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Syntheses and Antibacterial Activity of Testosterone Succinate-Vitamin B_1 Conjugate

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In this study, a straight forward route for the synthesis of testosterone succinate-vitamin B_1 conjugate and their antibacterial activities on *S. aureus, E. coli* and *K. pneumoniae* are reported. This first step was achieved by reacting 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium chloride (vitamin B_1) with testosterone-succinate resulting in amide bond formation. The results showed that the ^{1}H NMR spectra of the testosterone succinate-vitamin B_1 conjugate shows upfield shifts at 0.80 and 1.19 ppm for methyls present in the heterocyles rings at 3.18 and 3.90 ppm for methylenes of the hydroxietilen moiety bonded to thiazol ring. The hydrogens of the methylene between the pyrimidine and thiazol rings appears at 5.68 ppm. In addition, the proton of methyl bound to pyrimidine ring at 5.69 ppm was found. The antibacterial activity of testosterone succinate-vitamin B_1 conjugate on *S. aureus, K. pneumoniae* and *E. coli* was evaluated by means of dilution method and the minimum inhibitory concentration (MIC). The results showed that bacterial growth of *S. aureus* was blocked in presence of the steroid-derivative (MIC = 2.57×10^{-3} mmol). In addition, the bacterial growth of *E. coli* and *K. pneumoniae* in presence of testosterone succinate-vitamin B_1 conjugate (MIC = 1.48×10^{-3} mmol) was blocked. The experimental data suggest that quaternary amine group involved in the testosterone succinate-vitamin B_1 conjugate require only positive charge together with a hydrophobic region, in order to interact with the cell surface and perturb bacterial growth.

Key Words: Syntheses, Testosterone succinate-vitamin B_1, Conjugate, Antibacterial activity.

INTRODUCTION

Several causal agents, such as *S. aureus, K. pneumoniae* and *E. coli* have been shown to accelerate the progression of infectious diseases[1-4]. There are many therapeutic agents for treating them[5-7]. Nevertheless, data exist showing that prolonged

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antibiotic therapy induce bacterial-resistance[8,9], because some bacteria have developed ways to circumvent the effects of antibiotics[10,11] for example, several studies indicate that β-lactamic antibiotics (*i.e.* methicilin/oxacillin) induce resistance in *S. aureus*[12,13]. Other reports showed that antibiotic-resistant strains have emerged among Gram-negative bacilli such as *K. pneumoniae*[14] and *E. coli*[15]. Therefore, antibiotic resistance can be considered as a serious threat for health and an international approach to its management is required. In this sense, new drugs have been developed for control of bacterial resistance[16-18] for example, the synthesis of cholic acid-derivate compounds that have antibacterial activity by increasing the permeability of the outer membrane of Gram negative bacteria have shown promising results[19]. In addition, other studies showed a correlation of the antibacterial activities between cationic peptides and cationic steroids on Gram-negative and Gram-positive bacteria[20]. Cationic steroid-antibiotics were developed to *mimic* the antibacterial behaviour of endogenous peptide antibiotics, this task include selective association of the steroid-antibiotic with disruption of bacterial membranes[21,22]. The association involves structural characteristics of the steroids-antibiotic such as cationic forms and facially amphiphilic conformations, which appear to be the key requirement for antibacterial activity and membrane selectivity is primarily derived from ionic recognition of negatively charged bacterial membranes[23]. In this work our initial design included the synthesis of testosterone succinate-vitamin B_1 conjugate that contains in the D-ring of the steroid nucleus a spacer arm with both ester and amide functional groups (-O-C=O-$(CH_2)_2$-CO-NH-) coupled to pyrimidine ring and involve a quaternary amine in the thiazole ring with positive charge. This compound was made with the purpose to evaluate their antibacterial activity on *S. aureus, K. pneumoniae* and *E. coli* using the microbial minimal inhibitory method[24], because several data exist indicating that quaternary amine compounds exert antibacterial activity against both Gram-positive and Gram-negative bacteria by the perturbation of the bacterial membrane[25]. In addition to evaluate this premise, we used as pharmacological tool the 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxy-ethyl)-4-methyl-thiazolium chloride compound (vitamin B_1), since the nature of functional groups contained in the chemical structure of this compound involve a quaternary amine in the thiazole ring.

