Biochemistry and Biophysics Reports 33 (2023) 101427

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Antibacterial activity of 18β-glycyrrhetinic acid against *Neisseria gonorrhoeae* in vitro

Yuanyuan Zhao *, Xiaohong Su

Sexually Transmitted Disease Clinic, Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing, China

ARTICLE INFO

Keywords:
Neisseria gonorrhoeae
GRA
Antimicrobial
Anti-biofilm

ABSTRACT

Gonorrhea is the second most common sexually transmitted diseases worldwide. Chronic infection of *Neisseria gonorrhoeae* (*N. gonorrhoeae*) can lead to severe complications. Presently, *N. gonorrhoeae* has developed resistance to almost all antibiotics used for the treatment of gonorrhea. Thus, it's urgent to explore new approaches to treat gonorrhea. Presently, nontraditional treatment method as an alternative to antibiotic use is getting more and more attention. Here we demonstrated that 18β-glycyrrhetinic acid (GRA) exhibited robust antimicrobial activity against *N. gonorrhoeae* in vitro. GRA led to a significant decline in viable *N. gonorrhoeae* in a dose dependent manner compared with DMSO treatment ($P < 0.001$). Addition of GRA resulted in a significant reduction in viable bacteria within 2 h post-inoculation ($P < 0.001$). Minimum inhibitory concentrations (MICs) to GRA ranged from 3.9 to 62.5 μg/ml overall, with MIC50 and MIC90 values of 31.25 μg/ml and 62.5 μg/ml, respectively. There was no significant difference of MIC 50 and MIC90 between multi-drug resistant (MDR) strains and non-MDR strains. Minimum bactericidal concentration (MBC) ranges were 3.9–125 μg/ml, basically consistent with MIC values. GRA inhibited biofilm formation and diminished pre-formed biofilm. These data suggested that GRA could be a candidate for gonorrhea treatment.

1. Introduction

Gonorrhea is the second most common bacterial sexually transmitted infection (Chlamydia is first), with a worldwide estimated incidence of new gonococcal infections in 2020 of 82.4 million cases [1]. If untreated, gonorrhea could result in severe complications, such as pelvic inflammatory disease and infertility [2]. However, *N. gonorrhoeae* has developed resistance against all antibiotics used for the treatment of gonorrhea, raising the possibility that in the future, gonorrhea may become untreatable [3]. Thus, it's urgent to explore new treatment for gonorrhea.

Presently, nontraditional treatment methods as an alternative to antibiotic use is getting more and more attention, among which, glycyrrhizin (GA) is of special importance. GA is a triterpenoid saponin isolated from Glycyrrhiza spp., of the licorice family and is considered metabolically inactive. GA is hydrolyzed to its biologically active metabolite 18β-glycyrrhetinic acid (GRA) in vivo [4] and GRA has been demonstrated to have antiviral, anti-inflammatory and antioxidant activities [5–8]. In addition, GRA has now been confirmed to have antibacterial effects against *Staphylococcus epidermidis*, *methicillin-resistant Staphylococcus aureus (MRSA)* and *Pseudomonas aeruginosa* [9–11]. However, the antimicrobial activity of GRA against *Neisseria gonorrhoeae* remains unknown. Now, our study aims to demonstrate the activity of GRA against clinical isolates of *N. gonorrhoeae* collected from 2019 to 2020 in Nanjing, China in vitro.

2. Materials and methods

2.1. Bacterial isolates

Cotton swabs were used to collect urethral or cervical exudates from the patients attending the STD Clinic at the Institute of Dermatology, Chinese Academy of Medical Sciences, Nanjing, China. Then the swabs were plated onto Thayer-Martin medium (Zhuhai DL Biotech, China) immediately and cultured in candle jars at 36 °C for 24–48 h. Once bacterial colony formed, the colony morphology, oxidase testing and Gram's stain were used to identify *N. gonorrhoeae*. Gonococcal colonies were then cultured on GC chocolate agar base (Difco, Detroit, MI) with 1% IsoVitaleX (Oxoid, USA). Antimicrobial susceptibility testing was then performed. According to the criteria proposed by Tapsall et al., in

* Corresponding author.
E-mail address: zyy1014@pumcderm.cams.cn (Y. Zhao).

https://doi.org/10.1016/j.bbrep.2023.101427
Received 25 November 2022; Received in revised form 4 January 2023; Accepted 9 January 2023

