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Antimicrobial activity of liposome encapsulated cyclo(L-tyrosyl- L-prolyl)

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Various studies have shown the potentially beneficial biological activities of cyclic dipeptides and in particular, cyclo(L-tyrosyl-L-prolyl) (cyclo(Tyr-Pro)) has shown fair antibacterial activity *in vitro*. This study aimed to determine if liposome encapsulation would have any significant effects on the antibacterial activity of this compound. The thin-film hydration method with extrusion was used to produce small unilamellar vesicles containing cyclo(Tyr-Pro) that were shown to have an average encapsulation of 9.4% with a mean particle size of 160.4 nm. Minimum inhibitory concentrations tested against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Bacillus subtillis* were shown to be lower in liposome encapsulated cyclo(Tyr-Pro) than for the free form, while no antimicrobial activity was noted for either encapsulated nor non-encapsulated drug against the fungus *Candida albicans* or two methicillin-resistant strains of *Staphylococcus aureus* (MRSA). A positive control of liposome encapsulated amoxicillin was shown to be extremely active against both MRSA strains. The results confirm that liposome encapsulation has the potential to enhance activity as well as to overcome bacterial resistance towards current antibacterial agents.

1. Introduction

The potential biological activity of diketopiperazines have been reported extensively with a wide range of activities being identified (Prasad 1995). Recently, however, more intensive investigations have been conducted that explore this group of compounds' antimicrobial activity, where cyclic dipeptides such as cyclo(Tyr-Pro), cyclo(Phe-Pro), and cyclo(Leu-Pro) have shown promising benefits in the treatment of infections with a wide variety of bacteria (Graz et al. 1999; Rhee 2004). Apart from this, some studies have isolated this group of compounds from bacterial supernatants of *Pseudomonas spp* and have concluded that these molecules in quorum sensing, being able to inhibit or promote bacterial functions such as swarming and overgrowth (Holden et al. 1999).

Apart from its effects in bacteria, cyclo(L-tyrosyl-L-prolyl), also known as maculosin, has also been found to exhibit phytotoxic actions, being produced by the fungus *Alternaria alternata* (Park and Strobel 1994). This makes this molecule of particular interest in terms of its biological activity, however, limitations in terms of permeability may result in therapeutically limited potential (van der Merwe et al. 2008).

Efforts to improve cell entry through modification to enhance its physicochemical properties is not the only means to improve cell entry and hence its biological activity. Encapsulation of compounds into liposomes has been an established method of improving cellular entry, enhancing pharmacological activity while reducing systemic side effects and apart from these properties, the fact that liposomes are taken up by the body's reticuloendothelial system and concentrate in inflamed tissues makes them ideal drug delivery systems for antibacterials (Smith 2005; Hillery 1997; Allen and Moase 1996).

Apart from being ideal drug delivery systems for antibacterial agents as they improve entry into bacteria by membrane fusion (Rukholm et al. 2006), liposomes may also play an important role in the treatment of infection by resistant strains of bacteria. Recent reports suggest a vast increase in resistant strains of *Staphylococcus aureus* known as methicillin resistant *Staphylococcus aureus* (MRSA) with the number of isolates in the United States rising from 35.9% in 1992 to 64.4% in 2003 (Klevens et al. 2006). Besides MRSA, several other bacteria with pathogenic potential, such as *Pseudomonas aeruginosa*, have also shown resistance to antibacterial agents, whether it be inherent or by mutation (Rukholm et al. 2006) or through the development of biofilms which inhibit antibacterial penetration (Smith 2005). The application of liposome technology in the treatment of infection by resistant strains of bacteria has shown positive outcomes (Smith 2005; Rukholm et al. 2006; Huang et al. 2011) which justifies the use of liposomes in improving the antibacterial activity of cyclic dipeptides such as cyclo(Tyr-Pro). The aim of this study was, therefore, to determine the minimum inhibitory concentrations (MIC) of liposome encapsulated cyclo(Tyr-Pro) against several Gram positive and Gram negative bacteria, as well as clinical isolates of MRSA.

2. Investigations, results and discussion

2.1. Liposome characterization

Particle size for liposomes was found to be 160.4 ± 9.8 nm which is within normal limits for extruded liposomes (Berger et al. 2001). Encapsulation efficiency varied from batch to batch but on average was shown to be 9.44%. All batches produced were physically stable and showed no signs of coagulation.

