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ORIGINAL ARTICLE

Evaluation and characterization of antimicrobial properties of pregnenolone-derivatives on *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Escherichia coli*

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ABSTRACT. Recently, steroid-antibiotic conjugates have been developed as potential therapeutic agents for infectious diseases. In this work the antibacterial activity of two pregnenolone-derivatives was evaluated on *S. aureus, K. pneumoniae* and *E. coli*, using the dilution method and the minimum inhibitory concentration (MIC). The results indicate that bacterial growth of *S. aureus* was inhibited with cefotaxime (MIC = 0.25 mg/ml), gentamicin (MIC = 0.0125 mg/ml), hemisuccinate-pregnenolone (MIC = 1 mg/ml), ethylenediamine-hemisuccinate-pregnenenolone (MIC = 0.25 mg/ml) and the mixture of the two pregnenolone-derivatives (MIC = 0.5 mg/ml). Other results, showed that bacterial growth of *E. coli* was also inhibited with cefotaxime (MIC = 0.25 mg/ml), gentamicin (MIC = 0.00625 mg/ml), hemisuccinate-pregnenolone (MIC = 1 mg/ml), ethylenediamine-hemisuccinate-pregnenenolone (MIC = 0.5 mg/ml) and the mixture of two pregnenolone-derivatives (MIC = 0.5 mg/ml). Alternative experiments showed that bacterial growth of *K. pneumoniae* was inhibited with cefotaxime (MIC = 0.125 mg/ml), gentamicin (MIC = 0.0.125 mg/ml), hemisuccinate-pregnenolone (MIC = 1 mg/ml), ethylenediamine-hemisuccinate-pregnenenolone (MIC = 0.5 mg/ml) and the mixture of two pregnenolone-derivatives (MIC = 0.5 mg/ml). Our results suggest that effect induced by pregnenolone-derivatives could be by the interaction with some bacterial membrane factors that are specific for bacterial resistance. In this sense, the antibacterial activity of pregnenolone-derivatives can depend of the nature of functional groups involved in their chemical structure that seems to be the key required for antibacterial activity.

Key words: Ethylenediamine-hemisuccinate-pregnenenolone, *S. aureus*, *K. pneumoniae*, *E. coli*.

RESUMEN. Recientemente han sido desarrollados conjugados esteroide-antibiótico como agentes potenciales terapéuticos para enfermedades infecciosas. En este trabajo fue evaluada la actividad antibacteriana de dos derivados de pregnenolona sobre *S. aureus, K. pneumoniae* y *E. coli* usando el método de dilución y la concentración mínima inhibitoria (CMI). Los resultados obtenidos indican que el crecimiento bacterial de *S. aureus* fue inhibido con cefotaxima (MIC = 0.25 mg/ml), gentamicina (MIC = 0.0125 mg/ml), hemisuccinato-pregnenolona (MIC = 1 mg/ml), etilenediamina-hemisuccinato-pregnenenolona (MIC = 0.25 mg/ml) y la mezcla de los dos derivados de pregnenolona (MIC = 0.5 mg/ml). Otros resultados mostraron que el crecimiento bacterial de *E. coli* fue también inhibido con cefotaxima (MIC = 0.25 mg/ml), gentamicina (MIC = 0.00625 mg/ml), hemisuccinato-pregnenolona (MIC = 1 mg/ml), etilenediamina-hemisuccinato-pregnenenolona (MIC = 0.5 mg/ml) y la mezcla de los dos derivados de pregnenolona (MIC = 0.5 mg/ml). Experimentos alternativos, mostraron que el crecimiento bacterial de *K. pneumoniae* fue inhibido con cefotaxima (MIC = 0.125 mg/ml), gentamicina (MIC = 0.0.125 mg/ml), hemisuccinato-pregnenolona (MIC = 1 mg/ml), etilenediamina-hemisuccinato-pregnenenolona (MIC = 0.5 mg/ml) y la mezcla de los dos derivados de pregnenolona (MIC = 0.5 mg/ml). Nuestros resultados sugieren que el efecto inducido por los derivados de pregnenolona podría ser por la interacción con algunos factores de la membrana bacterial que son específicos para la resistencia bacterial. En este sentido, la actividad antibacterial de los derivados de pregnenolona puede depender de la naturaleza de los grupos funcionales involucrados en su estructura química que parece ser la llave requerida para la actividad antibacterial.

