. Chemical structure of azithromycin via ChemDraw19.1®

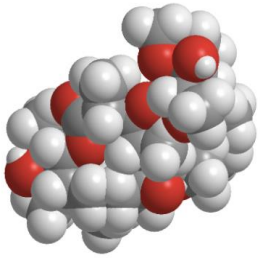

Figure 3. Space Filling model of azithromycin via Chem3D 19.1®

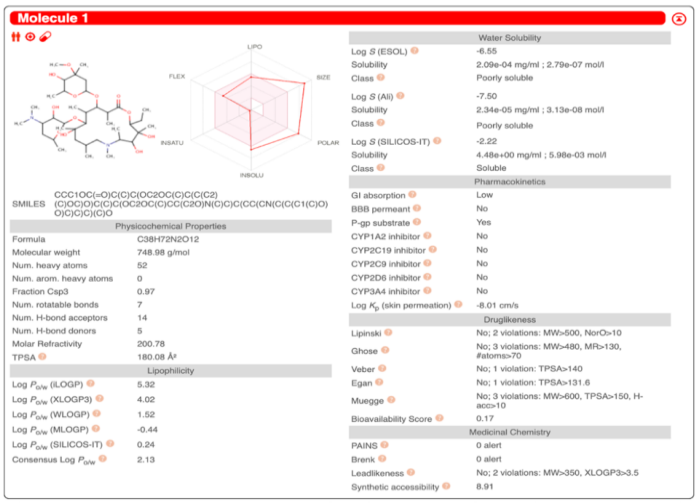

Figure 4: PK and physiochemical properties of azithromycin derived from SwissADME®.

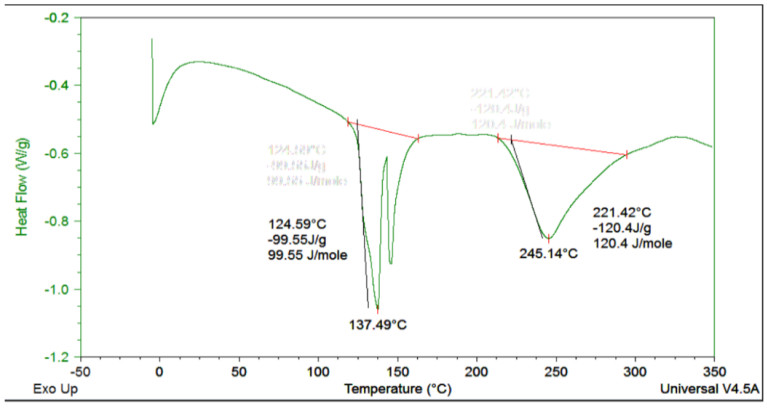

Figure 5: DSC thermogram of azithromycin.

