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In vitro comparative cytotoxicity study of a novel biocidal iodo-thiocyanate complex

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ABSTRACT

Novel biocides, which avoid the induction of cross-resistance to antibiotics, are an urgent societal requirement. Here, we compared the cytotoxic and bactericidal effects of a new antimicrobial agent, the iodo-thiocyanate complex (ITC), with those of the common antiseptics, hydrogen peroxide (H_2O_2), povidone iodine (PVP-I) and Lugol's iodine (Lugol). The antimicrobials were co-incubated for 10 min with HeLa and *Escherichia coli* cells in the presence and absence of organic matter (Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum). The cytotoxic concentrations of ITC were equivalent to its bactericidal concentrations (7.8 μg ml^{-1}). By contrast, cytotoxic effects of H_2O_2, PVP-I and Lugol were apparent at concentrations lower than their bactericidal concentrations (250, 250 and 125 μg ml^{-1}, respectively). The cellular effects of ITC were not quenched by organic matter, unlike the other antiseptics. ITC, PVP-I and Lugol had hemolytic effect on horse erythrocytes at high concentrations, while H_2O_2 showed no hemolysis. ITC, at 30 or 300 μg ml^{-1}, did not cause DNA breakage in HeLa cells as assessed by an *in vitro* comet assay in the absence of S9 metabolic activation, whereas H_2O_2 caused extensive single-strand DNA breaks. The pronounced antimicrobial potency of ITC and its favorable cytotoxicity profile suggests that ITC should be considered for antiseptic applications.

1. Introduction

The increasing occurrence of antibiotic resistance in pathogenic bacteria, coupled with a dramatic decline in the number of newly approved antibiotics, represents a major societal challenge. In the context of growing resistance to antibiotics, wound infection control and management, along with the control of potentially pathogenic bacteria in healthcare environments, is becoming challenging and represents a major healthcare burden (WUWHS, 2008).

Naturally occurring peroxidase-catalyzed antimicrobial systems provide a possible future direction. A peroxidase enzyme together with hydrogen peroxide, inorganic ion substrate and generated oxidized products is known as a peroxidase system (Kussendrager and van Hooijdonk, 2000). These systems are part of the host defense network and provide an immediate defense against invading microorganisms (Davies et al., 2008). A large number of studies have investigated and demonstrated the antimicrobial potential of peroxidase-catalyzed systems, as extensively reviewed elsewhere (Bafort et al., 2014; Davies et al., 2008; Kussendrager and van Hooijdonk, 2000; Naidu, 2000; Seifu et al., 2005). Peroxidase/hydrogen peroxide/halide systems have a dual role, however, acting as both a friend and a foe (Davies et al., 2008; Klebanoff, 2005). As they possess a non-specific, broad-spectrum target mechanism, aside from mediating bacterial killing, destroying invading parasites, combating fungal infections and inactivating viruses, they can also attack a variety of mammalian cells (Clark and Klebanoff, 1977; Edelson and Cohn, 1973), including tumor cells (Clark and Klebanoff, 1975; Henderson et al., 1981; Jong and Klebanoff, 1980).

Peroxidase-catalyzed systems may also inspire the development of a new generation of antimicrobial agents. Our previous research evaluated three such antimicrobial agents (Tonoyan et al., 2017). The agents, all produced without a peroxidase enzyme, were formed by the reactions of H_2O_2 with iodide (I^-) salt (H_2O_2/KI); or H_2O_2 with thiocyanate (SCN^-) salt (H_2O_2/KSCN); or H_2O_2 with both ion substrates (I^- and SCN^-). The latter reaction forms the iodo-thiocyanate complex (ITC). In the ITC, H_2O_2 serves as a source of oxidation for two substrates, generating reactive oxygen and iodine species within the

Abbreviations: ITC, iodo-thiocyanate complex; PVP-I, povidone iodine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; cfu, colony-forming units; hRBD, horse red blood cells; DAPI, 4′,6-diamidino-2-phenylindole; SD, standard deviation

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reaction mixture, which possess powerful antimicrobial properties. The mixture was shown to contain low concentrations of antimicrobial species such as hypoiodite, hypothiocyanite and hydroxyl radical; with molecular iodine being the major cidal component. The biocidal ITC formulation effectively and rapidly killed Gram-negative and Gram-positive bacteria, including a multidrug-resistant *Staphylococcus aureus*. It was shown that ITC was capable of eradicating mono- and dual-species bacterial biofilms within short exposure times (10 min and 30 s, respectively). In addition, we were unable to induce resistance in bacteria to the ITC, likely due to the presence of multiple cellular targets (Okano et al., 2017). These characteristics suggested the potential for use of ITC as a new antimicrobial to prevent, and possibly treat, bacterial infections. In addition to antimicrobial activity, however, the cytotoxic potential of a new agent must also be evaluated.

A variety of approaches can be used to evaluate and screen the *in vitro* toxicity of antimicrobial substances. Testing the effects of compounds on the viability of mammalian cells grown in culture (*in vitro* cytotoxicity tests) is widely used as an indicator of potential toxic effects in animals (Riss et al., 2011). Among the different cytotoxicity tests, the methyl tetrazolium (MTT) viability assay is the most popular, low-cost and convenient method (Fotakis and Timbrell, 2006). Considering that antimicrobials may potentially come into direct contact with blood, an evaluation of hemotoxicity is also worthwhile. The standard measure of blood compatibility is hemolytic activity or the lysis of red blood cells (Li et al., 2012). Genotoxicity testing is also an important aspect of the safety assessment of broad-spectrum substances, including pharmaceuticals and biocides (Corvi and Madia, 2016). Our previous research (Tonoyan et al., 2017) indicated that the ITC contains reactive species, which may have the potential to induce bacterial DNA breakage; the detection of DNA breaks by single cell gel electrophoresis (comet assay), would thus be informative.

