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Novel Approaches to Delivery of Biomacromolecules

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Novel Approaches to Delivery of Biomacromolecules

by
David Jeffrey Larwood

DISSERTATION
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DOCTOR OF PHILOSOPHY

in

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of the
UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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Acknowledgements

This work is the culmination of a long-sought goal and a stepping stone on my path to improving my skills as a scientist and my efforts to be a humanitarian and improve health in the world. My first thanks go to my parents who raised me and inspired me. My father Tom Larwood's selfless devotion to medicine and caring for the poor and underserved was an inspiration to our family, our community, and to me. My mother Patricia Larwood's tireless focus on raising five rambunctious children to be healthy, well-rounded contributors yielded a pretty balanced brood. Sadly, she died of cancer when I was just 16. Two years later my wonderful stepmother Pauline Larwood entered our lives, and for decades was an inspiration as a community leader, business executive, college board member, and wife to my father, all while raising seven children.

My science career became more grounded with my MA under Lars Helberg who taught me patience and focus in designing and running highly-moisture-sensitive acylation reactions. My skills improved dramatically under the tutelage of Ramachandran Ranganathan as a bench chemist at UC San Diego where I made my first later-commercial molecule, an X-ray contrast agent.

I extend tremendous thanks to Frank Szoka, as his first graduate student at UC San Francisco in my primary four years of PhD studies. He was endlessly supportive of my fishing for an interesting project. I thank him deeply for his continuing support, guidance, attention to detail, training me to be a more careful scientist, and expanding my perspective on to how to do quality research. My studies with Dr. Szoka led to the publication of what is here Chapter 1, some quite early work in a field that evolved to become fundamental in pharmaceutical

chemistry. Along the way we made some interesting compounds which were later commercialized.

I took a break from my science studies and switched to law for some time.

After a corporate transition left me without an obvious next legal position, I teamed up with John Galgiani MD, a brilliant clinician studying valley fever (coccidioidomycosis). Timing on that project allowed me to get a double MBA, completed just as we secured our first $~1M grant and our project needed my closer attention. Through two more large grants I helped him manage development of nikkomycin Z ("NikZ"), an antifungal compound. As co-Principal-Investigator, for many years I managed million-dollar annual budgets and directed our research efforts to make NikZ more effectively at multi-kilogram scale, and coordinated and participated in an FDA post-Phase 1 meeting.

I thank deeply the Valley Fever Americas Foundation for their years of support in my advancing NikZ development and my ideas for a better dosing protocol. David Stevens MD of Stanford and the California Institute for Medical Research (CIMR) was kind to give me an initial hearing, then became quite supportive when my crazy idea for a dosing protocol giving mice drug in water to spread out the intake over time worked extremely well. This became the basis for an extended line of studies. reported in several papers discussed here in Chapter 2. He also put me on important committees at CIMR that taught me more about operating a research institute. He taught me a great deal about anti-fungal research and biological diversity that makes drug development so challenging. His colleagues Gabriele Sass and Ioly Kotta-Loizou were inspirational as scientific leaders, always willing to share time and educate and improve my own science perspective and approach.

I extend tremendous thanks to Bill DeGrado who supported my interest to complete my PhD in his group and under his amazing guidance. He taught me about working in a large group of creative scientists and a vast range of cutting-edge chemistry and design. He taught me to open my mind yet remain focused on what is working, and figure out and refine what is not working. I hope to reflect and share his training with others as I step out to make new therapeutics and perhaps some science tools.

Finally, I thank my wife Suzie for endless support and encouragement, and tolerance for my many interests and projects.

Acknowledgement of Previously Published Materials

The following papers included as Chapters 1 and 3 in this dissertation were written by Mr. Larwood about his original work, in collaboration with the respective co-authors.

The first project reported in Chapter 1 was part of graduate study directed by co-author FC Szoka, PhD. This was published [1]. This is included here with permission of the authors and publisher.

Larwood DJ and Szoka FC, Synthesis, characterization, and *in vivo* disposition of iodinatable polyethylene glycol derivatives: Differences *in vivo* as a function of chain length. J Label Compd Radiopharm, 1984, 21: 603-614. https://doi.org/ucsf.idm.oclc.org/10.1002/jlcr.2580210703

The following paper included as Chapter 3 in this dissertation was written by Mr. Larwood about his original work in collaboration with the respective co-author. This project was initiated and directed by Mr. Larwood.

This was published on April 28, 2024 [3] with minor changes after reviewer comments. This is included here with permission of the authors and publisher. An earlier version was published in March as a preprint [2].

Larwood DJ and Stevens DA, Antifungal Activity of Brilacidin, a Nonpeptide Host Defense Molecule. Antibiotics 2024, 13, 405, https://doi.org/10.3390/antibiotics13050405

Contributions

This work was performed under the guidance and supervision of Francis Szoka PhD, David Stevens MD, and William DeGrado PhD and teams of skilled scientists working in the groups of each of these investigators. This study was completed under the guidance of my advisor and Principal Investigator William DeGrado, after early work under the guidance of my advisor and Principal Investigator Francis Szoka. This thesis contains reproduction of material previously published and contains contributions from collaborators cited. Chapter 1 is reproduced with permission from D. Larwood, F. Szoka, and the publisher. Chapter 3 is reproduced with permission from D. Larwood, D. Stevens, and the publisher.

Epigraph

“(And by that destiny) to perform an act,

Whereof what's past is prologue; what to come,

In yours and my discharge.”

Attributed to William Shakespeare, The Tempest, Act 2, Scene 1.

Novel Approaches to Delivery of Biomacromolecules

David Jeffrey Larwood

Abstract

Nature challenges healthy mammals with constant risk of infection by a wide variety of pathogens and with degradation of healthy tissue and control systems. I have been interested in drug delivery and improved delivery of therapeutics primarily to attack pathogens but with incidental value in assessing vital functions of a healthy mammal. My recent work uses protein design to approach such problems. Delivering biomacromolecules remains important both for therapeutics and in discerning and shaping functions of cells.

My first thesis project focused on designing a better glomerular filtration rate (GFR) marker to facilitate assessment of renal function and seeking a marker reflecting water distribution in the body, which is relevant to distribution of highly soluble drugs [1]. Tritiated polyethylene glycol (PEG) was known to clear the kidneys effectively and correlated well with a GFR standard assay. A better radioactive label for PEG would allow for easy detection, including imaging. At the time of the project, only one biological conjugation with PEG was reported.

Attaching an iodinatable moiety to polyethylene glycol (PEG) polymers of different sizes enabled tracking the compound using radioactive iodine. I made a series of related compounds and studied the pharmacokinetics (PK) and pharmacodynamics (PD) of these in rodents and ultimately in a dog. Using relatively long PEG polymers of molecular weight (MW) 5,000 to 6,000 daltons, the PEG dominated the behavior of the compounds, clearing rapidly through the kidneys. With shorter PEG polymers, the chemistry of the iodinatable group was more

significant and the compounds were more likely to clear through the bile, to a degree making them unsuitable for a GFR marker but possibly useful to study liver function.

