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## Bis(*N*-amidinohydrazones) and *N*-(amidino)-*N*-aryl-bishydrazones: New classes of antibacterial/antifungal agents

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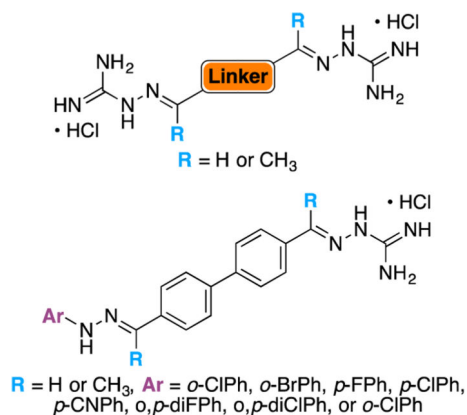
### Abstract

The emergence of multidrug-resistant bacterial and fungal strains poses a threat to human health that requires the design and synthesis of new classes of antimicrobial agents. We evaluated bis(*N*-amidinohydrazones) and *N*-(amidino)-*N*-aryl-bishydrazones for their antibacterial and antifungal activities against panels of Gram-positive/Gram-negative bacteria as well as fungi. We investigated their potential to develop resistance against both bacteria and fungi by a multi-step, resistance-selection method, explored their potential to induce the production of reactive oxygen species, and assessed their toxicity. In summary, we found that these compounds exhibited broad-spectrum antibacterial and antifungal activities against most of the tested strains with minimum inhibitory concentration (MIC) values ranging from <0.5->500  $\mu\text{M}$  against bacteria and 1.0->31.3  $\mu\text{g/mL}$  against fungi; and in most cases, they exhibited either superior or similar antimicrobial activity compared to those of the standard drugs used in the clinic. We also observed minimal emergence of drug resistance to these newly synthesized compounds by bacteria and fungi even after 15 passages, and we found weak to moderate inhibition of the human *Ether-à-go-go*-related gene (hERG) channel with acceptable  $\text{IC}_{50}$  values ranging from 1.12-3.29  $\mu\text{M}$ . Overall, these studies show that bis(*N*-amidinohydrazones) and *N*-(amidino)-*N*-aryl-bishydrazones are potentially promising scaffolds for the discovery of novel antibacterial and antifungal agents.

### Graphical Abstract

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## Keywords

Bishydrazones; *Candida albicans*; Cytotoxicity; hERG channel; Resistance; *Staphylococcus aureus*

## 1. Introduction

The emergence of multidrug-resistant bacteria and fungi as human pathogens warrants a continued focus on the development of new pharmacophores for the treatment of these devastating and often fatal infections. The rise of multidrug-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE), adversely affects the efficacy of many known, standard-of-care, antibacterial agents.<sup>1</sup> Evidence of the impact of these multidrug-resistant strains appears in a 2011 report from the Centers for Disease Control and Prevention (CDC) that estimates that the national incidence of invasive MRSA infections was 80,461 cases and 650 deaths. This mortality rate is among the highest recorded for bacterial infections.<sup>2</sup> Likewise, listeriosis, which is a common foodborne illness caused by *Listeria monocytogenes*, represents a serious illness afflicting elderly people, newborns, and those with impaired immune systems. Estimates are that 19% of deaths associated with the consumption of contaminated foods in the United States are due to *L. monocytogenes*.<sup>3</sup>

The incidence of invasive fungal infections is also on the rise due to an increasing population of critically ill patients as a result of the human immunodeficiency virus (HIV), systemic diseases such as cancer, and the increasing occurrence of organ transplantation.<sup>4</sup> The National Healthcare Safety Network (NHSN) at the CDC reported that *Candida* spp. ranked fifth amongst hospital-acquired pathogens.<sup>5</sup> *Candida* spp. are reported as the fourth most common causative pathogens of nosocomial and of fatal bloodstream infections.<sup>6</sup> Eukaryotic *C. albicans* shares a close evolutionary relationship, as well as many cellular mechanisms, with their human hosts, and represents challenges for treatment of systemic fungal infections. There is a clear need for new antimicrobials that selectively inhibit these microorganisms without causing host toxicity.

Pentamidine represents an archetypical, biscationic antibiotic with a symmetrical structure containing two amidinium functional groups separated by a flexible 1,5-diphenoxypentane spacer.<sup>7</sup> Developed initially as an antiprotozoal agent, pentamidine currently finds applications in both the treatment of protozoan diseases, such as *Trypanosoma brucei gambiense*

(West African trypanosomiasis), as well as systemic fungal infections caused by *Pneumocystis jirovecii*, often seen in patients with HIV. Related compounds include symmetrical bisamidines, (e.g., furimidazoline),<sup>8</sup> developed principally as topoisomerase inhibitors for cancer treatment. In addition to these biscationic compounds, other hydrazone-containing and guanidine-containing molecules possess a range of promising biological activities, including antitubercular,<sup>9,10</sup> anti-HIV,<sup>11,12</sup> anticonvulsant,<sup>13</sup> anticancer,<sup>14</sup> anti-inflammatory,<sup>15,16</sup> antimalarial,<sup>17,18</sup> antibacterial,<sup>19,20</sup> and antifungal activities.<sup>21,22</sup> Recently, bis(*N*-amidinohydrazones) were reported to inhibit the calcium-dependent serine endoprotease, furin, which activates immature proteins to their functional, mature forms.<sup>23</sup>

Inspired by the above successes, we were intrigued by the possibility that biscationic pharmacophores in either the bis(*N*-amidinohydrazones) or the *N*-(amidino)-*N*-aryl-bishydrazone family with either a flexible or a rigid spacer would offer advantages in terms of antibacterial and antifungal activities not found in the flexible bisamidines, as well as in the hydrazone- and guanidine-containing molecules currently in the literature. To investigate the potential of such compounds, we synthesized nine symmetrical bis(*N*-amidinohydrazones) (**3A-B** and **4A,C-H**) and eight asymmetrical *N*-(amidino)-*N*-aryl-bishydrazones (**7Aa-Ag** and **8Aa**) (Scheme 1). Further, we evaluated the antibacterial and antifungal activities of these novel compounds against panels of bacterial strains (four Gram-positive, six Gram-negative, and one mycobacterial) and seven *Candida albicans* strains. Because the development of resistance represents a crucial problem in antimicrobial drug development, we also established the potential for resistance development by bacteria and fungi against these compounds. Additionally, we measured the production of reactive oxygen species (ROS) of some of these compounds in yeast cells, determined their *in vitro* cytotoxicity and their affinity for the human *Ether-à-go-go*-related gene (hERG) potassium channel. The combination of testing new antimicrobial agents and early screening for resistance and toxicity represents an avenue that may produce pharmacophores of potential utility in the treatment of diseases.

## 2. Results and discussion

### 2.1. Design and chemical synthesis of biscationic compounds with two chemically identical termini

The reported syntheses of biscationic compounds containing two identical termini bearing guanidinium, amidinium, or pyridinium groups attached to aryl or alkyl spacers typically involved the modification of spacers with termini bearing primary halides, carbonyl, or nitrile groups.<sup>24-26</sup> Alkylation of  $\alpha,\omega$ -dihaloalkanes, for example, with 2-aminopyridine led to bis(2-aminopyridinium) salts.<sup>24</sup> Modification of the nitrile group in 5-(4-cyanophenyl)furan-2-carboxylic acid and completion of the spacer by linking the carboxylate groups led to bisamidinium agents.<sup>27</sup> In the same fashion, we modified the

carbonyl groups in either bisaldehyde **1** or bisketone **2**, which in some cases were in regiochemically distinct positions in the spacer (*e.g.*, **1B**, **2B**, **2H**), with *N*-aminoguanidine hydrochloride to obtain the biscationic products **3** and **4**, respectively (Scheme 1A).