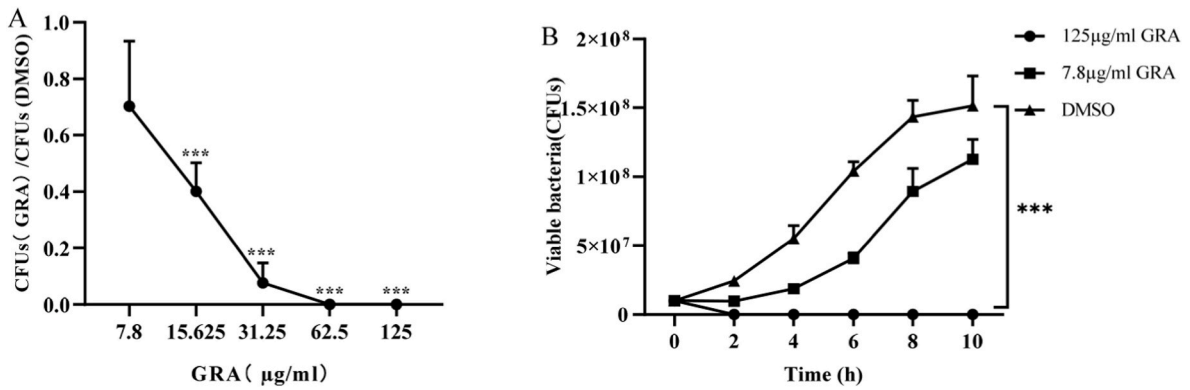

Fig. 1. GRA inhibited *N. gonorrhoeae* growth. (A) *N. gonorrhoeae* growth was decreased in a dose dependent manner upon treated with GRA. Results were expressed as percentage of viable bacteria from aliquots of GRA-treated bacteria with respect to viable bacteria from aliquots of DMSO-treated bacteria after 6 h incubation. (B) CFUs recovered every 2-h following incubation of 10^7 CFUs *N. gonorrhoeae* with varied concentrations of GRA over a 10-h time course. The error bars indicated the standard deviations. n = 3, ***P < 0.001, ANOVA test followed by Bonferroni's multiple comparison test.

2009 [12], MDR isolates were defined as those resistant or with decreased susceptibility to one or more widely used antimicrobials (ceftriaxone and cefixime) and resistant to two or more antimicrobials which are used less frequently (penicillin, ciprofloxacin and azithromycin).

2.2. Growth inhibitory assay

N. gonorrhoeae strains in logarithmic growth phase were incubated in a 96-well tissue culture plate (Corning, USA) with varied concentrations of GRA (ACMEC, China). After 6 h incubation, cultures were diluted and plated onto chocolate agar plates. Colony-forming units (CFUs) were then photographed and enumerated after colony formation. For the growth curves, *N. gonorrhoeae* strains were treated with 125 μg/ml, 7.8 μg/ml GRA or DMSO (sigma, Germany). CFUs values were then enumerated every 2 h over a 12 h time course.

2.3. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

MIC and MBC were determined via broth microdilution method according to the previous study [13]. *N. gonorrhoeae* strains were resuspended in varied concentrations of GRA ranging from 3.9 to 125 μg/ml in a 96-well tissue culture plate. The MIC was defined as the lowest concentration of GRA that inhibited visible growth of *N. gonorrhoeae*. The MBC was recorded as the lowest concentration of GRA that killed at least 99.9% *N. gonorrhoeae*.

2.4. Inhibition of biofilm formation

FA19 and clinical *N. gonorrhoeae* isolates in GCB liquid medium were incubated with varied concentrations of GRA (1/8 MIC, 1/4 MIC, 1/2 MIC and MIC) or DMSO in 96-welltissue culture plates (corning, USA). After incubated for 24 h, wells were washed twice with phosphate buffer saline (PBS) solutions, stained with Crystal Violet (Beyotime, China) and then measured at 570 nm [14]. Relative biofilm formation was calculated as percentage of biofilm formed with respect to DMSO control.

2.5. Biofilm dispersion assay

When biofilms were formed after 24 h, 100 μL of GRA with varied concentrations (1/4 MIC, 1/2 MIC, MIC, 2MIC and 4MIC) or DMSO were added to biofilm wells. Proteinase K (0.8 μg/ml, ACMEC, China) was treated as positive control. After incubated for 6 h at 37 °C, the wells were washed twice with PBS and the remaining biofilms were examined as described above [14].

2.6. Statistical analyses

Statistical analyses were performed using SPSS 23.0. One-way analysis of variance (ANOVA) with Bonferroni's multiple comparison tests were used to determine the level of significance. Significance was defined as P < 0.05 for all comparisons.