Chapter 1, the published manuscript from my first thesis project, is cited in 20 scientific publications and 51 issued US patents. Variations on the design principles of my project have been used widely in the pharmaceutical industry.

During a break in my PhD studies, I improved the formulation of a human Phase-2-ready antifungal drug and designed and organized extensive testing in mice and dogs to show that a sustained-release formulation would overcome PK limitations and made the drug much more potent. This work is discussed briefly in Chapter 2.

My second thesis project studied brilacidin activity against 40 fungal isolates from 20 different species, showing useful activity against several important human pathogens [2, 3]. The human and many other innate immune systems include a variety of peptides known as defensins that weaken or kill a variety of pathogens, including bacteria, fungi, and viruses. Brilacidin is a synthetic defensin-mimic, designed to exhibit the physicochemical properties of defensins as a class. Brilacidin is in human Phase 2 trials. Despite its potential, Brilacidin's efficacy against fungi had not been comprehensively explored until my studies, which showcased its viability as a therapeutic agent against challenging-to-treat fungal infections, thereby offering a beacon of hope for future clinical interventions.

Chapter 3, the published manuscript based on this second thesis project, has recently been published in Antibiotics after peer review [3].

An earlier version is available online in Preprints [2].

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Chapter 1

This paper was written by Mr. Larwood about his original work, in collaboration with co-author and advisor Francis Szoka [1].

Larwood DJ and Szoka FC, Synthesis, characterization, and *in vivo* disposition of iodinatable polyethylene glycol derivatives: Differences *in vivo* as a function of chain length.

J Label Compd Radiopharm, 1984, 21: 603-614. https://doi-org.ucsf.idm.oclc.org/10.1002/jlcr.2580210703

Synthesis, Characterization, and *in vivo* Disposition of Iodinatable Polyethylene Glycol Derivatives: Differences *in vivo* as a Function of Chain Length

Summary

A series of iodinatable water-soluble polyethylene glycol (PEG) derivatives were prepared for use as model hydrophilic drugs. Polyethylene glycol diamine 6000 was coupled to methyl p-hydroxybenzimidate, and PEG 1900- and PEG 5000- monomethyl ethers were coupled to tyramine and histamine. The derivatives underwent facile iodination with the chloramine-T reaction and were stable under a wide range of conditions. The larger derivatives showed rapid renal clearance, but the 1900 MW compounds underwent significant clearance via the bile.

1. Introduction

To facilitate studies on the *in vivo* disposition of particulate drug delivery systems, we sought to prepare an easily measurable, hydrophilic model drug. Our requirements were identical with those of a glomerular filtration rate (GFR) marker, namely, "a) physiologically inert and non-toxic, b) not protein bound, completely filterable at the glomerulus, c) not reabsorbed or secreted,

d) not subject to destruction, synthesis, or storage within the kidney, … , f) have constant clearance over a wide range of plasma concentrations [4].”

Polyethylene glycol (PEG, MW = 400-6000) appeared to be a logical candidate for such a compound since it has been proposed as a GFR marker and can be specifically derivatized. As a GFR marker in dogs, its clearance matched that of creatinine over a 2-fold range of plasma concentrations [5]. Moreover, PEG's of various sizes have been used to estimate the pore size in the glomeruli of dogs [6]. Renal clearance of PEG-1000 in rats was found to be consistently greater than inulin clearance, and to vary with PEG plasma levels [7, 8], however the markers still appear useful to follow renal excretion.

PEG can be derivatized specifically via the two terminal hydroxyls (one on the mono-methyl ethers). The structure is chemically stable and it appears to be biologically inert. Its properties have been exploited in a variety of ways:

PEG-400 has been activated with phosgene and coupled with procaine to yield a longer acting anesthetic [9]; PEG-6000 has been activated with cyanuric chloride and coupled with bovine serum albumin to reduce antigenicity [10]; PEG-10,000 has been activated with carbonyldiimidazole or dicyclohexylcarbodiimide and coupled to various amino acids as a soluble support for peptide synthesis [11, 12]. In addition, the hydroxyl groups can be readily converted to amino functions for alternative coupling schemes [13,14].

We prepared phenolic and histamine-containing PEG derivatives that could be iodinated with chloramine-T as the final step in the synthesis. The iodinated PEG compounds are easily purified by anion exchange or gel filtration, thus any desired isotope of iodine can be introduced for various applications. The synthesis, characterization, and disposition as a function of PEG molecular weight is described herein.

2. Materials and Methods

Polyethylene glycols were obtained from Polysciences, Inc., Warrington,PA. Ion exchange resins and some chromatography supports were obtained from Bio-Rad, Richmond, CA. Thin layer chromatography plates were silica gel, hard layer plates from Analtech, Newark, DE. Sephadex chromatography supports were obtained from Sigma Chemicals, St. Louis, MO. Carbonyl-diimidazole was purchased from Pierce Chemical Company, Rockford, IL. Tritiated PEG MW 900 and MW 4000 were obtained from New England Nuclear, Boston, MA as a dry powder and were dissolved in phosphate buffered saline. All other chemicals and solvents were reagent grade or better. Ultraviolet spectra were recorded on a Cary-18. NMR spectra were recorded on a Varian FT-80 using D_2O or $CDC1_3$ containing 3-(trimethylsilyl)-l-propanesulfonic acid (Aldrich, Milwaukee, WI) or tetramethylsilane as internal standard.

Three TLC solvent systems were used:

A- chloroform/methanol/ammonium hydroxide/water 34/14/1/1;

B-chloroform/methanol/acetic acid/water 34/14/1/1; and C- x% methanol in chloroform. TLC plates were routinely developed in iodine vapor, which is strongly adsorbed by PEG, and with one of the following sprays: $CoCl_2$, 1% in acetone; ninhydrin, 1% in acetone; or Folin reagent.

Polyethylene Glycol Diamine 6000 (**2**) -- **2** was prepared according to Mutter [15]. Assay of **2** with fluorescamine [16] showed quantitative conversion to the diamino compound. TLC system A, $R_f = 0.9$, detected with ninhydrin.

Polyethylene Glycol 6000 Bis (p-hydroxybenzamidine) (3) -- A mixture of 56.9 mg (9.5 mol) **2** and 4.3 mg (23 mol) methyl p-hydroxybenzimidate, hydrochloride [17], was suspended in 1 ml of chloroform. Addition of 20 µ1 (140 µmol) triethylamine gave a clear solution, which was stirred overnight at ambient temperature. The product was precipitated with 6 ml diethyl ether, and, after recrystallization from chloroform/diethyl ether, gave a white solid (53.4 mg, 91% yield). TLC system B, R_f = 0.95, and system C-10, R_f = 0.1, detected with Folin reagent. UV: λ_{max} 255 nm, ε = 10,700, NMR: 7.32 (d x d), 3.69 (s).

