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Evaluation of the toxicity of 2-aminoimidazole antibiofilm agents using both cellular and model organism systems

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Abstract

Biofilm formation is a ubiquitous bacterial defense mechanism and has been shown to be a primary element in the antibiotic resistance of many human diseases, especially in the case of nosocomial infections. Recently, we have developed several compound libraries that are extremely effective at both dispersing preexisting biofilms and also inhibiting their initial formation. In addition to their antibiofilm properties, some of these molecules are able to resensitize resistant bacterial strains to previously ineffective antibiotics and are being assessed as adjuvants. In this study, we evaluated the toxic effects of three of our most effective 2-aminoimidazole compounds (dihydrosventrin, RA, and SPAR) using a rapid pipeline that combines a series of assays. A methylthiazolyldiphenyl-tetrazolium assay, using the HaCaT keratinocyte cell line was used to determine epidermal irritants and was combined with *Caenorhabditis elegans* fecundity assays that demonstrated the effects of environmental exposure to various concentrations of these molecules. In each case, the assays showed that the compounds did not exhibit toxicity until they reached well above their current biofilm dispersion/inhibition concentrations. The most effective antibiofilm compound also had significant effects when used in conjunction with several standard antibiotics against resistant bacteria. Consequently, it was further investigated using the *C. elegans* assay in combination with different antibiotics and was found to maintain the same low level of toxicity as when acting alone, bolstering its candidacy for further testing as an adjuvant.

Keywords

2-aminoimidazole; adjuvant; biofilm; *Caenorhabditis elegans*; toxicity; Toxicity of 2-aminoimidazole antibiofilm agents

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Declaration of interest

The authors report no other declarations of interest.

Introduction

As the understanding of bacterial multidrug resistance expands, the importance of developing effective treatments has driven pharmaceutical research to explore various alternatives, driving a shift in paradigm from a planktonic model of bacterial disease to a biofilm-based model (Costerton et al., 1999; Lynch and Abbanat, 2010). Biofilms are found in practically every environmental niche. They are surface-associated communities of bacterial microcolonies encased in a protective extracellular polymeric matrix, which offers a high level of resistance to current microbicides and antibiotics. For pathogenic species, the formation of these communities typically results in a chronic infection of the host surface and is implicated in more than three quarters of human bacterial infections (Costerton et al., 1999; Stoodley et al., 2002; Parsek and Singh, 2003; Musk and Hergenrother, 2006; Davies, 2003). Many of these infections develop in immunocompromised patients, such as those with human immune-deficiency virus, certain types of cancer, or recipients of indwelling medical devices (Costerton et al., 1999; Donlan and Costerton, 2002; Costerton et al., 2003; Davies, 2003).

Currently, the most promising avenue for the treatment of biofilm-based infections has been the exploitation of compounds able to modulate the formation of these defensive barriers without killing the bacteria (Pace et al., 2006). Recently, we have developed several libraries of compounds inspired by bromoageliferin, a 2-aminoimidazole-containing compound from the marine sponge, *Agelas conifera*, that are able to inhibit the formation of biofilms and disperse preexisting biofilms, a more challenging undertaking, because, by definition, biofilms form to provide enhanced bacterial protection (Ballard et al., 2008; Richards et al., 2008b; Rogers and Melander, 2008). More recent studies have shown that in addition to having antibiofilm properties, many of our compounds also act in combination with antimicrobial treatments by reinitiating antibiotic sensitivity in resistant bacterial strains, such as multidrug resistant *Pseudomonas aeruginosa* (MDRPA), methicillin resistant *Staphylococcus aureus* (MRSA), and multidrug resistant *Acinetobacter baumannii* (MDRAB) (Huigens et al., 2007; Rogers et al., 2010). Although this sublibrary of compounds is extremely promising, it is imperative to define the potential toxicity of these molecules before they can be used.