EXPERIMENTAL

Testosterone-succinate and 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium chloride (vitamin B_1) were purchased from Sigma-Aldrich Co., Ltd..The melting points for the different compounds were determined on an Electrothermal (900 model). Ultraviolet spectroscopy (UV) was carried out in dry methanol on a Perkin-Elmer model 552 spectrophotometer and infrared spectra (IR) was recorded using KBr pellets on a Perkin-Elmer Lambda 40 spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Varian VXR-300/5 FT NMR spectrometer at 300 and 75.4 MHz in $CDCl_3$ using TMS as internal standard. EIMS

spectra were obtained with a Finnigan Trace GCPolaris Q. spectrometer. Elementary analysis data were acquired from a Perkin-Elmer Ser. II CHNS/0 2400 elemental analyzer.

Synthesis of 3-{4-[3-(10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15, 16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-17-yloxycarbonyl)-propionylami-no]-2-methyl-pyrimidin-5-ylmethyl}-5-(2-hydroxy-ethyl)-4-methyl-thiazol-3-ium; chloride: Testosterone-succinate (100 mg, 0.24 mmol) was added to a solution of 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxy-ethyl)-4-methylthiazolium chloride (122 mg, 0.37 mmol) and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (55 mg, 0.28 mmol) in acetonitrile-water (15 mL, 2:1). The mixture was stirred at room temperature for 48 h, the solvent was removed under vacuum and the crude product was purified by crystallization from methanol:hexane:water (3:2:1) to give 73 %, m.p. 148 ºC; UV (MeOH) λ_{max} (log ε) 220 (0.16) 203 (0.23) nm; IR (KBr, ν_{max}, cm^{-1}) 3330, 3204, 1735; ^{1}H NMR $CDCl_3$ (300 MHz) δ_H; 0.80 (3H, s, 19-CH_3), 0.91-1.17 (4H, m), 1.19 (3H, s, 18-CH_3), 1.45-1.94 (10 H, m), 2.01 (1 H, m), 2.20-2.40 (4H, m), 2.52 (3H, s, thiazol ring-CH_3), 2.55-256 (4H, s), 2.58 (3H, s, pyrimidine ring-CH_3), 3.18 (2H, t, CH_2-CH_2-OH; J = 6 Hz), 3.90 (2H, t, CH_2-CH_2-OH; J = 6 Hz), 4.63 (1H, m, -CH-O), 5.68 (1H, s, -CH=C-), 5.68 (1H, s, N^+-CH-C), 7.62 (1H, s, O=C-NH-C), 7.62 (1H, s, -OH), 7.92 (1H, s, -CH=N), 9.76 (1H, s, S-CH=N^+). ^{13}C RMN 75.4 MHz δ_C; 11.85 (thiazol ring-CH_3), 12.08 (19-CH_3), 17.51 (18-CH_3), 21.11 (14), 24.13 (15), 25.31 (pyrimidine ring-CH_3), 26.70 (CH_2-CH_2-OH), 27.64 (16), 29.50 (O=C-CH_2), 31.44 (CH_2-C(=O)-NH), 32.12 (8), 32.80 (7), 34.23 (1), 35.86 (9), 36.11 (6), 37.14 (13), 38.34 (5), 42.64 (12), 51.60 (11), 53.72 (-CH-N^+), 54.34 (10), 63.66 (-CH_2-OH), 82.43 (17), 119.27 (N^+-C=C), 124.32 (3), 130.20 (N^+-C-CH_3), 140.61 (S-C=C), 151.32 (-CH-N), 160.68 (NH-C-N), 161.23(S-CH-N^+), 167.91 (N=C-CH_3), 170.43 (4), 172.24 (O-C=O), 174.81 (-C(=O)-NH), 197.8 (C=O, ketone). EI-MS, m/s 670.17 (M^+-Cl^-, 8), 310.12 (25), 290.04 (40). 204.10 (32). Anal. calcd. (%) for $C_{35}H_{47}N_4O_5SCl$; C, 62.62, H, 7.06, Cl, 5.28, N, 8.35, O, 11.92 S, 4.78. Found (%): C, 62.59, H, 7.04, N, 8.30.

