Published in final edited form as:

Int J Antimicrob Agents. 2016 October ; 48(4): 367–372. doi:10.1016/j.ijantimicag.2016.06.009.

Antibacterial activity of resazurin-based compounds against *Neisseria gonorrhoeae* in vitro and in vivo

Deanna M. Schmitt^a, Kristie L. Connolly^b, Ann E. Jerse^b, Melinda S. Detrick^a, and Joseph Horzempa^{a,*}

^aDepartment of Natural Sciences and Mathematics, West Liberty University, West Liberty, WV, USA

^bDepartment of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

Abstract

Neisseria gonorrhoeae is the cause of the second most common sexually transmitted bacterial infection, with ca. 80 million new cases of gonorrhoea reported annually. The recent emergence of clinical isolates resistant to the last monotherapy against this bacterium, the cephalosporins, illustrates the need for new antigonococcal agents. Here we have characterised a new group of antimicrobials based on the compound resazurin that exhibit robust activity against *N. gonorrhoeae* in vitro. Resazurin inhibits the growth of a broad range of *N. gonorrhoeae* isolates, including those resistant to multiple antibiotics. Furthermore, treatment of human endometrial cells infected with *N. gonorrhoeae* with resazurin significantly reduces the number of intracellular bacteria. Whilst resazurin exhibited potent in vitro antimicrobial activity, in vivo resazurin did not limit the colonisation of mice with *N. gonorrhoeae* following vaginal infection. The ineffectiveness of resazurin in vivo is likely due to its interaction with serum albumin, which completely diminishes its antimicrobial activity. However, treatment of mice with a resazurin analogue (resorufin pentyl ether) that maintains its antimicrobial activity in the presence of serum albumin approached a significant decrease in the percentage of mice vaginally colonised. This treatment also decreased vaginal colonisation by *N. gonorrhoeae* over time. Together, these data suggest that resazurin derivatives have potential for the treatment of gonorrhoea.

Keywords

Neisseria gonorrhoeae; Resazurin; Gonorrhoea; Resorufin; Antimicrobial; Antibiotic

*Corresponding author. Tel.: +1 304 336 8284; fax: +1 304 336 8266. joseph.horzempa@westliberty.edu (J. Horzempa).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Competing interests: None declared.

Ethical approval: All work was performed under an approved protocol of the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences (Bethesda, MD).

1. Introduction

Gonorrhoea, caused by the bacterium *Neisseria gonorrhoeae*, is one of the most prevalent sexually transmitted diseases, with ca. 80 million new cases reported annually [1]. Individuals infected with *N. gonorrhoeae* are often asymptomatic and do not seek treatment, which can result in severe complications such as disseminated gonococcal infection, pelvic inflammatory disease and infertility [2]. Moreover, *N. gonorrhoeae* has developed resistance to almost all antibiotics that have been used clinically to treat this bacterium [3]. The emergence of such multidrug-resistant *N. gonorrhoeae* strains underscores the need for novel antimicrobials against this pathogen.

Over the past 70 years, a variety of antibiotics have been used to treat gonorrhoea. Sulfonamides were the first antimicrobials used to combat *N. gonorrhoeae* infections in the late 1930s but were quickly replaced by penicillin owing to the development of widespread resistance [4]. Penicillin remained the 'gold standard' for treating gonococcal infections for the next 40 years [3].

Resistant isolates did develop during this time, however, resulting in the use of other antibiotics such as aminoglycosides, macrolides and tetracycline to treat gonorrhoea [5]. In the 1980s, broad resistance to tetracycline led to its removal as a first-line therapy for *N. gonorrhoeae* infection [3]. In the 1990s through 2000s, fluoroquinolones became the recommended course of treatment for gonorrhoea until *N. gonorrhoeae* also developed resistance to this class of antimicrobials [6]. As of 2007, only one class of antibiotics, the cephalosporins, remained effective and available for the treatment of gonorrhoea [6].

The recent emergence of extensively drug-resistant *N. gonorrhoeae* strains with high-level resistance to ceftriaxone has raised global concern that gonorrhoea will become an untreatable disease in the near future [7].