Although animal models have been a gold standard, they tend to be lengthy and expensive; therefore, the use of *in vitro* cellular assays is desirable as a preliminary screening method. In this work, we explored a cost-effective screening pipeline for three of our leading antibiofilm molecules referred to as DHS, RA, and SPAR (Table 1). An earlier generation of these molecules (TAGE and CAGE) was shown to lack the mammalian cytotoxicity of the parent molecule, bromoageliferin, contributing to the development of a large library of derivative compounds (Bickmeyer, 2005; Huigens et al., 2008). This pipeline is straightforward, robust, and enables the “first-stage” analysis of the general toxic effects of many compounds in a rapid fashion. From these initial studies, promising subsets of compounds can be further subjected to more rigorous investigations. Here, the aim was to determine whether our compounds would demonstrate low enough toxicity to become viable candidates for further development 1) as antibiofilm agents and 2) as an adjuvant to antibiotic action against resistant bacterial strains, thereby restricting the number of animal experiments that would subsequently be undertaken.

A simple, highly reproducible test was selected first as a screening procedure to evaluate the skin irritation potential of the three compounds using human keratinocytes—the HaCaT cell line (Boukamp et al., 1988; Wilhelm et al., 1994; Wilhelm et al., 2001; Hirsch et al., 2009; George et al., 2010). For this study, HaCaT cells were tested in a traditional methylthiazolyldiphenyl-tetrazolium (MTT) assay, a colorimetric assay based on the

presence of metabolically active cells and the metabolites, nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate, in culture (Mosmann, 1983; Denizot and Lang, 1986). To complement and bolster these assays, we examined the effects these molecules had on reproduction and development via a *Caenorhabditis elegans* fecundity assay Richards et al., 2009. *C. elegans* is a nonparasitic nematode species found in soil and has become one of the most extensively studied model organisms (Wood, 1988; Riddle et al., 1997; Hope, 1999; Leung et al., 2008). The use of *C. elegans* in toxicological assays has become increasingly common for small-molecule studies to observe the effects of the compounds on physiology and stress responses that are highly conserved among higher organisms, including humans (NRC, 2000; Artal-Sanz et al., 2006; Kaletta and Hengartner, 2006; Kwok et al., 2006; Boyd et al., 2010).

From previous work, we had already established that the molecules, dihydrosventrin (DHS), RA, and SPAR, were all able to both effectively inhibit biofilm formation and also disperse robust preformed biofilms (Ballard et al., 2008; Richards et al., 2008b; Rogers and Melander, 2008). Each molecule was selected as the lead compound from extensive libraries: DHS is from our oroidin library, RA from our reverse amide library, and SPAR from our 2-aminoimidazole-triazole library (Ballard et al., 2008; Richards et al., 2008a; Rogers and Melander, 2008). Each of the compounds was selected based on its ability to effectively disperse and prevent biofilm formation. Representative IC₅₀ values (concentration required for 50% biofilm inhibition) and EC₅₀ values (concentration required for 50% biofilm dispersion) for the three compounds against *P. aeruginosa* strain PA14 are shown in Table 1 (Ballard et al., 2008; Richards et al., 2008b; Rogers and Melander, 2008). Of these antibiofilm compounds, the molecule referred to as SPAR also readily resensitizes highly resistant bacterial strains to the effects of conventional antibiotics, to which those strains are no longer susceptible (Rogers et al., 2010). With this in mind, SPAR was subjected to a *C. elegans* assay in a separate set of experiments in the presence of six different antibiotics. The aim was to evaluate whether SPAR, when used in an adjuvant capacity, maintained the same levels of toxicity as when acting alone. If this is the case, then it strengthens its candidacy for further testing as a combination therapy.

Methods

Compound preparation

The three compounds under investigation, referred to as DHS, RA, and SPAR (Table 1), represent some of our leading therapeutic candidates—as defined by their activity against biofilms and their ability to resensitize resistant bacteria. DHS, RA, and SPAR were maintained in 100-mM stocks at –20°C. Because the storage solvent for the small molecules, dimethyl sulfoxide (DMSO), is known to have severe developmental and lethal effects on *C. elegans* at greater than 2% (v/v), all test samples were diluted to have a final solvent concentration between 0.8 and 1.0% (Burns et al., 2006; Mukai et al., 2008). Before their application to the assay, all of the compounds were diluted from the 100-mM stock to a 20× final assay concentration (i.e., compound A was diluted to 20 mM for 1-mM assay volume), using serial dilutions with ddH₂O and a small volume of DMSO to maintain consistency during the assay. The final assay concentrations ranged from 1.0 μM to 2.5 mM for all compounds tested.