Polyethylene Glycol 6000 Bis(315-diiodo-4-hydroxybenzamidine) (4) -- Compound **3** (25 mg, 4.5 µmol) was dissolved in 100 µ1 borate buffer (100 mM, pH 8.0), and mixed with 32 µl (32 µmol) 1 M potassium iodide. Addition of 0.64 ml (32 µmol) chloramine-T (50 mM in borate buffer) turned the reaction briefly brown, then pale yellow. After 30 min at ambient temperature, the reaction was quenched by the addition of 0.5 ml (l00 µmol) sodium bisulfite (200 mM). Gel filtration on Bio-Gel P-2 (1.5 x 27 cm), eluting with water, yielded **4** in the void volume. Lyophilization gave 48.2 mg of a white powder, quantitative recovery. TLC system B, R_f 0.95, and system C-l0%, R_f= 0.1, detected with Folin reagent. UV: λ_{max} 318 nm, ε = 11,4000, NMR: 8.18(s), 3.64(s).

Polyethylene Glycol 5000 Carbonylimidazole Methyl Ether (7a) [18] -- Dry PEG 5000 monomethyl ether (6a, 2.15 g, 0.43 mmol) was dissolved in 5·ml dichloromethane. The sodium salt of imidazole was prepared by the action of 3.5 mg (l.5 mmol) freshly cut sodium on 122 mg (l.8 mmol) imidazole in 10 ml dry THF. After addition of 352 mg (2.2 mmol) carbonyldiimidazole and 0.5 ml (0.09 mmol) sodium imidazolide solution, the mixture was stirred overnight under nitrogen. The solvent was evaporated and the white solid was crystallized from chloroform/diethyl ether to give 2.36 g of a white powder.

Polyethylene Glycol 5000 2-(p-Hydroxyphenyl)ethylcarbamate Methyl Ether (8a)

-- Tyramine (79 mg, 0.58 mmol), dissolved in 3 ml warm, absolute ethanol, was added to 1 g (0.19 mmol) **7a** and 80 µl (0.57 mmol) triethylamine in 4 ml dichloromethane. After stirring overnight, the solvent was removed to give a pale yellow solid (quantitative yield). The crude product was purified in several batches by chromatography on BioGel P-6, eluting with water and lyophilizing the fractions. Final yield, 79% white powder. TLC system C 50%, R_f = 0.9, detected with Folin reagent. UV: λ_{max} 276 nm, ε = 1373, NMR: 7.l(d), 6.78(d), 3.64(s), 2.72(t), l.30(t).

Polyethylene Glycol 5000 2-(315-Diiodo-4-hydroxyphenyl)ethylcarbamate Methyl Ether (11a) -- Compound 8a was iodinated as for 3, purified on P-2 (1.0 x 28 cm) in borate buffer (l00 mM), desalted on P-2, and lyophylized to give 195 mg white powder, quantitative recovery. TLC system C-50%, R_f = 0.9, detected with Folin reagent.

UV: λ_{max} 291 nm, ε = 2066, NMR: 7.49(s), 7.17(s), 3.64(s), l.23(t).

Polyethylene Glycol 1900 Carbonylimidazole Methyl Ether (7b) -- To 530 mg (0.28 mmol) dry PEG 1900 monoethyl ether( ) in 2 ml dry CH2Cl2 was added 75 mg (0.46 mmol) carbonyldiimidazole and 10 mg (0.11 mmol) imidazole, sodium salt. After stirring overnight, 6 ml CH_2Cl_2 was added and the mixture was extracted with 1.75 ml water, then dried with anhydrous sodium sulfate. After filtration, solvent was removed (quantitative yield). Alternatively, the solvent was removed, and the resulting oil was recrystallized from chloroform/diethyl ether at -20°C. The white crystals were filtered through a chilled funnel, rinsed with a little diethyl ether, and used immediately.

Polyethylene Glycol 1900 2-(p-Hydroxyphenyl)ethylcarbamate Methyl Ether (8b) -- To 193 mg (0.1 mmol) **7b** in 2 ml dichloromethane was added 31 mg (0.23 mmol) tyramine in 3 ml

absolute ethanol, plus 57 µl (0.4 mmol) triethylamine. After stirring overnight, the solvent was removed. The resulting oil was taken up in 30 ml water, and extracted with 3 x 75 ml chloroform. The combined chloroform layers were dried over anhydrous sodium sulfate and evaporated to give 173 mg clear oil. This was purified by gel filtration on Sephadex G-25 (1.5 x 49 cm), eluting with water. Fractions from 26 to 44 ml were pooled and lyophilized to give 137 mg (68%) of a clear oil, pure by TLC. TLC system C-20%, R_f = 0.75, detected with Folin reagent. UV: λ_{max} 276 nm, ε = 1390, NMR: 6.88 (d x d), 3.63(s), 3.37(s), 2.70(t), 1.30(t).

Polyethylene Glycol 1900 2-(315-Diiodo-4-hydroxyphenyl)ethylcarbamate Methyl Ether (11b) was prepared in a similar fashion to **11a**.

Polyethylene Glycol 1900 2-(4-Imidazolyl)ethylcarbamate Methyl Ether) (**9**) -- To 600 mg (30 µmol) **7b** in l ml dichloromethane was added 50 mg (0.45 mmol) histamine in 1.5 ml ethanol and 70 µl (0.5 mmol) triethylamine. After stirring overnight, the solvent was removed. The oil was taken up in 5 ml dichloromethane and extracted with 5 x 1 ml 0.5 M Na2HPO4 to remove histamine. An NMR spectrum taken after the third extraction was virtually identical with a spectrum taken after the fifth extraction, showing one histamine per PEG. The product was pure by TLC, 79% yield. TLC system A, R_f = 0.9, and system C-50%, R_f = 0.2. detected with Folin reagent. NMR: 7.57(s), 6.81(s), 3.63(s), 3.36(s), 2.79(t), l.30(t).

^{125}1-Iodinations (to give **5**, **11a,b**, and **13**) -- To 1.5 mg (0.3 - 0.7 µmol) substrate (0.3 - 0.7 µmol) substrate (**3**, **8a,b** and **9** respectively) in 0.1 ml 100 mM borate buffer (pH 8.0) was added 3 µl (300 µCi, 15 pmol) Na^{125}I [New England Nuclear, Boston, MA] and 10 µl (14 nmol) 50 mM Chloramine-T, then kept at room temperature for 15 min. Addition of 10 µl (390 nmol) 200 mM sodium bisulfite stopped the reaction. The reaction mixture was purified on a small (5 x 70 mm) AG1-X4-Cl- column, eluting with normal saline. The first 1-2 ml contained the compound, while

99.95% of ^{125}I was retained on the column. Typical reactions incorporated 60-80% of ^{125}I. The products were judged pure by a second anion exchange column, a Bio-Gel P-2 column, and by TLC.

Stability Studies - The new compounds were tested for stability by incubating at 37°C in buffers (100 mM) at pH 2.0, 4.5, 7.4, or 11.0 for 18 days, or with mouse liver homogenate or lysosomes for 24 h.

Liver Homogenate and Lysosomes - Four mouse livers (6.4 g) were homogenized in 20 ml chilled (4°C) Tris/sucrose buffer (10 mM Tris/HCl, pH 7.5, 250 mM sucrose, 0.1 mM toluenesulfonyl fluoride as antipeptidase). The homogenate was assayed for protein by the modified Lowry method [19].