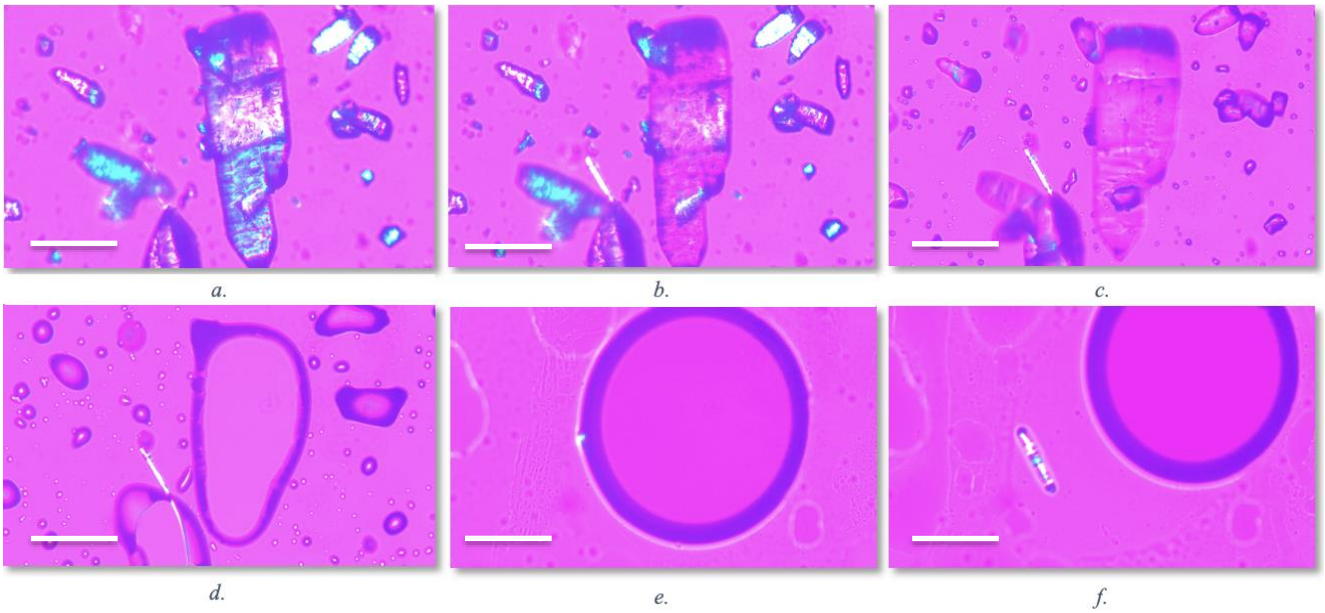

Figure 6. HSM micrograph of azithromycin at (a)37 °C (b)75 °C (c)125°C (d)130 °C (e)230 °C (f)245 °C (scale bar = 10µm).

Bacitracin (Bacilim®)

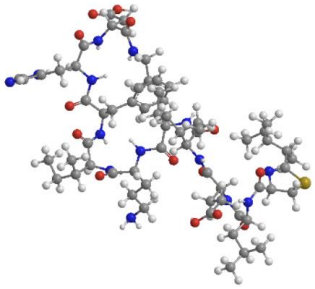

Figure 7. Ball and Stick model of bacitracin via Chem3D 19.1®

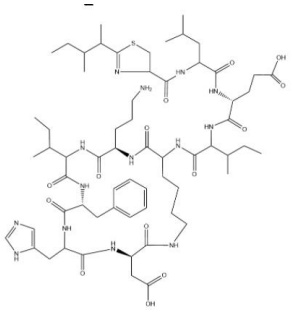

Figure 8. Chemical structure of bacitracin via ChemDraw19.1®

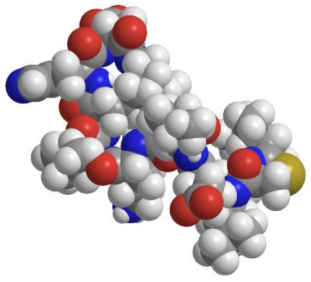

Figure 9. Space Filling model of bacitracin via Chem3D 19.1®

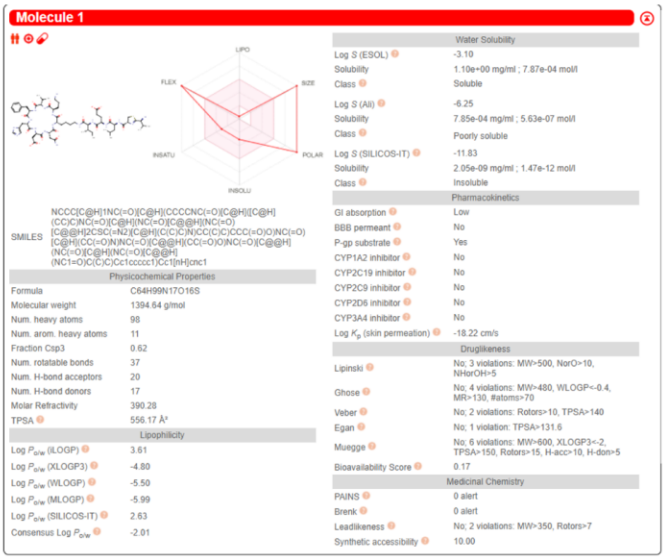

Figure 10: PK and physiochemical properties of bacitracin derived from SwissADME®.