MTT assay with HaCaT cells

Human adult, low-calcium, high-temperature (HaCaT) keratinocyte cells were obtained from Dr. Lisa Plano (University of Miami, Miami, Florida, USA) who initially obtained the cells from Professor Norbert Fusenig (University of Heidelberg, Heidelberg, Germany) (Boukamp et al., 1988). Keratinocytes were cultivated in 10% fetal bovine serum/

Dulbecco's modified Eagle's medium (FBS-DMEM) media (Invitrogen, Carlsbad, California, USA) with 100 IU/mL of penicillin and 100 µg/mL of streptomycin (Invitrogen) at 37°C and 5% CO₂ until 80–90% confluent. The HaCaT cells were transferred to a 96-well microtiter plate (CellBIND; Corning, Corning, New York, USA) in 100-µL aliquots at a density of 8×10^4 cells/mL in 10% FBS-DMEM complete media (Invitrogen) and incubated overnight at 37°C and 5% CO₂. Once the cultures were 80–90% confluent, the compounds were added to the cells using serial dilutions across the plate in duplicate (from 2.5 mM to 1.22 µM). Treated wells and controls (media dilutions, 10% DMSO dilutions, and azide dilutions) were incubated overnight. Using the Roche[®] Cell Proliferation Kit I (Roche Diagnostics Corp., Indianapolis, Indiana, USA), 10 µL of MTT (5 mg/mL in phosphate-buffered saline; Roche) were added to each well, and the plate was incubated in the dark for 4 hours. Next, 100 µL of MTT-solubilizing reagent (10% sodium dodecyl sulfate in 0.01 M of HCl; Roche) was added to each well and incubated for 12–18 hours. Once the incubation was complete, the plate was scanned at 550 nm, with a reference absorbance of 670 nm, using a TECAN[®] Sunrise plate reader with a rainbow filter (Tecan, Durham, North Carolina, USA).

C. elegans fecundity assay

The nematode species used in the assay was the wild-type *C. elegans* N2 Bristol strain (Brenner, 1974). Two *Escherichia coli* strains, OP50 and HB101, were used for *C. elegans* maintenance and performance of this bioassay. *C. elegans* N2 worms were maintained at 15°C on Nematode Growth Medium (NGM) agar plates with the *E. coli* OP50 strain as a bacterial food source (Lewis, 1995). Before the assays, the worms were harvested and synchronized using an alkaline bleach solution (25% NaOCl and 12% 4N NaOH in M9 media). The synchronized L1s were recovered via centrifugation and plated on NGM agar for incubation at 20°C until they reached the L4 stage. Using a 96-well plate, each assay was set up in triplicate with two compound ranges tested per plate along with controls. Two L4-stage larvae were transferred from the plates to a single well containing 25 µL of S medium, followed by 70 µL of a 5% (m/v) HB101 suspension in S medium and 5 µL of the compound dilution to be tested, yielding a total assay volume of 100 µL/well. Plates were incubated at 22.5°C on an orbital shaker for 6–7 days. Observations were made on day 4 and day 6 or 7 (depending on growth evaluation on day 4), and the plates were scored based on the clarity of the wells on the final day. If the well was cleared and was found to have a large population of worms (comparable to the worm-only control), it was scored as a nontoxic concentration; on the other hand, if the well was still opaque, found to have small populations of worms, or found to have worms with visible abnormalities, the concentration was scored as having possible toxicity. Starting with 100-µM steps, the assays were narrowed down based on the range of possible toxic concentrations found by the previous run until a threshold concentration range could be established (Figure 1). Controls consisted of various compound concentrations with bacteria only, worms with bacteria only, and a known nematocide (Ivermectin, product no.: I8898; Sigma-Aldrich, St. Louis, Missouri, USA) at matching concentrations of compound and DMSO (Omura and Crump, 2004).