## 2.2. Design and chemical synthesis of biscationic compounds with chemically non-identical termini

Synthetic approaches to bicationic agents with two chemically non-identical termini often involved the construction of the spacer as the ultimate step. For example, the coupling of an amidino-substituted naphthol with a guanidine-substituted benzoic acid secured biscationic esters with an amidinium group at one terminus and a guanidinium group at the other.<sup>28</sup> Our approach differed from this strategy in that we utilized a stepwise, chemoselective modification of bisaldehydes **1** or bisketones **2** in order to arrive at biscationic systems with different cationic groups at each terminus. Condensations of one equivalent of *N*-aminoguanidine hydrochloride with **1A** and **2A** led to the efficient production of monosubstituted *N*-amidinohydrazones **5A** and **6A**, respectively. The hydrochloride salts of these monocationic products were readily crystallized free from starting materials. The subsequent treatment of **5A** and **6A** with *N*-arylhyaazines provided the desired, biscationic agents **7Aa-Ag** and **8Aa**, respectively, which we describe as *N*-(amidino)-*N*-aryl-bishydrazones (Scheme 1B). Cavallini described the monocationic *N*-amidinohydrazones as antibacterial agents some years ago, but the range of organisms and MIC<sub>50</sub> values were, in general, unimpressive.<sup>29</sup>

With these first generations of bis(*N*-amidinohydrazones) **3** and **4** and *N*-(amidino)-*N*-aryl-bishydrazones **7** and **8** in hand, we aimed to answer the following eight *a priori* questions in terms of structure-activity-relationship (SAR). We included the numbers of the compounds used to answer these questions into parentheses following the questions. The eight questions are as follows: (i) are linkers comprised of only one aromatic ring sufficient to confer antimicrobial activity? (compounds **4G** and **4H**); (ii) is the presence of methyl groups on the carbons alpha to the linker beneficial for antimicrobial activity? (compounds **3A versus 4A**, and **7Aa versus 8Aa**); (iii) how does the substitution pattern of the biphenyl linker affect antimicrobial activity? (compounds **3A** and **3B**); (iv) how does the length of the flexible linear alkyl spacer between the two phenyl rings affect antimicrobial activity? (compounds **4A**, **4C**, and **4D**); (v) is the introduction of rigidity in the linker beneficial for antimicrobial activity? (compound **4C versus 4F**); (vi) is the presence of an oxygen atom in the linker beneficial for antimicrobial activity? (compound **4C versus 4E**); (vii) how does the identity of the substituent, when located at the same position on the aryl ring of the side chain, affect antimicrobial activity? (compounds **7Aa versus 7Ab**, **7Ac versus 7Ad versus 7Ae**, and **7Af versus 7Ag**); and finally, (viii) how does the substitution pattern of the aryl ring (*ortho versus para*-monosubstituted *versus ortho,para*-disubstituted) affect antimicrobial activity? (compounds **7Aa versus 7Ad versus 7Ag**).

## 2.3. Antibacterial activity

To determine if our novel bis(*N*-amidinohydrazones) and *N*-(amidino)-*N*-aryl-bishydrazones displayed antibacterial activity, we first evaluated compounds **3A-8Aa** against a panel of Gram-positive (4 strains), Gram-negative (6 strains), and one mycobacterial strain

in a concentration range of 0.5-500  $\mu\text{M}$  (Table 1). We used the aminoglycoside amikacin (AMK), the  $\beta$ -lactam ampicillin (AMP), and the fluoroquinolone ofloxacin (OFX) as positive controls. In general, the novel compounds displayed excellent (MIC values 0.5-7.8  $\mu\text{M}$ ), intermediate (also referred to as moderate) (15.6-31.3  $\mu\text{M}$ ), or low (also referred to as poor) (62.5- 500  $\mu\text{M}$ ) antibacterial activity against the bacterial strains tested. It is important to note that these MIC value ranges are used from here on to qualitatively described all compounds tested, including the positive controls.

Overall, when analyzing the MIC data obtained against the Gram-positive bacterial strains (strains **A-D**), we note that most of the novel bis(*N*-amidinohydrazones) (**3A,B**, **4C,D**, **4F**) and *N*-(amidino)-*N*-aryl-bishydrazones (**7Aa-Ad**, and **7Af-Ag**) showed excellent antibacterial activity against *Listeria monocytogenes* ATCC 19115 (strain **B**, *Lmo*) (MIC <0.5-7.8  $\mu\text{M}$ ), methicillin-resistant *Staphylococcus aureus* (strain **C**, MRSA) (MIC = 1.0-7.8  $\mu\text{M}$ ), and vancomycin-resistant *enterococcus* (strain **D**, VRE) (MIC = 1.0-7.8  $\mu\text{M}$ ). All of the novel compounds displayed poor activity against *Bacillus subtilis* 168 (strain **A**, *Bsu*), with the exception of compound **4F**, which displayed excellent activity (MIC = 7.8  $\mu\text{M}$ ) against this strain. Likewise, compound **7Ae** exhibited excellent antibacterial activity against *L. monocytogenes* ATCC 19115 (strain **B**, *Lmo*; MIC = 7.8  $\mu\text{M}$ ) and VRE (strain **D**; MIC = 2.0-3.9  $\mu\text{M}$ ), but only moderate antibacterial activity against *B. subtilis* 168 (strain **A**, *Bsu*; MIC = 31.3  $\mu\text{M}$ ) and MRSA (strain **C**; MIC = 15.6  $\mu\text{M}$ ), respectively. In general, compounds **4A**, **4E** (except against strain **D**, VRE), **4G**, **4H**, and **8Aa** (except against strain **D**) showed poor antibacterial activity (MIC = 62.5- 500  $\mu\text{M}$ ) against all four Gram-positive bacterial strains tested. Overall, compound **4F** was the most active against Gram-positive bacteria.

On the other hand, the majority of the novel compounds remained inactive against most of the Gram-negative bacterial strains that we tested with MIC values ranging from 62.5->500  $\mu\text{M}$ . As one of several exceptions, compound **4A** exhibited excellent activity against *P. aeruginosa* ATCC 27853 (strain **I**, *Pae*; MIC = 2.0  $\mu\text{M}$ ) and moderate activity against *Klebsiella pneumoniae* ATCC 27736 (strain **H**, *Kpn*; MIC = 15.6  $\mu\text{M}$ ). Similarly, compound **4F** also displayed excellent antibacterial activities against *Pae* strain **I** (MIC = 1.0-2.0  $\mu\text{M}$ ) and *S. enterica* ATCC 14028 (strain **J**, *Sen*; MIC = 3.9  $\mu\text{M}$ ), as well as moderate to low activity against *Kpn* strain **H** (MIC = 31.3-62.5  $\mu\text{M}$ ). Additionally, **7Ae** showed only moderate antibacterial activities against *Kpn* strain **H** (MIC = 15.6-31.3  $\mu\text{M}$ ), strain **I** (MIC = 31.3-62.5  $\mu\text{M}$ ), and *Sen* strain **J** (MIC = 15.6  $\mu\text{M}$ ). Compounds **3B**, **4C-D**, **4F**, **7Aa-Ad**, and **7Af-Ag** exhibited excellent antibacterial activity (MIC = 1.0-3.9  $\mu\text{M}$ ) against *M. smegmatis* MC2-155 (strain **K**, *Msm*), whereas compounds **4H** and **7Ae** showed only moderate activity (MIC = 15.6-31.3  $\mu\text{M}$ ) and compounds **3A**, **4A**, **4E**, **4G**, and **8Aa** showed low activity (MIC = 125->500  $\mu\text{M}$ ) against this mycobacterial strain. It is noteworthy that when compared to clinically relevant antibacterial drugs, such as AMK (MIC = 3.9-125  $\mu\text{M}$ ), AMP (MIC = 250  $\mu\text{M}$ ), and OFX (MIC = 0.5-31.3  $\mu\text{M}$ ), the novel compounds showed either superior or comparable antibacterial activity against all bacterial strains tested.