The aim of the present study was to investigate the *in vitro* cytotoxicity, hemolytic activity and DNA damaging effect of the novel antimicrobial ITC in relation to its antimicrobial activity. The potential suitability of ITC as an antiseptic was then considered and compared with H_2O_2, PVP-I and Lugol's iodine.

2. Materials and methods

2.1. Antimicrobial agents and preparation

All the materials were purchased from Sigma-Aldrich, unless otherwise stated. The antimicrobial agents evaluated in this study were H_2O_2/KI, H_2O_2/KSCN and H_2O_2/KI/KSCN [ITC; Tonoyan et al. (2017)]. The stock solutions were prepared by combining concentrated solutions of H_2O_2 (v/v) with KI (w/v) and/or KSCN (w/v) to obtain 1% final concentrations for each agent. These solutions were considered as 1% according to the concentration of H_2O_2 present in the mixtures, as KI and KSCN do not possess antimicrobial activity alone (data not shown). The antiseptics H_2O_2 (30% v/v stock solution), PVP-I (10% w/v available iodine stock) and Lugol's iodine (1.25% w/v available iodine stock) were also used in this study. The stock solutions were diluted to the desired working antimicrobial concentration range using sterile deionized water (dH_2O).

2.2. Test bacteria, cell cultures, nutrient solutions and growth conditions

The bacterial strain used in this study was *Escherichia coli* ATCC 25922 (from the American Type Culture Collection), which was cultured aerobically on Lennox agar and lysogeny broth at 37 °C. HeLa human cervical epithelial cell lines were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures) and were grown as a monolayer in complete DMEM (Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) at 37 °C, 5% CO_2.

2.3. Cytotoxicity studies

2.3.1. Determination of simultaneous cellular and bacterial toxicities of antimicrobials towards human and bacterial cells

The cellular toxicity of the test antimicrobials and antiseptics towards human epithelial HeLa cells was assessed utilizing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, whereas, their antimicrobial potential towards bacterial *E. coli* cells was assessed using viable bacterial cell counts.

The simultaneous cytotoxic and bactericidal effects of antimicrobials were determined by co-incubation of HeLa and *E. coli* cells, for 10 min, with the antimicrobials, at a range of concentrations, in two different media [phosphate-buffered saline (PBS) and DMEM supplemented with 10% FBS without addition of antibiotics (DMEM + FBS)], at room temperature. HeLa cells were seeded at a concentration of 8×10^3 cells $well^{-1}$ in 96-well plates in complete DMEM media. After 72 h incubation, the confluent cell monolayer was washed with PBS and was covered with 200 μl PBS, or DMEM + FBS, containing 10^6 cfu ml^{-1} *E. coli* cells and the antimicrobial agent. Eight two-fold dilutions of the selected antimicrobials were tested, and the concentrations in the wells ranged from 7.8 to 1000 μg ml^{-1}. As it was shown previously (Tonoyan et al., 2017) that ITC at this specified concentration range could eradicate *E. coli* biofilms within 10 min, this was thus considered an appropriate duration of exposure to demonstrate antimicrobial activity. Following the 10 min incubations, 10 μl aliquots were taken from the supernatant and ten-fold serially diluted in PBS. Aliquots (5 μl) of the dilutions were then replica (three per sample dilution) spot-plated on agar plates to enumerate viable *E. coli*. This method can be used to enumerate bacteria on a high-throughput scale in a fast, easy-to-use, labor-efficient and cost-efficient manner (Sieuwerts et al., 2008). Bactericidal activity (expressed as a minimum bactericidal concentration; MBC) was defined as a > 3 $\log_{10}$ reduction (99.9% kill) in cfu ml^{-1} from the starting *E. coli* inoculum concentration (Rose and Poppens, 2009). The HeLa monolayers were washed twice with PBS and incubated with complete DMEM, supplemented with 0.6 mg ml^{-1} MTT, for 4 h at 37 °C. The medium containing MTT was aspirated, formazan crystals were dissolved in 150 μl dimethyl sulfoxide and the absorbance was measured at 595 nm (Tecan GENios, Salzburg, Austria). Cellular viability was expressed as a percentage of the untreated controls.

2.3.2. Visualization of morphological changes in HeLa cells

The impact of the antimicrobial treatments on HeLa cells was also evaluated microscopically. Untreated cells, cells lysed with 10% Triton X-100, and cells exposed to the highest concentrations of the antimicrobials (1000 μg ml^{-1}) in either PBS, or DMEM + FBS, were visualized in 96-well plates, after 10 min antimicrobial treatments, using bright-field optic microscopy on an inverted microscope (Leica DM IL LED, Leica Microsystems Ltd.) fitted with a DFC420C digital camera using LAS at 20× objective magnification.

2.3.3. Recovery of HeLa cells after withdrawal of antimicrobials

To assess whether the cytotoxic effects of the antimicrobials on human cells were reversible or irreversible, cytotoxicity was measured either immediately after 10 min exposure of HeLa cells to antimicrobials, or after recovery based on a previously published protocol (Müller and Kramer, 2007). Briefly, HeLa cells were exposed to antimicrobials in PBS, or DMEM + FBS media, for 10 min, and the MTT assay was performed. Subsequently, another set of HeLa cells were exposed to antimicrobials in PBS, or DMEM + FBS, for 10 min, carefully washed twice to remove the antimicrobials, and then cultured for a further 24 h. Following incubation, the MTT assay was performed and cell viability was determined.