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CHARACTERIZATION OF THE ANTIMICROBIAL ACTIVITY OF TANREQING (TRQ) USED IN TRADITIONAL CHINESE MEDICINE AS ALTERNATIVE THERAPEUTIC STRATEGY AGAINST HUMAN BACTERIAL PATHOGENS

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Clinical data demonstrated that the TanReQing (TRO) formulation, used in traditional Chinese medicine (TCM), is safe and well tolerated. For example, TRQ effectively treats bacterial infections of the respiratory tract, which are frequently associated with bacterial biofilm formation. Thus, TRQ treatment could be a promising alternative strategy to cope with the worldwide emerging antibiotic resistance infections by bacterial pathogens. Moreover, biofilm-associated (e.g. implant-associated) infections pose severe global problems in modern implant medicine as they are difficult to treat by antibiotic therapy. We recently demonstrated that TRQ inhibits the growth of planktonic Staphylococcus aureus, destroys the 3D-structure of its biofilms, and effectively kills bacterial cells within biofilms. Moreover, pilot studies of our consortium indicate an antimicrobial activity of TRQ against Pseudomonas aeruginosa including its biofilm-state. However, a detailed characterization of the antimicrobial potency against other clinically relevant bacteria, which are associated with high incidence of antibiotic resistance and/or biofilm formation during infection is currently lacking. We will therefore analyze the antimicrobial potency of TRQ against important human pathogens, which show high incidence for antibiotic resistance and/or in vivo biofilm formation during infection is considered to be a virulence factor (i.e. Clostridium difficile, Acinetobacter baumannii, P. aeruginosa, Campylobacter ssp., Helicobacter pylori, Propionibacterium acnes, Staphylococcus epidermidis and ESBL-producing Enterobacteriaceae). Assessment of TRQ efficacy against bacterial pathogens associated with in vivo biofilm formation and increasing antibiotic resistance will also provide first evidence for successful use of TRQ against implant associated infections. Moreover, we try to elucidate the active ingredients of the TRQ formulation to obtain insights in the antimicrobial mechanisms of TRQ and its bacterial target structures.

Experimental Plan and expected results

1. Characterize if the TRQ treatment can be extended to other human pathogens

Determine the minimal inhibitory and bacteriocidal concentrations of TRQ against bacterial pathogens in the planktonic and biofilm state (High-throughput 96-well plate assays)

Bacterial pathogens (i.e. C. difficile, A. baumannii, P. aeruginosa, Campylobacter spp., H. pylori, P.acnes, S. epidermrdrs and ESBL-producing Enferobacteriaceae) will be grown in liquid broth in test tubes or in 96-well plates to obtain planktonic- or biofilm-derived bacteria, respectively.

Due to our expertise and previous studies by S. Schild & S. Kienesberger-Feist have established protocols including the optimal cultivation media and condition to obtain these samples. Minimal inhib & bacteriocidal concentration as MIC & MBC a inst TRQ as well as effects of TRO on metabolic activity (XTT assays) will be performed as described previously to't'tl. MIC,MBC & XTT assays against antibiotics routinely used in conventional therapy will serve as controls.

In summary, these assays will elucidate whether TRQ shows higher, equal or lower efficacy compared to conventional antibiotics for the prioritized bacterial pathogens.

Assess additive/synergistic effects of TRQ in combination with conventional antibiotic therapy

After assessment of the MIC and MBC against TRQ as well as its impact on metabolic activity, combinations of TRQ with antibiotics routinely used in conventional therapy to treat the bacterial infections can be tested. A recent report by our consortium members Profs. Yang & Wang indicates synergetic effects of TRQ combined with vancomycin to treat methicillin-resistant S. aureus infections. This project part will reveal potential additive/synergistic effects of TRQ and conventional antibiotics highlighting a potential combinatory therapy.