## 2.4. Antifungal activity

Having established the antibacterial activity profile of the novel bis(*N*-amidinohydrazones) and *N*-(amidino)-*N*-arylbishydrazones, we next determined their antifungal activity against a panel of seven *Candida albicans* strains using a concentration range of 0.5-31.3 µg/mL (Table 2). We used the common antifungal agent amphotericin B (AmB) as a positive control testing in the same concentration range of 0.5-31.3 µg/mL. For antifungal activity, we use the following descriptors: excellent (MIC = 0.5-3.9 µg/mL), moderate (MIC = 7.8-15.6 µg/mL), and poor (MIC = 31.3 µg/mL). We found that compounds **3B**, **4F**, **7Aa**, **7Ab**, and **7Af** exhibited excellent antifungal activities against all fungal strains tested (MIC = 1.0-3.9 µg/mL), with **4F** being overall the most active. The only exception to the aforementioned statement was **7Af**, which displayed only moderate activity against *C. albicans* ATCC 90819 (MIC = 7.8 µg/mL). Similarly, compound **4A** also displayed excellent antifungal activities (MIC = 2.0-3.9 µg/mL) against most strains, except against *C. albicans* ATCC 1003 (MIC = 7.8 µg/mL) and *C. albicans* ATCC 1237 (MIC = 31.3 µg/mL). Compounds **7Ad**, **7Ae**, **7Ag**, and **8Aa** showed only moderate fungal growth inhibition (MIC = 7.8-15.6 µg/mL) against the majority of the fungal strains, with the exception of *C. albicans* ATCC 64124 (MIC = 31.3 µg/mL for **7Ae**, **7Ag**, and **8Aa**), *C. albicans* ATCC 90819 (MIC = 31.3 µg/mL for **7Ag**), *C. albicans* ATCC 1003 (MIC = 3.9 µg/mL for **7Ad**) and *C. albicans* ATCC 2310 (MIC = 3.9 µg/mL for **7Ad**). Compounds **7Ac** also exhibited excellent growth inhibition against most of the fungal strains tested, except against *C. albicans* ATCC 10231 (moderate activity, MIC = 7.8 µg/mL) and *C. albicans* ATCC 64124 (poor activity, MIC = 31.3 µg/mL). Just as observed against all bacterial strains, compounds **4G** and **4H** displayed no activity (MIC = 31.3 µg/mL) against all fungal strains tested. It is important to emphasize that most of our bis(*N*-amidinohydrazones) and *N*-(amidino)-*N*-aryl-bishydrazones displayed either superior or comparable antifungal activities against the majority of the fungal strains tested, when compared to the clinically-relevant antifungal agent, AmB (MIC = 2.0-3.9 µg/mL).

## 2.5. SAR analysis

Using the antibacterial and antifungal results presented above (Tables 1 and 2), we addressed the eight *a priori* questions posed:

- (i) Are linkers comprised of only one aromatic ring sufficient to confer antimicrobial activity? We concluded that bis(*N*-amidinohydrazones) with linkers comprised of a single phenyl ring, regardless of its substitution pattern (*para* or *meta*, as in compounds **4G** and **4H**, respectively), displayed insufficient activity as antimicrobial agents to warrant further study of their antibacterial or antifungal activity.
- (ii) Is the presence of methyl groups on the carbons alpha to the linker beneficial for antimicrobial activity? Comparing the MIC values of compounds **3A** and **4A** as well as **7Aa** and **8Aa**, which only differ by the absence or presence of a methyl group on the carbons alpha to the phenyl rings, led us to conclude that the methyl group was detrimental to the antibacterial activity of these compounds. However, the presence of the methyl group appeared beneficial in



terms of antifungal activity. Compound **4A** was overall a much more active antifungal agent than compound **3A**.

- (iii) How does the substitution pattern of the biphenyl linker affect antimicrobial activity? When evaluating compounds **3A** and **3B**, the antibacterial MIC values were similar in all cases, except against strain **K** where the MIC value for compound **3B** (MIC = 1  $\mu$ M) was 500 times lower than that for compound **3A** (MIC >500  $\mu$ M). From these data, we deduced that the substitution pattern of the biphenyl linker had minimal effect on antibacterial activity. However, we found the substitution pattern of the biphenyl linker to have a substantial effect on antifungal activity. For example, compound **3B** was a much more active antifungal agent than compound **3A**.
- (iv) How does the length of the flexible linear alkyl spacer between the two phenyl rings affect antimicrobial activity? When comparing the MIC values of compounds **4A**, **4C**, and **4D**, again we observed opposing trends in antibacterial and antifungal activities. Longer flexible alkyl linkers between the two phenyl rings correlated with a decrease in antibacterial MIC values, but correlated with an increase in antifungal MIC values.
- (v) Is the introduction of rigidity in the linker beneficial for antimicrobial activity? By contrasting the MIC value profile of compound **4C** containing a flexible linker to that of compound **4F** containing a rigid linker, we concluded that introducing rigidity in the linker was overall beneficial for antimicrobial activity.
- (vi) Is the presence of an oxygen atom in the linker beneficial for antimicrobial activity? A comparison of the MIC value profiles of compounds **4C** and **4E** indicated that replacing the methylene bridging the two phenyl groups of the linker by an oxygen atom had no effect on antifungal activity, but was detrimental to antibacterial activity.
- (vii) How does the identity of the substituent, when located at the same position on the aryl ring of the side chain, affect antimicrobial activity? By comparing compounds **7Aa-7Ag**, we observed that the nature of the substituent in the *ortho* position of the mono-substituted aryl group of compounds **7Aa** and **7Ab** did not affect antibacterial or antifungal activity, whereas the nature of the substituent in the *para* position of the mono-substituted aryl group of compounds **7Ac-7Ae** had opposing effects on antibacterial (CN > halogen) and antifungal (halogen > CN) activity. We also observed that the *o,p*-difluorinated and *o,p*-dichlorinated aryl moieties of compounds **7Af** and **7Ag** had similar overall antibacterial activity, but different antifungal activity, in which the *o,p*-difluorinated group possessed improved antifungal activity.
- (viii) How does the substitution pattern of the aryl ring (*ortho versus para*-monosubstituted *versus ortho,para*-disubstituted) affect antimicrobial activity? Finally, comparison of the activity resulting from different chlorination patterns in compounds **7Aa** (*ortho*), **7Ad** (*para*), and **7Ag** (*o,p*-diCl), revealed

minimal effects on antibacterial activity, but substantial effects on antifungal activity. The *o*-Cl *N*-(amidino)-*N*-aryl-bishydrazone was superior to the *p*-Cl analogue, which in turn was much better than *o,p*-diCl analogue. In summary, the similarity and differences in the trends observed in this promising, preliminary SAR will be used to lay the foundation for the future discovery of compounds capable of selectively targeting either bacteria or fungi.

