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Nylon-3 Polymers with Selective Antifungal Activity

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Supporting Information

ABSTRACT: Host-defense peptides inhibit bacterial growth but show little toxicity toward mammalian cells. A variety of synthetic polymers have been reported to mimic this antibacterial selectivity; however, achieving comparable selectivity for fungi is more difficult because these pathogens are eukaryotes. Here we report nylon-3 polymers based on a novel subunit that display potent antifungal activity (MIC = 3.1 μg/mL for *Candida albicans*) and favorable selectivity (IC_{10} > 400 μg/mL for 3T3 fibroblast toxicity; HC_{10} > 400 μg/mL for hemolysis).

Natural strategies to fend off microbial infection include the production of relatively small peptides that manifest antimicrobial activity, part of the innate immune response.[1] These "host-defense peptides" have diverse sequences and bioactive conformations, and their biological effects appear to arise from multiple mechanisms.[2] Many host-defense peptides can adopt amphiphilic structures in which lipophilic and hydrophilic (usually cationic) side chains are segregated to distinct regions of the molecular surface.[3] This global amphiphilicity is widely believed to underlie the ability of host-defense peptides to compromise bacterial membrane barrier function and thereby kill prokaryotes or inhibit their growth.[4] Numerous reports describe synthetic peptides or peptidomimetic oligomers designed to be globally amphiphilic that can serve as tools to elucidate the origins of host-defense peptide function and as candidates for therapeutic applications.[5] The evaluation of synthetic systems has recently expanded to include random copolymers that contain both hydrophilic and lipophilic subunits, which are much more readily prepared than are sequence-specific peptides or other oligomers.[6]

Antimicrobial agents have the highest potential for application when their deleterious effects are specific for microbial cells relative to human cells. Such selectivity has been achieved with a variety of compounds for bacterial growth inhibition versus human cell destruction;[6h,m,7] the latter property is often assessed as lytic activity toward red blood cells ("hemolysis").[5e,8] Fundamental differences between prokaryotic and eukaryotic cellular membranes, including lipid composition and external surface charge density, seem to facilitate this selectivity.[2,8b] In contrast, it is difficult to target fungal pathogens selectively relative to human cells because fungi are eukaryotes.[9] For example, many host-defense peptides are not effective inhibitors of fungal growth at physiological ionic strength,[10] and only modest antifungal versus hemolytic selectivity has been achieved with sequence-specific oligomers.[11] Here we describe a new family of nylon-3 polymers (poly-β-peptides) that display significant and selective toxicity toward the most common fungal pathogen among humans, *Candida albicans*.[12]

Nylon-3 materials are readily prepared via ring-opening polymerization of β-lactams,[13] and we have previously reported that sequence-random copolymers containing lipophilic and cationic subunits can manifest significant antibacterial activity but low hemolytic activity if the subunit identities, lipophilic/cationic subunit proportion, and other parameters are optimized.[6h,m,14] The copolymer shown in Figure 1, for example, displays a particularly favorable antibacterial activity profile.[6h] However, antifungal activity among previously reported nylon-3 copolymer families proved to be inseparable from hemolytic activity (unpublished data). The present studies began with the preparation of a new β-lactam, **NM** ("no methyl"; Figure 2), which provides a cationic subunit at or below neutral pH. We were drawn to this subunit because it contains fewer saturated carbon atoms and therefore should have a lower hydrophobicity than the previously examined cationic nylon-3 subunits derived from β-lactams, **MM** ("monomethyl") and **DM** ("dimethyl").[6m] The synthesis of **NM** (Figure 3) involves cycloaddition of chlorosulfonyl isocyanate to an alkene, as in previous cases, but this route differs from the precedents in that the side-chain nitrogen is introduced after β-lactam formation.[6h,13f,15] Although the yield of the iodo-β-lactam is only modest, this potentially versatile

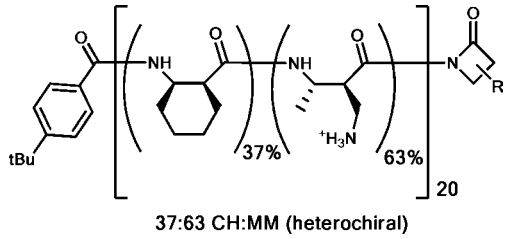

Figure 1. Representative sequence- and stereorandom nylon-3 copolymer (~20-mer average length) containing subunits derived from racemic *cis*-cyclohexyl-β-lactam (**CH**) and racemic β-monomethyl-α-aminomethyl-β-lactam (**MM**). R represents the side-chain group for either **CH** or **MM**. This copolymer inhibits the growth of several bacterial species at relatively low concentrations but is only weakly hemolytic.[6h]

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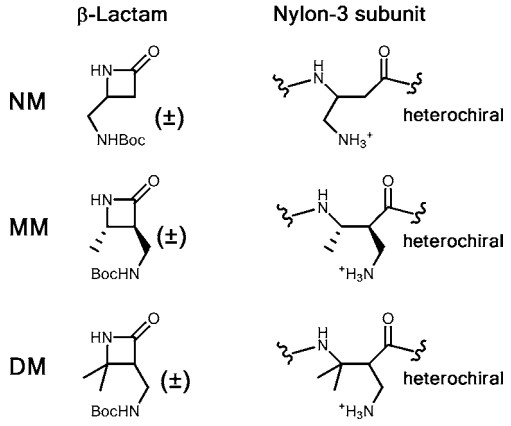

Figure 2. β-Lactams and corresponding hydrophilic (cationic) subunits within the nylon-3 polymer chain. All of the β-lactams were racemic, and the resulting polymers were heterochiral.