We recently identified resazurin, a commonly used viability dye, as a novel antimicrobial against *Francisella tularensis* and *Neisseria* spp. [8]. In this study, the efficacy of this compound against *N. gonorrhoeae* was evaluated in vitro and in vivo to determine its potential use as a treatment for gonorrhoea.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Neisseria gonorrhoeae strains used in this study are listed in Table 1. Bacteria were cultured on chocolate II agar or gonococcal (GC) agar supplemented with Kellogg's supplement I and ferric nitrate [9] and were incubated at 37 °C with 5–7.5% CO₂ for 18–22 h. Resazurin sodium salt (Acros Organics, Morris Plains, NJ; dissolved in water) was added to chocolate II agar at a concentration of 11 µg/mL where indicated. For mouse infection experiments, GC agar with vancomycin, colistin, nystatin, trimethoprim sulfate (VCNT supplement) (product #202408; Difco BD, Sparks, MD) and 100 µg/mL streptomycin sulfate (Sigma-Aldrich, St Louis, MO) was used to isolate *N. gonorrhoeae* from murine vaginal swabs. For inoculation of broth cultures, bacteria from chocolate II agar plates were suspended in

brain–heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) to an optical density at 600 nm (OD₆₀₀) of 0.3 and then 100 µL of inoculum was transferred to 10 mL of fastidious broth (FB) (Remel, Lenexa, KS). FB cultures were incubated at 37 °C with shaking at 100 rpm for 24 h.

2.2. Growth inhibition assays

Overnight broth cultures were used to inoculate FB containing resazurin sodium salt, resorufin sodium salt (Acros Organics; dissolved in water), resorufin acetate [Marker Gene Technologies, Eugene, OR; dissolved in dimethyl sulfoxide (DMSO)], resorufin butyrate (Sigma-Aldrich; dissolved in DMSO) or resorufin pentyl ether (AnaSpec, Fremont, CA; dissolved in DMSO). In select experiments, FB was also supplemented with 3 g/dL bovine serum albumin (BSA) (Fisher Scientific, Fair Lawn, NJ). Immediately following inoculation and 24 h later, cultures were serially diluted and were plated onto chocolate II agar. Plates were incubated at 37 °C in 5% CO₂ and individual colonies were enumerated. The limit of detection was 100 CFU/mL.

2.3. Determination of the minimum inhibitory concentration (MIC)

The MICs for resazurin and resorufin pentyl ether were determined by a modified broth microdilution assay. Serial two-fold dilutions of resazurin and resorufin pentyl ether were prepared using FB in 96-well microtitre plates (Corning Inc., Kennebunk, ME) beginning at initial concentrations of 22.4 µg/mL and 24.8 µg/mL, respectively. *Neisseria gonorrhoeae* bacteria from chocolate II agar plates were suspended in BHI to an OD₆₀₀ of 0.3 (ca. 4×10^7 CFU/mL) and were then diluted to 2×10^6 CFU/mL. Five microliters of this suspension (1×10^4 CFU) was added to each well and the microtitre plates were incubated overnight at 37 °C in 5% CO₂. Bacteria from each well were then transferred to chocolate II agar plates using a 48-pin microplate replicator (Dan-Kar Corp., Woburn MA). Plates were incubated overnight at 37 °C in 5% CO₂. The MIC reported for each strain was the lowest concentration of each compound that prevented visible growth in duplicate tests. No isolate differed by more than one dilution in duplicate tests.

2.4. Disk diffusion assays

Bacteria from chocolate II agar plates were suspended in BHI and were diluted to an OD₆₀₀ of 0.3. Then, 100 µL of this suspension was spread plated onto chocolate II agar. Sterile Whatman filter disks (BD Biosciences, Sparks, MD) infused with ampicillin (Akron Biotechnology, Boca Raton, FL) (0.1 µg or 1 µg), resazurin (11 µg or 55 µg) or resorufin (10 µg or 50 µg) were placed onto the surface of these plates, which were subsequently incubated overnight at 37 °C in 5% CO₂. The diameters of the zones of inhibition were measured using a metric ruler.

2.5. Infection of HEC-1-B cells with *Neisseria gonorrhoeae*

The human endometrial cell line HEC-1-B cells (ATCC HTB-113) was cultured in Eagle's minimum essential medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA). To assess invasion by *N. gonorrhoeae*, a gentamicin protection assay was performed [10,11]. HEC-1-B cells were

seeded in Primaria 96-well culture dishes (Corning Inc.) at a density of 2×10^5 cells/well. *Neisseria gonorrhoeae* strain FA1090 or MS11 cultured on chocolate agar was suspended in BHI to an OD₆₀₀ of 0.3 (ca. 4×10^7 CFU/mL) and was diluted to achieve a multiplicity of infection (MOI) of 1. The actual MOI was measured by plating serial dilutions of the inoculum on chocolate II agar plates. Cells were incubated with this MOI for ca. 14 h. After this time period, cells were washed twice with phosphate-buffered saline (PBS) (Mediatech) and were then incubated with gentamicin (100 µg/mL) (US Biological, Salem, MA) for 1 h to kill extracellular bacteria. Cells were washed twice more with PBS and then fresh culture medium (with or without resazurin 11 µg/mL and resorufin 10 µg/mL) was added and the cells were incubated for another 6 h at 37 °C in 5% CO₂. At the indicated time points, cells were lysed with 0.5 mM ethylene diamine tetra-acetic acid (EDTA) (Boston BioProducts, Ashland, MA) in PBS and the lysates were serially diluted and plated onto chocolate II agar. Plates were incubated at 37 °C in 5% CO₂ and individual colonies were enumerated. The limit of detection was 100 CFU per 2×10^5 cells.

