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Short communication

Resveratrol is cidal to both classes of *Haemophilus ducreyi*

Erin M. Nawrocki, Hillary W. Bedell, Tricia L. Humphreys*

Allegheny College Department of Biology, 520N. Main St., Meadville, PA 16335, USA

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ABSTRACT

Resveratrol, a polyphenolic phytoalexin, is produced by plants in response to infection and has antibacterial activity. *Haemophilus ducreyi* is a Gram-negative bacterium that is the causative agent of the sexually transmitted disease chancroid. This study employed minimum cidal concentration (MCC) assays to evaluate the potential of resveratrol as a microbicide against *H. ducreyi*. Five class I and four class II strains of *H. ducreyi* tested had MCCs ≤500 μg/mL. Resveratrol was also tested against *Lactobacillus* spp., part of the natural vaginal flora. Representative strains of *Lactobacillus* were co-cultured with *H. ducreyi* and 500 μg/mL resveratrol; in all cases, *Lactobacillus* was recovered in greater numbers than *H. ducreyi*. These results show that resveratrol is not only bacteriostatic but is bactericidal to *H. ducreyi*, confirming the compound's potential for use as a topical microbicide to prevent chancroid.

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a polyphenolic phytoalexin produced by a variety of flowering plants in response to unfavourable environmental conditions such as fungal infection or injury [1]. It is commonly found in dietary products, including peanuts, grapes and red wine [2]. Resveratrol has demonstrated antimicrobial activity against numerous Gram-negative and Gram-positive bacterial pathogens, including carcinogenic strains of *Helicobacter pylori* [2,3]. The antifungal and antiviral properties of resveratrol have also been demonstrated [4,5]. The compound is thought to work by inhibiting the activity of ATPases, thus preventing the production or utilisation of energy [3].

Haemophilus ducreyi is a fastidious Gram-negative bacillus and is the aetiological agent of chancroid, a sexually transmitted genital ulcer disease [6]. *Haemophilus ducreyi* strains are grouped into two classes based on their outer membrane components [7]. Chancroid facilitates the acquisition and transmission of human immunodeficiency virus type 1 (HIV-1) [6]. Like other sexually transmitted infections (STIs), chancroid can interact with HIV via genital ulcers, which increase the infectiousness of HIV-positive individuals and the susceptibility of HIV-negative individuals [8]. Chancroid is endemic to regions of Africa and Asia, but because these areas lack diagnostic tests for *H. ducreyi* the actual prevalence of chancroid is unknown [6].

In recent decades, strains of *H. ducreyi* have acquired plasmid-mediated mechanisms of resistance to several classes of antibiotics [9]. The recommended treatment regimens for chancroid rely on azithromycin, ceftriaxone, ciprofloxacin or erythromycin, but *H. ducreyi* isolates with intermediate resistance to either ciprofloxacin or erythromycin have been identified [10]. Although a single dose of azithromycin (1 g orally) or ceftriaxone (250 mg intramuscularly) remains effective in treating chancroid, their high costs evoke concerns that inadequate doses will be taken, supporting the selection of resistance to these drugs [8]. The rise of antibiotic resistance and the prevalence of chancroid in resource-poor environments have necessitated the development of alternative treatment and prevention strategies.

Unlike antibiotics, microbicides prevent transmission rather than treating infection; microbicides must also reduce a microbe's infectivity within minutes rather than days [11]. An ideal microbicide would prevent transmission of pathogens while maintaining an environment of healthy normal flora [11]. In most women, the vaginal environment is dominated by the genus *Lactobacillus*, including species such as *Lactobacillus crispatus*, *Lactobacillus jensenii*, *Lactobacillus gasseri*, *Lactobacillus iners* and *Lactobacillus vaginalis* [12]. In addition, a microbicide designed to prevent STIs should have activity against multiple STI-causing pathogens. Resveratrol has been shown to selectively inhibit the growth of *Neisseria gonorrhoeae*, the causative agent of gonorrhoea, although Docherty et al. did not test resveratrol against natural vaginal flora [13].

This study tested the microbicidal activity of resveratrol against both classes of *H. ducreyi* as well as several of the most common vaginal strains of *Lactobacillus*. Minimum cidal concentration (MCC) assays were performed on monocultures and co-cultures of *H. ducreyi* and lactobacilli to assess the viability of resveratrol as a microbicide for chancroid prevention. Resveratrol was found to be

* Corresponding author. Tel.: +1 814 332 2967; fax: +1 814 332 2789.
E-mail address: thumphre@allegheny.edu (T.L. Humphreys).

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more bactericidal to *H. ducreyi* than to lactobacilli, recommending its use as a topical microbicide for chancroid prevention.

2. Materials and methods

2.1. Resveratrol

Trans-resveratrol (MegaResveratrol®; Candlewood Stars Inc., Danbury, CT) was dissolved in ethanol and stored protected from light at 4 °C until use. To prevent isomerisation to *cis*-resveratrol, which can occur after 30 days under fluorescent lighting, fresh stocks of resveratrol solutions were made monthly [1].