Table 1: Minimum inhibitory concentrations (MIC) of free and liposomal cyclo(Tyr-Pro) and amoxicillin against several bacterial strains (n = 4 for each value)

	cyclo(Tyr-Pro)	Lip. cyclo(Tyr-Pro)	Amoxicillin	Lip. Amoxicillin
E. coli	>10 mg/ml	5 mg/ml	–	–
K. pneumoniae	>10 mg/ml	0.625 mg/ml	–	–
B. subtillis	>10 mg/ml	0.3125 mg/ml	–	–
C. albicans	>10 mg/ml	>10 mg/ml	–	–
S. aureus	>10 mg/ml	0.3125 mg/ml	0.0625 mg/ml	0.0625 mg/ml
MRSA 1	>10 mg/ml	>10 mg /ml	2 mg/ml	0.0625 mg/ml
MRSA 2	>10 mg/ml	>10 mg/ml	2 mg/ml	< 0.008 mg/ml

Lip. = Liposome encapsulated

2.2. Minimum inhibitory concentration

Minimum inhibitory concentrations (MIC) for free and encapsulated cyclo(Tyr-Pro) and amoxicillin is shown in Table 1. The activity previously reported by Milne et al. (1998) as well as Graz et al. (1999) was calculated as a percentage inhibition as compared to a positive control or determined using the Kirby-Bauer disk diffusion assay. Percentage inhibition as compared to a negative control may not be the most effective means of determining whether an agent may be clinically useful as it simply states that growth was slower than the negative control but does not clearly indicate at what concentration bacterial growth was stopped (or inhibited). The Kirby-Bauer disk diffusion assay is also considered to provide a mostly qualitative assessment of antibacterial activity as it relies on the extent to which a drug diffuses through the agar to form a logarithmic concentration gradient radiating from the disk (Kiska 1998). It is possible that this may be the reason why the results presented by these authors were not congruent with those obtained in this study. The MIC results indicated growth at all concentrations tested for free cyclo(Tyr-Pro), which may indicate that this agent, on its own, may not show benefit in the treatment of infection by the organisms tests. However, once encapsulated into the liposome formulation, significant differences in inhibition were noted with inhibition being as low as 0.3125 mg/ml for *B. subtillis* and *S. aureus*, while still showing improved activity against *E. coli* and *K. pneumoniae*. As was reported in the previous studies, no activity was noted for cyclo(Tyr-Pro) against the fungus *C. albicans*, in either the free or liposomal forms.

With the rise in the incidence in bacterial resistance being well known (Gould 2007), it was also valuable to assess the influence of liposomal encapsulation on clinical isolates of resistant forms of *S. aureus* (MRSA 1 and 2). No difference in the activity was noted (in the concentration range tested) for free or liposomal cyclo(Tyr-Pro) on either of the two clinical isolates of MRSA. It was extremely interesting to note, however, that although some limited activity of amoxicillin against these strains was noted (MIC = 2 mg/ml for both strains), encapsulation of amoxicillin in the liposome formulation resulted in a surprising increase in activity, reducing the MIC against MRSA 1 to 0.0625 mg/ml while no growth was detected within the concentration range tested for liposomal amoxicillin against MRSA 2. This result further justifies the need to investigate alternative drug delivery strategies for known antibiotics against resistant strains of bacteria, particularly with the increase in resistance noted for bacteria such as *S. aureus* as well as *M. tuberculosis* resulting in the emergence of extensively resistant tuberculosis (XDR TB) (Shah et al. 2007).

2.3. Conclusion

Rising bacterial resistance is a growing concern and with very few new antimicrobial agents having emerged in the last decade and even fewer in the pipeline (Gould 2007) it may be necessary to re-look at the current agents available from a different drug delivery perspective. As has been shown in this study, agents that had shown limited activity against a range of bacteria, proved to be significantly more effective when encapsulated in a liposome vesicle. Reasons for the increase in activity could be as a result of improved cell entry or even protection of the antimicrobial agent from enzymatic degradation. Further investigations are necessary to evaluate the reasons for improved activity as well as to evaluate how the liposome formulation can be optimized for improved activity.

3. Experimental

3.1. Chemicals

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA) while cholesterol was purchased from Northern Lipids (Barnaby, BC, Canada). Protamine sulphate was purchased from Sigma (St Louis, MO, USA). The cyclic dipeptide, cyclo(L-tyrosyl-L-prolyl) or cyclo(Tyr-Pro) was synthesized according to the method of Milne et al. (1998). Structural elucidation of the cyclic dipeptide was achieved through standard methods and included NMR spectroscopy, mass spectrometry and infrared spectroscopy. All other chemicals and reagents were obtained from local suppliers and were of analytical grade.