Biological evaluation

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: The steroids derivatives and the other compounds studied were dissolved in methanol and diluted with distilled water. Cefotaxime, gentamycin 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.*[24].

The bacterial species were incubated on McConkey (*E. coli* and *K. pneumoniae*) and *Staphylococcus* 110 (*S. aureus*) agars for 24 h at 37 ºC. After 24 h, it was 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 soye) 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 (9×10^8 cells/mL) and all the tubes were incubated at 37 ºC for 24 h. Subsequently, a loop was taken from each of them and inoculated into the appropriate cultures for different bacterial organisms and were incubated for 24 h at 37 ºC.

RESULTS AND DISCUSSION

In this study we report a straight forward route for the synthesis of testosterone succinate-vitamin B_1 conjugate (Fig. 1) and their antibacterial activity on *S. aureus, E. coli* and *K. pneumoniae* was evaluated. This first step was achieved by reacting 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium chloride (vitamin B_1) with testosterone-succinate resulting in amide bond formation. It is important to mention that many procedures for the formation of amide groups are known in the literature[26-28]. The most widely practiced method employs carboxylic acid chlorides as the electrophiles which react with the amino group in the presence of an acid scavenger[29]. Despite its wide scope, the former protocol suffers from several drawbacks. The most notable are the limited stability of many acid chlorides and the need for hazardous reagents for their preparation (thionyl chloride)[30]. Nevertheless, there are reports which indicate that 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride catalyzed amidation reaction with high yielding[31]. Therefore, in this work was used carbodiimide-derivative for amide bond formation in the testosterone succinate-vitamin B_1 conjugate. ^{1}H NMR spectra of the testosterone succinate-vitamin B_1 conjugate shows upfield shifts at 0.80 and 1.19 ppm for methyls present in the heterocyles rings; at 3.18 and 3.90 for methylenes of the hydroxietilen moiety bonded to thiazole ring; the hydrogens of the methylene between the pyrimidine and thiazol rings appears at 5.68 ppm. In addition, the proton of methyl bound to pyrimidine ring at 5.69 ppm was found. At down field a signal at 7.62 for the protons involved in both amide and hydroxyl groups. Finally, another signal at 9.76 ppm for the proton in the thiazol ring (S-CH=N^+) was found.

On the other hand, ^{13}C NMR spectra displays chemical shifts at 11.85 for the carbon of the methyl bound to pyrimidine ring; at 12.08 and 17.51 for methyl groups presents in the steroid rings. Another chemical shifts at 25.31 ppm for methyl group

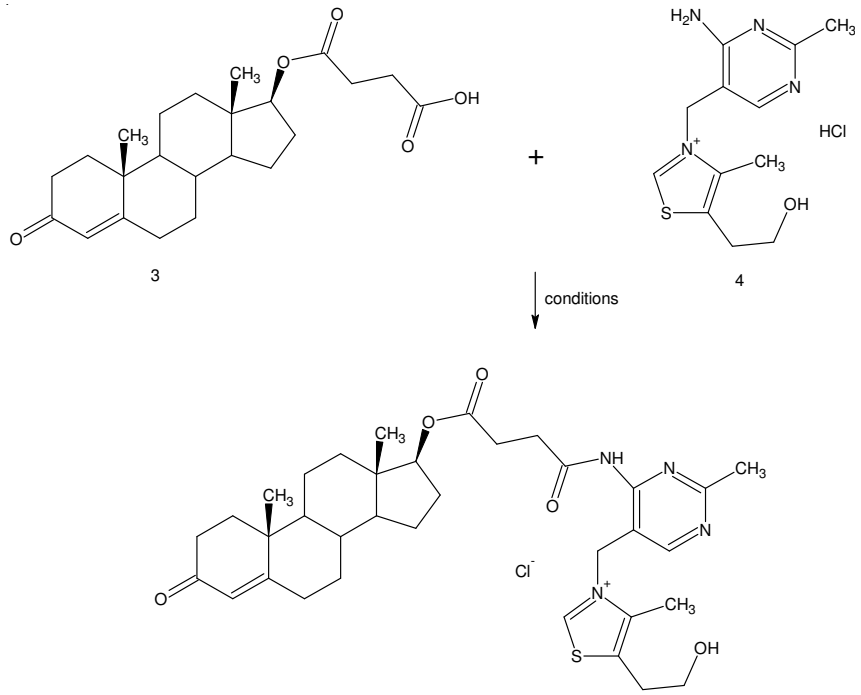

Fig. 1. Synthesis of testosterone-vitamine B_1 conjugate, Conditions; 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride, acetonitrile/water