2.2. Bacterial cultures

The following strains of *H. ducreyi* (kindly provided by Stan Spinola, Indiana University School of Medicine, Indianapolis, IN) were used in this study (class in parentheses): 35000HP (I); HMC56 (I); HD85-023233 (I); HD188 (I); HD183 (I); HMC112 (II); DMC64 (II); 33921 (II); and CIP542ATCC (II). The following vaginal *Lactobacillus* isolates (kindly provided by Sharon Hillier, University of Pittsburgh, Pittsburgh, PA) were also used: *L. vaginalis* Lac08(vl)-5 7/14/04; and *L. vaginalis* Lac11(vl)-b 7/14/04. One gastrointestinal *Lactobacillus* strain (*Lactobacillus reuteri* CF48-3A HM-102) was obtained from BEI Resources (Manassas, VA).

All *H. ducreyi* strains were cultured on chocolate agar (GC Agar Base, 1% haemoglobin; Alpha Biosciences, Baltimore, MD) supplemented with 1% IsoVitaleX™ (Becton Dickinson, Franklin Lakes, NJ) at 33 °C in a 10% CO_2 incubator. Lactobacilli strains were grown on de Man–Rogosa–Sharpe *Lactobacillus* agar (Becton Dickinson) at 35 °C in a 5% CO_2 incubator. Co-cultures were grown on chocolate agar at 35 °C in a 5% CO_2 incubator. All cultures were revived from frozen stocks and were subcultured once before an assay was performed.

2.3. Minimum cidal concentration assay

An MCC assay was used to determine the microbicidal activity of resveratrol against various strains of bacteria [11]. Bacteria were suspended in phosphate-buffered saline (PBS) to match the turbidity of a 0.5 McFarland standard. The suspension was then diluted 1:10 in PBS. Resveratrol was added to brain–heart infusion (BHI) medium (EMD Millipore, Billerica, MA) supplemented with 0.1% potato starch (Difco Laboratories, Detroit, MI), 5% haemin (Spectrum Chemicals and Laboratory Products, New Brunswick, NJ) and 1% IsoVitaleX™ (Becton Dickinson) to give a test solution of the desired concentration of resveratrol, and 80 μL was added to each well of a 96-well microtitre plate (USA Scientific, Ocala, FL). In monocultures, 20 μL of the bacterial suspension was added to each well to yield a total volume of 100 μL per well. In co-cultures, 10 μL of the *H. ducreyi* suspension and 10 μL of the *Lactobacillus* suspension were added to each well.

After 30 min of incubation, an aliquot from each well was serially diluted ten-fold in saline and the dilutions were plated and incubated for 48 h. Colonies were then counted to determine CFU/mL. The log (CFU/mL) of the resveratrol test solution was compared with the log (CFU/mL) of the bacterial solution without resveratrol added. The MCC was defined as the lowest concentration causing a 4-log (99.99%) decrease in viable bacteria. Because 500 μg/mL resveratrol was required for cidal activity against class I *H. ducreyi*, this concentration was used for all co-culture assays.

Table 1
Minimum cidal concentration (MCC) of resveratrol against *Haemophilus ducreyi* strains.

Strain	Class	MCC (μg/mL)[a]
35000HP	I	500
HMC56	I	500
HD85-023233	I	500
HD188	I	500
HD183	I	500
HMC112	II	250
33921	II	250
DMC64	II	250
CIP542ATCC	II	250

[a] The MCC is the lowest concentration required to cause a 4-log decrease in viable bacteria relative to a control without resveratrol after 30 min of exposure; nine replicates for all strains except CIP542ATCC, which had six replicates.

3. Results and discussion

Class II strains were more susceptible to resveratrol than class I strains (Table 1). A control solution of 5% ethanol in BHI–starch complete medium was tested to confirm that the decrease in viable bacteria was due to resveratrol and not its solvent; in no case did ethanol cause more than a 1-log decrease in CFU/mL (data not shown).

Because class I *H. ducreyi* required 500 μg/mL resveratrol to be killed in vitro, this concentration was tested against species of the *Lactobacillus* genus to determine any deleterious effects a microbicide might have on natural flora. Each of the three *Lactobacillus* strains in this study was treated with 500 μg/mL resveratrol in monoculture; in no case did the treatment cause a decrease >2-log (data not shown). The *Lactobacillus* strains were then co-cultured with *H. ducreyi* to determine the interaction effects among the two species and the resveratrol test solution. The results were consistent with those of the monocultures, with *H. ducreyi* being more susceptible than *Lactobacillus* to the cidal effects of resveratrol. Except in the case of the HMC112 co-cultures, 500 μg/mL resveratrol was cidal (i.e. caused a 4-log decrease) to *H. ducreyi* but had very little effect on lactobacilli, causing <1-log decrease (Table 2). When co-cultured with HMC112, the two *Lactobacillus* strains were more susceptible to resveratrol, but neither decreased >4-log relative to the untreated control (Table 2).