Palabras clave: Etilenediamina-hemisuccinato-pregnenenolona, *S. aureus*, *K. pneumoniae*, *E. coli*.

INTRODUCTION

Epidemiological and clinical studies suggest that infectious diseases are one of the main causes of mortality in the world.[1-3] Several causal agents, such as *S. aureus*,[4] *K. pneumoniae*[5] and *E. coli*[6] among others,[7] have been shown to accelerate the progression of these pathologies. Although there are many therapeutic agents for the treatment of these bacterial microorganisms,[8-10] unfortunately, prolonged antibiotic therapy may induce bacterial-resistance,[11,12] because some bacteria have developed ways to circumvent the effects of antibiotics.[13,14] For example, several studies indicate that β-lac-

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tam antibiotics (methicilin/oxacillin) induced resistance in *S. aureus*.[15,16] Other studies showed that antibiotic-resistant strains have emerged among Gram-negative bacilli such as *K. pneumoniae*[17] and *E. coli*.[18] Therefore, antibiotic resistance can be considered a serious threat for the human health; this fact requires an international approach to its management. In this sense, new drugs have been developed for control of bacterial resistance.[19-21] For example, there has been a resurgence of interest in steroids as potential therapeutic agents for infectious diseases.[22] In this context, several steroid-antibiotics have been developed to mimic the antibacterial behavior of endogenous peptide antibiotics.[23] This task includes selective association of the steroid-antibiotic with disruption of bacterial membranes.[24] The association relates to the chemical structural characteristics of the steroid-antibiotic agents such as, cationic forms and facially amphiphilic conformations, which seems to be the key required for antibacterial activity. It has also been suggested that membrane selectivity is primarily derived from ionic recognition of negatively charged bacterial membranes.[25] In addition, several studies suggest that functional groups of steroid-derivative are involved in the bacterial activity.[26] Therefore, in this work our initial design included two pregnenolone-derivatives; the first steroidal compound (hemisuccinate-pregnenenolone) has a spacer arm in the A-ring with both: ester and carboxyl groups *(-O-C=O-$(CH_2)_2$-COOH)*; the second steroid-derivative (ethylenediamine-hemisuccinate-pregnenenolone) has the presence of the *-O-C=O-$(CH_2)_2$-C=O-NH-$(CH)_2$-NH_2* fragment in the A-ring. These pregnenolone-derivatives were used to asses the antibacterial activity on *S. proteus*, *K. pneumoniae* and *E. coli* using the microbial minimal inhibitory (MIC_{90}) method described by Chiong and coworkers.[27] Our aim was to have new drugs that can be used for treatment of infectious disease.

MATERIAL AND METHODS

General methods

Strains. The microorganisms in this study belonged to the strain bank at the Department of Pharmaco-Chemistry at the Facultad of Ciencias Quimico-biologicas of the Universidad Autonoma de Campeche. The strains are certified by the Center for Disease Control in Atlanta and were as follows. S. aureus (ATCC 25923), *K. pneumoniae* (ATCC 700603) and *E. coli* (ATCC 25922). The strains are kept under refrigeration at 4 ºC in special gel (BBL).

Antimicrobial agents. Pregnenolone-hemisuccinate (5-Pregnen-20-one, 3-(3-carboxy-1-oxopropoxy) and ethylenediamine-hemisuccinate-pregnenolone (N-(2-aminoethyl)-succinamic acid 17-acetyl-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradeca- hydro-1H-cyclopenta [α]phenanthren-3-yl-ester) (Fig. 1) were synthesized by the method reported by Figueroa.[28] The compounds were dissolved in methanol and diluted with distilled water. Cefotaxime, gentamicin and methicillin were used as control drugs.