2.6. Experimental murine infection

Female 6–8-week-old BALB/c mice (Charles River Laboratories Inc., Wilmington, MA; NCI Frederick strain of inbred BALB/cAnNCr mice, strain code 555) in the dioestrus stage of the oestrus cycle were implanted with a 5-mg, 21-day controlled-release oestradiol pellet (Innovative Research of America, Sarasota, FL) subcutaneously and were treated with streptomycin and trimethoprim throughout the infection to increase their susceptibility to *N. gonorrhoeae* as described previously [9,12]. Two days after pellet implantation, mice were inoculated intravaginally with *N. gonorrhoeae* strain MS11 (ca. 2×10^6 CFU). Beginning 2 days post-infection (antibiotic treatment Day 0), mice were administered either resazurin (11 mg/kg in PBS), resorufin (11 mg/kg in PBS) or resorufin pentyl ether (15 mg/kg in 50% DMSO) intraperitoneally for five consecutive days. As controls, mice were administered either 50% DMSO intraperitoneally for five consecutive days or a single intraperitoneal dose of ceftriaxone (15 mg/kg in water). Vaginal swabs were collected by gently inserting a rayon swab (Puritan, Guilford, ME) into the vagina and suspending the swab in 1 mL of GC broth. Samples were serially diluted and plated on GC agar supplemented with VCNT and streptomycin using an Autoplate 4000 Plating Machine (Spiral Biotech, Norwood, MA). The number of viable bacteria was determined using Spiral Biotech Q-Counter Software. The limit of detection was 20 CFU/mL. A small portion of the sample was also cultured on heart infusion agar to monitor the presence of potentially inhibitory facultatively anaerobic commensal flora. All work was performed under an approved protocol of the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences (Bethesda, MD).

2.7. Statistical analyses

Data were analysed for significant differences using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). The statistical tests used are indicated in the figure legends.

3. Results

3.1. Resazurin has bactericidal activity against *Neisseria gonorrhoeae*

An assortment of *N. gonorrhoeae* clinical isolates (Table 1) were plated on chocolate II agar plates supplemented with resazurin (11 µg/mL). None of these bacterial strains grew in the presence of resazurin, including multidrug-resistant strains as well as those isolated from patients with disseminated gonococcal infection (FA1090 and FA19). FA1090 has been used in the majority of experimental infection studies conducted over the past 20 years [13]; therefore, this isolate was used in the current study to further characterise the bactericidal activity of resazurin against *N. gonorrhoeae*. In disk diffusion assays, *N. gonorrhoeae* was similarly susceptible to resazurin and ampicillin, an antibiotic commonly used to treat gonorrhoea (Fig. 1A). Resorufin, the reduced derivative of resazurin, also inhibited the growth of *N. gonorrhoeae*, however the zones of inhibition were significantly smaller ($P < 0.05$) than those observed with ampicillin at a high dose of this compound (Fig. 1A). In broth culture, resazurin concentrations as low as 2.8 µg/mL resulted in a significant reduction in viable *N. gonorrhoeae* compared with growth medium alone (Fig. 1B; Table 1). To determine how quickly resazurin exhibits an antimicrobial effect on *N. gonorrhoeae*, viable bacteria were measured over time grown in the presence of resazurin and resorufin. Addition of resazurin resulted in a significant decline in viable bacteria within 4 h post-inoculation (Fig. 1C). Moreover, a similar decrease in bacterial viability was observed in cultures treated with resorufin (Fig. 1C). By 24 h post-inoculation, no viable *N. gonorrhoeae* bacteria were detected in cultures treated with resazurin or resorufin.

Neisseria gonorrhoeae invades genital tract epithelial cells; therefore, resazurin must also be able to kill intracellular *N. gonorrhoeae* bacteria in order to be an effective therapeutic. To test this, HEC-1-B cells (human endometrial cell line) were cultured with either *N. gonorrhoeae* strain FA1090 or MS11 and gentamicin was added to kill extracellular bacteria as described previously [10,11]. Cells were then treated with resazurin or resorufin for a period of 6 h or were left untreated. Both resazurin and resorufin treatment resulted in a significant decrease in viable *N. gonorrhoeae* bacteria (Fig. 2). Visible observation of the HEC-1-B cells 6 h post-treatment indicated that resazurin was reduced to resorufin, although this phenomenon was not quantified (data not shown). This result suggested that the cells were still viable and that the combination of resazurin and *N. gonorrhoeae* did not culminate in undesirable toxicity. Collectively, these results suggest that resazurin could have potential for the treatment of gonorrhoea.