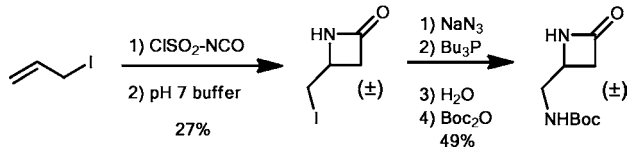

Figure 3. Synthesis of racemic β-lactam **NM**.

molecule can easily be prepared on a multigram scale.[15,16] The β-lactam bearing a Boc-protected amino group in the side chain was readily incorporated into nylon-3 copolymers via the base-catalyzed process we have previously employed, in which the N-terminal group on each polyamide chain is specified by the choice of polymerization coinitiator.[13f] All of the polymers discussed below were prepared with 20-mer average length because previous work indicated that this size range is generally favorable in terms of maximizing antimicrobial activity and minimizing hemolytic activity.[6m]

The antifungal activity of the new **NM**-containing copolymers (Figure 4) was evaluated with a clinically isolated strain of

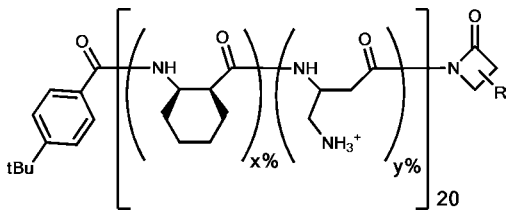

Figure 4. Structure of the **CH**:**NM** copolymers. All of the copolymers (~20-mer average length) were heterochiral and sequence-random. $x + y = 100$, $y = 40$, 50, 60, 70, 80, or 90. R represents the side-chain group of either **CH** or **NM**.

C. albicans (K1).[17] The minimum inhibitory concentration (MIC) was measured using a protocol suggested by the Clinical and Laboratory Standard Institute (previously known as the National Committee for Clinical Laboratory Standards).[18] To assess the effects of the new polymers on mammalian cells, we determined the concentration necessary for 10% lysis of human red blood cells (HC_{10}) and the concentration necessary to induce 10% cell death in NIH 3T3 fibroblasts (IC_{10}). We previously used the minimum hemolytic concentration (MHC) as a metric of red blood cell disruption, but we shifted to HC_{10} for the present studies because it was sometimes difficult to identify the lowest polymer concentration that displayed a nonzero extent of hemolysis.[6h,m] The fibroblast assays provided an alternative to hemolysis as a measure of toxicity toward mammalian cells. Amphotericin B (AmpB), which is used clinically for *C. albicans* infections but is associated with high toxicity toward mammalian cells, served as a positive control in these studies.[19] The results are summarized in Table 1.

Table 1. Physical and Biological Properties of the Nylon-3 Polymers[a]

polymer composition	DP[b]	PDI[c]	MIC (μg/mL)[d]	IC_{10} (μg/mL)[e]	HC_{10} (μg/mL)[f]
60:40 **CH**:**NM**	23	1.29	100	>400	100–200
50:50 **CH**:**NM**	23	1.29	50	>400	200
40:60 **CH**:**NM**	21	1.29	13	>400	>400
30:70 **CH**:**NM**	20	1.26	6.3	>400	>400
20:80 **CH**:**NM**	22	1.33	3.1	100–200	>400
10:90 **CH**:**NM**	17	1.24	3.1	>400	>400
NM	20	1.13	3.1	>400	>400
MM	22	1.03	200	>400	>400
DM	18	1.13	6.3	50	3.1
AmpB[g]	N/A	N/A	0.78	<1.5	ND

[a]All of the polymers bore an N-terminal *p-tert*-butylbenzoyl group. [b]Degree of polymerization [i.e., average polymer length (number of subunits)]. [c]Polydispersity index. [d]Minimum inhibitory concentration for fungal growth as determined for *C. albicans* in planktonic form. [e]Concentration necessary to induce 10% cell death in NIH 3T3 fibroblasts. [f]Concentration necessary for 10% lysis of human red blood cells. [g]Amphotericin B was dissolved in 1:1 DMSO/water as the stock solution for the bioassay. N/A denotes not applicable. ND indicates that HC_{10} was not determined.