Antimicrobial activity. The evaluation of antimicrobial effect of the different compounds on the bacterial species was made by method described by Chiong *et al*.[27] The bacterial species were incubated on Mc Conkey (*E. coli* and *K. pneumoniae*) and *Staphylococcus* 110 (*S. aureus*) agars for 24 hours at 37 ºC. After such time, it was be determined whether growth had taken place or not.

In addition, a series of tubes were prepared, the first of which contained 2 ml of culture medium (tripticase soy) at double concentration and the remainder (11 tubes), contained the same quantity of medium at single concentrations. From the first tube (double concentration) an aliquot of 2 ml of the studied compound (1 mg/ml) was added and stirred, from this tube an aliquot of 2 ml was taken and added to the following tube (simple concentration) and the process was successively repeated until the last 2 ml of dissolution had been used up. After this process, each tube was inoculated with 0.1 ml of the bacterial suspension, whose concentration corresponded to McFarland scale (9 x 10^8 cells/ml) and all the tubes were incubated at 37 ºC for 24 hours. Subsequently, a loop was taken from each of them and inoculated into the appropriate cultures for different bacterial organisms, and were incubated for 24 hours at 37 ºC. After such time, the minimum inhibitory concentration (MIC) was evaluated to consider the antimicrobial effect of the pregnenolone-derivatives.

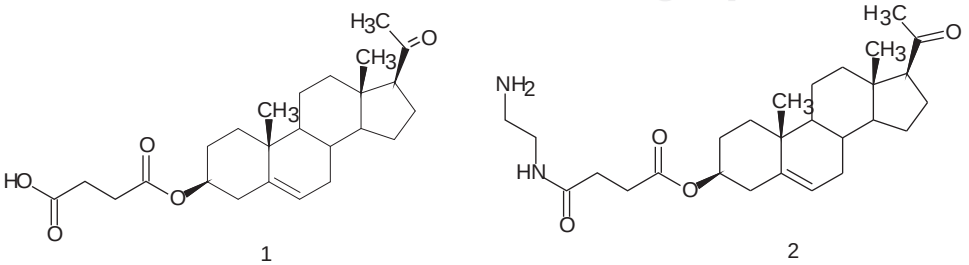

Figure 1. *Chemical structure of hemisuccinate-pregnenenolone (1) and ethylenediamine-hemisuccinate-pregnenenolone (2).*

In order to discard the effect of methanol (solvent) on the bacterial species studied, a series of the same number of tubes was prepared in parallel, to which 2 ml of methanol at 60% was added to the first and corresponding successive dilutions were added in the same way as before. In addition a control series was also performed using distilled water to pH 7.0.

RESULTS

The bacterial activity of pregnenolone-derivatives was compared with the antibacterial effect of cefotoxime, gentamicin and methicillin (controls). The results (Fig. 2) indicate that bacterial growth of *S. aureus* was inhibited with cefotaxime (MIC = 0.25 mg/ml, 5.23 x 10^{-4} mmol) and gentamicin (MIC = 0.0125 mg/ml, 2.68 x 10^{-5} mmol). It is important to mention that ampicillin did not inhibit the bacterial growth. Other results showed that in presence of hemisuccinate-pregnenolone (MIC = 1 mg/ml, 2.40 x 10^{-3} mmol) and ethylenediamine-hemisuccinate-pregnenolone (MIC = 0.25 mg/ml, 4.54 x 10^{-4} mmol) the bacterial growth was blocked in a dose-dependent manner. In addition, the obtained results using the mixture of the two pregnenolone-derivatives showed a MIC of 0.5 mg/ml (1.05 x 10^{-3} mmol) on bacterial growth of *S. aureus*.