Half of the homogenate was centrifuged for 12 min at 1000 x g. The supernatant was centrifuged at 4°C for 30 min at 20,000 x g. The pellet was resuspended in 6 ml sodium acetate buffer (150 mM, pH = 4.6), assayed for protein, and frozen at -40°C. The lysosomes were warmed to 37°C and refrozen three times to lyse the membranes, releasing lysosomal enzymes.

A typical assay is described below: 6.5 µg (10^6 cpm) ^{125}I-**5** was incubated with 200 µl liver homogenate or lysosomal enzymes (l2 mg or 4 mg protein respectively) in a sealed centrifuge tube at 37°C. After 0,1,2,4,8, and 24 h, aliquots (10 µl) were spotted in triplicate on a TLC plate. The plates were developed in 1 N HCl/2.5% potassium iodide [20] to resolve radioactive ^{125}I- (R_f= 0.9), free diiodobenzamidine (R_f = 0.7), and intact polymer (R_f = 0). Plates spotted with **11a** or **11b** were developed in chloroform/methanol/acetic acid/water: 34/14/1/1 to resolve intact marker (R_f =0.9), ^{125}I- (R_f= 0.6), or diiodotyrosine (R_f= 0). Autoradiography of the TLC plates showed very little breakdown, which was quantified by scraping and counting the bands.

pH Stability - A mixture of 1.0 mg (160 nmol) **4** and 20 µ1(2 x 10^6 cpm) ^{125}I -**5** in 130 µl 100 mM buffer was kept at 37°C for 25 h in a capped micro-centrifuge tube. The reactions were sampled at 0,1,2,4,8, and 24 h, spotted on TLC plates, and analyzed as above. Single values were taken for each time point. The buffers used were: pH 2 - HCl/KCl; pH 4.5-acetate; pH 7.4 - phosphate; and pH 11.0 - borate.

Organ Clearances - White ICR mice [Simonsen, Gilroy, CA], 25-30 grams, were fed and watered ad libitum, and kept on a 12 h per day light cycle. Each animal was injected via the tail vein with 0.1 ml per 10 g body weight (l4% of normal blood volume, assuming 7.3% by weight). A typical dose contained 2.5 µg **4** and 200,000 cpm ^{125}I-**5** per 0.1 ml. Three or four animals were injected per time point. Each animal was anesthetized with chloroform, and, after collecting 0.5 ml blood by cardiac puncture, the desired organs were dissected, rinsed with water, weighed and counted. After carefully removing the bladders, the carcasses were rinsed with water and counted.

Total Body Clearance - Three or four female, ICR mice were kept in a single metabolic cage [Model A4565, American Scientific Products, McGaw Park, IL] for 24 h. After 1,2,3,4,8, and 24 h, the urine and feces were collected. Water rinses of the cage were sampled, and counted (included with urine values). The entire feces were counted. Tritium-containing feces were soaked overnight in 0.5 ml 1N potassium hydroxide, mixed with 0.5 ml t-butylhydroperoxide, and soaked overnight again. All tritium containing samples were mixed with 15 ml PCS [Amersham, Arlington Heights, IL] and counted. Radioactivity was determined using a Beckman LS-7800 scintillation counter or a Beckman Gamma-8000 gamma counter.

Bile Duct Cannulation - The femoral vein and bile duct of a female, Sprague Dawley rat [Simonsen, Gilroy, CA] (under light ether anesthesia) were cannulated. Injections (0.5 ml saline

containing the marker) were made via the femoral cannula, and bile was collected. The bladder was not catheterized, but urine was collected when possible. The rat, maintained for 22 h without anesthetic, appeared not to be under duress. Subsequent doses were given after 3.5-5 h, when < 0.1% of the last dose could be detected. The clearance of **13** was essentially the same whether given as the initial dose or after 18 h.

3. Results and Discussion

The preparation of the derivatives, outlined in **Figure 1**, was straightforward. The larger PEG derivatives could be precipitated with diethylether from chloroform solutions, which facilitates intermediate purification. The PEG-1900 derivatives were very soluble in dichloromethane, while almost all impurities were more water soluble, leading to easy purification by extraction. All derivatives were purified by gel filtration and lyophilization prior to UV or NMR analyses.

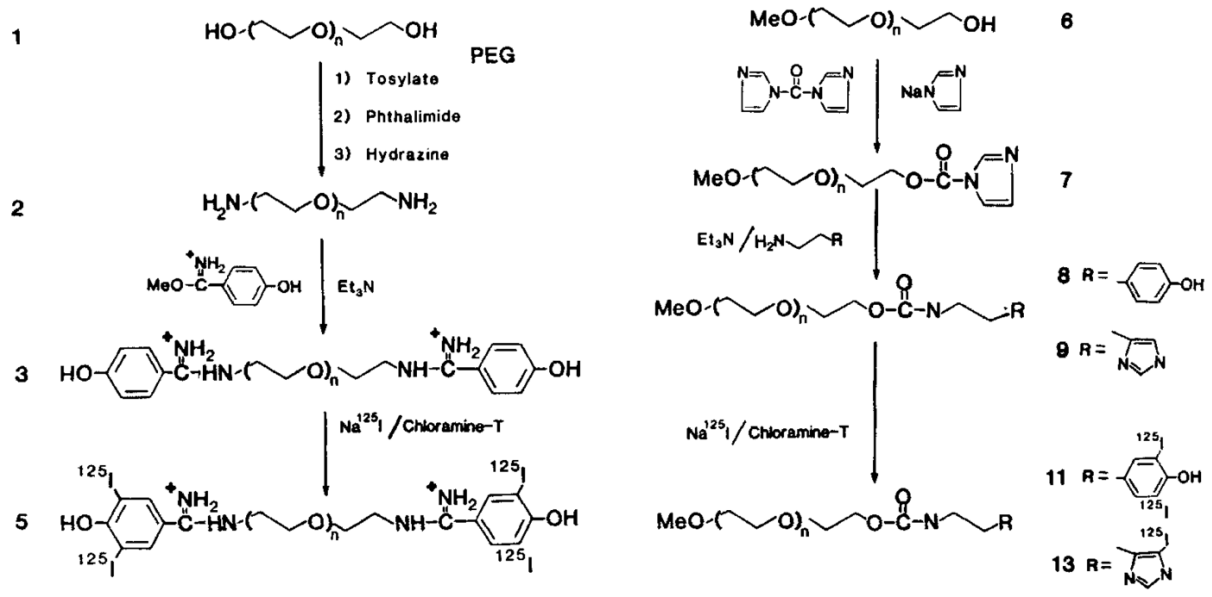

FIGURE 1: SYNTHETIC SCHEME AND STRUCTURE OF PEG DERIVATIVES

Two derivatives were tested for stability in liver homogenate (pH 7.5) or disrupted lysosomes (pH 4,6) at 37°C for 1 day. Very little breakdown of marker to either free I- or free reporter group (e.g. diiodo-benzimidate or -tyramine), was observed (**Table 1**), Derivatives **4** and **9a** were very stable, being essentially unchanged after incubation in 100 mM buffers at several pH's at 37°C for 18 days.

