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Article

Antifungal Activity of Brilacidin, a Nonpeptide Host Defense Molecule

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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, 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 find antifungal activity at low concentrations for many pathogens, which indicates further screening for activity, particularly *in vivo*, is justified to evaluate this compound, and other mimetics, as attractive leads for development of effective antifungal agents.

Keywords: brilacidin; synthetic nonpeptide mimetics; antifungal activity; defensins; antimicrobial peptides; AMP

1. Introduction

Peptide antibiotics (e.g., vancomycin, daptomycin, polymyxin, echinocandins) have shown their value in clinical medicine. There are >2000 discovered natural "antimicrobial peptides" (AMPs), highly evolutionarily conserved, and present in microbes, plants, and all vertebrates [1,2]; >100 are known to be produced by humans [3,4]. A broad antimicrobial spectrum is a group characteristic, most are amphiphilic and cationic [3]. These peptides are better termed "host defense peptides" or defensins, because they are part of the host innate immune response, the first line of defense [5,6]. 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 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 development of antibodies in heterologous hosts [6–11]. 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 [12,13]. It was then discovered that the physicochemical properties of the synthetic molecules was more important than the sequence of the amino acids [10,11,14], 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 [10,11]. A lead candidate from this line of research is brilacidin, a cationic water-soluble amphiphilic helical arylamide, with discrete nonpolar hydrophobic and polar

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hydrophilic regions, and a polymer backbone [10]. 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. Materials and Methods

2.1. Drugs

Brilacidin (N4, N6-bis(3-(5-gaunidinopentanamido)-2-(R)-pyrrolidin-3-yl)oxy)-5-(trifluorormethyl)phenyl)pyrimidine-4,6-dicarboxamide tetrahydrochloride) ($C_{40}H_{50}F_6N_{14}O_6.4HCl$), MW 1082.7, sterile and >98% pure, was supplied by Innovation Pharmaceuticals, Wakefield, MA. 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; echinocandins by Merck, Inc., Rahway, NJ; and amphotericin B by Bristol-Myers Squibb Company, Princeton, NJ.

2.2. 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 [15]. It was this document that guided our selection of isolates, constrained by availability of isolates in our collections. The isolates were all recent clinical isolates, sent to our laboratories for clinical testing, with 3 exceptions (CN9759, Silv., 10AF), which were originally clinical isolates, but 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.

2.3. Testing

Testing was performed by standard broth dilution methods detailed elsewhere [16–18]. RPMI-1640 medium is desirable because it is fully defined and it would also allow microbial susceptibility testing in the presence of mammalian cells in the future. Testing of *Coccidioides* was performed under BSL3 conditions. 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 assays, and always reproducible. Every assay included a positive concurrent control, embodying a pan-susceptible *Candida kefyr* and fluconazole (MIC <0.5 μg/mL).

3. Results

The screening of 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 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 has potential clinical utility. These MIC values, in μg/mL are favorable compared to those of conventional antifungals.

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Table 1. Brilacidin activity against problem pathogens.

BRILACIDIN MICs			
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
"	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
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
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

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"	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
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 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 [19]. However, the clinical utility of azoles and echinocandins, which also do not produce even 100% fungal inhibition in vitro, suggest conclusions about efficacy of brilacidin in vivo needs 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 2 represent an initial screening effort to examine whether other groups of pathogens may be worthy of the broader screening displayed in Table 1. Several of these pathogens are in the favorable range discussed for pathogens studied as per Table 1, and should be more extensively screened in future; the initial results with *Nakaseomyces glabratus* and *Candida auris* do not as yet, unfortunately, give such indication.

