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Solid lipid nanoparticles of clotrimazole silver complex: An efficient nano antibacterial against *Staphylococcus aureus* and MRSA

Rahul S. Kalhapure, Sandeep J. Sonawane, Dhiraj R. Sikwal, Mahantesh Jadhav, Sanjeev Rambharose, Chunderika Mocktar, Thirumala Govender*

Discipline of Pharmaceutical Sciences, School of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa

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ABSTRACT

New and effective strategies to transform current antimicrobials are required to address the increasing issue of microbial resistance and declining introduction of new antibiotic drugs. In this context, metal complexes of known drugs and nano delivery systems for antibiotics are proving to be promising strategies. The aim of the study was therefore to synthesize a silver complex of clotrimazole and formulate it into a nano delivery system for enhanced and sustained antibacterial activity against susceptible and resistant *Staphylococcus aureus*. A silver complex of clotrimazole was synthesized, characterized and further encapsulated into solid lipid nanoparticles to evaluate its antibacterial activity against *S. aureus* and methicillin-resistant *S. aureus* (MRSA). An in vitro cytotoxicity study was performed on HepG2 cell lines to assess the overall biosafety of the synthesized clotrimazole silver complex to mammalian cells, and was found to be non-toxic to mammalian cells (cell viability >80%). The minimum inhibitory concentrations (MIC) of clotrimazole and clotrimazole-silver were 31.25 and 9.76 μg/mL against *S. aureus*, and 31.25 and 15.62 against MRSA, respectively. Clotrimazole SLNs exhibited MIC values of 104 and 208 μg/mL against both MSSA and MRSA at the end of 18 and 36 h, respectively, but thereafter completely lost its antibacterial activity. Clotrimazole-silver SLNs had an MIC value of 52 μg/mL up to 54 h, after which the MIC value was 104 μg/mL against both strains at the end of 72 h. Thus, clotrimazole-silver SLNs was found to be an efficient nanoantibiotic.

1. Introduction

The Gram positive bacterium, *Staphylococcus aureus*, causes several nosocomial infections in humans, ranging from skin lesions to lethal diseases, such as bacteremia, endocarditis, pneumonia, toxic shock syndrome, osteomyelitis, and septicaemia [1]. It asymptomatically colonizes normal people (with about 30% asymptomatic nasal carriers), which makes them at higher risk of infections, and is an important source for spreading *S. aureus* [2]. This bacteria has developed resistance to a number of antibiotics [3], with methicillin resistant *S. aureus* (MRSA) being one of the most prominent pathogens that causes healthcare, community and livestock acquired infections [4]. Global attempts have been made to control the spread of MRSA due to the associated increased morbidity and treatment costs when compared to infections by methicillin-susceptible *S. aureus* (MSSA) [5].

The increased microbial resistance to antibiotics and subsequent current global antibiotic crisis, as well as the decline in new antibiotics being introduced into the market, is regarded as a serious threat to effectively treating infectious diseases [6–8] and a global danger to human health. A major focus area of research priority internationally is therefore the search for new and effective strategies to enhance drug therapy with current antimicrobials [8].

Clotrimazole (CTM) is a highly lipophilic antimycotic agent, its use as a broad spectrum drug being to treat fungal infections of the

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; CTM, clotrimazole; RT, room temperature; CTM- Ag, clotrimazole-silver complex; MTT, 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MHA, Mueller-Hinton agar; MHB, Mueller-Hinton broth; NMR, nuclear magnetic resonance; bs, broad singlet; m, multiplet; HRMS, high resolution mass spectrometry; DMSO, dimethyl sulfoxide; MIC, minimum inhibitory concentration; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PI, polydispersity index; ZP, zeta potential; TEM, transmission electron microscopy; HPLC, high performance liquid chromatography; DSC, differential scanning calorimetry; XRD, x-ray diffraction; PBS, phosphate buffer saline; MW, molecular weight; MWCO, molecular weight cut-off; RMSE, root mean square error; MDT, mean dissolution time; PSA, polar surface area; SD, standard deviation; ANOVA, analysis of variance; M.p., melting point; MHz, megahertz; ESI- TOF, electrospray ionization-time of flight; kDa, kilodalton; nm, nanometer; DLS, dynamic light scattering.

* Corresponding author. Fax: +27 31 260 7792.
E-mail address: govenderth@ukzn.ac.za (T. Govender).

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skin as well as for mucus diseases [9]. These include tinea pedis, tinea crusis, vaginal yeast infections, oral thrush or candidiasis, ringworm, mycotic infections of the genito-urinary tract, as well as also cancer and sickle cell anemia [10–14]. Although there are few detailed evaluations of CTM as an antibacterial agent, it has been reported to be active against Gram-positive *staphylococci* and *streptococci* [15]. Silver is also a potent antimicrobial, capable of disturbing key functions in a microorganism that causes antimicrobial resistance [16]. Various silver co-ordinates of metronidazole have been reported to be effective antimicrobials against numerous bacteria and fungi, including *S. aureus* [17]. It is also reported that the challenge of antimicrobial resistance can be addressed effectively by using novel nanosized drug carriers to efficiently deliver antibiotic [8,18]. The hypotheses of the present investigation were therefore: (1) the reaction of CTM with silver nitrate could result in a CTM-silver complex (CTM-Ag) with enhanced antibacterial activity, acting by more than one mechanism of action, and (2) the performance of CTM-Ag could be further improved by formulating it into solid lipid nanoparticles (SLNs). The development of resistance to such a nanosystem containing an antibacterial agent acting by more than one mechanism of action would be difficult for bacteria, as this will require multiple simultaneous mutations in the same bacterial cell [18,19]. While the antibacterial activity of silver and its nano form has been well reported in the literature [20], there are very limited reports, if any, on the antibacterial activity of an antimicrobial drug-silver complex. To the best of our knowledge, CTM-Ag complex and its subsequent encapsulation into a nanoparticlulate system has not been reported. In this work, we report for the first time on the synthesis of CTM-Ag and its solid lipid nanoparticles (SLNs) for enhanced and sustained antibacterial activity against MSSA and MRSA.