All of the derivatives were rapidly cleared from the body (**Table 2**). **Table 2** lists the amount (%) of dose remaining in several tissues after various times.

Each blood clearance curve could be described by a two-term exponential decay, with half lives of 5-10 minutes (initial) and 200-300 minutes (terminal).

TABLE 1: STABILITY OF PEG MARKERS UNDER VARIOUS CONDITIONS[a]

Compound	pH 2.0[b]	pH 4.5[b]	pH 7.4[b]	pH 11.0[b]	Liver[c]	Lysosomes[c]
5	98.7	99.6	101.5	97.7	97	92
11a	100	ND	96.8	93.8	100	99
13	ND	ND	97d	ND	ND	ND

a % of compound unchanged (2-3 determinations, ± < 4%)
b after 18 days, 37ºC
c after 24 hours, 37ºC
d after 5 days, 37ºC
ND = not determined

The larger derivatives, **5** and **11a**, were cleared almost exclusively by the kidneys (**Tables 2 and 3**). Although the residual levels of marker in individual organs dropped rapidly, the amount remaining in the whole animal was judged to be excessive for use as a hydrophilic model drug compound when compared to ^{14}C inulin (1.4% total *in vivo* after 5 hours) [21].

Decreasing the chain length of PEG to MW = 1900 led to substantial biliary excretion (**Tables 3 and 4**). Although PEG-400 has been reported to be quantitatively recovered in canine urine [5], we found significant biliary excretion (l2%) of ^{3}H-PEG-900 in the rat. Biliary clearance of **11b** and **13** was several-fold higher which must be a result of the head group. However, the head group probably does not dominate the process since ^{125}I-histamine was found to be 95% excreted in urine, 75% after 2 hours, with little evidence for excretion via the bile. Moreover, diiodophenols resemble several thyroid hormones, which are not significantly excreted. The attachment of tyramine to polyhydroxyethyl-aspartamide at a mole percentage of 20% results in both a more rapid and greater accumulation of the polymer in the kidney than the non-modified polymer [22]. *In vitro* experiments demonstrate that the phenolic residues enhance the rate of pinocytotic capture of the polyhydroxyethylaspartamide [23]. In our studies, the attachment of tyramine and histamine modified the *in vivo* distribution of only the 1900 MW PEG, not the 5000 MW, and the 1900 MW derivatives accumulated in the bile, not the kidney. Thus it appears the effect of modification of polymers by small ligands on the *in vivo* behavior of the polymers is a complex function of the ligand, polymer, and ligand/polymer ratio.

TABLE 2: ORGAN DISTRIBUTION OF PEG DERIVATIVES IN MICE[a]

Hours[b]	N[c]	Blood	Liver	Spleen	Lung	Kidney	Stomach	Intestine	Carcass
5 Polyethylene Glycol 6000 Bis(3,5-^{125}I-diiodo-4-hydroxybenzamidine)									
l	12	0.89±0.04	0.61±0.03	0.01±0.001	0.07±0.02	1.19±0.18	0.24±0.08	0.62±0.03	7.10±0.37
4	3	0.282±0.03	0.31±0.04	0.02±0.01	0.05±0.02	0.42±0.07	0.23±0.17	0.51±0.55	ND
24	3	bkg	bkg	bkg	bkg	bkg	bkg	bkg	bkg
11a Polyethylene Glycol 5000 2-(3,5-^{125}I-Diiodo-4-hydroxyphenyl)ethylcarbamate Methyl Ester									
0.5	4	2.98±0.16	2.30±0.51	0.06±0.01	ND	1.58±0.19	0.70±0.10	1.40±0.04	15.3±15.4
l	7	1.39±0.06	1.31±0.17	0.05±0.01	0.38±0.18	0.89±0.09	0.64±0.03	1.16±0.2	17.9±2.3
4	4	0.48±0.10	0.53±0.05	0.01±0.00	ND	0.60±0.18	0.53±0.34	0.40±0.13	4.9±1.5
11b Polyethylene Glycol 1900 2-(3,5-^{125}I-Diiodo-4-hydroxyphenyl)ethylcarbamate Methyl Ester									
0.5	8	0.59±0.09	5.12±1.7	0.02±0.00	0.09±0.03	0.28±0.17	1.01±0.57	30.6±7.3	14.0±2.9
l	12	0.99±0. 10	2.04±0.95	0.02±0.00	0.12±0.02	0.64±0.37	0.61±0.07	27.5±12.3	16.5±9.3
4	8	0.04±0.01	0.21±0.10	0.01±0.02	0.02±0.01	0.03±0.01	0.89±0.97	1.39±1.2	19.8±9.7
24	4	0.02±0.01	0.06±0.02	bkg	bkg	0.02±0.01	0.09±0.1	0.88±0.12	0.65±0.18
13 Polyethylene Glycol 1900 2-(4-^{125}I-Imidazolyl)ethylcarbamate Methyl Ester									
0.25	2	1.4,1.7	4.45,4.62	0.03,0.05	0.15,0.19	0.41,5.7	0.93,1.20	32.0,39.5	8.2,18.6
4	2	1.5,1.6	0.71,3.1	0.02,0.05	0.09.,0.10	0.21,0.21	3.3,3.7	0.85,1.5	35.2,38.8

a % of dose remaining per organ (mean ± standard deviation)
Blood values are % of dose remaining in total blood compartment
b Hours after administration of dose
c Number of animals per determination
d Duplicates
ND not determined, bkg: background

TABLE 3: CLEARANCE OF PEG DERIVATIVES FROM MICE[a]

Compound	% in feces	% in Urine	Time to Peak (hr)
^{3}H-PEG	0.87	73.3	1
5	1.63	101.3	3
11a	0.87	73.3	3
11b	57.3	21.6	8
13	33.7	64.1	2

a Mice were kept in metabolic cages, output (% of dose) determined as outlined in Materials and Methods, Values are from a single representative experiment.

TABLE 4: PERCENT OF DOSE EXCRETED INTO THE BILE[a]

Compound	% in bile	Time to Peak (hr)	Collection period (h)	% in Urine	Peak (h)
11b	68.0	15	3:40	4.3	5
13	43.1	7	5	35.7	3
^{3}H-PEG	12.1	7	4	44.8	0:20
13	56.5	7	3	33.5	4:30
11b	78.6	7-15	5	--	--

a Values are from a single representative experiment with consecutive runs

PEG's of molecular weight 4000-6000 were initially chosen because inulin, a standard GFR marker, has a molecular weight of 5000. However, the fraction of the dose remaining *in vivo* at 5 hours appeared to us to be unacceptable for a useful model drug compound. The lower molecular weight PEG-1900 derivatives were prepared to assure total filtration in small animals, such as

the mouse [6]. Surprisingly, the reporter group (tyramine, histamine) altered the properties of the polymer to the point where the derivatives were cleared mainly in the bile. Biliary excretion of the PEG-1900 derivatives is very rapid, comparable at least in time course to imino-diacetic acids used for liver imaging [24], which may make the 1900 MW PEG derivatives useful markers for studying liver function and bile formation.