Table 2. 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
" *(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

4. Discussion

Activity of AMPs have been described against bacteria, protozoa, and viruses [2,20–22]. Several theoretical models exist to explain their interactions with cells [2,22,23]. Antifungal activity of other

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AMPs and their analogues has previously been demonstrated [3,4,12,13,24–28], including, in our prior study, against pathogens resistant to specific antifungals [13] and with cidal activity sometimes demonstrated [13,27]. A topically applied AMP has already shown antifungal efficacy in patients [23]. In the present study, conidia or yeasts are used as the inoculum. The conidia develop during the assay to hyphae; thus, in the case of filamentous organisms, antifungal activity against conidia themselves, transformation to hyphae, or on hyphal development could produce positive test results. Prior studies have indicated AMP activity against all these phases [4,29]. Our results, with our testing methods, are consistent with observed rapid antifungal action of AMPs [13,24]. The present study shows brilacidin activity in vitro against several problem fungal pathogens, studies that for possible clinical interest must be expanded to further study brilacidin pharmacology, tissue penetration, and toxicology. Not yet understood is why there are the species differences in susceptibility we demonstrate, 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 [30], our results present a difference from the inhibitory activity against *A. fumigatus* demonstrated for some AMPs [4]. A caution in this subject is that some AMPs have also been shown to increase *A. fumigatus* growth [4,27].

Prior studies have indicated synergy in vitro of AMPs and their analogues with conventional antimicrobials and antifungals [7,8,26,28,30], even with host AMPs [8], an avenue for further exploration. One possible mechanism for any such synergy is AMP increase of permeability of, and depolarization of, the pathogen membrane, allowing greater penetration of the conventional drug [6,31,32]. Brilacidin synergy with an antifungal in vivo has been shown [30].

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 conditions of pH, ionic concentration, oxygenation and buffer [29]. 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 [33]. Testing against fungal biofilms may be more relevant than against planktonic growth for many clinical situations [34], and AMPs have been demonstrated to inhibit biofilms [1,8,13,26,35].

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, regulation of cell death, binding the anionic nucleic acids and/or affecting their synthesis, preventing biofilm formation, regulating iron metabolism, inhibition of cellular enzymes, activation of cell wall lytic enzymes, binding of glucan and/or chitin, modulation of the cell wall to expose beta glucan, and degradation of cell walls [1–4,6–8,12,23,25,27,28,36,37]. Given AMP effects on regulation of many genes in their targets [6] and all these possible mechanisms of action, many effects on host function have also been described for them, including affecting host cell differentiation, immunomodulation, regulation of cytokines, opsonization, regulation of inflammation, increase of phagocytosis, stimulation of chemotaxis (for neutrophils, monocytes and lymphocytes), activation of eosinophils, angiogenesis, and activation of epithelial cells [1–3,7,27,38]. It is likely these possible host effects would come into play if brilacidin were used as an antifungal in vivo, and this may make MIC absolute values, or differences, in vitro less important for the effect on the outcome.

Development of resistance to AMPs has been shown generally difficult for microbes to achieve [6], and that has been corroborated for peptide AMPs [4], synthetic peptides [13] and brilacidin [10]. AMP action on several different microbial processes, as detailed above, may explain AMP breadth of microbial spectrum [3], our results here with various species, and AMP defense against resistance development [1]. Previous observations of development of resistance to AMPs have included development of microbial efflux pumps, which may be lessened for the nonpeptide mimetics [8]. The cationic nature of brilacidin, and its water solubility, may relate to its ability to target charged fungal

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membranes [2,11]. Brilacidin depolarization of microbial membranes, and induction of membrane and cell wall stress, has been demonstrated [10].

The structure of the nonpeptide mimetics preserves the AMP theme of such biologically active molecules having both a charged face and a hydrophobic face [6,39]. The activity of these mimetics is more closely linked to their physicochemical properties than the details of the structures [40]. This nature of this class of molecules allows for studies of molecular modifications that could improve efficacy and decrease undesirable effects [12]- manipulation of charge, amphiphilicity, hydrophobic-hydrophilic balance and folding properties create possibilities for the future. Presently, brilacidin is being studied in clinical trials in non-mycologic conditions.

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Data Availability Statement: Tables of the original raw data are available from the corresponding author.

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Conflicts of Interest: David J. Larwood is employed by Valley Fever Solutions and is a PhD candidate at the Univ. of California San Francisco. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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