One explanation for altered susceptibilities to resveratrol is differential production of ATPases, as hypothesised by Martini et al. [3]. Certain *H. pylori* strains use ATPases to adapt to acidic conditions, whilst strains colonising a less acidic environment may be more vulnerable to resveratrol because of reduced ATPase production [3]. Lactobacilli, being accustomed to the low vaginal pH, may employ ATPases in a similar strategy. If *H. ducreyi* expresses fewer ATPases than *Lactobacillus* spp., this could account for its greater susceptibility to resveratrol.

Although these *Lactobacillus* strains are a few of many that may make up the vaginal environment, their lower susceptibility to resveratrol supports the compound's potential as a microbicide for preventing STIs. Variation in the cidal and inhibitory activity of resveratrol against lactobacilli suggests that testing a broader sample of natural flora strains could be beneficial.

Resveratrol has previously been described as an antimicrobial compound based on its minimum inhibitory concentrations (MICs) against other organisms. The MICs of resveratrol against the Gram-positive species *Bacillus cereus*, *Staphylococcus aureus* and *Enterococcus faecalis* ranged from 50 μg/mL to 200 μg/mL [2]. Paulo et al. were unable to determine the MICs of Gram-negative bacteria, which exceeded 400 μg/mL, because resveratrol was insoluble in their culture medium above this concentration [2]. The current study achieved higher concentrations of resveratrol in vitro and was able to confirm the compound's antimicrobial activity against

Table 2
Log decrease of resveratrol-treated organisms relative to untreated control[a].

Organisms co-cultured		Log decrease (mean ± S.D.; $n = 9$)	
Haemophilus ducreyi	*Lactobacillus* spp.	*H. ducreyi*	*Lactobacillus* spp.
35000HP	*L. vaginalis* 08	8.59 ± 0.31	0.01 ± 0.42
	L. vaginalis 11	8.67 ± 0.50	0.01 ± 0.39
	L. reuteri	8.54 ± 0.92	0.57 ± 0.51
HMC56	*L. vaginalis* 08	7.92 ± 0.87	−0.25 ± 0.87
	L. vaginalis 11	7.81 ± 1.04	0.35 ± 0.67
	L. reuteri	8.67 ± 0.91	−0.39 ± 0.41
HD85-023233	*L. vaginalis* 08	8.59 ± 0.31	0.23 ± 0.52
	L. vaginalis 11	9.46 ± 0.13	0.34 ± 0.61
	L. reuteri	8.83 ± 0.26	0.05 ± 0.86
HD188	*L. vaginalis* 08	8.33 ± 0.50	−0.76 ± 0.73
	L. vaginalis 11	8.48 ± 0.64	0.23 ± 0.68
	L. reuteri	8.67 ± 0.50	0.34 ± 0.49
HD183	*L. vaginalis* 08	7.97 ± 1.03	0.15 ± 0.37
	L. vaginalis 11	8.33 ± 1.00	−0.11 ± 0.59
	L. reuteri	7.59 ± 0.78	0.28 ± 0.75
HMC112	*L. vaginalis* 08	3.97 ± 0.00	2.55 ± 0.34
	L. vaginalis 11	4.03 ± 0.00	3.30 ± 0.07
DMC64	*L. vaginalis* 08	6.27 ± 0.00	−0.22 ± 0.16
	L. vaginalis 11	6.61 ± 0.00	−0.28 ± 0.39

S.D., standard deviation.
[a] Co-cultures were exposed to 500 μg/mL resveratrol for 30 min. One *H. ducreyi* species and one *Lactobacillus* sp. were co-cultured in each assay. Decreases of ≥4 log are considered cidal.

H. ducreyi. In addition, we show that unlike traditional antibiotics, which have much lower MICs against *H. ducreyi* but are designed to treat rather than prevent infections [9], resveratrol is not only inhibitory but is bactericidal to *H. ducreyi*.

Future research should consider the efficacy and toxicity of resveratrol when present in the vaginal environment. A microbicide should remain active in the presence of blood and/or semen, which may be present in the vagina as a result of infection, menstruation or intercourse and which change the local pH [14]. Furthermore, in the past, microbicidal candidates have demonstrated cytotoxicity to epithelial cells and irritation to the mucus membrane [15]. If resveratrol is to be used as a microbicide applied directly to the vaginal wall, its effects in vivo must be addressed.

Microbicides are a promising method of STI and HIV prevention because they are female-controlled. Although the female condom is one such current and effective method of STI prevention, its disadvantages include high cost, difficulty of use, pre-planning intercourse, and reaction of partners [8]. Microbicides present a method of protection that may not require the knowledge or acceptance of a partner. The prevalence of coercive sexual intercourse where it is difficult for women to negotiate for safer sex necessitates an inconspicuous female-controlled method of protection against *H. ducreyi* infection and HIV.

These in vitro results indicate that a solution of 500 μg/mL resveratrol is cidal to both classes of *H. ducreyi* but not to three representative species of *Lactobacillus*. Assays with co-cultures of *H. ducreyi* and lactobacilli indicated similar results as the monocultures of each species, indicating that the killing of *H. ducreyi* by resveratrol is not affected by the presence of lactobacilli and affirming the potential of resveratrol as a topical microbicide for the prevention of chancroid.

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