4. Acknowledgments

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Chapter 2

Interim Studies – Antifungal Drugs

I am second author on three papers since 2020 reporting continuous dosing antifungal mouse studies that I organized, participating closely in the design, execution and analysis. I secured funding for these and some related, ancillary studies. I am a coauthor on a fourth mouse study that elaborated on a study that I initiated. The references here include 13 of my publications since 2020, noted by ResearchGate to have 113 citations. These do not include my conference abstracts and presentations.

For some years I worked with a group devoted to improving therapies for the rare but concerning disease coccidioidomycosis ("valley fever"), managing $7M NIH support as co-PI making and testing nikkomycin Z ("NikZ"). I was invited to write a review of NikZ and therapy [25]. This has been cited more than 37 times as of this writing.

In 2019 I was reminded that a 1998 report of highly effective NikZ therapy against *Candida albicans* when dosing by continuous infusion suggested benefits warranting testing a sustained dosing oral formulation. From my extensive studies of NikZ manufacturing and purification I recognized that a solution of NikZ at pH 3.3 might be stable for extended periods and suitable for oral administration. I asked David Stevens, MD, to try my idea of providing drug in drinking water to spread out the dosing administration for mice at relatively low burden on the operators and the animals [26]. I worked with his group extensively from late 2019 until starting classes again at UCSF in 2022, and we still are collaborating.

The first 2019 test dosing NikZ in drinking water was used to treat mice infected with *Coccidioides posadasii*. This proved so successful that our lowest test dose level of 200 mg/kg/day essentially cleared the disease, where a similar dose given twice a day (BID, bis in die) would be expected to only help. We repeated the study with a 4 times higher inoculation challenge and a lowest dose reduced by >95% to 8 mg/kg/day in drinking water. That lowest dose proved more effective than 100 mg/kg/day fluconazole, standard of care [27]. The medium high doses almost eliminated detectable residual infection.

This encouraged NIAID to support a study dosing mice with brain disease induced by intracranial injection of Coccidioides, extending older studies dosing at 40, 160 and 600 mg/kg/day divided BID by adding a third dose three times a day (TID) for 75, 300 and 900 mg/kg/day for 12 days. The best survival was 80%, whether dosing at 600 or 900 mg/kg/day, with barely significant reduction of fungal burden at the highest test dose. Support from a foundation let us repeat this test with NikZ in drinking water, achieving equivalent fungal burden reduction at 30 mg/kg/day (3.3% of 900 TID) for 12 days with 70% survival after 40 days. Superior to any previous NikZ observation, survival of 90% and 100% was achieved at manageable doses of 100 and 300 mg/kg/day with no detectable residual fungus, effectively sterilizing most survivors at all three dose levels [28].

Human meningeal coccidioidomycosis in the brain or central nervous system (CNS) is 100% fatal in 18 months without therapy, and all of the available therapies are unpleasant. About 300 new human cases annually require patients to take drugs literally for life. Valley fever in dogs is very similar to the disease and response to therapy in humans. A dog with severe brain infection

failing conventional therapy for years was treated for four months with NikZ in our spread dosing protocol, showing almost complete recovery from CNS valley fever symptoms. After stopping all antifungal therapy, the dog regressed only slightly and has been stable for more than a year with no antifungal drugs. This is an unprecedented outcome in this disease and shows great promise for human relief.

Lacking sophisticated formulation (too expensive for early testing), the experimental spread dosing protocol for dogs asks owners to divide a daily dose into 12 units and dose every 2 hours, with some pre-and post-loading around sleep periods. Several dedicated dog owners have done this for months, with dramatic improvement essentially clearing serious natural valley fever infection. We reported these findings in April 2024 at the 69th annual Coccidioidomycosis Study Group meeting. We feel this validates our approach and justifies making and testing a one-a-day (QD) sustained release formulation. Finding support remains challenging.

Infections caused by *Candida spp*. costs the US some $1.2B per year and kill more than 10,000 people. Mirroring the 1998 report of treating virulent *Candida* infection with NikZ by continuous IV infusion mentioned above, treating a similar *Candida* infection with NikZ in drinking water increased survival and reduced fungal burden significantly [29].

Planktonic *Candida* grows by forming germ tubes, a growth phase with high chitin formation that is sensitive to NikZ in many strains. An *in vitro* study showed suppression of this growth was variable between strains and that combinations of NikZ and conventional drugs were highly synergistic, enabling better suppression of the more virulent strains [30].

Sporothrix is a dimorphic fungus, as is *Coccidioides*. Expanding on a study that I conceived and initiated with Dr. Stevens, some collaborators ran a more extensive study in mice showing NikZ benefit dosed by our drug in water protocol against sporotrichosis, a dangerous epidemic in Brazil [31].

I am co-author on several additional *in vitro* studies of nikkomycin Z [32-36]. I am co-author on a paper reporting on an FDA workshop where I presented 20% of a one-hour panel on the status and commercial prospects for drugs used to treat valley fever [37]. I am an inventor of three published patent applications [26, 38, 39], none of which have issued as patents.

I have directed or collaborated on additional studies which are not published.

Chapter 3

The following paper included as Chapter 3 in this dissertation was written by Mr. Larwood about his original work in collaboration with the respective co-author.

PrePrint [2]:

Larwood DJ and Stevens DA, Antifungal Activity of Brilacidin, a Nonpeptide Host Defense Molecule. Preprints 2024, 2024021664, https://doi.org/10.20944/preprints202402.1664.v1

Peer reviewed, with minor changes [3] (*starting here on this page 19***):**

Larwood DJ and Stevens DA, Antifungal Activity of Brilacidin, a Nonpeptide Host Defense Molecule. Antibiotics 2024, 13, 405, https://doi.org/10.3390/antibiotics13050405

ANTIFUNGAL ACTIVITY OF BRILACIDIN, A NONPEPTIDE HOST DEFENSE MOLECULE

Abstract: Natural host defensins, also sometimes termed antimicrobial peptides, are evolutionarily conserved. They have been studied as antimicrobials, but some pharmaceutical properties, undesirable for clinical use, have led to the development of synthetic molecules with constructed peptide arrangements and/or peptides not found in nature. The leading development currently is synthetic small-molecule nonpeptide mimetics, whose physical properties capture the characteristics of the natural molecules, and share their biological attributes. We studied brilacidin, an arylamide of this type, for its activity *in vitro* against fungi (40 clinical isolates, 20 species) that the World Health Organization has highlighted as problem human pathogens. We found antifungal activity at low concentrations for many pathogens, which indicates that further screening for activity, particularly *in vivo*, is justified to evaluate this compound, and other mimetics, as attractive leads for the development of effective antifungal agents.

1. Introduction

Peptide antibiotics (e.g., vancomycin, daptomycin, polymyxin, echinocandins) have shown their values in clinical medicine. There are >2000 discovered natural "antimicrobial peptides" (AMPs), which are highly evolutionarily conserved, and present in microbes, plants, and all vertebrates [40, 41]; >100 are known to be produced by humans [42, 43]. A broad antimicrobial spectrum is a group characteristic, most are amphiphilic and cationic [42]. These peptides are better termed "host defense peptides" or defensins because they are part of the host's innate immune response, and are the first line of defense [44, 45]. Many of these appear to have broad biological functions, as will be further discussed.