## 2.6. Development of bacterial and fungal resistance studies

The emergence of antibiotic resistance by microbes is an inevitable process; however, the frequency at which resistance develops varies from one antibiotic to another. It is critical to assess the ability of new antimicrobials to evade, as long as possible, the development of bacterial and fungal resistance early in the drug discovery process. To establish if the bis(*N*-amidinohydrazones) and *N*-(amidino)-*N*-aryl-bishydrazones evade resistance in bacteria, we first performed a multi-step resistance selection experiment with compounds **4D**, **4F**, and **7Aa** as well as AMK as a reference drug against four bacterial strains (Figure 1). *L. monocytogenes* ATCC 19115 (strain **B**, *Lmo*), MRSA (strain **C**), VRE (strain **D**), and *P. aeruginosa* ATCC 27853 (strain **I**, *Pae*) were exposed to sub-inhibitory concentrations of compounds **4D**, **4F**, **7Aa**, and AMK, and were sub-cultured for 15 serial passages to determine if an increase in MIC values occurred for each compound against the strains tested. *L. monocytogenes* ATCC 19115 (strain **B**, *Lmo*), MRSA (strain **C**), VRE (strain **D**), and *P. aeruginosa* ATCC 27853 (strain **I**, *Pae*) did not develop resistance to compound **4F**, as demonstrated by the one-fold increase in MIC values after 15 serial passages. Likewise, strains **B** (*Lmo*) and **D** (VRE) did not develop resistance to compound **7Aa**, but strains **C** (MRSA) and **I** (*Pae*) developed resistance to this compound after twelve serial passages. In contrast, we observed the rapid development of resistance to compound **4D** in strains **C** (MRSA) and **D** (VRE) with a 16-fold increase in MIC values after fifteen serial passages. This development of resistance against compound **4D** was observed also in strains **B** (*Lmo*) and **I** (*Pae*), where increases in relative MIC values by 8- and 4-folds, respectively, were observed after 15 passages. Interestingly, in strains **D** (VRE) and **I** (*Pae*), resistance to the control antibiotic, AMK, was observed also after 3 and 5 passages, respectively; whereas in strains **C** (MRSA) and **B** (*Lmo*), resistance developed after 12 and 15 passages, respectively. Overall, these results indicate that there is a low probability of emergence of resistance to these promising novel biscationic compounds **4D**, **4F**, and **7Aa**.

We next investigated the development of drug resistance in *C. albicans* ATCC 10231 to our best antifungal compounds **7Aa** and **4F**, and we found that *C. albicans* ATCC 10231 did not develop drug resistance to either **7Aa** or **4F**, despite repeated treatments with sub-MIC drug concentrations. Only a slight 2-fold shift in MIC values was observed after 13 passages. These results also suggest the low probability of onset of drug resistance by fungi to these novel compounds.

## 2.7. Measurement of ROS induction in fungal cells

Various antifungal drugs, such as AmB and miconazole, as well as novel antifungal agents, were shown to mediate their inhibitory effect by inducing intracellular ROS production.<sup>30-32</sup> Universally, eukaryotic cells produce basal amount of ROS in mitochondria as a byproduct



of cellular metabolism. In response, the cellular enzymatic antioxidants, including superoxide dismutase and glutathione peroxidase, scavenge ROS in cells. However, overproduction of deleterious ROS perturbs the delicate, intracellular equilibrium between ROS production and scavenging, and the result is cellular damage.

In order to investigate the ability of compounds **4F** and **7Aa** to alter ROS production in *C. albicans* ATCC 10231 (strain **A**), we performed a fluorescent-based assay using a 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) dye.<sup>33</sup> We found that treatment of *C. albicans* ATCC 10231 (strain **A**) with compounds **4F** and **7Aa** at their 1× and 2× MIC values increased intracellular ROS production in this fungal strain (Figure 2). As expected, the H<sub>2</sub>O<sub>2</sub> positive control (at 1 mM) also induced ROS production in yeast cell, whereas no ROS induction was observed with untreated yeast cells (negative control). Although it remains to be determined if ROS production is critical for growth inhibition and death of yeast cells, these results suggest one direction for future mechanistic exploration, which is outside of the scope of the current proof-of-principle exploration for the first generations of bis(*N*-amidinohydrazones) and *N*-(amidino)-*N*-aryl-bishydrazones as antimicrobials.

## 2.8. Cytotoxicity

Another important aspect to consider during the development of antimicrobial agents is their potential toxicity towards mammalian cells. Having established the potent antimicrobial activities of the novel bis(*N*-amidinohydrazones) and *N*-(amidinohydrazones)-*N*-aryl-bishydrazones against bacteria and fungi, we determined the toxicity profile of these compounds against two mammalian cell lines (A549 and BEAS-2B; Figure 3) and measure the IC<sub>50</sub> values for these compounds (Table 3). The majority of the novel compounds showed concentration-dependent toxicity with IC<sub>50</sub> values of 1.7-6.7 µg/mL. Compounds **3B**, **7Ac**, **7Ad**, and **7Af**, with excellent antibacterial MIC values of <0.4-3.3 µg/mL, displayed IC<sub>50</sub> values of 4.4-13.9 µg/mL against both mammalian cell lines. It is important to note that compounds **7Ac** and **7Af** also displayed excellent antifungal MIC values, indicating that these compounds are important leads for future evaluation.

## 2.9. hERG inhibition assay

The hERG encodes a voltage gated potassium channel that plays an essential role in regulating heart rhythm.<sup>34</sup> Inhibition of the hERG channel disrupts the heart rhythm and may lead to cardiac death. Recently, drugs (*e.g.*, terfenadine and cisapride) were withdrawn from the market due to their interaction with the hERG channel.<sup>35</sup> Currently, the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) require evaluation at hERG for drug candidates.

We performed a [<sup>3</sup>H]-dofetilide competition binding assay using HEK-293 cell membranes stably expressing the hERG channel to evaluate the activity of our most potent bis(*N*-amidinohydrazones) and *N*-(amidino)-*N*-aryl-bishydrazones at hERG (Figure 4). Compounds exhibiting IC<sub>50</sub> values of >10 µM are considered to have low affinity for the hERG channel. Compounds displaying IC<sub>50</sub> values in the range of 1-10 µM are considered to have moderate affinity, whereas compounds exhibiting IC<sub>50</sub> values of <1 µM are considered to have high affinity for the hERG channel.<sup>36</sup> The majority of the novel

compounds displayed  $IC_{50}$  values within the acceptable range of 1-10  $\mu\text{M}$  (Figure 4). Although compound **7Ab** displayed excellent antifungal activities (2-3.9  $\mu\text{g/mL}$ ), it exhibited *in vitro* mammalian cell toxicity and was found to potently interact with the hERG channel ( $IC_{50} = 0.44 \pm 0.10 \mu\text{M}$ ), suggesting that the *o*-bromophenyl substituent is an unfavorable structural feature. However, the *p*-fluorophenyl and the 2,4-difluorophenyl groups in compounds **7Ac** and **7Af** displayed only moderate interaction with hERG ( $IC_{50} = 3.29 \pm 0.63 \mu\text{M}$  and  $2.24 \pm 0.70 \mu\text{M}$ , respectively). Strikingly, both of these compounds displayed excellent antifungal activities and also exerted low mammalian cell toxicity. Similarly, compounds **4F** and **7Aa** displayed moderate affinity for hERG ( $IC_{50} = 1.12 \pm 0.32 \mu\text{M}$  and  $1.14 \pm 0.27 \mu\text{M}$ , respectively); however, they displayed some toxicity against mammalian cells. Therefore, we concluded that compounds **7Ac** and **7Af** are the most promising *N*-(amidino)-*N*-aryl-bishydrazone compounds.