3.2. Resazurin has no therapeutic effect in a mouse model of gonorrhoea

Given the promising in vitro data on the antimicrobial activity of resazurin against *N. gonorrhoeae*, we next sought to evaluate its efficacy in vivo. Although *N. gonorrhoeae* is a strict human pathogen, oestradiol-treated female mice can be infected with *N. gonorrhoeae* and murine infection mimics several aspects of human infection [12]. Mice were infected intravaginally with *N. gonorrhoeae* strain MS11 and were then administered resazurin or resorufin intraperitoneally 2 days later at a dose of 11 mg/kg. Mice were treated with the compounds for five consecutive days (Days 0–4 of antibiotic administration) and were then monitored for vaginal colonisation by *N. gonorrhoeae*. There was no significant difference in

the number of mice that cleared the infection following resazurin (5/9 mice) or resorufin (4/8 mice) treatment compared with those treated with vehicle (6/9 mice) by 8 days after the start of treatment administration. All mice treated with a single dose of ceftriaxone (15 mg/kg), an antibiotic used clinically to treat gonorrhoea, cleared the infection within 24 h of treatment (9/9 mice).

3.3. The poor in vivo efficacy of resazurin is due to binding to serum albumin

The robust in vitro bactericidal activity of resazurin against *N. gonorrhoeae* did not translate to effective clearance in a mouse model of gonococcal infection. One possible reason for the poor efficacy of resazurin in vivo is its binding to plasma proteins, specifically serum albumin. Serum albumin binding significantly impairs the antimicrobial activity of a number of β -lactam antibiotics and fluoroquinolones [14–17]. Therefore, we tested whether resazurin would retain its antibacterial properties in the presence of 3 g/dL BSA (near normal physiological levels [18]). Without BSA, almost no viable *N. gonorrhoeae* bacteria were detected 24 h post-inoculation in broth cultures containing resazurin (Fig. 3). Resorufin also significantly reduced the number of bacteria compared with growth medium alone (Fig. 3). In medium supplemented with BSA, however, an equivalent number of viable *N. gonorrhoeae* bacteria was observed in all cultures 24 h post-treatment (Fig. 3). A similar result was observed with all other *N. gonorrhoeae* clinical isolates listed in Table 1 (data not shown). Addition of BSA to the medium did not alter *N. gonorrhoeae* growth over 24 h (Fig. 3). This result suggests that resazurin failed in vivo owing to its interaction with serum albumin.

3.4. Resorufin pentyl ether exhibits anti-*Neisseria gonorrhoeae* activity in the presence of serum albumin

We hypothesised that particular modifications of resazurin may result in an analogue that does not bind serum albumin but retains the anti-*N. gonorrhoeae* activity associated with the parent molecule. Therefore, a variety of commercially available resazurin derivatives were tested for antimicrobial activity in the absence and presence of BSA (Fig. 4A). Both resorufin acetate and resorufin pentyl ether significantly reduced the number of viable *N. gonorrhoeae* bacteria in culture 24 h post-inoculation; however, only resorufin pentyl ether retained its antimicrobial activity when BSA was added (Fig. 4B). As low as 3.1 μ g/mL resorufin pentyl ether was sufficient to inhibit the growth of most *N. gonorrhoeae* clinical isolates tested (Table 1). A third resazurin analogue, resorufin butyrate, did not limit replication of *N. gonorrhoeae*, indicating that certain modifications will inactivate the antimicrobial capacity of resazurin (Fig. 4B).

3.5. Resorufin pentyl ether exhibits in vivo efficacy

Based on the observed antimicrobial activity in the presence of serum albumin (Fig. 4B), resorufin pentyl ether was selected for in vivo testing. Female mice infected intravaginally with *N. gonorrhoeae* strain MS11 were treated with resorufin pentyl ether (15 mg/kg) for five consecutive days beginning 2 days post-infection. By 24 h following the final dose of resorufin pentyl ether, the percentage of mice that had cleared the gonococcal infection was approaching significance (6/9 mice) compared with the vehicle controls (2/9 mice) (Fig. 5A). All mice treated with a single dose of ceftriaxone (15 mg/kg), an antibiotic used

clinically to treat gonorrhoea, cleared the infection within 24 h of treatment (9/9 mice) (Fig. 5A,B). Lower numbers of CFU were recovered in mice treated with resorufin pentyl ether compared with mice treated with the vehicle (DMSO), however this difference was not statistically significant (Fig. 5B). When comparing changes in bacterial burden over time in mice treated with resorufin pentyl ether, a significant reduction in *N. gonorrhoeae* bacteria was observed beginning 4 days following the start of treatment (Fig. 5B). Control treatment with the vehicle (DMSO) did not reduce gonococcal colonisation, whilst the positive control, ceftriaxone, rapidly decreased CFU over time (Fig. 5B). Together, these data provide a 'proof of concept' that structural modifications of resazurin which do not alter the antimicrobial capabilities of the parent molecule but limit interaction with serum albumin have real potential for use to treat gonorrhoea.