3.2. Preparation of liposomes

Liposomes containing cyclo(Tyr-Pro) were prepared by the thin-film hydration method with extrusion through polycarbonate membranes. Briefly, DPPC and cholesterol in a molar ratio of 2:1 (90 μmol total lipid) were dissolved in 5 ml of a 4:1 mixture of chloroform and methanol in a 50 mL round-bottom flask and dried to a lipid film with a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) at 60 °C under controlled vacuum for 3 h. The lipid film was flushed with nitrogen to remove traces of chloroform and hydrated with 5 mL of a 20 mg/mL solution of either cyclo(Tyr-Pro), chloramphenicol or amoxicillin dissolved in 5 % DMSO in normal saline. The drug-lipid suspension was shaken in a water bath at 60 °C for 3 h to allow for full hydration of the lipid film. The multilamellar liposomes formed were then extruded 15 times through a 0.1 μm polycarbonate filter (Millipore, Billerica, USA) in a stainless steel pressure extrusion device (Avanti Polar Lipids, Alabaster, USA) in order to homogenize the size and size distribution of the liposomes. The lipid content of the final suspensions were measured using the Stewart assay. Just prior to use, encapsulated drug was separated from free drug through centrifugation (19500 × g for 3 h).

3.3. Liposome characterization

Encapsulation efficiency was determined indirectly through separation of free drug using protamine to aggregate liposome vesicles. To 50 μl of suspension was added 50 μl of protamine solution (10 mg/ml) and incubated for 10 min at 37 °C. The mixture was made to 1 ml with saline and centrifuged at 5000 × g for 20 min. Total drug was established by lysing of liposomes by adding 50 μl of 10% Triton X-100 to 50 μl of suspension and making to 1 ml with saline. Concentration of cyclo(Tyr-Pro) in either the protamine aggregated supernatant (free drug) or Triton X-100-lysed solution (total drug) was determined using HPLC on a C18 column (Waters Symmetry C18, 4.6 × 150 mm) and a mobile phase of 20% methanol in water utilizing an LC2020 HPLC system incorporating a binary pump and photodiode array detector and chromatograms recorded on the software Package, LC Solutions (Shimadzu, Tokyo, Japan).

Table 2: Resistance patterns for MRSA strains in relation to *Staphylococcus aureus*. 'R' denotes resistant while 'S' denotes sensitive

Bacteria	Cloxacillin	Penicillin/ Ampicillin	Erythromycin	Clindamycin	Co-trimoxazole	Vancomycin
ATCC *S. aureus*	S	S	S	S	S	S
MRSA 984444	R	R	R	R	S	S
MRSA 985531	R	R	S	S	R	S

Particle size was determined using transmission electron microscopy (TEM). Samples were prepared on Formvar®-coated 300-mesh copper grids (SPI Supplies, West Chester, USA) where one drop of liposome suspension diluted 1:100 with de-ionised water was dropped onto the grid and blotted with Whatman No. 1 filter paper. The grids were then transferred to the surface of a freshly poured, 1% agarose gel plate to facilitate de-salting and drying of the sample (Wei and Smith 1994). TEM images were then recorded on a JEOL JEM 1210 transmission electron microscope (Tokyo, Japan) and the average particle size of 20 liposomes measured to determine mean particle size.

3.4. Bacterial strains and culture

The following ATCC strains were utilized in this study: *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Bacillus subtillis* and *Candida albicans*. Two clinical isolates of MRSA were also used (MRSA 984444 and MRSA 985531) and their resistance profiles are shown in relation to ATCC *Staphylococcus auereus* in Table 2. All bacterial strains were received as a donation from the Department of Biomedical Technology at the Nelson Mandela Metropolitan University. Bacteria were cultured at 37 °C in Mueller Hinton broth while the fungus was cultured in Sabouraud broth at 30 °C. Bacteria and fungi were all standardized to the 0.5 McFarland standard at 540 nm prior to inoculation into test solutions.

3.5. Minimum inhibitory concentration

Minimum inhibitory concentrations were determined according to the broth microdilution method. All equipment and media were sterilized by autoclaving at 121 °C for 15 min while drug solutions and liposome suspensions were filter sterilized through sterile 0.20 μm cellulose acetate filters (GEMA Medical S.L, Barcelona, Spain).

Into each well of a sterile 96-well microtiter plate (Sarstedt, Nümbrecht, Germany) was added 100 μl sterile Mueller Hinton broth for bacteria or Sabouraud broth for fungi. 100 μl of liposome suspensions or free drug was then added and serially diluted such that the concentration of drug tested ranged from 6.25 μg/ml to 10 mg/ml. 10 μl of bacterial culture containing 5×10^4 CFU were then added to each well and incubated at 37 °C (for bacteria) or 30 °C (for fungi) for 24 h. Free and liposome encapsulated amoxicillin and chloramphenicol were used as positive controls while sterile saline was used as a negative control.

As a result of liposome suspensions being turbid, standard methods of optically evaluating wells for growth was not possible. For this reason, MTT was used as an indicator of bacterial viability. To each well, 50 μl of MTT solution (5 mg/ml) was added and incubated for a further 6 h. After incubation, the plates were centrifuged at 3000 rpm for 15 min and the supernatants aspirated and replaced with 150 μl DMSO to solubilise the formazan precipitate formed. The MIC was defined as the lowest concentration at which no purple colour was visible. Each concentration was tested in quadruplicate and the average concentration at which no growth was present was reported.

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