### 3. Conclusions

In summary, we discovered nine bis(*N*-amidinohydrazones) and eight *N*-(amidino)-*N*-aryl-bishydrazones as first-generation antibacterial and antifungal agents. These compounds displayed low potential for the development of resistance and were found to induce ROS production in a *C. albicans* strain. They also were found to display reasonable toxicity profiles against two mammalian cell lines as well as acceptable levels of interaction with hERG channels. However, as these compounds display both antibacterial and antifungal activity, it is possible that they aim at a target shared by prokaryotes and eukaryotes and potentially display lower selectivity. However, in light of our SAR study, we conclude that compounds **4F**, **7Ac**, and **7Af**, which showed the most promise, warrant further optimization. Such studies are currently underway in our laboratories.

### 4. Experimental section / Supplementary material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

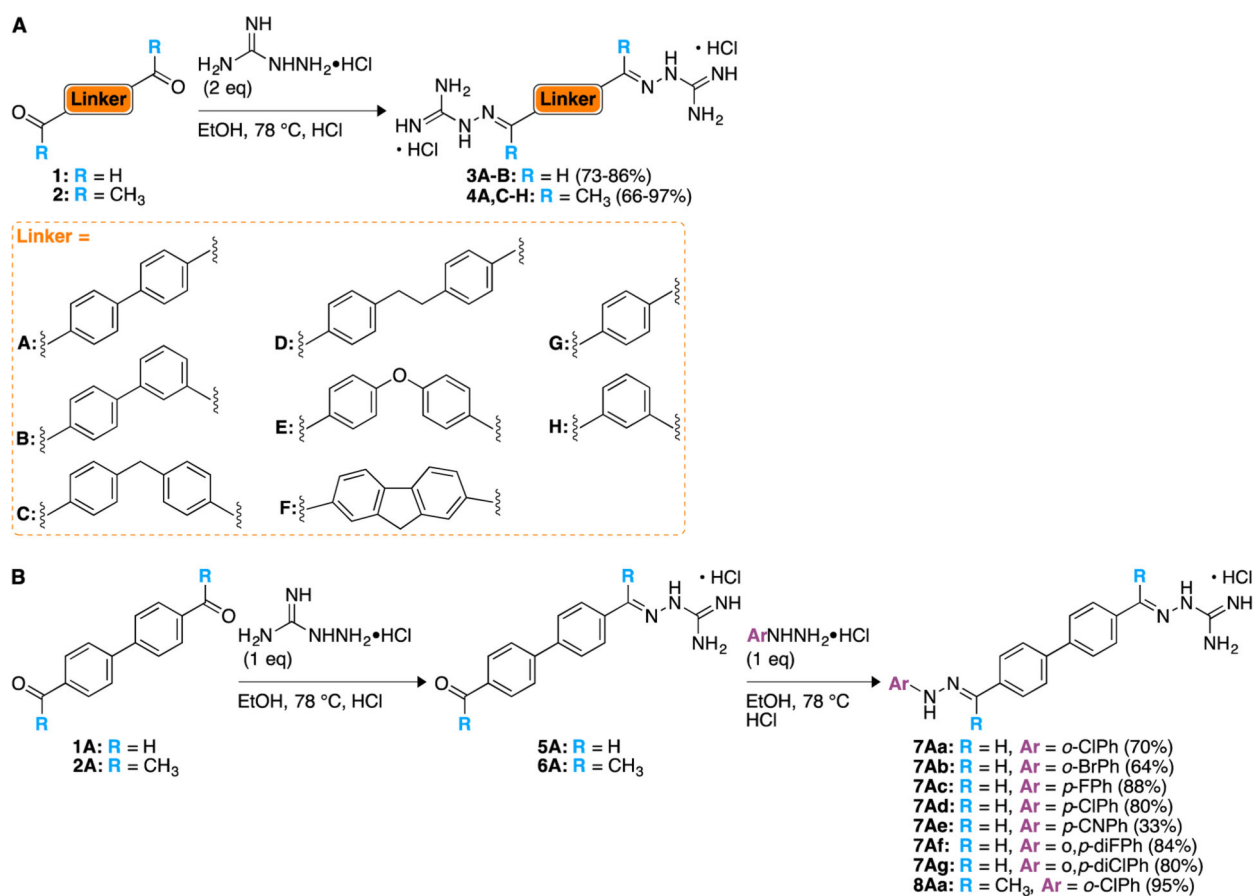
This work was supported by startup funds from the College of Pharmacy (to S.G.-T.) and by the Office of the Dean of the College of Medicine (to D.S.W.) at the University of Kentucky. It was also supported by NIH grant AI090048 (to S.G.-T.); and NIH grants U01 DA013519, UL1TR000117 and T32 DA016176 (to L.P.D. and J.R.N.). D.S.W. was also supported in part through collaborations involving NIH grants P20 RR020171 (to L. Hersh), CA172379 (to C. Liu), and CA187273 (to V. Rangnekar). The manuscript's contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH, NIAID, or NIGMS.

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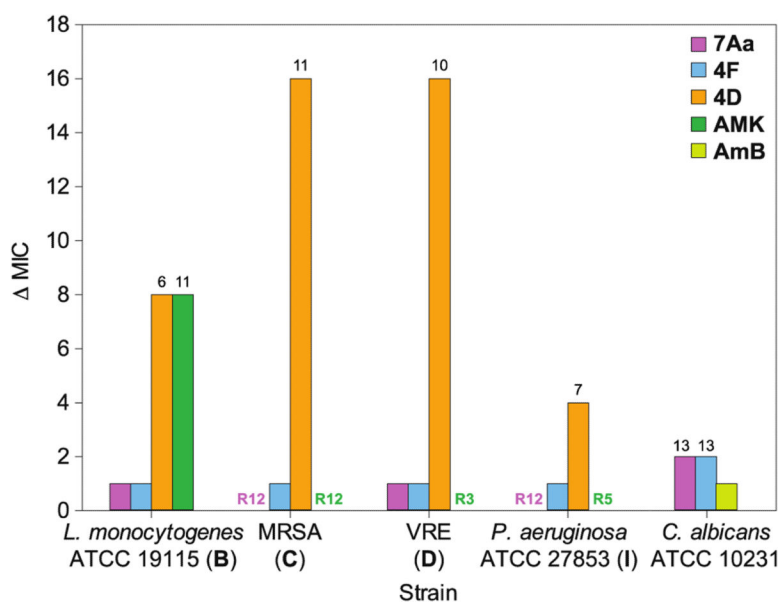
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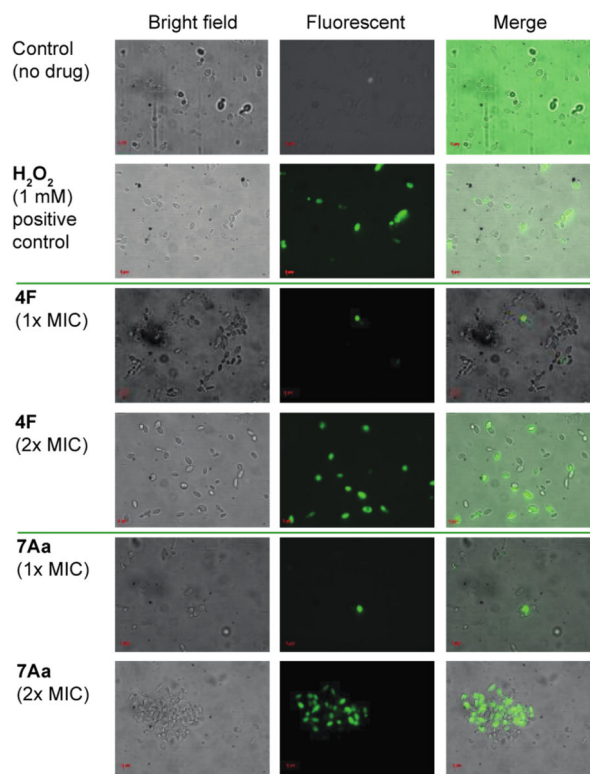
**Scheme 1.**

Synthetic scheme for the preparation of **A**, the bis(*N*-amidinohydrazones) and **B**, *N*-(amidino)-*N'*-aryl-bishydrazones.