4. Discussion

The extensive antibiotic resistance of *N. gonorrhoeae* has severely limited our ability to successfully treat infections with this pathogen. Third-generation cephalosporins are now the sole antimicrobial class recommended for treatment of gonorrhoea. Here we have identified a new group of antimicrobials based on the compound resazurin, which exhibits robust activity against *N. gonorrhoeae* in vitro. Resazurin inhibits the growth of a broad range of *N. gonorrhoeae* isolates, including those resistant to multiple antibiotics. Furthermore, resazurin is capable of killing *N. gonorrhoeae* bacteria residing within genital epithelial cells. The antibacterial activity of this compound is not surprising given its similarity in chemical structure to phenoxazine. Phenoxazine derivatives have been shown to inhibit the growth of a variety of bacterial species, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae* and *Helicobacter pylori* [19,20]. To date, resazurin has only been shown to be antimicrobial against *F. tularensis* and *Neisseria* spp. [8], although its activity against *H. pylori* is not known.

The selective toxicity of resazurin for only *F. tularensis* and *Neisseria* spp. is ideal to limit the development of resistance against this compound in the future. In addition, the narrow spectrum of organisms targeted by resazurin suggests this compound has a unique mechanism of action. Whilst these two bacteria are taxonomically distinct from one another, both possess lipoprotein sorting machinery that is unique from other Gram-negative bacteria [21]. Typically, sorting to the outer membrane is mediated by recognition of mature triacylated lipoproteins by the ABC transporter LolCDE [21]. *Francisella tularensis* and *N. gonorrhoeae* possess a novel ABC transporter, LolFD, that allows for the transport of both diacylated and triacylated proteins to the outer membrane [22]. Resazurin may specifically interact with LolFD, blocking lipoprotein transport, and resulting in death of the bacterium. The effect of resazurin treatment on lipoprotein expression in the outer and inner membrane of *N. gonorrhoeae* is currently being investigated.

Interestingly, resazurin was not effective at limiting vaginal colonisation by *N. gonorrhoeae* in a mouse model of infection. A possible explanation for this is the binding of resazurin to serum albumin. Protein binding significantly reduces the MIC of numerous β -lactam antibiotics and fluoroquinolones [14–17]. In support of this hypothesis, the antimicrobial activity of resazurin was completely abolished when *N. gonorrhoeae* was cultured in the

presence of BSA. Moreover, binding to serum albumin may prevent the penetration of resazurin into host tissues, further limiting its effectiveness in vivo [23]. Another explanation for the poor efficacy of resazurin is modification of this compound during its metabolism by the host. Lipophilic drugs such as resazurin commonly undergo glucuronidation, which involves the addition of glucuronic acid by uridine diphosphate glucuronosyltransferases to convert the compounds into more water-soluble metabolites that can be excreted [24]. Resorufin β -D-glucopyranoside, a compound similar to glucuronidated resazurin, does not inhibit the growth of *N. gonorrhoeae* on chocolate agar (data not shown). Therefore, we hypothesise that modifying resazurin or resorufin to inhibit glucuronidation will increase the in vivo therapeutic efficacy of this drug.

A different resazurin derivative, resorufin pentyl ether, did exhibit bactericidal activity in vitro and in vivo in a mouse model of gonococcal infection. The augmented in vivo activity of this compound compared with resazurin is likely due to its lack of binding to serum albumin. In support of this, resorufin pentyl ether maintains bactericidal activity against *N. gonorrhoeae* in the presence of BSA in vitro. Whilst there was a trend towards a significant reduction in the number of mice colonised with *N. gonorrhoeae* within a day following completion of resorufin pentyl ether treatment, treatment with this compound did not result in complete clearance of the infection. In comparison, no viable *N. gonorrhoeae* bacteria were recovered from mice administered the conventional antibiotic ceftriaxone within 48 h of treatment. However, the total number of vaginal *N. gonorrhoeae* bacteria was significantly reduced over time in mice treated with resorufin pentyl ether beginning 4 days following the initiation of treatment, supporting the potential therapeutic effect of this compound. This reduction in bacterial burden may also have significantly reduced inflammation within the vaginal mucosa. Future studies will focus on comparing cytokine levels and neutrophil infiltrates in *N. gonorrhoeae*-infected mice treated with resorufin pentyl ether to controls.