Before running the adjuvant version of the *C. elegans* assay, an antibiotic control assay was performed to confirm that the concentrations used would not provide any bias. The assay was performed as described above, except the following antibiotic solutions that were substituted for compound in triplicate: ampicillin (50 µg/mL, 25 µg/mL), kanamycin (50 µg/mL, 25 µg/mL), chloramphenicol (170 µg/mL, 85 µg/mL), streptomycin (25 µg/mL, 12.5 µg/mL), rifampicin (100 µg/mL, 50 µg/mL), tetracycline (50 µg/mL, 25 µg/mL), methicillin (37.5 µg/mL, 18.75 µg/mL), and tobramycin (50 µg/mL, 25 µg/mL). For the adjuvant fecundity assays, the lead compound, SPAR, was added at 50 µM and up to 1 mM in 100-µM steps (in triplicate) to wells containing one of the following antibiotic solutions:

ampicillin (50 µg/mL), kanamycin (50 µg/mL), chloramphenicol (170 µg/mL), streptomycin (25 µg/mL), methicillin (37.5 µg/mL), and tobramycin (50 µg/mL). Plates were incubated at 22.5°C on an orbital shaker for 6–7 days.

Results

Determination of compound epidermal exposure toxicities

During the MTT portion of the pipeline, keratinocytes were externally exposed to varying concentrations of each of the three compounds to determine whether any cytotoxic effects could be observed through simulated epidermal exposure. Results were calculated as the concentrations of the compounds at which 50% toxicity (TCED₅₀) and no toxicity (endpoint) were observed, as compared to the controls. Based on these assays, all three compounds had TCED₅₀ values that were much higher than their PA14 inhibition concentrations, ranging from 4× for DHS to greater than 200× for SPAR, whereas only SPAR and RA had endpoint values greater than their IC₅₀ values. When compared to their PA14 EC₅₀ values, the TCED₅₀ values for both DHS and SPAR were found to be greater than their dispersion concentrations, but only SPAR had a higher endpoint (Table 1).

Evaluation of exposure toxicity using *C. elegans*

The *C. elegans* fecundity assay used in this study was a qualitative assay designed to determine an estimated concentration threshold (ECT) for each compound that could be used as a conservative comparison in a quick, straightforward manner. The ECT was defined as the compound concentration at which the media (*E. coli* HB101/S medium) was no longer sufficiently transparent to see the worms, even under a microscope (Figure 1). Media clearing indicated that the worms were able to feed and reproduce across more than one generation without experiencing any detrimental effects from their exposure to the compound at the tested concentration. Beyond the ECT for a compound, the wells remained opaque.

For each compound, the fecundity assays were carried out in three stages: 1) low exposure (<100 µM), 2) high exposure (100 µM–1.0 mM), and 3) threshold exposure (ECT ± 100 µM). During the low-exposure stage, all of the antibiofilm compounds were found to have no toxicity at every tested concentration (Figure 2). Most important, the controls were able to confirm that the clearing was the result of the feeding activity of the large number of *C. elegans* present in the well. After the success of the low-concentration assays, the concentration range of the assay was expanded to search for the ECT of the candidate molecules at higher exposures. According to the results of this assay, DHS had an apparent ECT between 400 and 500 µM, SPAR had an apparent ECT of ~800 µM, and RA did not have any observable toxic effects at any of the tested concentrations (Figure 3). Using the results of the second-stage assay as a guide, a more refined set was run to help narrow the concentration value for each compound's ECT (Table 1). Similar to the MTT assay results, the ECT values for all three compounds were much higher than their IC₅₀ values against *P. aeruginosa* and also proved to be considerably higher than the equivalent EC₅₀ values.

