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Antibacterial and Antifungal Activity of the Triiodide $[\text{Na}(\text{12-Crown-4})_2]\text{I}_3$ on the Pathogens *Escherichia coli*, *Streptococcus pyogenes*, *Streptococcus faecalis*, *Bacillus subtilis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Candida albicans*

Zehra Edis *, Samir Haj Bloukh and Hamed Abu Sara

College of Pharmacy and Health Sciences, Ajman University, P.O. Box 346, Ajman, UAE; z.edis@ajman.ac.ae; jrac.bloukh5@ajman.ac.ae; Jrad.hamed_a@ajman.ac.ae

* Correspondence: z.edis@ajman.ac.ae; Tel.: +97-16-705-6137

Abstract: New antibacterial agents are needed to overcome the increasing number of infectious diseases caused by pathogenic microorganisms due to the emergence of multi-drug resistant strains. In this context, halogens, especially Iodine is known since ages for its antimicrobial activity. Therefore, especially triiodides encapsulated in organometallic complexes can be helpful as new agents against microorganisms. The aims of this work was to study the biological activity of $[\text{Na}(\text{12-Crown-4})_2]\text{I}_3$ against gram positive *Streptococcus pyogenes*, *Streptococcus faecalis*, the spore forming bacteria *Bacillus subtilis* and gram negative bacteria *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, as well as the yeast *Candida albicans*. The antimicrobial and antifungal activities of the triiodide were determined by zone of inhibition plate studies. $[\text{Na}(\text{12-Crown-4})_2]\text{I}_3$ exhibited potent antimicrobial activity on gram positive Streptococci and the yeast *C. albicans*. Furthermore, the gram negative bacteria *P. aeruginosa* and *K. pneumoniae* were less effectively inhibited, while *E. coli* and *P. mirabilis* proved to be even resistant.

Keywords: triiodide; antibacterial activity; antifungal activity; sodium; crown ether complex

1. Introduction

Polyiodide anions are built through attachment of iodine molecules on iodide ions and can be incorporated next to large cations into crystalline solids [1–6]. They contain $[\text{I}_{2k+n}]^{n-}$ -units, which are formed through donator-acceptor-interactions of the combined iodine molecules and iodide ions. Within the last few years new polyiodide structures were investigated demonstrating a positive effect of crown ethers and metal cations on the structure and stability of the polyiodides [7]. Anion- π -interactions in combination with electron-poor, polarized salts showed a stabilizing effect of polyiodides in the solid state [8]. The I–I-distances in the I_3^- -anion and the $\text{I}_3^- \cdots \text{I}_3^-$ -interactions in its dimers were investigated in the gaseous phase and in theoretical studies [9]. Further importance is in the use of cations as stabilizing factor in polyiodide structures, their effect on halogen bonding and asymmetry in triiodides [10].

With increasing number of infectious diseases caused by pathogenic microorganisms, the widespread use of antibiotics and the emergence of resistance towards antibiotics, there is a serious need to find new antibacterial compounds [11]. Many new strains of the known bacterial pathogens are highly multi-drug resistant since several years [12]. The increase in bacterial infection-associated morbidity and mortality worldwide through drug and multidrug-resistant bacterial strains needs to be addressed by limiting bacterial growth [13, 14].

In this context, inorganic antimicrobial agents, like the elements iodine, copper [15-18], and very recently both elements combined [19, 20], as well as Li^+ , Na^+ , K^+ , other metal cations [21, 22] and transition metals [23] have gained a growing interest in drug development and biocidal applications [24-27]. Especially iodine and copper are very essential to human health, nontoxic in most mammals,

including humans, but have been approved and registered by the US Environmental Protection Agency (EPA) as antimicrobial agents and are used in many combinations in the food industry [17].

