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ORIGINAL ARTICLE

Enhancement of the efficacy of erythromycin in multiple antibiotic-resistant gram-negative bacterial pathogens

S. Saha[1], P.B. Savage[2] and M. Bal[1]

1 Department of Physiology, University College of Science and Technology, University of Calcutta, Kolkata, West Bengal, India
2 Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA

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Correspondence
Manjusri Bal, Microbiology Section, Department of Physiology, University College of Science and Technology, University of Calcutta, 92, Acharya Prafulla Chandra Road, Kolkata-700009, West Bengal, India.
E-mail: ayoshee_ms@yahoo.co.in

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Abstract

Aims: To improve the efficacy of erythromycin, a hydrophobic antibiotic, against multiple antibiotic-resistant gram-negative bacterial pathogens by enhancing their outer membrane permeability.

Methods and Results: Fifty-one nonrepeat gram-negative bacterial pathogens of various genera, resistant to multiple antibiotics, including erythromycin, were selected by disc agar diffusion tests. The amphiphilic cationic steroid antibiotic, Ceragenin CSA-13, a potent permeabilizer of bacterial outer membranes, reduced the minimum inhibitory concentration of erythromycin in 92% of the bacterial pathogens selected for the test, when supplemented with erythromycin. A synergistic effect of Ceragenin CSA-13 and erythromycin in combination was also observed. Spectrofluorimetric study confirmed that Ceragenin CSA-13 acts by depolarizing the bacterial outer membrane. The toxicity of Ceragenin CSA-13 was evaluated to be insignificant by measuring 'median lethal dose' (LD_{50}) on mouse model.

Conclusions: Ceragenin CSA-13 may be useful as an agent to make erythromycin effective against infections caused by multiple antibiotic resistant gram-negative bacteria.

Significance and Impact of the Study: The outcome of the study suggests erythromycin–Ceragenin combination as a new approach to overcome the problem associated with the rapid emergence of multi-drug-resistant pathogens. The insignificant toxicity of Ceragenin CSA-13, as found, supports the possibility of the application of this compound for human therapeutics.

Introduction

Resistance of bacteria to antibiotics, particularly human pathogens, appeared within a few years after the onset of the antibiotic era (Kirby 1944), and this problem has reached epic proportions owing to overuse and improper use of antibiotics (Hellinger 2000). Microbial resistance to antibiotics currently spans all known classes of natural and synthetic compounds (D'Costa *et al.* 2006). Increasing resistance of pathogenic bacteria to many commonly used antibiotics highlights the need for either the development of new and novel antibiotics (Bax *et al.* 2001; Livermore 2004) or the improvement of efficacy of established antibiotics by the development of new agents capable of enhancing antibiotic activity (Neu 1992).

The outer membrane of gram-negative bacteria acts as a relatively effective permeability barrier (Nikaido 2003). Gram-negative bacteria are inherently resistant to hydrophobic antibiotics, such as macrolides, as their outer membrane limits the entry of these antibiotics into the cell (Poole 2002). The hydrophobic macrolide antibiotic erythromycin is used extensively in treating gram-positive bacterial infections owing to its selective toxicity on bacterial protein synthesis machinery (Champney and Tober 2000). However, it has very limited use against gram-negative bacteria (Vaara 1993) owing to permeability barriers of their outer membrane. The lack of susceptibility of

gram-negative bacteria to erythromycin has also been suggested owing to the action of efflux pumps (Chollet *et al.* 2004). It is likely that both mechanisms cause resistance to hydrophobic antibiotics, and effective permeabilization of the outer membrane may overcome these resistance pathways.

Ceragenins are synthetically produced cholic acid derivatives with amine groups appended on one face of the steroid (Savage *et al.* 2002). Studies with a series of Ceragenins revealed that the hydrophobic chains present in their structures facilitate their movement through the outer membrane to the cytoplasmic membrane (Li *et al.* 1999). In addition, Ceragenins sensitize gram-negative bacteria to hydrophobic antibiotics by effectively permeabilizing the outer membranes of these organisms (Ding *et al.* 2002).