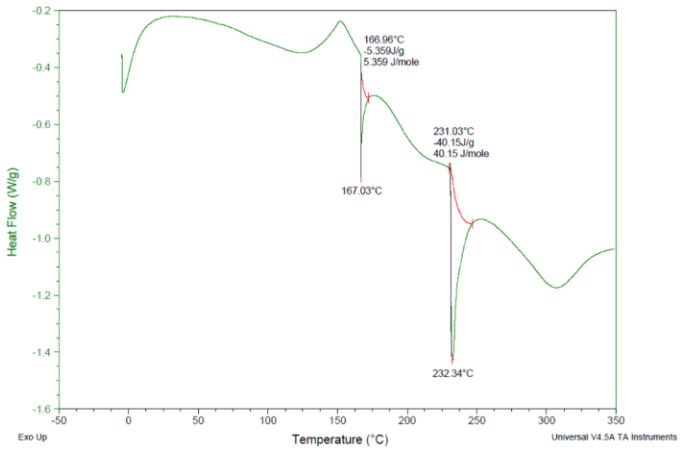

Figure 11: DSC thermogram of bacitracin.

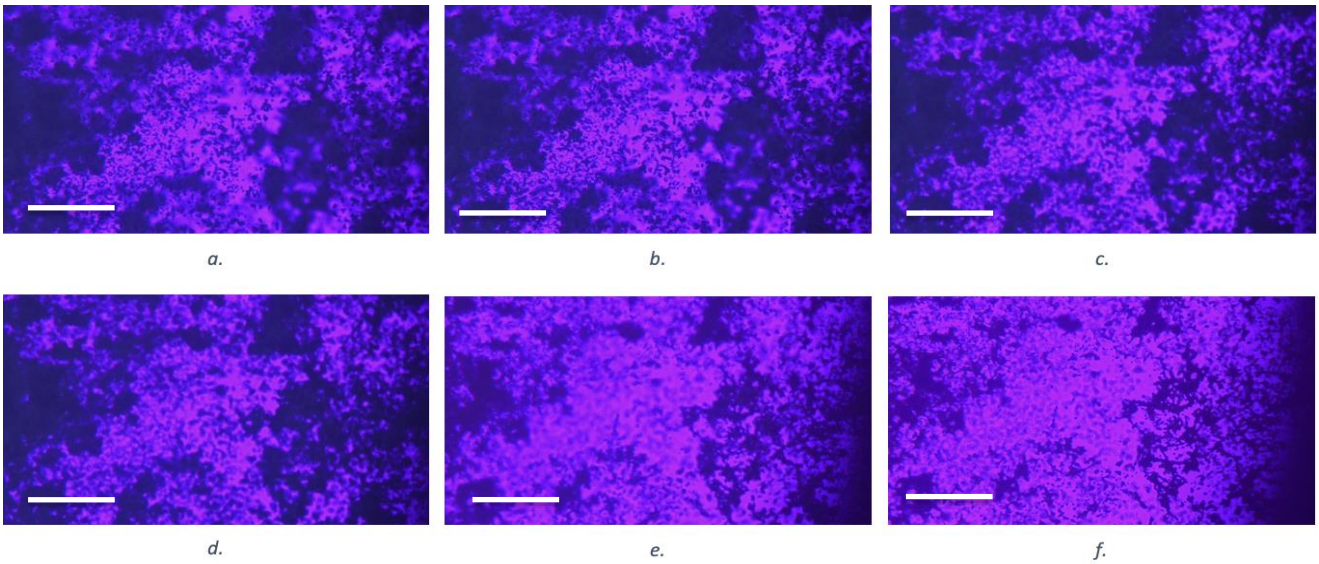

Figure 12. HSM micrograph of bacitracin at (a)37 °C (b)75 °C (c)125°C (d)130 °C (e)230 °C (f)245 °C (scale bar = 10μm).