There has been longstanding interest in exploiting such molecules, and their analogues, as clinical anti-infectives, with stimulation to expand our armamentarium owing to the development of resistance to current chemically synthesized molecules and other natural products. Natural AMPs may be undesirable for clinical therapeutics because of instability, degradation by host proteases, low solubility, reduced activity in the presence of salts or DNA, short half-lives *in vivo*, difficult and expensive manufacturing issues, and the possibility of the development of antibodies in heterologous hosts [45-50]. This led to the development of synthetic AMPs, using amino acid sequences and/or amino acids not found in nature, which ameliorated some of these problems [51, 52]. It was then discovered that the physicochemical properties of the synthetic molecules were more important than the sequence of the amino acids [49, 50, 53], and, with attention to the secondary structure, charge and folding, that totally synthetic non-peptide molecules could recapitulate the structural properties of AMPs and mimic their activities [49, 50]. A lead candidate from this line of research is brilacidin, a cationic water-soluble amphiphilic

helical arylamide, with discrete nonpolar hydrophobic and polar hydrophilic regions, and a polymer backbone [49]. The present study is an initial exploration of the antifungal spectrum of brilacidin, with particular attention to pathogens for which there is a huge present clinical burden (e.g., cryptococcosis in Africa in the wake of the AIDS epidemic) and those pathogens for which present clinically available antifungals provide insufficient efficacy.

2. Results

The screening of the selected fungal pathogens of great interest is displayed in **Table 1**. The low MIC values (largely <4 μg/mL) of all in this group, except *A. fumigatus*, suggests that brilacidin is worthy of study in animal models, to ascertain whether this level of potency *in vitro* will translate into efficacy *in vivo*, and thus have potential clinical utility. These MIC values, in μg/mL are favorable compared to those of conventional antifungals.

TABLE B1. BRILACIDIN ACTIVITY AGAINST PROBLEM PATHOGENS

BRILACIDIN MICs µg/mL

Pathogen	Strain	50% Inhibition	100% Inhibition
Coccidioides posadasii	Silv.	4	>64
Coccidioides sp.	22-50	2	>64
	22-40	2	>64
	22-35	2	>64
	22-33	2	>64
Aspergillus fumigatus	18-31	>64	>64
	13-130	>64	>64
	19-12	>64	>64
	21-23	64	>64
	09-03	>64	>64
	18-32	64	>64
	18-117	>64	>64
(A. fumigatus)	13-30	>64	>64
	11-13	>64	>64
	09-117	>64	>64
	10AF	64	>64
Aspergillus lentulus (voriconazole resistant)	14-39	32	>64
Aspergillus terreus	12-70	>64	>64
Aspergillus niger	22-4	8	16

(Table B1)

Pathogen	Strain	50% Inhibition	100% Inhibition
Lomentospora prolificans	15-101	4	8
	15-99	4	8
	15-97	4	8
	15-98	4	8
	94-58	8	16
	10-03	4	8
	15-100	8	16
Scedosporium apiospermum complex	12-13	4	8
	98-38	2	8
	01-48	4	16
	10-23	2	4
	18-46	8	16
Fusarium species	07-144	4	16
	22-51	8	16
	07-136	2	16
	00-137	2	32
	19-171	2	32
	12-22	1	64
	22-1	2	32

(Table B1)

Pathogen	Strain	50% Inhibition	100% Inhibition
Mucorales			
Rhizopus species	16-88	4	16
	20-235	16	32
	21-01	8	16
	13-91	2	8
	94-2	2	32
	21-85	4	64
Mucor species	20-177	16	32
	15-64	4	64
	13-39	4	32
	13-127	4	>64
Unspeciated zygomycete	07-140	2	16
Sporothrix brasiliensis	20-18	8	64
	20-19	16	64
	20-20	16	64
Sporothrix schenckii	20-45	4	16
	20-46	8	32

(Table B1)

Pathogen	Strain	50% Inhibition	100% Inhibition
Cryptococcus neoformans	*00-288*	*1*	*2*
	01-126	*1*	*1*
	06-71	1	1
	00-289	1	2
	97-370	2	2
	CN9759	1	8
	17-66	2	2

There is a disparity between this 50% inhibition and the elevated 100% inhibition MICs, for *Coccidioides*, *Mucorales*, *Sporothrix*, and *Fusarium*, suggesting that for those pathogens, brilacidin's antimicrobial activity is unlike that of polyenes. Polyenes, such as amphotericin B, typically have similar concentrations for 50% and 100% inhibition, and even for cidal activity [54]. However, the clinical utility of azoles and echinocandins, which also do not produce even 100% fungal inhibition *in vitro*, suggests that conclusions about the efficacy of brilacidin *in vivo* need to be deferred until animal models are explored. The most striking, consistent results are those against *C. neoformans*, where brilacidin appears to have unique antifungal activity among these pathogens assayed.

The studies displayed in **Table B2** represent an initial screening effort to examine whether other groups of pathogens may be worthy of the broader screening displayed in **Table B1**. Several of these pathogens are in the favorable range discussed for pathogens studied as per **Table B1** and should be more extensively screened in the future. The initial results with *Nakaseomyces glabratus* and *Candida auris* unfortunately do not as yet give such indication.

TABLE B2. INITIAL SCREEN OF BRILACIDIN ACTIVITY AGAINST OTHER PROBLEM PATHOGENS

BRILACIDIN MICs

Pathogen	Strain	50% inhibition	100% inhibition
Candida albicans	20-132	1	4
	5	4	>64
Candida albicans (fluconazole-resistant)	21-76	32	>64
Candida auris	20-253	>64	>64
Candida krusei (fluconazole-resistant)	03-287	8	16
Candida lusitaniae (amphotericin-intermediate)	22-16	8	8
Torulopsis glabrata *(Nakaseomyces glabratus)*	22-21	64	>64
Acremonium species (resistant to azoles, polyenes, echinocandins)	18-51	4	>64
Exserohilum species	19-48	1	16

3. Materials and Methods

3.1. Drugs

Brilacidin (N4, N6-bis(3-(5-gaunidinopentanamido)-2-(R)-pyrrolidin-3-yl)oxy)-5-(trifluoromethyl)phenyl)pyrimidine-4,6-dicarboxamide tetrahydrochloride) (C40H50F6N14O6.4HCl), MW 1082.7, sterile and >98% pure, was supplied by Innovation Pharmaceuticals, Wakefield, MA, USA. It was supplied as a solid and was readily soluble in water and liquid media, such as RPMI 1640. To convert μg/mL, as expressed in this paper, to millimolar, multiply μg/mL by 0.924.

In prior studies for some isolates as mentioned, azoles were supplied by Pfizer Inc, Groton, CT, USA; echinocandins by Merck, Inc., Rahway, NJ, USA; and amphotericin B by the Bristol-Myers Squibb Company, Princeton, NJ, USA.