In this study, Ceragenin CSA-13 was used to facilitate the entry of erythromycin through the outer membrane of gram-negative bacteria so that effective *in vivo* bactericidal concentrations were reached.

As depolarization of the bacterial outer membrane is accompanied by an increase in fluorescence of cyanine dyes (Sims *et al.* 1974), the outer membrane-depolarizing activity of Ceragenin CSA-13 was determined using a cyanine dye.

The health hazards likely to arise from the exposure of human being to Ceragenin CSA-13 were tested on mouse model. The toxic effect of Ceragenin CSA-13 was determined from the value of its 50% lethal dose (LD_{50}) and therapeutic index (TI) (Zbinden and Flury-Roversi 1981); it did not show any significant toxic effect on mice.

The results of this study suggest that a combination of erythromycin and Ceragenin CSA-13 may be used as an effective approach for controlling gram-negative bacterial pathogens.

Materials and methods

Bacterial strains

Two hundred and fifty-six nonrepeat, pathogenic clinical isolates were collected from different hospitals of Kolkata, India. Among these, 110 (43%) were gram positive. From the rest, 146 (57%) gram-negative bacterial pathogens, multiple antibiotic-resistant strains were selected for this study on the basis of their antibiotic-resistance profiles. The selected bacterial strains isolated from different specimens, e.g. pus, urine, blood, sputum, throat swab, burn, tracheal secretion, abdominal drain fluid etc. were: *Escherichia coli* (22), *Klebsiella* spp. (12), *Proteus* spp. (four), *Pseudomonas* spp. (12) and *Salmonella typhi* (one). Bacterial strains were identified by gram staining and various conventional biochemical tests (Forbes *et al.* 1998).

Media and chemicals

For the preservation and growth of bacteria, Mueller–Hinton broth (MHB; Himedia, Mumbai, India) and agar (Merck Specialities, Mumbai, India), whenever necessary, were used. Antimicrobial agents used in this study were ampicillin (AMP; Lyka Hetero Healthcare, Thane, India), chloramphenicol (CHL; Sigma, St Louis, MO, USA), gentamicin (GEN; Nicholas Piramal, Dhar, India), ciprofloxacin (CIP; Ranbaxy Laboratories, Gurgaon, India), trimethoprim : sulfamethoxazole (1 : 5) (TMP; Piramal Health Care, Mumbai, India), cephalexin (LEX; Lupin Ltd., Mumbai, India), cefuroxime (CXM; Glaxo Smithkline Pharmaceuticals, Nashik, India), cephotaxime (CTX; Alkem Laboratories, Mumbai, India), streptomycin (STR; Synbiotics, Vadodara, India) and erythromycin (ERY; Sigma-Aldrich, St Louis, MO, USA). All the antibiotic solutions except chloramphenicol and erythromycin were prepared in sterile water. These two antibiotics were first dissolved in alcohol and then diluted to appropriate concentrations with sterile water. Ceragenin CSA-13 is a product of Ceragenix Pharmaceuticals Inc., Denver, Colorado, USA. The stock solution of Ceragenin CSA-13 was prepared in sterile water and stored at −20°C. The fluorimetric assay of membrane-depolarizing activity of Ceragenin CSA-13 was performed using the cyanine dye 3,3′-dietylthiodicarbocyanine iodide (DiS-C_2-[5]; Alfa aesar, MA, USA). The stock solution of the dye was prepared freshly in sterile water and stored in a dark place throughout the experiment. Tris buffer (Spectrochem, Mumbai, India), sucrose (Qualigens Fine Chemicals, Mumbai, India) and magnesium sulfate ($MgSO_4$, $7H_2O$; Qualigens Fine Chemicals, Mumbai, India) were used to prepare buffer solution for fluorimetric assay.