Levofloxacin (Levaquin®)

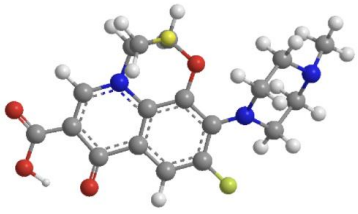

Figure 13. Ball and Stick model of levofloxacin via Chem3D 19.1®

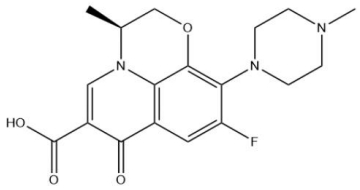

Figure 14. Chemical structure of levofloxacin via ChemDraw19.1®

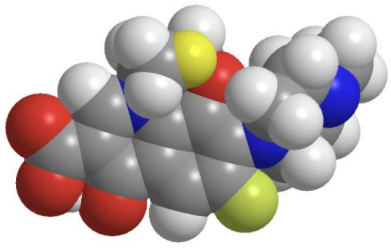

Figure 15. Space Filling model of levofloxacin via Chem3D 19.1®

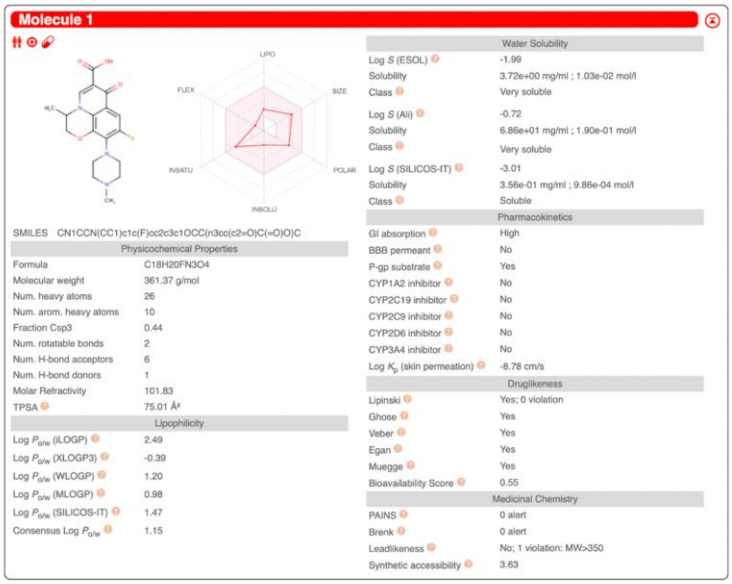

Figure 16: PK and physiochemical properties of levofloxacin derived from SwissADME®.

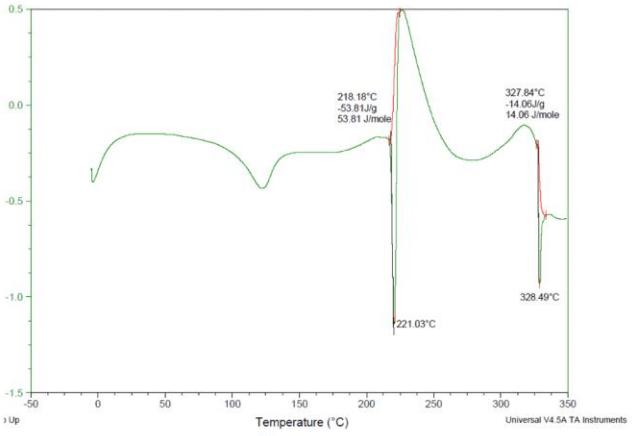

Figure 17: DSC thermogram of levofloxacin.