connected to pyrimidine ring and at 26.70 ppm for the methylene involved in the hydroxy ethylene moiety were found. In addition, two signals also observed at 29.59 and 31.44 for ethylene joined between steroid nucleus and pyrimidine ring. The chemical shift of the methylene joined the pirimidine and thiazole rings is find out at 53.72 ppm. Another chemical shifts at down field showed several signals (119.27-170.43 ppm) corresponding to the carbons of the heterocycles. Finally, two signals at 174.81 for amide group and at 197.02 for carbon of ketone group were found. Additionally, the mass spectra displays a molecular ion of m/z 670.17 corresponding to M^+-Cl^- which confirm the structure of the testosterone succinate-vitamin B_1 conjugate obtained.

The antibacterial activity of testosterone succinate-vitamin B_1 conjugate on *S. aureus, K. pneumoniae* and *E. coli* was evaluated by means of dilution method and the minimum inhibitory concentration (MIC), using gentamycin, ampicillin and cefotaxime as control in this study. The results (Fig. 2) indicate that bacterial growth of *S. aureus* was inhibited with cefotaxime (MIC = 5.23×10^{-4} mmol) and gentamycin (MIC = 2.68×10^{-5} mmol). In presence of ampicillin, the bacterial growth of *S. aureus* was not blocked (data not shown). In addition, the bacterial growth of *S. aureus* in presence of testosterone succinate-vitamin B_1 conjugate (MIC = 7.44×10^{-4} mmol) was blocked. These data indicate that testosterone succinate-vitamin B_1 compound

had antibacterial potency different in comparison with cefotaxime (β-lactam antibiotic) and gentamycin (inhibitor of synthesis of protein) on this pathogen microorganism. This phenomenon can be due mainly to the different molecular mechanism involved and the characteristic chemical structure of the compounds studied. Therefore, in this work was interesting to consider the different molecular mechanisms involved in the antibacterial activity induced by the testosterone succinate-vitamin B_1 conjugate. It is important to mention that this compound contains in the D-ring of the steroid nucleus a spacer arm with both characteristics, ester and amide groups (-O-C=O-$(CH_2)_2$-CO-NH-) coupled to pyrimidine ring of vitamin B_1, in addition to involve a quaternary amine in the thiazole ring with positive charge. It is important to mention that several reports showed that quaternary amine compounds exert antibacterial activity against both Gram-positive and Gram-negative bacteria through perturbation of lipid bi-layer membranes that constitute the bacterial cytoplasmic membrane and the outer-membrane of bacteria[32]. In order to evaluate this premise, we used the 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methyl-thiazolium chloride (vitamin B_1), since the nature of functional groups contained in the chemical structure involved a quaternary amine in the thiazole ring. The results showed that in presence of vitamin B_1 the bacterial growth of *S. aureus* was not blocked (data not showed). These experimental data suggest that quaternary amine of free vitamin B_1 by itself, does not have antibacterial activity on this pathogen microorganism and suggested that the steroid-derivative moiety could the only responsible for antibacterial activity. In order to analyze this possibility, we evaluated the effect exerted by the testosterone-succinate compound on the bacterial growth of *S. aureus* and compared with the antibacterial effects induced by testosterone succinate-vitamin B_1 conjugate. The results showed that bacterial growth of *S. aureus* was blocked in presence of the steroid-derivative compound (MIC = 2.57×10^{-3} mmol). These experimental data suggest that antimicrobial effect induced by the testosterone succinate compound could depend on the nature of the free carboxyl group contained in its chemical structure, which is a membrane-perturbing agent whose antibacterial activity is induced, possibility, by the interaction with the positively charged amino groups contained in the D-alanyl incorporated in the teichoic acids, essential polymers that plays a vital role in the growth and development of the gram-positive bacteria[33]. This premise is supported by the studies[34] reported for another steroids type, which indicate that carboxyl groups involved in their chemical structure are specific to inhibit the growth bacterial of *S. aureus*. All these experimental data suggest that when the testosterone-succinate compound is bound with vitamin B_1 to form the testosterone-succinate-vitamin B_1 conjugate, the antibacterial activity seems to be greater, possibly because the quaternary amine compounds require only a strong positive charge together with a hydrophobic region in order to interact with the cell surface and integrate into the cytoplasmic membrane. Such integration into the membrane is sufficient to perturb bacterial growth to cause the membrane to lose fluidity and for the cell to die.