Only few years ago, Copper-Nanoparticles were also introduced against many microorganisms, offering new prospects for antimicrobial treatments [21], even in combination with chitosan, the derivative of the naturally occurring polymer chitin [25] leading to the emergence of polymer based antimicrobial applications [26]. Polymeric antibacterial agents, the new field of investigations, have the ability to increase the long-term effectiveness and the stability of the biocidal agent [27]. In this regard, Iodine in combination with natural occurring polymeric biocides like starch are toxic against microorganisms [2, 27, 28]. Long term effectiveness and stability of the biocidal agent are important requirements. This can be achieved by using polyiodide-structures as a complex, molecular backbone, releasing the biocidal agent slowly and effectively over a long period of time [28]. Our goal is to find the most effective antimicrobial and antifungal compound fulfilling the major requirements by utilizing polyiodide-structures in combination with sodium iodide, stabilized by crown ether complexes. Previous investigations show the stability and structure of these compounds [29]. In $[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$ the structure is triclinic, and contains one independent complex cation without crystallographic symmetry, while the anionic part consists of two crystallographically independent, isolated, linear triiodide anions I_3^- with crystallographic inversion symmetry. The cations are distorted sandwich-like complexes with two 12-crown-4 molecules surrounding the alkali metal atom in the center [29]. These matrices may enhance the antimicrobial activity on our target microorganisms through long term, controlled effectiveness by slowly releasing iodine from the stable triiodide unit resulting in increased exposure times.

These preliminary findings indicate that, triiodides of crown-ether-complexes of NaI have antimicrobial activity against a selection of gram negative (*K. pneumoniae*, *E. coli*, *P. mirabilis*) and pathogenic gram positive bacteria (*S. faecalis*, *S. pyogenes*, *B. subtilis*), as well as *C. albicans*.

E. coli is a pathogen, which causes serious outbreaks and is commonly present in water, raw meats, dairy products and vegetables [17, 30]. *P. aeruginosa* leads to nosocomial infections and some strains showed high level of antibiotic resistance [23]. However, recent findings suggest, that *E. coli*, *P. aeruginosa* and the gram positive bacteria *B. subtilis* are susceptible to combinations of organic acids and copper or other metals [23]. This synergy between transition metals and organic acids inhibited the growth of these bacteria by increase in the membrane permeability to transition metal ions leading to elevated intracellular metal accumulation [23]. Furthermore, the investigations of ions that are generally membrane-impermeable metals (sodium, magnesium, calcium, potassium) showed only a mild effect for sodium and no effects for magnesium, calcium or potassium [23].

The aim of this study was to investigate antibacterial activity of the sodium-ion containing triiodide $[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$ against four gram negative bacteria and three gram positive bacteria, as well as the antifungal activity against *C. albicans*.

2. Results

Testing the inhibitory effect of $[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$, I_2 , 12-crown-4 and NaI on the selected gram positive and gram negative pathogens, as well as the fungi *C. albicans* gave the expected results. All microorganisms were most strongly inhibited by Iodine. The gram positive bacteria and *C. albicans* showed the second largest inhibition zones for 12-crown-4 followed by $[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$ and NaI except for *B. subtilis*. The gram negative bacteria responded strongest to NaI, then 12-crown-4 and finally to $[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$ (Table 1).

Table 1. Antimicrobial activity of I₂, NaI, 12-crown-4 and [Na(12-Crown-4)₂]₃I₃.

	I ₂ [mm]	NaI [mm]	12-crown-4 [mm]	[Na(12-Crown-4) ₂] ₃ I ₃ [mm]
<i>S. faecalis</i>	80	0	24	18
<i>S. pyogenes</i>	60	0	12	10
<i>B. subtilis</i>	80	16	18	12
<i>K. pneumoniae</i>	65	30	18	9
<i>P. aeruginosa</i>	58	30	18	9
<i>E. coli</i>	58	27	17	0
<i>P. mirabilis</i>	64	22	16	0
<i>C. albicans</i>	30	0	-	13

Resistant=0 mm

The gram positive bacteria and *C. albicans* are all resistant against NaI except *B. subtilis*, while the gram negative pathogens are resistant against [Na(12-Crown-4)₂]₃I₃ except *K. pneumoniae* and *P. aeruginosa* (Table 1).