Disc agar diffusion (DAD) test

The test bacterium taken from an overnight culture (inoculated from a single colony) was freshly grown for 4 h having approx. 10^6 CFU ml^{-1}. With this culture, a bacterial lawn was prepared on Mueller–Hinton agar.

Filter paper discs of 6-mm size were used to observe antibiotic susceptibility patterns against 10 antibiotics (amount of antibiotic per disc in microgram [μg]; AMP [10], CHL [30], GEN [10], CIP [5], TMP [5], LEX [30], CXM [30], CTX [30], STR [10], ERY [15]). Antibiotic discs were prepared according to Bauer *et al.* (1966). The diameter of zone of bacterial growth inhibition surrounding the disc (including the disc), was measured and compared with a standard for each drug. This gave a profile of drug susceptibility *vis-à-vis* antibiotic resistance (Bauer *et al.* 1966). *Staphylococcus aureus* ATCC 25923 and

E. coli ATCC 25922 were taken as the quality control strains in DAD test.

Minimum inhibitory concentration (MIC)

The MIC values of Ceragenin CSA-13 and erythromycin alone and in combination were determined by two-fold serial broth dilution method (Erricson and Sherris 1971).

Fractional inhibiting concentration (FIC)

The ability of Ceragenin CSA-13 to sensitize gram-negative bacteria to erythromycin was quantified using FIC values (Eliopoulos and Moellering 1991), calculated using the formula:

$$\mathrm{FIC} = [A]/\mathrm{MIC_A} + [B]/\mathrm{MIC_B}$$

where, $\mathrm{MIC_A}$ and $\mathrm{MIC_B}$ are the respective MIC of erythromycin and Ceragenin CSA-13, while $[A]$ and $[B]$ are the respective concentrations erythromycin and Ceragenin CSA-13, respectively, at which they inhibit bacterial growth, in combination. In this study, $[B]$ was kept constant (0·5 μg ml^{-1}), and $[A]$ for the bacterial strains was determined. Synergy is defined by FIC < 0·5 (Schmidt *et al.* 2001).

Measurement of membrane depolarization

Bacterial culture grown up to early exponential phase (optical density at 620 nm was 0·2–0·3) was harvested by centrifugation, washed twice in 0·1 mol l^{-1} Tris buffer, pH 7·6 containing 0·25 mol l^{-1} sucrose and 0·005 mol l^{-1} $MgSO_4$, $7H_2O$ and suspended in the same buffer at an optical density of 0·09 (620 nm). The cyanine dye was added giving 1·0 μmol l^{-1} concentration and allowed to incorporate into the cells for 15 min. Ceragenin CSA-13 was added to its final concentration of 0·5 μg ml^{-1}. Excitation and emission wavelengths of 600 and 660 nm, respectively, were used to monitor depolarization. The fluorescence of DiS-C_2-(5) was measured at ambient temperature setting the fluorescence intensity of the cell suspension plus dye solution at zero. The sample was slowly stirred during the measurements that were taken every 30 s using a Jasco FP-6200 Spectrofluorimeter.

Experimental animals, housing and feeding conditions

For toxicological testing, healthy and young male Swiss-albino mice having 20–25 g body weight were used. The temperature of the animal room was 22°C (±3°). Mice were kept in sufficiently spacious cages so that there was no difficulty in observing each animal clearly. Conventional laboratory feeding and unlimited drinking water were provided to the animals.

Approval for working on animals was taken from the Institutional Animal Ethical Committee (Registration No. 820/04/ac/cpcsca, dated 06.08.2004), Department of Physiology, University of Calcutta.

Determination of LD_{50}

Animals were acclimatized to the laboratory conditions for 7 days prior to the test. Doses were selected carefully to exclude extremely toxic doses. Overnight fasted mice were divided into six groups (five test groups and one control group), each group consisting of eight mice. Individual weight of each animal was determined shortly before administration of the test sample. Animals of test groups were injected with 0·1 ml aqueous solution of Ceragenin CSA-13 of five different concentrations by intraperitoneal route. Animals of the control group were injected with an equal volume of sterile water. Food was withheld for further 3 h after injection. Following administration, individual animals were monitored for 14 days according to the guidance for single-dose acute toxicity testing provided by Center for Drug Evaluation and Research (http://www.fda.gov/cder/guidance/pt1.pdf/1998/february). The time of onset of toxic symptoms, duration of recovery, change in food consumption rate, number of animals killed and the precise time of death were recorded. LD_{50} was determined as a mean of observations of five repeated experiments.