2. Materials

Silver nitrate ($AgNO_3$), CTM and Lutrol F68 were procured from Sigma–Aldrich Co. Ltd., (USA), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Merck Chemicals (Germany). Compritol 888 ATO was a generous gift sample from Gattefossé (France), Mueller–Hinton agar (MHA) and Nutrient broth were purchased from Biolab Inc. (South Africa), and Mueller–Hinton broth (MHB) from Oxoid Ltd. (England). All other reagents and solvents were purchased from Sigma–Aldrich Co. Ltd., (USA). The water was obtained through an Elix® water purification system by Milli-pore Corp. (USA). *S. aureus* (ATCC 25922) and *S. aureus* Rosenbach (ATCC®BAA-1683TM) (MRSA) bacteria were used in antibacterial studies.

3. Methods

3.1. Synthesis and characterization of CTM-Ag (Fig. 1a)

A solution of CTM (0.68 g, 2 mmol) in ethanol (20 mL) was added to a solution of $AgNO_3$ (0.17 g, 1 mmol) in ethanol (20 mL). The reaction mixture was stirred at room temperature (RT) for 24 h, then filtered and concentrated under vacuum. The crude product was purified by recrystallization in 95% ethanol and characterized by NMR spectroscopy (^1H and ^{13}C) and HRMS.

3.2. Cell toxicity assay

The MTT assay was used to determine the in vitro cytotoxicity of CTM-Ag. Briefly, the cells were seeded equivalently (2.5×10^3) and cultured for 24 h at 37 °C. An appropriate amount of test compound was added to the wells to achieve final concentrations (20–100 μg/mL). The cells were further incubated for 48 h, and the culture medium was replaced with the fresh medium (100 μL) and 5 mg/mL MTT solution in PBS (100 μL). The cells were then incubated at 37 °C for an additional 4 h. The reaction was stopped by lysing the cells with DMSO (100 μL), and the optical density of each well was measured on a microplate spectrophotometer (Mindray MR-96A) at a wavelength of 540 nm. The percentage cell viability was calculated as follows:

$$\%\text{Cell survival} = \frac{[A_{540\text{nm}}\text{treated cells}]}{[A_{540\text{nm}}\text{untreated cells}]} \times 100$$

3.3. Antibacterial assay of CTM-Ag

In order to assess the antibacterial potential of CTM and CTM-Ag, the minimum inhibitory concentration (MIC) values were determined against MSSA and MRSA using a broth dilution method [21]. The bacterial cultures were grown overnight in nutrient broth at 37 °C and diluted to 0.5 McFarland. The serial dilutions of the test samples were incubated with these cultures for 18 h in a shaking incubator at 37 °C. These dilutions (10 μL) were spotted on MHA plates and incubated for 18 h. The minimum concentration at which no bacterial growth was observed was considered as the MIC. All the experiments were performed in triplicate.

3.4. Gel electrophoresis

The mode of action of CTM-Ag was studied by gel electrophoresis of bacterial outer membrane proteins. The SDS-PAGE method reported previously was adopted for this purpose (Supplementary Information) [22].

3.5. Preparation of SLNs

SLNs were prepared by an ultrasound dispersion technique as described elsewhere, with slight modifications [21]. Briefly, Compritol 888 ATO (500 mg) and CTM-Ag (25 mg) were heated at 80 °C in a water bath, and a solution of Lutrol F68 (300 mg) in milli-Q water heated separately at 80 °C was added. The resulting mixture was homogenized for 15 min at 6000 rpm with an Ultra Turrax T-25 homogenizer (IKA Labortechnik, Germany), probe sonicated (30% amplitude) for 30 min and cooled to 20 °C. CTM SLNs were prepared in a similar way for antibacterial activity comparison.

3.6. Determination of size, polydispersity index (PI) and zeta potential(ZP)

The size, PI and ZP were measured using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK) equipped with a 633 nm laser and 173° detection optics.

3.7. Morphology

The surface morphology of SLNs was examined using a transmission electron microscope (TEM) (Jeol, JEM-1010, Japan). A drop of appropriately diluted SLNs was placed on 3 mM forman (0.5% plastic powder in amyl acetate) coated copper grid (300 mesh), allowed to dry and visualized at an accelerating voltage of 100 kV.

3.8. Encapsulation efficiency and drug loading

The SLN dispersion (500 μL) was transferred to Nanosep® centrifuge tubes (MWCO 10 KDa, Pall Corporation, Ann Arbor, USA) and centrifuged at 15,000 rpm for 20 min. The amount of free CTM-Ag in the aqueous phase was determined by an HPLC method at