3. Discussion

The antibacterial action on the studied pathogens are different depending on the characteristics of the bacterial cell walls. In gram positive bacteria the outer structure is more compact with a cytoplasmic membrane enveloped by the cell wall, which consists of negatively charged peptidoglycans containing inclusions of teichoic acid and lipoteichoic acid. The outer structure of the gram negative bacteria are more complicated, composed of cell membrane, cell wall and an outer membrane. The outer membrane is basically consisting of lipopolysaccharides that result in higher negative charge on the cell surface than in gram positive microorganisms. Furthermore, this outer membrane of gram negative bacteria forms an additional barrier leading to more resistance against antimicrobial agents. All studied microorganisms are affected without much difference by the known antimicrobial activity of free iodine in I₂ through microbial membrane penetration leading to immediate intracytoplasmic protein oxidation [31]. The gram positive bacteria showed the highest

inhibition zones followed by the gram negative bacteria with their additional outer barrier and finally *C. albicans*. Here, free I_2 penetrates bacterial membrane channels (porins) and causes oxidation of proteins within the bacterial cytoplasm [31]. Large molecules like chitosan, even the complex $[Na(12-Crown-4)_2]I_3$, 12-crown-4 and the well known antimicrobial chlorhexidine cannot pass through these porins and must adsorb to the microbial membrane before activity [31, 32]. Porins are present in the plasma membrane of Gram-positive bacteria, and in both the outer and plasma membranes of Gram-negative bacteria [31]. Furthermore, the antibacterial activity of chitosan is due to an interaction between the positively charged chitosan (protonated amine groups) and the negatively charged bacterial membrane (lipopolysaccharide in Gram-negative bacteria and lipoteichoic acid in Gram-positive bacteria) [33, 34]. Also, the hydrophobicity of the glucopyranoside rings of chitosan contributes to the insertion or translocation into the hydrophobic tail part of lipid [34]. In our studies, all the gram positive pathogens were inhibited by 12-crown-4. Like chitosan, the complexing agent 12-crown-4 is also able to chelate metals surrounding the bacteria, interrupting the needed flow of nutrients [32]. Moreover, like chitosan, 12-crown-4 is attracted through electrostatic interactions due to its partially positively charged carbon atoms to the negatively charged peptidoglycans in the more compact bacterium cell membrane of the gram positive Streptococci [32, 33]. Through this mechanism, electrostatic interactions can lead to hydrolysis of peptidoglycans or even changes in the cell wall permeability due to osmotic differences resulting in inhibition of bacterial growth and leakage of intracellular components [32, 33]. The disruption of inner and outer cell membranes changes the bacterial metabolism and leads to bacterial cell death [32, 33].

Within the gram positive microorganisms the Streptococci (*S. faecalis* and *S. pyogenes*) responded to $[Na(12-Crown-4)_2]I_3$ more than to NaI, while *B. subtilis* shows opposite results. Here, the slow release of free Iodine from the stable triiodide units surrounding the complex cation is the main antimicrobial characteristic of the crown ether complex $[Na(12-Crown-4)_2]I_3$. The free Iodine penetrates the microbial membrane and leads to intracytoplasmic protein oxidation [31].

However, the gram negative bacteria are less affected by 12-crown-4 and $[Na(12-Crown-4)_2]I_3$ because of their stronger, outer barrier preventing such electrostatic interactions. Especially the gram negative pathogens are resistant against $[Na(12-Crown-4)_2]I_3$ except *K. pneumonia* and *P. aeruginosa*. Here, sodium iodide proves to be more effective through attraction of the positively charged sodium ion to the more negatively charged outer membranes of the gram negative microorganisms. Mechanisms of bacterial resistance include the occurrence of diminished protein channels on the bacterial outer membrane to decrease drug entry and/or the presence of efflux pumps to decrease the amount of drug accumulated within the cells [12]. The inhibitory action of $[Na(12-Crown-4)_2]I_3$ on *K. pneumonia* and *P. aeruginosa* proves two possible mechanisms. An increased influx of the toxic components into the cell through porin protein channels or failure of the polyselective efflux pumps to eject the toxic compound from the intermembrane space [12]. There is an increasing number of strains producing these efflux pumps in *Enterobacter aerogenes* and *K. pneumoniae* clinical isolates [12]. Especially *P. aeruginosa* contains a large number of efflux pumps capable to eliminate toxic compounds from the periplasm and cytoplasm quickly [12]. This plays a key role in its multidrug resistance and happens usually in such a high rate that the drug concentrations are never enough to exert an antibacterial effect [12]. This action may have been impaired by the antimicrobial effect of $[Na(12-Crown-4)_2]I_3$ in *K. pneumonia* and *P. aeruginosa*.

4. Materials and Methods

4.1 Materials and Chemicals

Iodine ($\geq 99.0\%$), Potassium iodide, Sodium iodide, Dimethyl sulfoxide and Mueller Hinton Broth (MHB) were purchased from Sigma Aldrich (Gillingham, UK). 1,4,7,10-Tetraoxacyclododecan

(12-crown-4) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Ethanol (analytical grade) was purchased from Fisher Scientific (Loughborough, UK) used as received. All other reagents were of analytical grade.