Whilst resorufin pentyl ether did limit *N. gonorrhoeae* colonisation over time, this treatment was not as effective as ceftriaxone at completely clearing the infection. We are perhaps below the in vivo optimal dose, but attempts to try higher doses were unsuccessful due to insufficient solubility in DMSO (data not shown). Another potential explanation for the suboptimal efficacy of resorufin pentyl ether is that this compound is still being glucuronidated. We are currently working to identify a resazurin analogue that is neither glucuronidated nor binds serum albumin and is more soluble in the hope that we can create a novel antigonococcal agent with robust in vivo activity.

Acknowledgments

The authors thank Stephen Cutler for his helpful insight and suggestions.

Funding: This work was supported by a National Institutes of Health (NIH) grant [5P20RR016477] to the West Virginia IDeA Network for Biomedical Research Excellence (WV-INBRE), a grant from WV-INBRE [P20GM103434], a grant from the National Institute of Allergy and Infectious Diseases (NIH-NIAID) [5K22AI087703], and funding from the WV-NASA Space Grant Consortium.

References

1. World Health Organization. Sexually transmitted infections (STIs). Geneva, Switzerland: WHO; 2015.

2. US Centers for Disease Control and Prevention. Gonorrhea—CDC fact sheet (detailed version). Atlanta, GA: CDC; 2015.
3. Unemo M, Shafer WM. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st century: past, evolution, and future. Clin Microbiol Rev. 2014; 27:587–613. [PubMed: 24982323]
4. Lewis DA. The gonococcus fights back: is this time a knock out? Sex Transm Infect. 2010; 86:415–421. [PubMed: 20656721]
5. Unemo M, Shafer WM. Antibiotic resistance in *Neisseria gonorrhoeae*: origin, evolution, and lessons learned for the future. Ann N Y Acad Sci. 2011; 1230:E19–E28. [PubMed: 22239555]
6. Centers for Disease Control and Prevention (CDC). Update to CDC's sexually transmitted diseases treatment guidelines, 2006: fluoroquinolones no longer recommended for treatment of gonococcal infections. MMWR Morb Mortal Wkly Rep. 2007; 56:332–336. [PubMed: 17431378]
7. Unemo M, Nicholas RA. Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. Future Microbiol. 2012; 7:1401–1422. [PubMed: 23231489]
8. Schmitt DM, O'Dee DM, Cowan BN, Birch JW, Mazzella LK, Nau GJ, et al. The use of resazurin as a novel antimicrobial agent against *Francisella tularensis*. Front Cell Infect Microbiol. 2014; 3:93. [PubMed: 24367766]
9. Jerse AE, Crow ET, Bordner AN, Rahman I, Cornelissen CN, Moench TR, et al. Growth of *Neisseria gonorrhoeae* in the female mouse genital tract does not require the gonococcal transferrin or hemoglobin receptors and may be enhanced by commensal lactobacilli. Infect Immun. 2002; 70:2549–2558. [PubMed: 11953395]
10. Shaw JH, Falkow S. Model for invasion of human tissue culture cells by *Neisseria gonorrhoeae*. Infect Immun. 1988; 56:1625–1632. [PubMed: 3131248]
11. Minor SY, Banerjee A, Gotschlich EC. Effect of α -oligosaccharide phenotype of *Neisseria gonorrhoeae* strain MS11 on invasion of Chang conjunctival, HEC-1-B endometrial, and ME-180 cervical cells. Infect Immun. 2000; 68:6526–6534. [PubMed: 11083761]
12. Jerse AE. Experimental gonococcal genital tract infection and opacity protein expression in estradiol-treated mice. Infect Immun. 1999; 67:5699–5708. [PubMed: 10531218]
13. Hobbs MM, Sparling PF, Cohen MS, Shafer WM, Deal CD, Jerse AE. Experimental gonococcal infection in male volunteers: cumulative experience with *Neisseria gonorrhoeae* strains FA1090 and MS11mkC. Front Microbiol. 2011; 2:123. [PubMed: 21734909]
14. Zeitlinger MA, Sauermann R, Traunmuller F, Georgopoulos A, Muller M, Joukhadar C. Impact of plasma protein binding on antimicrobial activity using time–killing curves. J Antimicrob Chemother. 2004; 54:876–880. [PubMed: 15472003]
15. Schmidt S, Rock K, Sahre M, Burkhardt O, Brunner M, Lobmeyer MT, et al. Effect of protein binding on the pharmacological activity of highly bound antibiotics. Antimicrob Agents Chemother. 2008; 52:3994–4000. [PubMed: 18779351]
16. Jones RN, Barry AL. Antimicrobial activity of ceftriaxone, cefotaxime, desacetylcefotaxime, and cefotaxime–desacetylcefotaxime in the presence of human serum. Antimicrob Agents Chemother. 1987; 31:818–820. [PubMed: 3606081]
17. Zeitlinger M, Sauermann R, Fille M, Hausdorfer J, Leitner I, Muller M. Plasma protein binding of fluoroquinolones affects antimicrobial activity. J Antimicrob Chemother. 2008; 61:561–567. [PubMed: 18216178]
18. Oren R, Dabeva MD, Petkov PM, Hurston E, Laconi E, Shafritz DA. Restoration of serum albumin levels in nagase analbuminemic rats by hepatocyte transplantation. Hepatology. 1999; 29:75–81. [PubMed: 9862853]
19. Agbo SA, Igbum GO, Anoh VA, Swande PI. Synthesis and in vitro antimicrobial activity of some novel azaphenoxazine carboxamide derivatives. IOSR J Appl Chem. 2015; 8:21–25.
20. Hanawa T, Osaki T, Manzoku T, Fukuda M, Kawakami H, Tomoda A, et al. In vitro antibacterial activity of Phx-3 against *Helicobacter pylori*. Biol Pharm Bull. 2010; 33:188–191. [PubMed: 20118538]
21. Okuda S, Tokuda H. Lipoprotein sorting in bacteria. Annu Rev Microbiol. 2011; 65:239–259. [PubMed: 21663440]
22. LoVullo ED, Wright LF, Isabella V, Huntley JF, Pavelka MS Jr. Revisiting the Gram-negative lipoprotein paradigm. J Bacteriol. 2015; 197:1705–1715. [PubMed: 25755189]