3. Results

3.1. Effects of GRA on the survival of N. gonorrhoeae in vitro

To analyze the antimicrobial effects of GRA against *N. gonorrhoeae*, we incubated *N. gonorrhoeae* (FA19 and two clinical isolated strains) for 6 h with varied concentrations of GRA or DMSO (as control wells). The results showed that when treated with no less than 15.625 μg/ml concentrations of GRA, the number of CFUs were decreased significantly (Fig. 1A). In addition, the effect of GRA on the survival of *N. gonorrhoeae*

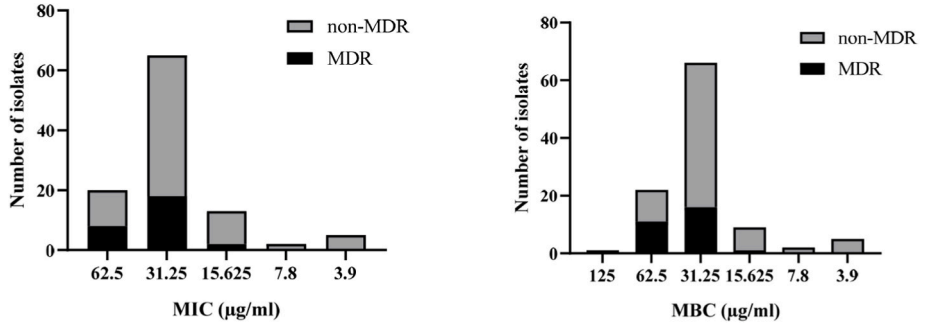

Fig. 2. MIC distribution of GRA for 105 clinical *N. gonorrhoeae* isolates. MIC50 and MIC90 values of MDR and non-MDR strains showed no difference.

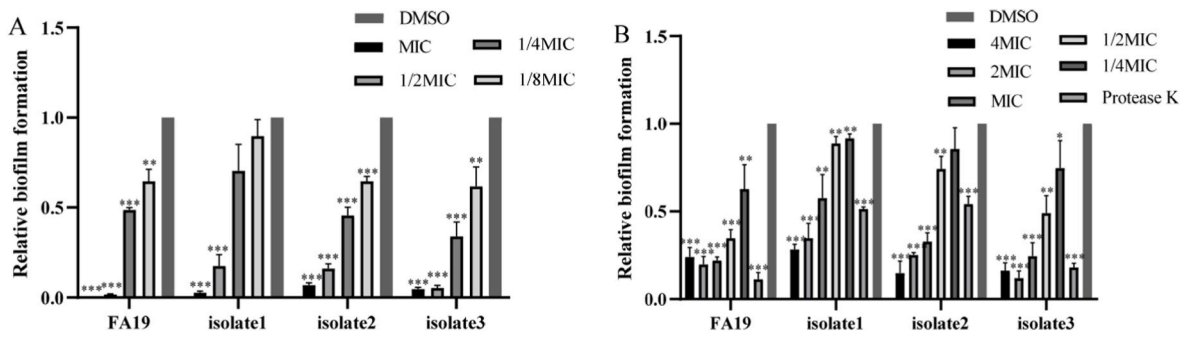

Fig. 3. Effect of GRA on biofilm formation and dispersal. (A) GRA inhibited biofilm formation in a dose-dependent manner. (B) GRA could disperse pre-formed biofilm in the presence of both sub-MIC and MIC levels of GRA. The error bars indicated the standard deviations between strains. Control bars indicate *N. gonorrhoeae* biofilms with DMSO, set as 100%. N = 3, *P < 0.05, **P < 0.01, ***P < 0.001, ANOVA test followed by Bonferroni's multiple comparison test.

displayed a dose-dependent patten. To investigate how quickly GRA exhibited an antimicrobial effect against *N. gonorrhoeae*, CFUs were measured over a 12 h time course in the presence of 125 μg/ml GRA, 7.8 μg/ml GRA or DMSO. It was shown that the survival rates of *N. gonorrhoeae* were significantly declined within 2 h post-inoculation when treated with 125 μg/ml GRA (Fig. 1B). Thus, GRA had an antimicrobial activity against *N. gonorrhoeae* in vitro.

3.2. Determination of MIC and MBC

Susceptibilities (MICs) of N. gonorrhoeae to GRA were confirmed with broth microdilution method for the 105 clinical isolates. FA19 (MIC 31.25 μg/ml) was regarded as a positive control. MICs to GRA ranged from 3.9 μg/ml to 62.5 μg/ml overall, with MIC_{50} and MIC_{90} values of 31.25 μg/ml and 62.5 μg/ml, respectively (Fig. 2). MBC ranges were 3.9–125 μg/ml. The MBC values were generally equal to or two-fold greater than those of the MIC values. In addition, these 105 isolates included 28 MDR strains and 77 non-MDR strains. MIC_{50} and MIC_{90} values showed no difference, illustrating GRA had bactericidal activity against both MDR and non-MDR strains.