Results

DAD test

The antibiotic-resistance profile, as determined by DAD test, revealed that out of 146 gram-negative bacterial pathogens, 51 strains were resistant to at least five of the 10 different antibiotics, and erythromycin resistance was common for all of them; all these 51 strains were chosen for this study.

MIC of erythromycin and Ceragenin CSA-13

The MIC values of erythromycin for various test bacterial strains were determined both in the absence and presence of Ceragenin CSA-13 that was added in very low concentration (0·5 μg ml^{-1}). In each set of experiment, control tubes showed no significant growth inhibitory effect of Ceragenin CSA-13 at this concentration. The MIC values of this compound for the selected 51 strains varied within 2 to 8 μg ml^{-1}; Table 1 shows MIC for 16 representative strains. The table also presents MIC values of erythromycin

Table 1 Effect of Ceragenin CSA-13 on minimum inhibitory concentration (MIC) of erythromycin

Bacterial strains	MIC (μg ml^{-1})				
	Ceragenin CSA-13 (MIC_B)	Erythromycin (MIC_A)	Erythromycin in combination* [A]	Fold reduction in erythromycin MIC values	FIC
Escherichia coli NRS-399	2	>1000	31·25	>32	0·28
E. coli ICH-410	4	250	31·25	8	0·25
E. coli AG-124	8	1000	31·25	32	0·09
E. coli AG-155	2	1000	31·25	32	0·28
E. coli SCRL-19	4	1000	31·25	32	0·15
E. coli SCRL-20	8	>1000	62·5	>16	<0·19
Klebsiella spp. NRS-318	4	>500	7·8125	>64	<0·15
Klebsiella spp. NRS-325	4	>500	15·625	>32	<0·14
Klebsiella spp. NRS-327	8	>500	15·625	>32	<0·09
Klebsiella spp. AG-42	4	>1000	31·25	>32	<0·15
Pseudomonas spp. NRS-238	2	500	62·5	8	0·37
Pseudomonas spp. ICH-295	6·4	2000	250	8	0·20
Pseudomonas spp. ICH-1351	6·4	>2000	250	>8	<0·20
Pseudomonas spp. AG-1003	8	500	31·25	16	0·12
Salmonella typhi RGK-7	2	500	31·25	16	0·31
Proteus vulgaris NRS-58	>8	500	500	–	≥1

The result is a mean of three different sets of experiments.

Name of different strain collection centres of Kolkata, India:

NRS, Nil Ratan Sirkar Medical College and Hospital; ICH, Institute of Child Health; AG, Apollo Gleneagles Hospital; SCRL, Scientific Clinical Research Laboratory; RGK, R. G. Kar Medical College and Hospital.

*Ceragenin CSA-13 was added in a constant amount of 0·5 μg ml^{-1} [*B*] with different concentrations of erythromycin.

for these strains. It was observed that a majority of the strains were highly resistant to erythromycin growing at concentrations >250 μg ml^{-1}. Ceragenin CSA-13 sensitized majority of the bacterial strains to erythromycin, which was reflected by the reduction in erythromycin MIC values in its presence (Table 1). Except the *Proteus* strains, CSA-13 exhibited its effect on all strains of the four other species tested (Table 1).