Evaluation of toxicity under adjuvant conditions

Because SPAR exhibits the ability to resensitize antibiotic-resistant bacterial strains to previously ineffective antibiotics, it is a particularly appealing candidate for use as an adjuvant therapy (Rogers et al., 2010). With this in mind, a variation of the fecundity assay was developed to confirm that the synergy between SPAR and the antibiotics did not have any effect on the previously determined threshold. The assay was conducted with the addition of the six of antibiotic solutions (see above) and SPAR. After 1 week of incubation,

all of the antibiotic and adjuvant combinations showed remarkable clearing, confirming that the combination of SPAR with various antibiotics was nontoxic to *C. elegans* (Figure 4).

Discussion

Two important points can be ascertained from these studies: 1) the exposure toxicities for each compound (Table 1) was markedly higher than the current concentrations used for antibiofilm activity (Ballard et al., 2008; Richards et al., 2008b; Rogers and Melander, 2008), and 2) the use of these assays provides a quick way of determining which synthetic methods will yield the most effective, least hazardous molecules. These results suggest a notable safety window for each compound. Of the three compounds used, SPAR has been the most successful biofilm modulator against all tested strains. In addition, it also possesses the ability to resensitize highly resistant bacterial strains to the effects of common antibiotics (Rogers et al., 2010). As a result of these assays, it is now clear that not only does SPAR have no bactericidal activity, but it is also a relatively nontoxic molecule, especially at effective concentrations.

These compounds were derived from a single parent molecule and exhibit very different structural modifications (Ballard et al., 2008; Richards et al., 2008a). Even without knowing the exact mechanism of action for this family of modulators, it is clear that the chemical modifications not only have an effect on their activity against biofilms, but also contribute to their toxicity to a host organism (Ballard et al., 2008). This structural aspect of their toxicity is best exemplified by the vastly different results for RA. Though RA was completely nontoxic to the *C. elegans* reproduction with an ECT above 1 mM, it was found to be the strongest skin irritant because of its cytotoxic effect on HaCaT cells. By screening these candidate molecules against multiple organisms, we are able to better define which libraries and synthesis methods will yield the most effective compounds with the lowest potentials to be toxic to move into further testing. Because of the costly nature of drug discovery and screening, the use of high-throughput assays, such as those performed using *C. elegans*, allows for the maximum amount of information to be collected from a single inexpensive test, and when combined with the previously recorded activity data, it is easier to determine which molecules would make the strongest candidates for large-scale trials.

Conclusion

As newer generations of antibiofilm agents are generated, we feel that the use of this prescreening method will become a key tool to aid in sorting potential therapeutics from their hazardous relatives.

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Figure 1.

Example of the *C. elegans* fecundity assay. Each 96-well plate was divided into three sections: compound A, compound B, and controls. Results of these assays were evaluated based on clarity of the individual wells and the visible worm population. A toxic concentration could be identified by an opaque well with little to no living worms (based on movement), whereas a nontoxic concentration was defined by transparency in white light and a large population of viable worms. Controls consisted of media alone, compound and bacterial suspension alone, ivermectin dosages, and worms and bacterial suspension.

Figure 2. Results of low-concentration *C. elegans* fecundity assay. All of the compounds exhibited no toxic effects within this concentration range. The bacteria-only controls showed no erroneous clearing.

Figure 3.

Results of the high-concentration *C. elegans* fecundity assay. All three compounds were added in triplicate with additional DMSO (to maintain 1% v/v). RA (top) showed no toxic effects, whereas DHS (middle) and SPAR (bottom) were found to have toxic effects above 400 μ M. The bacterial suspension (Bact. Controls) and worm viability (W. Ctrl.) controls showed no visible sources of bias.

Figure 4.

Results of the adjuvant fecundity assay using SPAR. Six different antibiotics were used at selective concentrations: ampicillin (Amp), kanamycin (Kan), chloramphenicol (Chlor), streptomycin (Strept), methicillin (Met), and tobramycin (Tob). Chloramphenicol was used as a negative control because of its ethanol toxicity and remained opaque throughout the assay.

Comparison of activity and toxicity for each anti-biofilm compound.

IC50 and EC50 values were determined against *Pseudomonas aeruginosa* PA14 (Ballard et al., 2008; Richards et al., 2008b; Rogers and Melander, 2008).