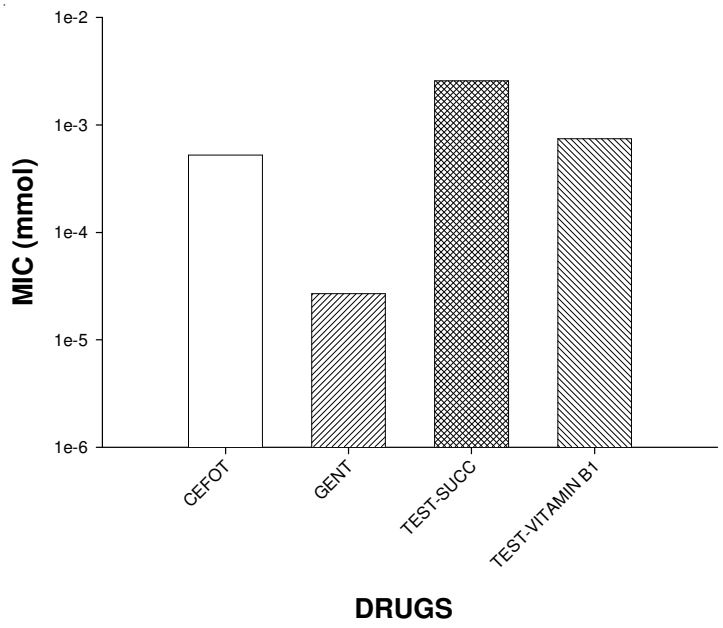

Fig. 2. Antibacterial effects induced by testosterone-derivates and control (cefotaxime, CEFOT and gentamycin, GENT) on *S. aureus*. Data showed that *S. aureus* was susceptibly to cefotaxime (MIC = 5.23×10^{-4} mmol) and gentamicin (MIC = 2.68×10^{-5} mmol). The bacterial growth of this microorganism in presence of the testosterone succinate compound (TEST-SUCC; MIC = 2.57×10^{-3} mmol) and the testosterone succinate-vitamin B_1 conjugate (TEST-VIT B_1; MIC = 7.44×10^{-4} mmol) was inhibited. MIC = minimum inhibitory concentration

On the other hand, analyzing data obtained and reports on the antibacterial activity induced by another steroid type on Gram negative bacteria[35], in this work the effect exerted by testosterone-succinate-vitamin B_1 conjugate on *E. coli* and *K. pneumoniae* was evaluated. The results (Fig. 3) indicate that bacterial growth of both *E. coli* was inhibited with cefotaxime (MIC = 5.23×10^{-4} mmol) and gentamycin (MIC = 1.34×10^{-5} mmol). Additionally, another results (Fig. 4) showed that bacterial growth of *K. pneumoniae* was blocked by administration of cefotaxime (MIC 2.61 $\times 10^{-4}$ mmol) and gentamycin (MIC = 2.68×10^{-4} mmol). In addition, the bacterial growth of *E. coli* and *K. pneumoniae* in presence of testosterone succinate-vitamin B_1 conjugate (Figs. 3 and 4; MIC = 1.48×10^{-3} mmol) was blocked. It is important to mention that bacterial growth of *E. coli* and *K. pneumoniae* in presence of ampicillin and testosterone succinate was not blocked (data not shown). These experimental data suggest that the antibacterial activity of testosterone succinate-vitamin B_1 conjugate possibly could be associated indirect through of the interaction of testosterone succinate-vitamin B_1 conjugate with the lipid A that is a primary component of the outer membrane of Gram-negative bacteria and plays an essential role in cell wall integrity[36]. This phenomenon can induce, as consequence, an increase in the permeability of the