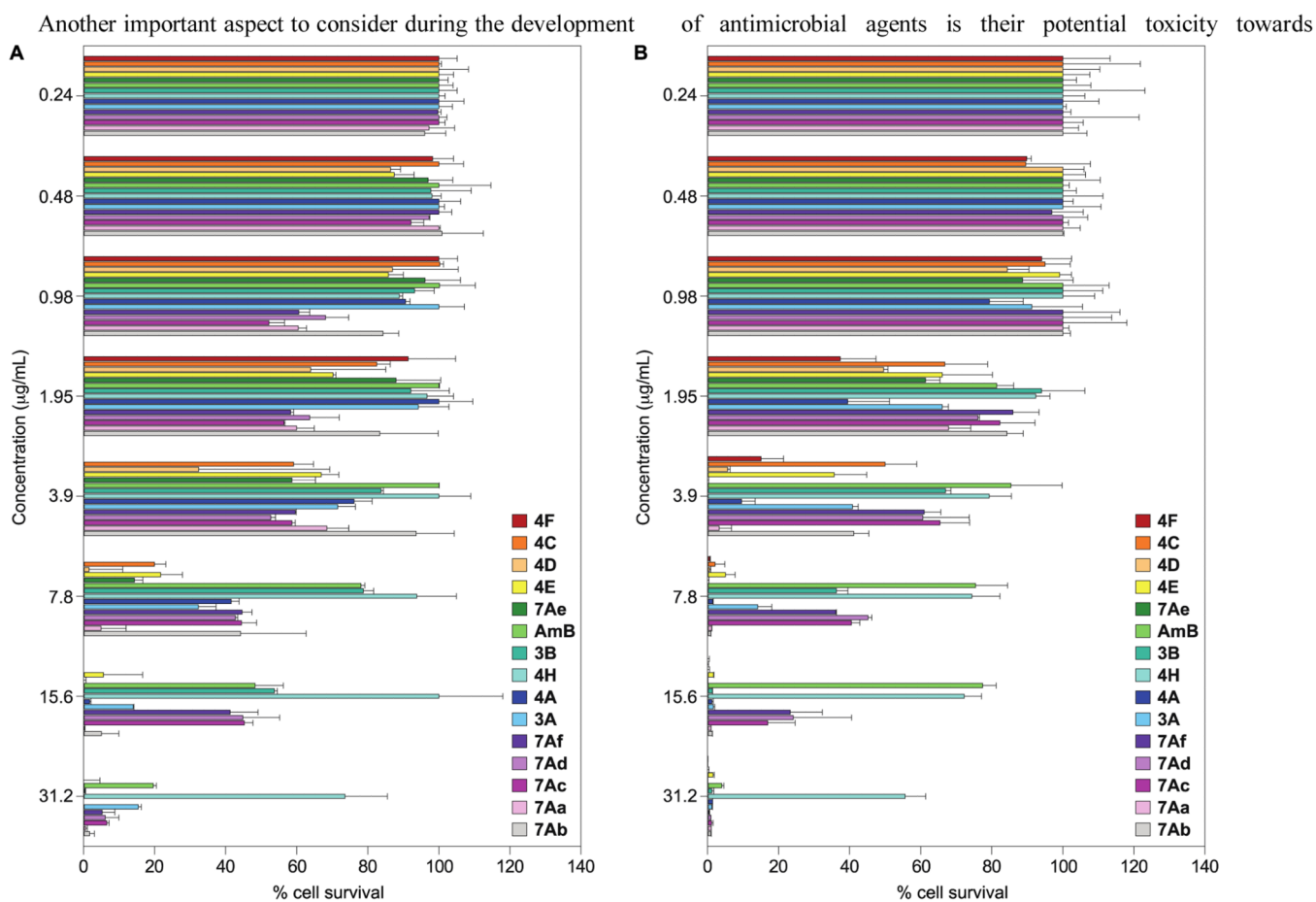
**Figure 1.** Changes in MIC values of *L. monocytogenes* ATCC 19115 (strain **B**), MRSA (strain **C**), VRE (strain **D**), and *P. aeruginosa* ATCC 27853 (strain **I**) treated with AMK (green), **7Aa** (purple), **4F** (blue), **4D** (orange), as well as *C. albicans* ATCC 10231 treated with AmB (yellow), **7Aa** (purple), and **4F** (blue) over 15 cycles. Numbers above the bars represent the passage when either bacterial or fungal cells developed resistance.



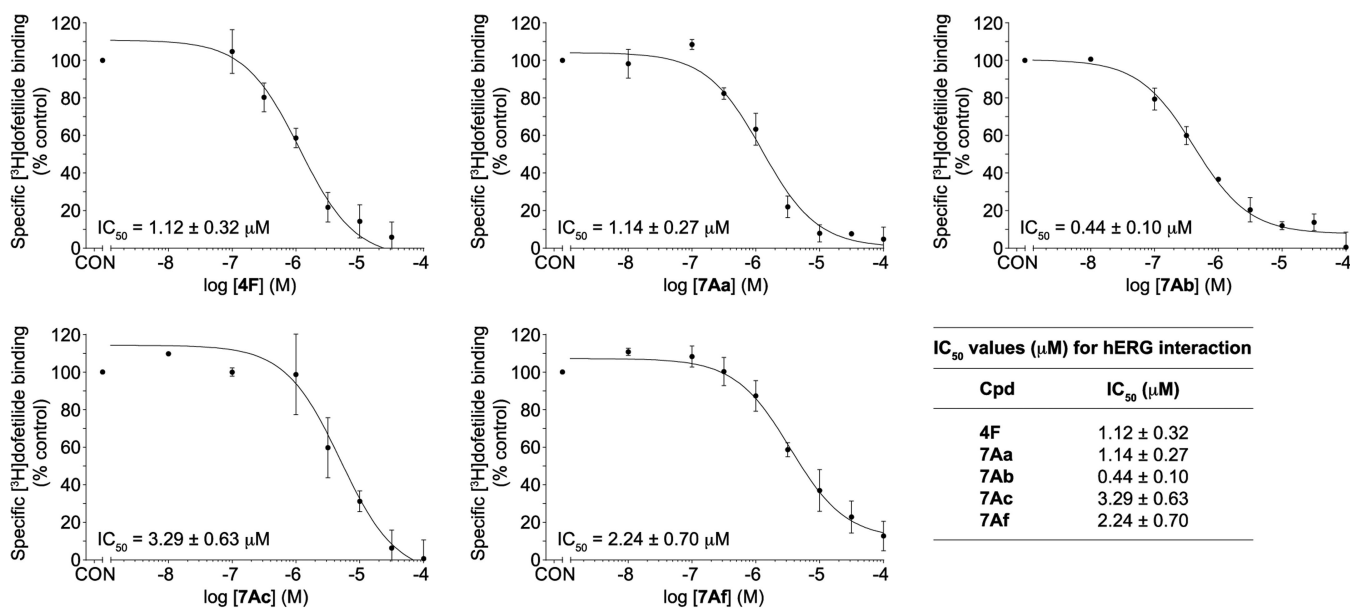


**Figure 2.**

Effect of compounds **4F** and **7Aa** on intracellular ROS production by *C. albicans* ATCC 10231. Yeast cells were treated in the absence of drug (negative control), 1 mM of H<sub>2</sub>O<sub>2</sub> (positive control), or **4F** and **7Aa**, at 1× and 2× respective MIC values for 1 h at 35 °C. After staining with DCFH-DA (20 µg/mL), the samples were analyzed using a Zeiss Axovert 200M fluorescence microscope.