Characterize alterations in biofilm formation upon presence of sublethal concentrations of TRQ

In parallel to1.2., we will analyze the interference in biofilm formation upon presence of TRQ in 96-well microplates for rapid quantification and flow cells for microscopical assessment of biofilms. Both, the 96- well plate and the flow cell assays are well established in our laboratory and have been successfully used in several studies t4' e'121. Along this project part the impact on biofilm formation can also be analyzed for the most efficient combinations of TRQ and antibiotics, which will be determined by 1.2.The 96-well plate biofilm assay is a high-throughput method to quantify the amount ol ljefilml Almation in diverse conditions (e.9. diverse concentrations of TRQ at different time points) by crystal violet staining in a 96-well microtiter plates format. The incubation time and cultivation conditions (media, temperature and oxygen availability) can be adjusted to the requirements of the respective bacterial pathogen tested. At a given time point growth of the respective bacterial pathogen will be determined by measuring the ODooo in an absorbance microplate reader (BMG Labtech Spectrostar nano) before the microtiter plates will be subjected to biofilm staining. After removal of the planktonic cells, biofilms can be stained with crystal violet, solubilized with 100% ethanol, and quantified by absorbance at 595 nm. We will used a established high throughput system using flat-bottom 96-well plates in combination with a microplate plate washer (Anthos Fluido 2) and an absorbance microplate reader (BMG Labtech Spectrostar nano).This allows rapid and reproducible quantification of biofilm formation in correlation to the growth dynamics of various conditions in parallel. We also have protocols to determine the colony forming units (CFU) and the metabolic activity [XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assaysl in the biofilms after removal of the planktonic cells.

As a complementary approach, the flow cell assay allows a comprehensive morphological analvsis of biofilms. Therefore biofilms will be grown anaerobically in enclosed flow-cell systems using appropriate conditions for the respective bacterial pathogen tested. Use of such flow cells allows direct microscopic investigation of biofilm development.

In addition, the setup allows the assembly in combination with a peristaltic pump, which is frequently used to analyze biofilm development under hydrodynamic conditions. In addition the flow system in combination with a pump allows efficient removal of planktonic cells before as well as removal of excessive fluorescent dye after staining of the biofilm. To allow visualization by confocal laser scanning microscopy (CLSM), mature three-dimensional biofilms will be stained with fluorescent d We have obtained results with d like SYTO 9 cell- permeable DNA dye) or nile red (lipid membrane dye), which stain biofilms within a few minutes have no observable effects on biofilm growth and cell viability. Images of optical sections will be taken by the inhouse confocal microscopes based on the specific filter requirements. Quantitative analysis of the image stacks will be performed using the computer program COMSTAT (http://www.comstat.dk; [t3' tal, which allows the calculation of important biofilm parameters like biomass, maximum thickness, average thickness, diffusion distance (density of the biofilm) and roughness coefficient (heterogeneity of the biofilm). In addition, attachment rates of the individual isolates can be assayed at earlier time points reflecting the monolayer stage of the biofilm by determination of the surface coverage. The use of commercially available live/dead kits comprising a cell permeable and cell-impermeable dye will be used to monitor the viability of the biofilm.

In summarv, this part will reveal the impact of TRO (in combination with antibiotics) on biofilm formation in complementary assays. This will deliver insights in a potential application of TRQ to prevent biofilm formation, being an important virulence marker for the bacterial pathoqens studied in this proposal.

Characterize effects on mature biofilms upon presence of therapeutic agents (e.9. biomass, viability & morphology)

It is well established in the scientific field, that mature biofilms are more resistant to antibiotic treatment. Pilot studies of our consortium indicate that TRQ has a higher efficacy on mature biofilms of S. aureus compared to conventional antibioticstal. Thus, TRQ treatment might be a promising therapeutic strategy to target mature bacterial biofilms, resulting in less viability and biofilm reduction. In order to address these questions, the disintegration of mature bacterial biofilms in presence of TRQ will be studied. We will allow the bacterial pathogens to form a mature biofilm in microtiter plates or flow-cells before the TRQ will be added. This time point for mature biofilms has already been established for all pathogens relevant to this proposal. Addition of buffer solution without TRQ will serve as a control. As outlined above, the 96-well format will allow a rapid screening of the TRQ to determine the efficacy of the TRQ in a dose-response, while alterations in biofilm morphology or viability (live/dead staining) will be analyzed by confocal microscopy of flow-cell grown biofilms. Along this project part the impact on biofilm disintegration can also be analyzed for the most efficient combinations of TRQ and antibiotics, which will be determined by 1.2.