23. Zeitlinger MA, Derendorf H, Mouton JW, Cars O, Craig WA, Andes D, et al. Protein binding: do we ever learn? *Antimicrob Agents Chemother*. 2011; 55:3067–3074. [PubMed: 21537013]
24. Kaivosaaari S, Finel M, Koskinen M. *N*-glucuronidation of drugs and other xenobiotics by human and animal UDP-glucuronosyltransferases. *Xenobiotica*. 2011; 41:652–669. [PubMed: 21434773]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Highlights

- Resazurin is effective against *Neisseria gonorrhoeae* in vitro but not in vivo.
- Serum albumin likely inactivates resazurin in vivo.
- Resorufin pentyl ether (RPE) exhibits activity in the presence of serum albumin.
- RPE reduces vaginal colonisation by *N. gonorrhoeae* over time.
- Resazurin-based therapies could have potential for the treatment of gonorrhoea.

Fig. 1.

Resazurin and resorufin have an antimicrobial effect on *Neisseria gonorrhoeae*. (A) *Neisseria gonorrhoeae* FA1090 bacteria were spread onto chocolate II agar plates and were subjected to disk diffusion assays with the indicated antibiotics. Data shown are the mean $\pm$ standard error of the mean (SEM) from three individual experiments. Statistically significant differences in zones of inhibition were determined by two-way analysis of variance (ANOVA) followed by Bonferroni's comparison of means (* $P < 0.05$ for ampicillin vs. resorufin). (B,C) *Neisseria gonorrhoeae* FA1090 was cultivated in fastidious broth with or without resazurin or resorufin at the designated concentrations. *Neisseria gonorrhoeae* cultures were diluted and plated to determine the number of viable bacteria at 24 h (B) or at the indicated time points (C). Data shown are mean $\pm$ SEM from three individual experiments. The limit of detection was 100 CFU/mL. Statistically significant differences in growth post-inoculation were determined by (B) one-way ANOVA followed by Dunnett's post-hoc test (***) $P < 0.001$ compared with tryptic soy broth supplemented with cysteine) or (C) two-way ANOVA followed by Bonferroni's comparison of means ($P < 0.01$ for control vs. resazurin and for control vs. resorufin for 4–24 h).

Fig. 2.

Resazurin kills intracellular *Neisseria gonorrhoeae* in an infection model. HEC-1-B cells were infected with *N. gonorrhoeae* FA1090 or MS11 using a gentamicin protection assay. Cells were then treated with 11 $\mu\text{g/mL}$ resazurin or resorufin for 6 h or were left untreated. Data shown are the mean $\pm$ standard error of the mean (SEM) and are representative of three independent experiments. The limit of detection was 100 CFU per 2×10^5 cells. Statistically significant differences in growth at 24 h post-infection were determined by two-way analysis of variance (ANOVA) followed by Bonferroni's comparison of means (** $P < 0.01$ for control vs. resazurin and for control vs. resorufin).