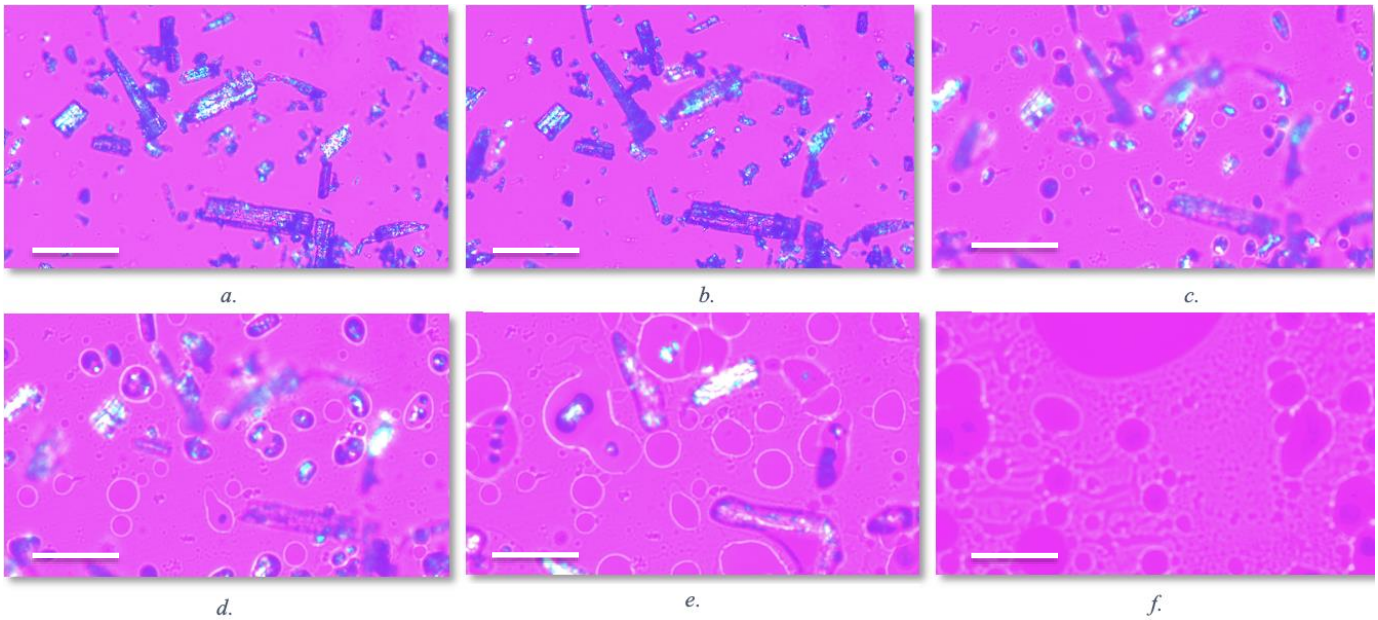

Figure 18. HSM micrograph of levofloxacin at (a)37 °C (b)122 °C (c)220 °C (d)222 °C (e)240 °C (f)265 °C (scale bar = 10μm).

DISCUSSION

Pharmacokinetic and physicochemical parameters demonstrate the important factors that help us understand how and why drugs work at their targeted sites. Compounds that abide by the laws of polar surface area (PSA) are molecules less than 120 angstroms (Å) which can saturate the gastrointestinal (GI) tract. Molecules less than 70Å have the capability of passing the blood brain barrier (BBB). Azithromycin has a PSA of 748.98Å and bacitracin has a PSA of 457.96Å. This indicates that the two molecules have low gastrointestinal absorption. The partition coefficient (LogP) and solubility (LogS) determine the relative drug lipophilicity and behavior in the body in terms of permeability, clearance, and metabolism. LogP values that are less than 5 tend to be more lipophilic, meaning they have aqueous solubility, which is less ideal for bioavailability. However, the more lipophilic a molecule is, the higher its chances for crossing the BBB. For the purposes of this study census LogP (cLogP) was utilized. Passive diffusion of the BBB states that a drug should have a PSA less than 70Å, H-bond donors 0-1, Log P 2-4, MW less than 450 g/mol, all though all three drugs have a LogP less than 4, none of these drugs will passively penetrate the BBB. According to Lipinski's Rule of 5, molecules that can passively diffuse the lipid bilayer of the small intestine can have no more than 5