**Figure 3.** Mammalian cell cytotoxicity of selected bis(*N*-amidinohydrazones) and *N*-(amidino)-*N*-aryl-bishydrazones, as well as AmB (as a control) against **A.** A549 cell line and **B.** BEAS-2B cell line.



**Figure 4.** IC<sub>50</sub> curves for hERG interaction by representative bis(*N*-amidinohydrazones) and *N*-(amidino)-*N*-aryl-bishydrazones along with a table (bottom right) summarizing these IC<sub>50</sub> values.

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Table 1

MIC values in µg/mL and (µM)<sup>a</sup> for compounds **3A**, **B**, **4A**, **C**, **H**, **7Aa**, **Ag**, and **8Aa** against various bacterial strains.

Cpd	Gram-positive					Gram-negative					
	<b>A</b> ( <i>Bsu</i> )	<b>B</b> ( <i>Lmo</i> )	<b>C</b> (MRSA)	<b>D</b> (VRE)	<b>E</b> ( <i>Aba</i> )	<b>F</b> ( <i>Ecl</i> )	<b>G</b> ( <i>Eco</i> )	<b>H</b> ( <i>Kpn</i> )	<b>I</b> ( <i>Pae</i> )	<b>J</b> ( <i>Sen</i> )	<b>K</b> ( <i>Msm</i> )
<b>AMK</b>	3.0-12.2 (3.9-15.6)	3.0 (3.9)	24.5 (31.3)	97.7 (125)	24.5 (31.3)	48.9 (62.5)	24.5 (31.3)	24.5 (31.3)	48.9 (62.5)	3.0-6.1 (3.9-7.8)	12.2 (15.6)
<b>AMP</b>	>92.8 (>250)	92.8 (250)	92.8 (250)	92.8 (250)	>92.8 (>250)	>92.8 (>250)	>92.8 (>250)	>92.8 (>250)	>92.8 (>250)	92.8 (250)	>92.8 (>250)
<b>OFX</b>	0.2 ( 0.5)	0.4 (1.0)	0.4 (1.0)	2.8 (7.8)	0.2 ( 0.5)	0.2 ( 0.5)	1.4-2.8 (3.9-7.8)	5.6-11.3 (15.6-31.3)	1.4 (3.9)	5.6-11.3 (15.6-31.3)	5.6 (15.6)
<b>3A</b>	>198 (>500)	0.4 (1.0)	1.5 (3.9)	0.8 (2.0)	>198 (>500)	>198 (>500)	>198 (>500)	>198 (>500)	>198 (>500)	>198 (>500)	>198 (>500)
<b>3B</b>	>198 (>500)	0.4 (1.0)	1.5 (3.9)	1.5 (3.9)	>198 (>500)	>198 (>500)	>198 (>500)	>198 (>500)	>198 (>500)	>198 (>500)	0.4 (1.0)
<b>4A</b>	>212 (>500)	>212 (>500)	>212 (>500)	>212 (>500)	>212 (>500)	52.9 (125)	26.5 (62.5)	6.6 (15.6)	0.8 (2.0)	>212 (>500)	>212 (>500)
<b>4C</b>	>219 (>500)	1.7 (3.9)	1.7 (3.9)	0.9 (2.0)	>219 (>500)	>219 (>500)	>219 (>500)	>219 (>500)	>219 (>500)	>219 (>500)	0.9 (2.0)
<b>4D</b>	>226 (>500)	0.5 (1.0)	0.5-0.9 (1.0-2.0)	0.5 (1.0)	>226 (>500)	>226 (>500)	>226 (>500)	>226 (>500)	>226 (>500)	>226 (>500)	0.5 (1.0)
<b>4E</b>	54.9 (125)	27.5 (62.5)	54.9 (125)	1.7 (3.9)	27.5 (62.5)	110 (250)	54.9 (125)	27.5 (62.5)	27.5 (62.5)	54.9 (125)	54.9-110 (125-250)
<b>4F</b>	3.4 (7.8)	<0.2 (<0.5)	0.4-0.9 (1.0-2.0)	0.4 (1.0)	218 (500)	218 (500)	218 (500)	13.6-27.2 (31.3-62.5)	0.4-0.9 (1.0-2.0)	1.7 (3.9)	0.4 (1.0)
<b>4G</b>	86.8 (250)	86.8 (250)	>173 (>500)	43.4 (125)	173 (500)	>173 (>500)	>173 (>500)	173 (500)	86.8 (250)	86.8-173 (250-500)	>173 (>500)
<b>4H</b>	86.8 (250)	86.8 (250)	>173 (>500)	43.4 (125)	>173 (>500)	>173 (>500)	>173 (>500)	>173 (>500)	173 (500)	21.7-86.8 (62.5-250)	5.4 (15.6)
<b>7Aa</b>	>215 (>500)	0.4-0.9 (1.0-2.0)	0.9 (2.0)	0.9 (2.0)	>215 (>500)	>215 (>500)	>215 (>500)	>215 (>500)	>215 (>500)	>215 (>500)	1.7 (3.9)
<b>7Ab</b>	>235 (>500)	0.9 (2.0)	0.9 (2.0)	0.9 (2.0)	>235 (>500)	>235 (>500)	>235 (>500)	>235 (>500)	>235 (>500)	>235 (>500)	1.8 (3.9)
<b>7Ac</b>	>205 (>500)	0.8 (2.0)	3.2 (7.8)	1.6 (3.9)	>205 (>500)	>205 (>500)	>205 (>500)	>205 (>500)	>205 (>500)	>205 (>500)	1.6 (3.9)
<b>7Ad</b>	>214 (>500)	1.7 (3.9)	3.3 (7.8)	1.7 (3.9)	>214 (>500)	>214 (>500)	>214 (>500)	>214 (>500)	>214 (>500)	>214 (>500)	1.7 (3.9)
<b>7Ae</b>	13.1 (31.3)	3.3 (7.8)	6.5 (15.6)	0.8-1.6 (2.0-3.9)	6.5-13.1 (15.6-31.3)	209 (500)	26.1-52.2 (62.5-125)	6.5-13.1 (15.6-31.3)	13.1-26.1 (31.3-62.5)	6.5 (15.6)	6.5-13.1 (15.6-31.3)
<b>7Af</b>	>214 (>500)	0.9-1.7 (2.0-3.9)	1.7 (3.9)	1.7 (3.9)	>214 (>500)	>214 (>500)	>214 (>500)	>214 (>500)	>214 (>500)	>214 (>500)	0.9-1.7 (2.0-3.9)
<b>7Ag</b>	>231 (>500)	3.6 (7.8)	3.6 (7.8)	3.6 (7.8)	>231 (>500)	>231 (>500)	>231 (>500)	>231 (>500)	>231 (>500)	>231 (>500)	1.8 (3.9)
<b>8Aa</b>	26.6-53.3 (62.5-125)	26.6-53.3 (62.5-125)	53.3 (125)	1.7 (3.9)	26.6 (62.5)	213 (500)	213 (500)	26.6-53.3 (62.5-125)	213 (500)	26.3-53.3 (62.5-125)	213 (500)