4.2 Preparation of $[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$

Reddish-brown crystals of $[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$ were formed through the reaction of 0.09 g (0.63 mmol) NaI and 0.16 g (0.63 mmol) I_2 in 25 ml ethanol under stirring at room temperature and addition of 0.2 ml (1.26 mmol) 12-crown-4. The crystals appear after 3 days at room temperature.

4.3 Bacterial strains and culturing

All the eight microorganisms used in this study are from our microbial culture collection unit in Ajman University. They were strains isolated from the patients in the Khalifa Hospital, Ajman, UAE. These strains were cultured at 37°C except *C. albicans*, which was grown at 30°C, and all were maintained at 4°C using MHB media. Selected bacterial strains were inoculated in MHB, and cultivated 24 h at 37°C. Antifungal activity of $[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$ was tested on *C. albicans*. The selected fungus was inoculated in YPD Broth (1% Peptone, 0.5% Yeast extract, 2% Glucose, Difco & Co, Corpus Christi, TX, USA) and cultivated 24 h at 30°C.

4.4 Investigation / Determination of Antibacterial and Antifungal Properties of $[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$

$[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$ was tested on gram positive *S. pyogenes*, *S. faecalis*, spore forming bacteria *B. subtilis* and gram negative bacteria *E. coli*, *P. mirabilis*, *P. aeruginosa* and *K. pneumoniae*. The bacterial cultures were incubated overnight in 5 mL Mueller Hinton broth in a Certomat BS-T incubation shaker (Sartorius Stedim Biotech, Aubagne, France) at 37°C and 150 rpm until the culture reached an optical density of 600 of 1.0 (Spekol UV VIS 3.02, Analytic Jena, Jena, Germany), which corresponds to 108 colony-forming units per ml. Antifungal activity of $[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$ was tested on *C. albicans*. The selected fungus was inoculated in YPD Broth (1% Peptone, 0.5% Yeast extract, 2% Glucose, Difco & Co, Corpus Christi, TX, USA) and cultivated 24 h at 30°C.

4.5 Procedure for Zone of Inhibition Plate Studies

The antimicrobial activities of $[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$ were evaluated against the above mentioned microorganisms, using the zone of inhibition plate method because the crown ether complex was only partly soluble in polar media. Therefore, we selected the method by White et al. [35]. 20 mL liquid-autoclaved Mueller Hinton agar (pH 7.3±0.2 at 25°C) was poured onto the disposable sterilized Petri dishes, solidified and dried in an incubator. By using a sterilized glass rod, 100 µL of the microbial suspensions in the Mueller Hinton broth was streaked over the dried surface of the agar plates, spread uniformly and then the plates were dried. A 6 mm diameter circular piece of MHB agar was removed by a sterile cork borer from the plate center and filled with 20 mg $[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$. Three other agar plates were each loaded in the same way with 20 mg Iodine, Sodium Iodide and 12-crown-4 as positive controls, while another was loaded with 50 µl of dimethyl sulfoxide (DMSO) as negative control. The plates were then incubated at 37 °C for 24 h. Diameter of zone of inhibition was measured to the nearest millimeter with a ruler. The experiments were replicated twice. Antimicrobial activity was evaluated based on the diameters of clear inhibition zone surrounding the sample. If there is no inhibition zone, it is assumed that there is no antimicrobial activity.

5. Conclusions

$[\text{Na}(12\text{-Crown-4})_2]\text{I}_3$ exerted antimicrobial effects on gram positive Streptococci (*S. faecalis* and *S. pyogenes*), as well as *C. albicans* through the release of free iodine from their triiodide units. The gram

negative bacteria *P. aeruginosa* and *K. pneumoniae* are inhibited slightly by $[\text{Na}(\text{12-Crown-4})_2]\text{I}_3$, while *E. coli* and *P. mirabilis* proved to be resistant towards it.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

12-crown-4	1,4,7,10-Tetraoxacyclododecan
NaI	Sodium iodide
I ₂	Iodine
I ₃ ⁻	Triiodide
MHB	Mueller Hinton Broth
DMSO	Dimethyl sulfoxide

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