3.2. Isolates

The World Health Organization has recently identified particular fungal pathogens as needing attention because of epidemiological reasons and/or resistance to many available drugs [55]. It was this document that guided our selection of isolates, constrained by the availability of isolates in our collections. The isolates were all recent clinical isolates, sent to our laboratories for clinical testing, with three exceptions (CN9759, Silv., 10AF), which were originally clinical isolates, but were maintained in the laboratory because they have desirable characteristics for animal model studies, which may be indicated in the future. All were tested using their CIMR accession numbers, without any patient identification.

3.3. Testing

Testing was performed by standard broth dilution methods detailed elsewhere [56-58]. The RPMI-1640 medium is desirable because it is fully defined and it also allows microbial susceptibility testing in the presence of mammalian cells in the future. Testing of *Coccidioides* was performed under BSL3 conditions. A stock solution was made of 640 µg/mL. The range of concentrations tested, in 2-fold dilutions, was 0.5-64 µg/mL. For the testing of a new drug, it is not clear whether a 50% inhibition endpoint for yeasts (equivalent to a Minimum Effective Concentration, that concentration producing a morphological change in filamentous fungi), as is used clinically for azoles and flucytosine, or a 100% inhibition endpoint (i.e., a tube as clear as

the starting inoculum), as is used clinically for polyenes, is most relevant, so both endpoints were determined for brilacidin. In isolated instances where relevant (mentioned in the tables), azole resistance was defined as 50% inhibition at ≥64 μg/mL, echinocandin resistance as 50% inhibition at ≥3.1 μg/mL, and amphotericin intermediate as 100% inhibition at ≥2 μg/mL. Testing was repeated in approximately 20% of the assays, and was always reproducible. Every assay included a positive concurrent control, embodying a pan-susceptible *Candida kefyr* and fluconazole (MIC <0.5 μg/mL).

4. Discussion

The activities of AMPs have been described against bacteria, protozoa, and viruses [41, 59-61]. Several theoretical models exist to explain their interactions with cells [41, 61, 62]. The antifungal activity of other AMPs and their analogues has previously been demonstrated [42, 43, 51, 52, 63-67], including, in our prior study, against pathogens resistant to specific antifungals [52] and with cidal activity sometimes demonstrated [52, 66]. A topically applied AMP has already shown antifungal efficacy in patients [62]. In the present study, conidia or yeasts were used as the inoculum. The conidia develop during the assay to hyphae; thus, in the case of filamentous organisms, antifungal activity against conidia themselves, during transformation to hyphae, or on hyphal development could produce positive test results. Prior studies have indicated AMP activity against all these phases [43, 68]. Our results, with our testing methods, are consistent with the observed rapid antifungal action of AMPs [52, 63]. The present study shows brilacidin activity *in vitro* against several problem fungal pathogens. For possible clinical interest these studies must be expanded to further study brilacidin's pharmacology, tissue penetration, and toxicology. What is not yet understood is why there are the species differences

in susceptibility that we have demonstrated, and this may relate to differences in susceptibility to the mechanism(s) of drug action. More studies, with other fungal species, are required. Although brilacidin has been shown to depolarize the *A. fumigatus* cell membrane, and to disrupt the cell wall [69], our results (minimal activity against this genus) present a difference from the inhibitory activity against *A. fumigatus* demonstrated for some AMPs [43]. A caution regarding this subject is that some AMPs have also been shown to increase *A. fumigatus* growth *in vitro* [43, 66].

Prior studies have indicated the synergy *in vitro* of AMPs and their analogues with conventional antimicrobials and antifungals [46, 47, 65, 67, 69], even with host AMPs [47], which is an avenue for further exploration. One possible mechanism for any such synergy is that AMPs increase the permeability of and depolarization of the pathogen membrane, allowing greater penetration of the conventional drug [45, 70, 71]. Brilacidin synergy with an antifungal *in vivo* has been shown [69].

It is unclear what *in vitro* test characteristics, aside from whether to use 50% or 100% endpoints, will be most useful to predict activity *in vivo*. Which medium is the best needs determination, as well as the conditions of pH, ionic concentration, oxygenation and buffer [68]. It may be most relevant to study these agents in the presence of host cells, and, depending on the target *in vivo*, to test in a milieu that reflects the tissue situation, such as artificial sputum medium, as we have done [72]. Testing against fungal biofilms may be more relevant than against planktonic growth for many clinical situations [73], and AMPs have been demonstrated to inhibit biofilms [40, 47, 52, 65, 74].

Mechanisms of action for AMPs and their analogues include insertion into pathogen (and host) membranes (with creation of pores) or other phospholipids, and/or into ribosomal subunits,

stress on protein folding, stress of cell membranes, increase of reactive oxygen species, affecting intracellular calcium concentrations, affecting the proteome, inactivation of cellular proteins, affecting cell signaling, the regulation of cell death, binding the anionic nucleic acids and/or affecting their synthesis, preventing biofilm formation, regulating iron metabolism, the inhibition of cellular enzymes, the activation of cell wall lytic enzymes, binding of glucan and/or chitin, the modulation of the cell wall to expose beta glucan, and the degradation of cell walls [40-43, 45-47, 51, 62, 64, 66, 67, 75, 76].

Given AMP's effects on the regulation of many genes in their targets [45] and all these possible mechanisms of action, many effects on host function have also been described for them, including affecting host cell differentiation, immunomodulation, the regulation of cytokines, opsonization, the regulation of inflammation, the increase of phagocytosis, the stimulation of chemotaxis (for neutrophils, monocytes and lymphocytes), the activation of eosinophils and angiogenesis, and the activation of epithelial cells [40-42, 46, 66, 77]. It is likely that these possible host effects would come into play if brilacidin were to be used as an antifungal *in vivo*, and this may make MIC's absolute values, or differences, *in vitro* less important for the effect on the outcome.

The development of resistance to AMPs has been shown generally difficult for microbes to achieve [45], and that has been corroborated for peptide AMPs [43], synthetic peptides [52] and brilacidin [49]. AMP action on several different microbial processes, as detailed above, may explain AMP's breadth of microbial spectrum [42], as shown in our results here with various species, and AMP's defense against resistance development [40]. Previous observations of the development of resistance to AMPs have included the development of microbial efflux pumps, which may be lessened for the nonpeptide mimetics [47]. The cationic nature of brilacidin, and

its water solubility, may relate to its ability to target charged fungal membranes [41, 50]. Brilacidin depolarization of microbial membranes and its induction of membrane and cell wall stress have been demonstrated [49].

The structure of the nonpeptide mimetics preserves the AMP theme of such biologically active molecules having both a charged face and a hydrophobic face [45, 78]. The activity of these mimetics is more closely linked to their physicochemical properties than the details of the structures [79]. This nature of this class of molecules allows for studies of molecular modifications that could improve efficacy and decrease undesirable effects [51]. Its manipulation of charge, amphiphilicity, hydrophobic-hydrophilic balance and folding properties create possibilities for the future. Presently, brilacidin is being studied in human clinical trials for other indications and is not yet focused on fungal infections.

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