3.3. Impact of GRA on biofilm formation and dispersal

Biofilm is a huge barrier to eradicate bacteria. Previous study has demonstrated *N. gonorrhoeae* could form biofilm in vitro and vivo. To test if GRA could inhibit biofilm formation, we incubated *N. gonorrhoeae* (FA19 and 3 clinical isolated strains) with varied concentrations of GRA (MIC, 1/2MIC, 1/4MIC and 1/8MIC) and the result showed that GRA inhibited biofilm formation in a dose-dependent manner. About 96.4%, 89.9%, 50.3%, 29.9% of the biofilm formation in average were inhibited when incubated with MIC, 1/2 MIC, 1/4 MIC and 1/8 MIC GRA respectively (Fig. 3A). In addition, GRA of both sub-MIC and MIC levels could diminish pre-formed biofilm (Fig. 3B), indicating GRA may help eradicate persist bacteria in biofilm.

4. Discussion

The extensive antibiotic resistance of *N. gonorrhoea*e has severely threatened current treatment for this pathogen. Presently, extensive spectrum cephalosporin (ESC) like ceftriaxone and cefixime is now the sole recommended antimicrobial class for gonorrhea treatment. With the dissemination of FC428 clones with high level resistance to ESCs, it becomes more and more difficult to treat gonorrhea effectively [15]. Here we reported a natural compound GRA which had bactericidal activity against *N. gonorrhoeae* in vitro and could be a promising agent for treating gonorrhea. GRA has been demonstrated to have antibacterial effects against *Staphylococcus aureus*, *Actinobacillus actinomycetemcomitans*, *Bacillus subtilis*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, with MIC values of 64, 8, 7.6, 12.5 and 160 μg/ml respectively [9,10,16]. Now, we confirmed that GRA killed *N. gonorrhoeae* in a dose-dependent manner and in a short time within 2 h. The MIC ranges of GRA tested against *N. gonorrhoeae* were 3.9 μg/ml to 62.5 μg/ml, without prominently exceeding MIC values against other bacteria. In addition, it was shown that the susceptibility of the MDR gonococcal strains and non-MDR strains to GRA was very similar. Previous study showed that GRA was distributed throughout the intracellular region of bacteria and then inhibited DNA, RNA and protein synthesis [16]. There may exist differences between the mechanisms conferring resistance to GRA and to antibiotics such as cephalosporins, which needs further exploration.

It has been demonstrated that *N. gonorrhoeae* can form biofilms over glass, plastic surfaces, primary urethral and cervical epithelial cells [17]. There is also evidence that biofilms are present during natural cervical infections [18]. Once biofilm formation, it becomes more difficult to eradicate *N. gonorrhoeae*. Thus, biofilm maybe an important factor for chronic infection and antibiotic resistance of *N. gonorrhoeae*. GRA has been demonstrated to inhibit biofilm formation of *Streptococcus sobrinus* [19], *Pseudomonas aeruginosa* [9] and *Streptococcus mutans* [20]. Our results showed that GRA in sub-MIC levels inhibited biofilm formation significantly. For preformed-biofilms, penetration of antimicrobials into the biofilm was restricted and may contribute to the resistance development. Here we found that GRA could not only inhibit biofilm formation, but also diminish pre-formed biofilm significantly. It was shown that GRA could reduce the expression of key virulence factors of MRSA [11], raising the possibility that GRA may inhibit some key genes expression associated with the biofilm formation in *N. gonorrhoeae*. We will further explore the anti-biofilm mechanism of GRA by RNA sequencing in the following experiments.

Ideally, new antimicrobial agents for gonorrhea would have antimicrobial activity against other coinfecting sexually transmitted pathogens. Gonorrhea has been reported to contribute to the transmission of other STIs, most notably HIV infection [21]. Previous study has demonstrated that glycyrrhizin inhibited the growth of herpes simplex type 1 (HSV-1) and inhibited HIV replication [6,22], indicating its property to be a candidate for the treatment of gonorrhea.

However, our experiments existed limits. Our study only revealed that GRA had antibacterial activity against *Neisseria gonorrhoeae* in vitro. Animal experiments are still needed to further verify whether GRA has bactericidal activity against *N. gonorrhoeae* in vivo.

Ethical approval

Not required.

Funds

This work was supported by grants from the Chinese Academy of Medical Sciences Initiative for Innovative Medicine (2016-I2M-3–021).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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