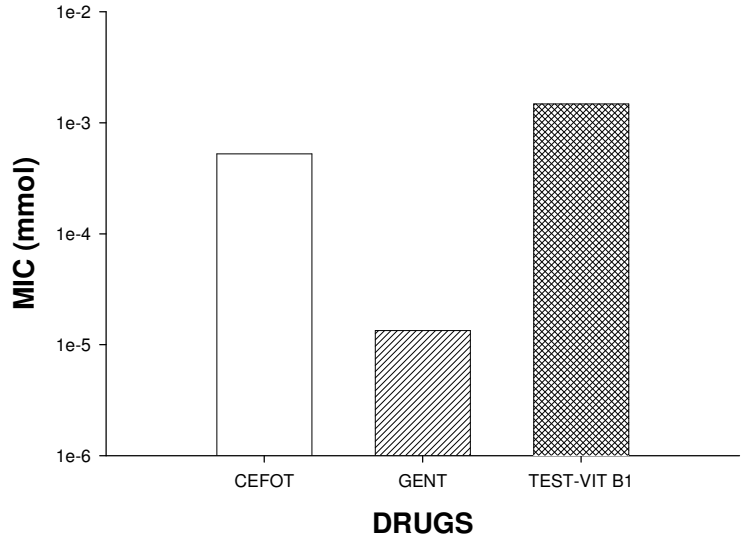

Fig. 3. Effect induced by testosterone-derivate and control (cefotaxime, CEFOT and gentamycin, GENT) on *E. coli*. It is showed that exist differences of antibacterial activity of CEFOT (MIC = 5.23×10^{-4} mmol) and GENT (MIC = 1.34×10^{-5} mmol) on *E. coli* in comparison with testosterone succinate-vitamin B_1 conjugate (TEST-VIT B_1; MIC = 1.48×10^{-3} mmol). MIC = minimum inhibitory concentration

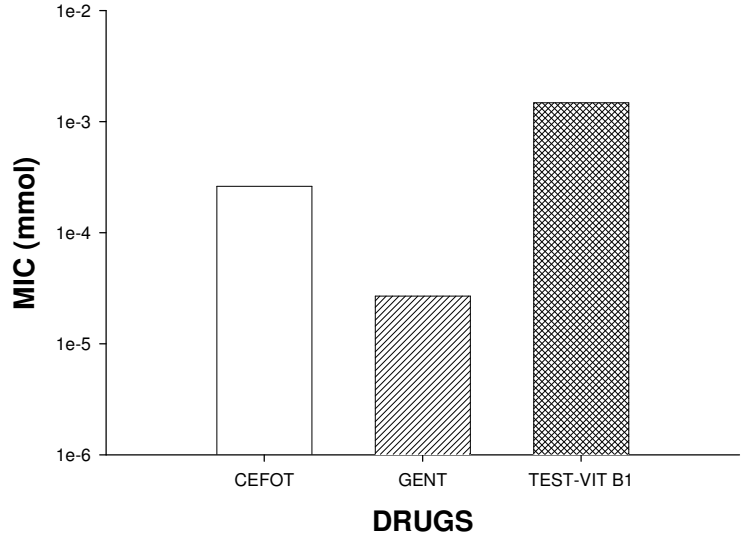

Fig. 4. Antibacterial effects exerted by testosterone-derivate and controls (cefotaxime, CEFOT and gentamycin, GENT) on *K. pneumoniae*. Experimental data showed that *K. pneumoniae* was susceptible to cefotaxime (MIC = 2.61×10^{-4} mmol) and gentamycin (MIC = 2.68×10^{-5} mmol). In presence of testosterone succinate-vitamin B_1 conjugate (TEST-VIT B_1) the MIC was of 1.48×10^{-3} mmol. MIC = minimum inhibitory concentration

outer membrane and induce growth bacterial inhibition on these pathogen microorganisms. This premise is supported by some mechanisms, based in experimental data, which proposed that steroid-antibiotics can adopt cationic conformations to induce bacterial death[37].

Conclusion

Experimental data suggest that quaternary amine group involved in the testosterone succinate-vitamin B_1 conjugate require only positive charge together with a hydrophobic region, in order to interact with the cell surface and perturb bacterial growth.

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