hydrogen bond donors, no more than 10 hydrogen bond acceptors, have a molecular weight less than 500 g/mol, and have a LogP value less than 5.0. From our selected drugs levofloxacin is the only one that abides by Lipinski's rule of 5.

SD is the measure of the amount of variation or dispersion of a set of values to the average. In our study the SD of Max Peak and corresponding enthalpies of azithromycin and bacitracin reflected low variation from the sample runs conducted. However, levofloxacin displayed the highest variation.

As for the DSC thermograms of each drug they all displayed very similar phase transitions peaks. The first and second peaks display an endothermic system by displaying a downward trough. This shows that energy (heat) is being absorbed by the system. The changes in enthalpy of the systems are overall positive. Another indicator that verifies that the system is absorbing heat. These peaks may mean that the drug is undergoing a possible solid-state transition. The major peaks of azithromycin, bacitracin and levofloxacin may be indicative of a melting point. The melting point range for azithromycin is 113-115 °C and bacitracin is 221-225 °C and levofloxacin is approximately 224 °C. This does not agree with the mean and SDs calculated for max peak temperatures and corresponding enthalpies for the drugs. Although the thermograms and thermogram analysis differ, the phase transitions may be further explained and identified via the HSM experiments.

Azithromycin and levofloxacin exhibited birefringence confirming their crystallinity. Birefringence can be defined as the use of crossed polarized lens to visualize the presence also known as crystallinity or the absence of crystals also known as amorphism during a phase transition. Both azithromycin showed a solid state to liquid state transition at about 125 °C and levofloxacin at 222 °C, corresponding to their melting points predicted with molecular modeling software. These images were in good agreement with the DSC thermograms previously described. Unfortunately, conclusions could not be drawn from the HSM data retrieved for bacitracin. Birefringence was not displayed in the bacitracin data, but we believe this may have been caused by conducting the HSM experiment with too much raw drug on the slide. Another HSM experiment could not be run, due to lab and scheduling restrictions.

There were several limitations to this study which included the limited number of persons allowed to be in the lab due to COVID-19 pandemic. In turn this also led to limited time in the lab and constraints with lab personnel for instructions with the laboratory instruments. The camera utilized for HSM needed to be repaired and replaced initially, this process took several months. *In-silico* methods were difficult to apply at times since working with raw products, most sources contain an added salt which could have construed data. Another limitation of this study was the limited selection of drugs in the lab, making it difficult to compare to one another due to differences in medication class. Lastly with all conducted experiments there is an assumed fraction of human error.

CONCLUSION

The primary findings of our study show the pharmacokinetic and physicochemical properties of azithromycin, bacitracin and levofloxacin were successfully predicted with *in-silico* molecular modeling software via SwissADME® and ChemDraw®. Based on the phase transitions displayed while conducting DSC, the individual runs for each of the drugs were in good agreement to the predicted values obtained. However overall, when comparing them to mean ± SD values for Max peak temperatures, they were not in good agreement. We believe this may have been due to error while conducting the DSC runs. HSM experiments concluded that azithromycin and levofloxacin displayed birefringence confirming that the drugs were crystalline. Conclusions could not be drawn for bacitracin regarding its crystallinity. Azithromycin showed a solid state to liquid state transition at about 125°C and levofloxacin at 222°C, corresponding to their melting points predicted with *in-silico* methods. These results were in good agreement with the DSC data previously described.