Gram-positive: **A** (*Bsu*) = *B. subtilis* 168, **B** (*Lmo*) = *L. monocytogenes* ATCC 19115, **C** = MRSA, **D** = VRE.Gram-negative: **E** (*Aba*) = *A. baumannii* ATCC 19606, **F** (*Ecl*) = *E. cloacae* ATCC 13047, **G** (*Eco*) = *E. coli* MC1061, **H** (*Kpn*) = *K. pneumoniae* ATCC 27736, **I** (*Pae*) = *P. aeruginosa* ATCC 27853, **J** (*Sen*) = *S. enterica* ATCC 14028.Mycobacterial: **K** (*Msm*) = *M. smegmatis* MC2-155. Note: All strains without an ATCC number are either clinical isolates or obtained from a different source than ATCC (source provided in supporting information).Control antibiotics: **AMK** = amikacin, **AMP** = ampicillin, **OFX** = ofloxacin.<sup>a</sup>These antibacterial MIC values were determined originally in µM using a range of 0.5 to 500 µM. These values are presented into parentheses. The values in µg/mL are presented for comparison with the antifungal MIC values, which were determined originally in µg/mL.

**Table 2**

MIC values in  $\mu\text{g/mL}$ <sup>a</sup> and ( $\mu\text{M}$ ) for compounds **3A,B**, **4A,C-H**, **7Aa-Ag**, and **8Aa** against various *C. albicans* strains.

Cpd	<i>C. albicans</i> ATCC strain #						
	1003 <sup>a</sup>	1237 <sup>b</sup>	2310 <sup>c</sup>	2876 <sup>c</sup>	10231 <sup>b</sup>	64124 <sup>b</sup>	90819 <sup>b</sup>
<b>AmB</b>	3.9 (4.2)	3.9 (4.2)	3.9 (4.2)	3.9 (4.2)	3.9 (4.2)	2.0-3.9 (2.1-4.2)	2.0 (2.1)
<b>3A</b>	7.8 (19.7)	15.6 (39.5)	>31.3 (>79.2)	7.8 (19.7)	15.6 (39.5)	>31.3 (>79.2)	>31.3 (>79.2)
<b>3B</b>	3.9 (9.9)	3.9 (9.9)	3.9 (9.9)	3.9 (9.9)	3.9 (9.9)	3.9 (9.9)	3.9 (9.9)
<b>4A</b>	7.8 (18.4)	>31.3 (>73.9)	3.9 (9.2)	2.0 (4.7)	2.0-3.9 (4.7-9.2)	2.0 (4.7)	3.9 (9.2)
<b>4C</b>	7.8 (17.8)	7.8 (17.8)	7.8 (17.8)	3.9 (8.9)	7.8 (17.8)	7.8 (17.8)	7.8 (17.8)
<b>4D</b>	15.6 (34.6)	15.6 (34.6)	31.3 (69.3)	3.9 (8.6)	7.8 (17.3)	>31.3 (>69.3)	7.8 (17.3)
<b>4E</b>	7.8 (17.8)	7.8 (17.8)	7.8 (17.8)	3.9 (8.9)	7.8 (17.8)	7.8 (17.8)	7.8 (17.8)
<b>4F</b>	1.0 (2.3)	2.0 (4.6)	1.0 (2.3)	1.0 (2.3)	1.0-2.0 (2.3-4.6)	1.0 (2.3)	2.0 (4.6)
<b>4G</b>	>31.3 (>90.1)	>31.3 (>90.1)	>31.3 (>90.1)	>31.3 (>90.1)	>31.3 (>90.1)	31.3 (90.1)	>31.3 (>90.1)
<b>4H</b>	>31.3 (>90.1)	31.3 (90.1)	31.3 (90.1)	7.8 (22.5)	>31.3 (>90.1)	>31.3 (>90.1)	>31.3 (>90.1)
<b>7Aa</b>	2.0 (4.6)	3.9 (9.2)	2.0 (4.6)	2.0 (4.6)	2.0 (4.6)	3.9 (9.2)	2.0 (4.6)
<b>7Ab</b>	2.0 (4.1)	3.9 (8.3)	3.9 (8.3)	3.9 (8.3)	2.0 (4.1)	3.9 (8.3)	3.9 (8.3)
<b>7Ac</b>	2.0 (4.7)	7.8 (19.0)	3.9 (9.5)	3.9 (9.5)	7.8 (19.0)	31.3 (76.2)	3.9 (9.5)
<b>7Ad</b>	3.9 (9.1)	15.6 (36.5)	3.9 (9.1)	7.8 (18.3)	7.8 (18.3)	15.6 (36.5)	7.8 (18.3)
<b>7Ae</b>	7.8 (18.7)	15.6 (37.3)	15.6 (37.3)	15.6 (37.3)	15.6 (37.3)	31.3 (74.9)	15.6 (37.3)
<b>7Af</b>	2.0 (4.5)	3.9 (9.1)	3.9 (9.1)	3.9 (9.1)	1.0 (2.3)	3.9 (9.1)	7.8 (18.2)
<b>7Ag</b>	7.8 (16.8)	15.6 (33.8)	7.8 (16.8)	15.6 (33.8)	7.8 (16.8)	31.3 (67.8)	31.3 (67.8)
<b>8Aa</b>	15.6 (34.3)	15.6 (34.3)	15.6 (34.3)	15.6 (34.3)	15.6 (34.3)	31.3 (68.7)	15.6 (34.3)

Control antifungal agent: **AmB** = amphotericin B.

<sup>a</sup>These antifungal MIC values were determined originally in  $\mu\text{g/mL}$  using a range of 0.5 to 31.3  $\mu\text{g/mL}$ . The values in  $\mu\text{M}$  into parentheses are presented for comparison with the antibacterial MIC values, which were determined originally in  $\mu\text{M}$ .

<sup>b</sup>Indicates strains that are resistant to FLC, ITC, and VOR according to ATCC.

<sup>c</sup>Indicates strains that are susceptible to FLC, ITC, and VOR according to ATCC.

**Table 3**

The cytotoxicity (IC<sub>50</sub>, µg/mL) of selected bis(*N*-amidinohydrazones) and *N*-(amidino)-*N'*-aryl-bishydrazones against A549 and BEAS-2B cell lines. Values are presented as mean ± SDEV.

Cpd	Mammalian cell line	
	A549	BEAS-2B
AmB	18.2 ± 4.5	17.7 ± 6.4
3A	6.6 ± 1.7	3.0 ± 0.7
3B	13.9 ± 4.1	5.7 ± 1.8
4A	6.3 ± 2.2	1.7 ± 0.5
4C	4.3 ± 1.3	2.9 ± 0.9
4D	2.9 ± 0.9	1.8 ± 0.6
4E	3.9 ± 0.9	2.9 ± 0.9
4F	2.5 ± 1.4	1.8 ± 0.6
4H	31.2 ± 7.2	33.0 ± 4.6
7Aa	2.8 ± 0.9	2.3 ± 1.0
7Ab	6.7 ± 2.3	3.4 ± 1.3
7Ac	4.4 ± 1.4	6.0 ± 1.2
7Ad	4.9 ± 1.1	6.0 ± 1.1
7Ae	4.2 ± 1.4	1.9 ± 0.8
7Af	4.8 ± 1.3	5.8 ± 1.2