FIC of erythromycin and Ceragenin CSA-13 combination

In this experiment, the concentration at which Ceragenin CSA-13 inhibited bacterial growth in combination with erythromycin was predetermined and the value was 0·5 μg ml^{-1}. It was calculated from the formula described earlier (Eliopoulos and Moellering 1991), that for various bacterial species tested and for different values of MIC_A, MIC_B and [*A*], FIC values were less than 0·5. The only exception was *Proteus* spp., which exhibited FIC values more than 0·5. Thus, the condition for synergistic effect (Schmidt *et al.* 2001) of erythromycin and Ceragenin CSA-13 combination was satisfied by all varieties of multiple antibiotic-resistant gram-negative bacterial pathogens tested, excluding *Proteus* spp. (results of few representative strains are shown in Table 1).

Fluorometric assay of bacterial membrane depolarization

The fluorescent membrane potential probe [DiS-C_2-(5)] was scanned to verify its excitation (600 nm) and emission (660 nm) maxima at experimental conditions. Except *Proteus* strains, rapid increase of fluorescence of the dye was observed after Ceragenin CSA-13 was added to the mixture of bacterial cell suspension and dye solution (Fig. 1). In case of *Proteus* strains, fluorescence intensity changed randomly (Fig. 1).

LD_{50} and TI of Ceragenin CSA-13

The mortalities of mice for doses 10, 20, 25, 30 and 40 μg g^{-1} of body weight were 0%, 33%, 55%, 79% and 87%, respectively, as determined by Reed and Muench's method (Gulati and Pal 1985). With the help of these values, LD_{50}, the statistically derived single dose expected to cause death in 50% of test animals expressed in terms of weight of test substance per unit weight of test animal (mg kg^{-1} or μg g^{-1}), of Ceragenin CSA-13 was determined to be 24·74 μg g^{-1} of body weight. The *in vitro* effective dose (ED), i.e. the minimal dose required for the desired beneficial effect of Ceragenin CSA-13 was 0·5 μg ml^{-1} (Table 1). Thus, the TI evaluated from the ratio of *in vitro* ED to LD_{50} was:

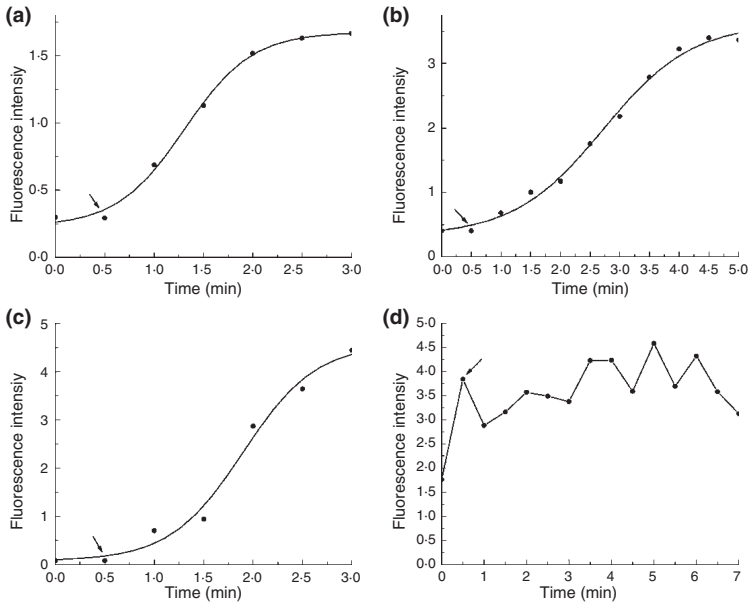

Figure 1 Membrane depolarization of bacterial cells by Ceragenin CSA-13 (0·5 μg ml^{-1}) as measured by change in fluorescence intensity of the dye DiS-C_2 (5). *Escherichia coli* NRS-399 (a), *Klebsiella* spp. AG-408 (b), *Pseudomonas* spp. ICH-1351 (c), and *Proteus mirabilis* NRS-300 (d). → indicates the time of Ceragenin CSA-13 addition.

$$TI = 24 \cdot 74/0 \cdot 5 = 49 \cdot 48$$

which is sufficient to indicate that the toxicity of Ceragenin CSA-13 is not considerable.