This study shows that in-silico methods are very valuable experiments in the drug discovery process because they give insight on molecular behavior of a pharmaceutical. The information derived from molecular modeling can help guide research and development efforts in the drug discovery process. This study furthermore

highlights the importance of further conducting thermal experiments like DSC and HSM above to confirm predictive software information. The use of predictive molecular modeling software in conjunction with *in-vitro* thermal experiments remain important and critical experiments in the drug development and discovery process.

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TABLES AND FIGURES

Table 1. Retrieval of pharmacokinetic and physicochemical properties of Azithromycin, Bacitracin (Bac) and Levofloxacin using SwissADME® and ChemDraw®. Data obtained from * Thermo Fisher Scientific Inc©, **ChemSpider©

Pharmacokinetic Properties	SwissADME®			ChemDraw®		
	Azith	**Bac**	**Levo**	**Azith**	**Bac**	**Levo**
Molecular Weight (g/mol)	748.98	1307.60	361.37	749	137.62	361.37
tPSA (Å²)	180.08	457.96	75.01	180.08	428.37	73.32
LogP	2.13	1.43	1.15	2.64	2.79	-0.51
LogS	-6.55	-6.82	-1.99	-4.08	-10.8	-3.85
Hydrogen Bond Donor	5	14	1	--	--	--
Hydrogen Bond Acceptors	14	27	6			
Gastrointestinal Absorption	Low	Low	High	--	--	--
Blood Brain Barrier Permeability	No	No	No	--	--	--
Lipinski Rule of 5	No, 2 Violations	No, 3 Violations	Yes, 0 Violations	--	--	--
Boiling Point (°C)	--	--	--	717*	571.5**	699.73
Melting Point (°C)	--	--	--	126*	224**	520.92

Table 2. Thermogram analysis of Azithromycin, Bacitracin and Levofloxacin (n=3, mean± SD)[4]

	Max Peak (C°)	Enthalpy (J/g)
Azithromycin	137.67 ± 1.07	86.15 ± 15.95
Bacitracin	168.78 ± 16.44	1.64 ± 3.82
Levofloxacin	173.82 ± 51.65	30.82 ± 19.74

APPENDICES

APPENDIX A: Literature Search Strategy

DATABASE	KEY WORDS
Access Pharmacy	● Differential Scanning Calorimetry ● Nanotechnology ● Hot Stage Microscopy
PubMed/Medline	● Differential Scanning Calorimetry in Nanotechnology ● Hot Stage Microscopy
PubMed	● 'Thermal analysis' ● 'Differential scanning calorimetry' ● 'Differential scanning calorimetry' + 'metformin' ● 'Hot stage microscopy'

APPENDIX B: Type of IRB Approval Required

Data Collection Site:	University of Arizona College of Pharmacy

Data source:	Patient charts
	Database
	Questionnaire/interviews/focus groups
	Published literature/studies
	Public website
	Laboratory (bench) research
	Laboratory animals
	Other (specify)

IRB form required (Complete as instructed by Dr. Nix):	Determination of Human Research
	Application for Human Research
	None—Research does not involve humans (e.g., systematic review, lab research)
	Other (specify)

Supplemental Forms Required (Complete as instructed by Dr. Nix):	Site permission letter
	Written informed consent form
	PHI authorization form
	List of Research Personnel
	CV's for key personnel (PI, Co-PI, adviser)

	Data collection instrument
	Other (specify)

APPENDIX C: Data Collection Forms/Data Dictionary

Appendix C1: Data Collection Forms

DSC Template			
Sample #	Empty pan	Filled Pan	Difference
Analyte 1 Sample 1			
Analyte 1 Sample 2			
Analyte 1 Sample 3			
Analyte 2 Sample 1			
Analyte 2 Sample 2			
Analyte 2 Sample 3			
Analyte 3 Sample 1			
Analyte 3 Sample 2			
Analyte 3 Sample 3			