In the other hand, alternative experiments in Gram-negative bacteria (*K. pneumoniae* and *E. coli*) using the same control-antibiotics and to evaluate the antibacterial effect of pregnenolone-derivatives were made. The results indicate that bacterial growth of *E. coli* was inhibited (Fig. 3) in presence of the hemisuccinate-pregnenolone compound (MIC = 1 mg/ml, 2.40 x 10^{-3} mmol) and ethylenediamine-hemisuccinate-pregnenolone conjugate (MIC = 0.5 mg/ml, 4.54 x 10^{-4} mmol). The mixture of the two pregnenolone-derivatives showed a MIC of 0.5 mg/ml (1.05 x 10^{-3} mmol) on *E. coli*. This experimental data showed different antibacterial activity in comparison with cefotaxime (MIC = 0.25mg/ml, 5.236 x 10^{-4} mmol) and gentamicin (MIC = 6.25 x 10^{-3} mg/ml, 1.34 x 10^{-5} mmol). In presence of ampicillin, the bacterial growth of *E. coli* was not blocked.

Hemisuccinate-pregnenolone compound (MIC = 1mg/ml, 2.4 x 10^{-3} mmol) and ethylenediamine-hemisuccinate-pregnenolone conjugate (MIC = 0.5 mg/ml, 9.09 x 10^{-4} mmol) (Fig. 4), blocked the growth of *K. pneumoniae* in a dose-dependent manner. In addition in presence of the mixture of the two pregnenolone-derivatives the bacterial growth was also inhibited (MIC = 0.5 mg/ml, 1.05 x 10^{-3} mmol). These results were compared with the antibacterial effect of controls, in this sense, cefotaxime showed a MIC of 0.125 mg/ml (2.61 x 10^{-4} mmol), and the MIC for gentamicin was of 0.0125 mg/ml (2.68 x 10^{-5} mmol).

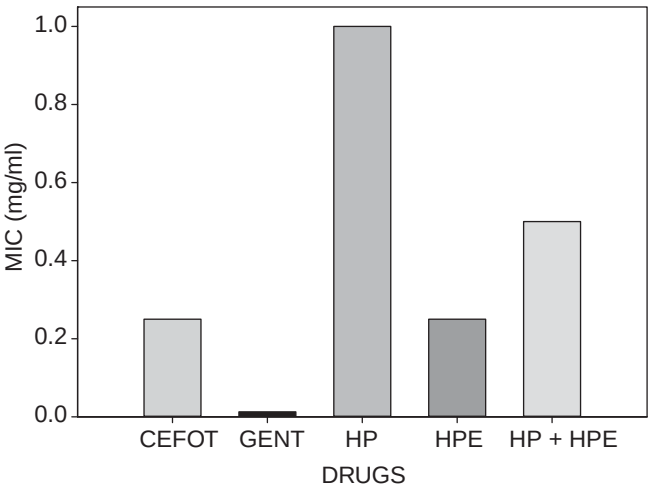

Figure 2. *Antibacterial effects induced by pregnenolone-derivatives and controls (cefotaxime, CEFOT and gentamicin, GENT) on* S. aureus*. The results showed that* S. aureus *was susceptibly to cefotaxime (MIC of 0.25 mg/ml, 5.23 x 10^{-4} mmol) and gentamicin (MIC of 0.0125 mg/ml, 2.68 x 10^{-5} mmol). In addition, the bacterial growth in presence of the hemisuccinate-pregnenolone compound (MIC of 1 mg/ml, 2.40 x 10^{-3} mmol) and the ethylenediamine-hemisuccinate-pregnenolone conjugate (MIC of 0.25 mg/ml, 4.54 x 10^{-4} mmol) was inhibited. This pathogen microorganism was inhibited by the mixture of two pregnenolone-derivatives with a MIC of 0.5 mg/ml (1.05 x 10^{-3} mmol).*