In summary, this part will reveal the impact of TRQ (in combination with antibiotics) on mature biofilms and deliver insights in a potential application of TRQ to treat bacterial infections associated with biofilm formation (e.g. implant-associated infections).

2. Elucidation of the active compound(s) in the TRQ formulation along with the molecular mechanism(s) of antimicrobial activity

Assess the minimal inhibitory/bacteriocidal activity of the identified ingredients of TRQ formulation or combinations thereof to pinpoint the active compound(s)

Along the ongoing research cooperation of Prof A. Brantner (University of Graz) and Prof. Wang Yi (China Academy of Chinese Medical Sciences) five ingredients (baicalin, ursodeoxycholic acid, chenodeoxycholic acid, chlorogenic acid, and caffeic acid) with potential antimicrobial activity have been identified. Pure substances can either be provided by Prof. A. Brantner/ Wang or are commercially available. In addition, the TRQ formulation is composed of five organic extracts IScutellariae radix (Huang Qin), Lonrberae flos (Jin Yin Hua), Forsythiae fructus (Lian Qiao), Ursi fel (Xiong Dan) and Naemorhedi cornu han Y Jiao which are available. In order to narrow down the9lts antimicrobial active component(s) we will perform MIC, MBC and XTT assays using the pure ingredients or individual organic extracts. As we cannot exclude additive or synergistic effects of components in the TRQ formulation, we will also include combinations in our analyses. As this is a very labor- & time-consuming screening approach, we will focus on two bacterial pathogens showing the highest susceptibility to TRQ in 1.1. Identified active ingredients or combinations thereof will be subjected to biofilm assays to elucidate their activity on bacterial biofilms.

In summary, this part will elucidate the active ingredients of the TRQ solution, which will allow an improved formulation solelv based on the active compounds in the future. This will not only reduce costs but also simplify the regulatory processes to get approval for this medical treatment on the international market.

Characterize the antimicrobial potential of bile salt-derivatives

Preliminary data support the hypothesis, that especially the bile salt-related ingredients (ursodeoxycholic & chenodeoxycholic acid) exhibit potent antimicrobial activity. Thus, in a complementary approach to 1.2. we will specificallv investiqate these bile salt-related inqredients of TRQ with regard to their antimicrobial potency. Due to their interest in gastrointestinal bacterial pathogens, the groups of S. Schild and S. Kienesberger-Feist have several strains with different bile salt tolerance available. These include Vibrio cholerae, Escherichia coli, H. pylori, and Campylobacter spp. including diverse surface mutants with less or more bile salt resistance, respectively t15-201. The bile salt resistance profile against commercially available bile salt mixtures as well as cholic acid & deoxycholic acid of these strains is already available. We will assess the MIC against TRQ, ursodeoxycholic & chenodeoxycholic acid for these strains. If bile salt-derivatives are the active compound of the TRQ formulation, the MIC against TRQ should follow the MIC against bile salts.

As bile salts are amphipathic molecules with hydrophobic and hydrophilic regions, they solubilize lipids from bacterial membranes and thereby inhibit bacterial cell division and growth t"1. This will be investigated and confirmed by live cell microscopy.are active in the formulation providing a first insight in the mechanism of action.

Sustainability

This proposal will assess the potential use of TRQ or individual compound(s) to serve as an alternative strategy to treat or prevent bacterial infections. A first targeted analysis of the active ingredients of the TRQ solution will allow to simplify the formulation and reduce costs, which is essential to get approval for this treatment on the international market.