We began by examining random copolymers (Figure 4) formed from the new β-lactam **NM** and *cis*-cyclohexyl-β-lactam (**CH**), because the latter had given rise to selective antibacterial copolymers when paired with the cationic subunit derived from **MM** (Figure 1).[6h] All of the new polymers bore a *p-tert*-butylbenzoyl group at the N-terminus, as in previous antibacterial examples. The polymer with the maximum proportion of **CH** that could be used without compromising aqueous solubility (60:40 **CH**:**NM**) exhibited weak antifungal activity and weak hemolytic activity (MIC and $HC_{10} \sim 100$ μg/mL). The antifungal activity steadily increased (i.e., the MIC decreased) as the proportion of **CH** declined, and no copolymer containing >50% **NM** manifested detectable hemolytic activity. Members of this polymer family were generally not toxic toward mouse fibroblasts. The activity levels observed for **CH**:**NM** copolymers with ≥80% **NM** (on a μg/mL basis) approached that of AmpB but were accompanied by substantially less fibroblast cytotoxicity than was observed for AmpB. Replacing the *p-tert*-butylbenzoyl end group with an acetyl end group did not alter the biological activity of poly-**NM**. The **NM** homopolymer displayed antifungal activity comparable to that of the most active **CH**:**NM** copolymers. Follow-up studies showed that poly-**NM** is fungicidal at the MIC rather than merely inhibitory toward fungal growth.[20]

The excellent activity profile observed for poly-**NM** contrasts with the behavior observed for two other cationic nylon-3 homopolymers, poly-**MM** and poly-**DM** (Table 1). Poly-**MM** showed very little antifungal activity, and this homopolymer also was not hemolytic or toxic toward 3T3 fibroblasts. Poly-**DM**, on the other hand, approximately matched poly-**NM** in

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activity against *C. albicans* but was hemolytic and moderately toxic toward 3T3 fibroblasts.

Poly-**NM** was evaluated for antibacterial activity against a panel of four species that we previously used to assess poly-**MM** and poly-**DM** as well as cationic/hydrophobic copolymers (Table 2).[6m] The antibacterial effects of poly-**NM** were

Table 2. Antibacterial Activities of Cationic Nylon-3 Homopolymers

	MIC (μg/mL)[a]			
polymer	*E. coli*	*B. subtilis*	*E. faecium*	*S. aureus*
NM	50	6.3	>200	100
MM	>200	6.3	>200	100
DM	100	3.1	100	50

[a]Minimum inhibitory concentration for bacterial growth.

generally comparable to those of the other two cationic nylon-3 homopolymers: significant activity was observed for *Bacillus subtilis*, which seems to be highly susceptible to a wide array of peptides and peptidomimetic oligomers and polymers, but all three homopolymers were considerably less active against *Escherichia coli*, *Enterococcus faecium*, and *Staphylococcus aureus*. The generally low antibacterial activity of poly-**MM** and poly-**DM** was previously rationalized in terms of their lack of hydrophobic subunits (e.g., the subunit derived from **CH**), which may limit their ability to disrupt bacterial membranes.[6m,14] From this perspective, the relatively low antibacterial activity of poly-**NM** is not surprising. The potent antifungal activity of poly-**NM** is noteworthy in the context of this limited antibacterial activity.

The data presented here show that nylon-3 polymers containing subunits derived from the new β-lactam **NM** display potent antifungal activity without a strong tendency to disrupt human red blood cell membranes or strong toxicity toward 3T3 fibroblasts. It is particularly intriguing that poly-**NM** displays such profound differences in biological activity relative to the structurally similar cationic nylon-3 homopolymers poly-**MM** and poly-**DM**. There are several differences among the subunits of these three polymers: (1) the added side-chain carbons in poly-**MM** and poly-**DM** relative to poly-**NM** cause a modest increase in hydrophobicity;[20] (2) the added carbons alter the backbone flexibility; (3) the point of attachment of the aminomethyl side chain in **NM** (β-carbon) differs from that in **MM** and **DM** (α-carbon). Further studies are necessary to determine the mechanism by which these seemingly subtle molecular-level changes exert such a substantial influence on biological activity. We previously proposed that nylon-3 copolymers exert antibacterial effects via disruption of prokaryotic cell membranes, and this hypothesis was supported by studies of the 40:60 **CH**:**MM** copolymer (Figure 1) with synthetic vesicles of varying lipid composition.[14] However, our finding that the maximal antifungal activity was manifested by poly-**NM**, the least hydrophobic nylon-3 polymer we have examined to date, raises the possibility that **NM**-containing polymers act via a mechanism that does not involve disturbance of lipid bilayers. The surprising biological activity profile discovered for **NM**-based nylon-3 suggests that antifungal applications of these new materials should be pursued.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details for the synthesis and characterization of nylon-3 polymers, antifungal and antibacterial assays, cytotoxicity toward 3T3 fibroblasts, and hemolysis of human RBCs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): B.W. and S.H.G. are co-inventors on a patent application that covers the polymers described here.

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