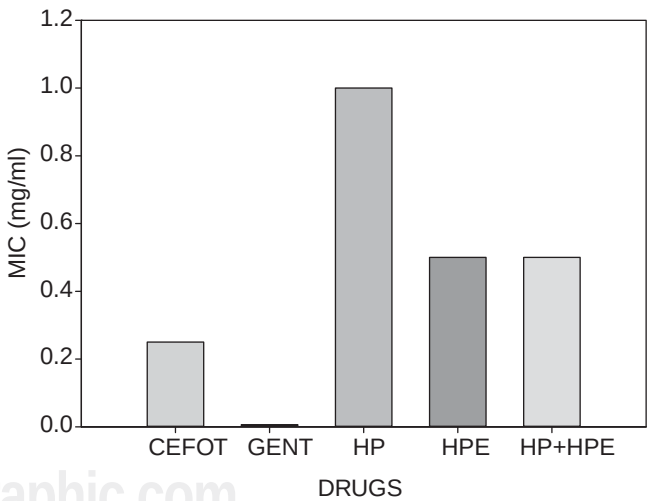

Figure 3. *Effects induced by pregnenolone-derivatives and control (cefotaxime, CEFOT and gentamicin, GENT) on* E. coli*. The results showed that bacterial growth of* E. coli *in presence of CEFOT (MIC = 0.25 mg/ml, 5.23 x 10^{-4} mmol), GENT (MIC = 6.25 x 10^{-3} mg/ml, 1.34 x 10^{-5} mmol), ethylenediamine-hemisuccinate-pregnenolone conjugate (MIC = 4.54 x 10^{-4} mmol) and the hemisuccinate-pregnenolone compound (MIC = 2.40 x 10^{-3} mmol) was inhibited. In addition, the mixture of two pregnenolone-derivatives (MIC = 0.5 mg/ml, 1.05 x 10^{-3} mmol) showed a similar antibacterial effect of ethylenediamine-hemisuccinate-pregnenolone conjugate.*

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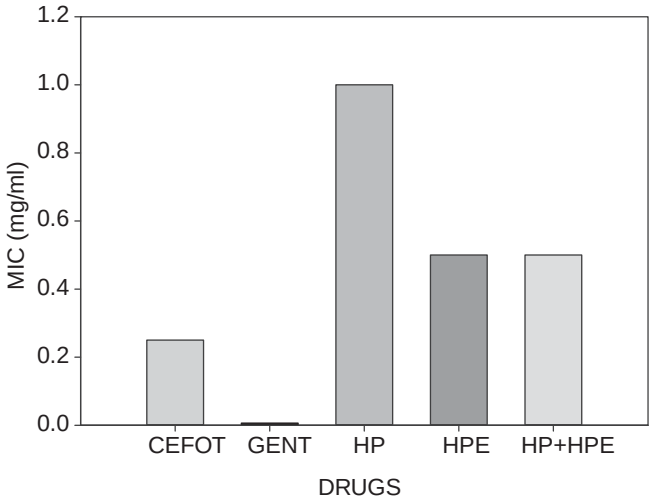

Figure 4. *Antibacterial effects induced by pregnenolone-derivatives and controls (cefotaxime, CEFOT and gentamicin, GENT) on* K. pneumoniae*. Experimental data showed that K. pneumoniae was susceptibly to cefotaxime (MIC = 0.125 mg/ml, 2.61 x 10^{-4} mmol) and gentamicin (MIC = 0.012 mg/ml, 2.68 x 10^{-5} mmol). Nevertheless, in presence of hemisuccinate-pregnenolone compound the MIC was of 1 mg/ml (2.40 x 10^{-3} mmol) and for ethylenediamine-hemisuccinate-pregnenolone conjugate the MIC it's of 0.5 mg/ml (9.09 x 10^{-4} mmol). In addition, this pathogen microorganism was inhibit by the mixture of two pregnenolone-derivatives with a MIC of 0.5 mg/ml (1.05 x 10^{-3} mmol).*