Fig. 3.

Serum albumin completely suppresses the bactericidal activity of resazurin against *Neisseria gonorrhoeae*. *Neisseria gonorrhoeae* FA1090 was cultivated in fastidious broth supplemented with or without bovine serum albumin (BSA) (3 g/dL). *Neisseria gonorrhoeae* cultures were treated with resazurin (11 µg/mL) or resorufin (10 µg/mL). After 24 h of incubation, cultures were diluted and were plated to determine the number of viable bacteria. Data shown are the mean ± standard error of the mean (SEM) from three individual experiments. The limit of detection was 100 CFU/mL. Statistically significant differences in growth post-inoculation were determined by two-way analysis of variance (ANOVA) followed by Bonferroni's comparison of means (***) $P < 0.001$ for control vs. resazurin and for control vs. resorufin).

Fig. 4.

Resorufin pentyl ether maintains antimicrobial activity against *Neisseria gonorrhoeae* in the presence of bovine serum albumin (BSA). (A) Structures of resorufin acetate (RA), resorufin butyrate (RB) and resorufin pentyl ether (RPE). (B) *Neisseria gonorrhoeae* FA1090 was grown in fastidious broth supplemented with or without BSA (3 g/dL). *Neisseria gonorrhoeae* cultures were treated with RA (11 $\mu\text{g/mL}$), RB (12.5 $\mu\text{g/mL}$), RPE (12.5 $\mu\text{g/mL}$) or vehicle control [dimethyl sulfoxide (DMSO)]. After 24 h of incubation, cultures were diluted and were plated to determine the number of viable bacteria. Data shown are the mean $\pm$ standard error of the mean (SEM) from three individual experiments. The limit of detection was 100 CFU/mL. Statistically significant differences in growth post-inoculation were determined by two-way analysis of variance (ANOVA) followed by Bonferroni's comparison of means (** $P < 0.01$ for control vs. select resazurin analogue).

Fig. 5.

Treatment of *Neisseria gonorrhoeae*-infected mice with resorufin pentyl ether (RPE) reduces the bacterial burden and the percentage of mice colonised. Oestradiol-treated female mice were infected intravaginally with *N. gonorrhoeae* strain MS11 and were then administered RPE (15 mg/kg) intraperitoneally 2 days later (antibiotic treatment Day 0). As positive and negative controls, mice were administered either ceftriaxone (CRO) (15 mg/kg in water, single dose on Day 0) or vehicle control [50% dimethyl sulfoxide (DMSO), five consecutive days], respectively. Mice were treated with the compound for five consecutive days (Days 0–4 of antibiotic administration, arrows) and then vaginal swabs were quantitatively cultured to determine the number of viable bacteria. Data shown are (A) the percentage of mice colonised with *N. gonorrhoeae* and (B) the mean bacterial burden over the course of 8 days following the start of treatment administration. (A) Changes in the percentage of mice colonised with *N. gonorrhoeae* were analysed using a Kaplan–Meier survival curve; log-rank (Mantel–Cox), $P = 0.077$ RPE vs. DMSO; $P < 0.0001$ CRO vs. DMSO. (B) The mean bacterial burden (CFU/mL) recovered by daily culture is shown for each treatment group. Error bars indicate the standard error of the mean (SEM). Differences in the mean number of *N. gonorrhoeae* recovered (CFU/mL) over the course of infection were determined by repeated measures two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test as a post-hoc analysis comparing the change in bacterial burden between treatment versus control groups ($P = 0.2347$, RPE vs. DMSO; $P = 0.0004$, CRO vs. DMSO) and over time for each treatment (* $P < 0.05$ CFU compared with Day 0).

Table 1Minimum inhibitory concentrations (MICs) for *Neisseria gonorrhoeae* strains used in this study

Strain	Description	MIC (µg/mL)	
		Resazurin	Resorufin pentyl ether
FA1090	Isolated from patient with disseminated gonococcal infection. Resistant to streptomycin	2.8	3.1
LGB-24	Isolated from urogenital tract. Resistant to tetracycline and penicillin; not a β -lactamase producer	2.8	6.2
NG886	Resistant to penicillin, tetracycline and fluoroquinolones	2.8	3.1
MS11	Isolated from a case of cervicitis. Overexpresses the MtrCDE multidrug efflux pump; resistant to azithromycin and penicillin	2.8	6.2
FA19	Isolated from patient with disseminated gonococcal infection	2.8	3.1
LG-16	Isolated from urogenital tract. Resistant to penicillin, tetracycline and azithromycin; β -lactamase producer	2.8	3.1