Discussion

Cellular impermeability is one of the major mechanisms of intrinsic resistance of bacteria to various antibiotics. Consequently, chemical agents that reduce this impermeability barrier have a significant role in enhancing bacterial susceptibility to those antibiotics (Ayres *et al.* 1999).

Erythromycin penetrates the outer membrane of gram-negative bacteria very poorly. For this reason, gram-negative bacteria express high-level resistance to this antibiotic. We, in this study, successfully employed Ceragenin CSA-13 to increase the activity of erythromycin against various species and various genera of pathogenic bacteria by means of outer membrane permeabilization. Ceragenins manifest a broad-spectrum bactericidal activity (Savage *et al.* 2002). The concentration of Ceragenin CSA-13 (0·5 μg ml^{-1}), chosen for this study, was at least four times lower than its MIC value for the test strains of bacteria (Table 1). It was tested in subsequent trial experiments that this particular concentration of Ceragenin CSA-13 is optimal for exhibiting the permeabilizing activity, and at the same time, did not show its bactericidal activity. The reductions of erythromycin MIC values, in presence of Ceragenin CSA-13, varied from strain to strain in the same species. No change in erythromycin MIC values was observed in case of *Proteus* strains tested. This observation is supported by the previous reports that *Proteus* spp. is resistant to the action of various outer membrane permeabilizers owing to the presence of high content of phosphate-linked 4-aminoarabinose in its lipopolysaccharides (Sidorczyk *et al.* 1983). This would also explain why synergistic effect of erythromycin and Ceragenin CSA-13 combination was not observed in *Proteus* spp. Owing to modifications in lipopolysaccharide composition, mutants of *E. coli* and *Salmonella* spp. also become resistant to various outer membrane permeabilizers, *viz.* polymyxin, EDTA etc. in comparison with their parent strains (Vaara 1992). Hence, Ceragenin CSA-13 might be very useful, as it is effective, more or less, on all the pathogenic bacterial strains (except *Proteus* spp.) we selected randomly for this study.

The fluorescence response of the cyanine dye DiS-C_2-(5) changes with the cell membrane potential. Cellular depolarization results in release of the dye molecules into the extra-cellular medium in which dye molecules fluoresce with higher intensity (Sims *et al.* 1974). In this experiment, the rapid increase of fluorescence intensity of the dye owing to the addition of Ceragenin CSA-13 (Fig. 1) demonstrates the membrane depolarizing activity of this compound. Hence, it can be stated that Ceragenin CSA-13 permeabilizes the outer membrane of the test bacterial strains by depolarizing their outer membrane. The observed irregular changes of fluorescence intensity in *Proteus* cells (Fig. 1) probably represent their intrinsic resistance to the outer membrane permeabilizing activity of Ceragenin CSA-13.

As toxicological testing of an investigational new drug is required prior to human application, the preliminary toxicity of Ceragenin CSA-13 was determined by testing

the acute toxicity in mice. Though the values of LD_{50} and TI are satisfactory, more intensive testing of acute and chronic toxicity of Ceragenin CSA-13 on higher animals is necessary.

In conclusion, Ceragenin CSA-13 may be used as an agent, which is expected to expand the choice of antibiotics for treatment of infections caused by multiple antibiotic resistant bacterial pathogens by enlarging the arsenal of antibiotics. The therapeutic crisis threatened by emerging antimicrobial resistance may be overcome partially by utilizing the alternative approach as described in this paper. Furthermore, previous reports suggest that ceragenins are useful for clinical application as they demonstrate affinity for prokaryotic cells over eukaryotes (Ding *et al.* 2004). Although the bacterial pathogens (*viz. E. coli*, *Klebsiella* spp., *Pseudomonas* spp. and *Salmonella* spp.) selected for our experiment were collected from various types of specimens from human body, all of them may cause enteric diseases. Our findings may therefore be of great significance in developing countries of the world, which account for more than 95% of enteric infections (http://www.meetingsmanagement.com/ved_2007).

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