DISCUSSION

The results indicate that pregnenolone-derivatives compounds had different antibacterial activity in comparison with the controls (cefotaxime and gentamicin) and this can be due mainly to the different molecular mechanism involved in their antibacterial activity. Possibly, the molecular mechanism of pregnenolone derivatives can mimic the actions of the derivatives of cholic acid.[25] In this sense, it has been suggested that steroids-derivatives, in general, adopt cationic, facially amphiphilic conformations involving the nature of functional groups contained in their chemical structure, which appears to be a key requirement for antibacterial activity.[28] Their structures allow them to disrupt bacterial membranes at relatively different concentrations by the recognized of some factors in the bacterial membrane as effective targets.[22]

In this work we found that antimicrobial effect induced by hemisuccinate-pregnenenolone can depend of the nature of the free carboxyl group contained in the chemical structure, which is an membrane-perturbing agent whose antibacterial activity is achieved possibility, by the interaction with the positively charged amino groups contained in the D-alanyl incorporated in the teichoic acids, essential polymers that play a vital role on the growth and development of the Gram-positive bacteria (*S. aureus*).[29] Here is important to mention that experimental data exist showing that D-alanyl can modulate cell envelope properties and function of teichoic acids[30] and consequently D-alanyl ester group contribute to bacterial resistance to several antimicrobial peptides.[31] The interaction of hemisuccinate-pregnenenolone compound with this molecule can blocked the bacterial growth of *S. aureus* in a dose-dependent manner.

The addition of -NH-$(CH)_2$-NH_2 fragment to hemisuccinate-pregnenenolone to generate ethylenediamine-hemisuccinate-pregnenenolone conjugate induced greater antibacterial effect on *S. aureus*. Therefore, the arm spacer with free amine group of this pregnenolone-derivative seems to be the key requirement for antibacterial activity. This suggestion is supported by the studies of Ding and coworkers[25] whom showed that amine groups contained in chemical structure of several cationic steroid-antibiotics to confer an antibacterial effect.

The antibacterial-induced effect of ethylenediamine-hemisuccinate-pregnenenolone compound on *S. aureus* is similar to glycopeptides antibiotics that do not permeate into the cytoplasm of the cell, but apparently form complexes with the D-Ala-D-Ala carboxyl termini of peptidoglycan precursors outside the cell membrane. This binding presumably interferes with transglycosylase activity and possibly transpeptidase activity as well,[32,33] both of which are essential for the synthesis of new cell wall. Therefore, the interaction of pregnenolone-derivative with D-Ala-D-Ala carboxyl termini of peptidoglycan precursors can brings as consequence, inhibition of bacterial growth.

In the other hand, the results obtained exploring the antibacterial activity of the pregnenolone-derivatives on Gram-negative bacteria, show that bacterial microorganism (*E. coli* and *K. pneumoniae*) were more sensitive to ethylenediamine-hemisuccinate-pregnenolone conjugate in comparison with the hemisuccinate-pregnenolone compound. The antibacterial molecular mechanism can be different compared with the proposed on *S. aureus*. This mechanism might involved the interaction of free amine group with the lipid A of Gram-negative bacteria, this premise is based on the works of Li[34] and Ding,[35] whose developed a class of cationic steroid antibiotics with the intent of mimicking the antibacterial activities of polymyxin B on Gram-negative bacteria. These authors proposed a compelling model of complex formation involving ionic interactions between the phosphates on lipid A and the amine groups on polymyxin B. This phenomenon can induce, as consequence, an increase on the permeability of the outer membrane and induced growth bacterial inhibition on this Gram-negative microorganism.

In conclusion, in this work bacterial microorganisms such as *S. aureus*, *K. pneumoniae* and *E. coli* were sensitive to both; cefotaxime and ethylenediamine-hemisuccinate-pregnenenolone compound and were less sensitive to that hemisuccinate-pregnenenolone conjugate. Nevertheless, our results suggest that effect induced by pregnenolone-derivatives could be by the interaction with some bacterial membrane factors that are specific for bacterial resistance. In this sense, the antibacterial activity of pregnenolone-derivatives can depend of the nature of functional groups involved in their chemical structure that seems to be the key required for antibacterial activity.

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