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Biological activity exerted by an adamantanyl-steroid derivative against ischemia/perfusion injury

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Some studies indicate that steroids may exert effects against ischemia/reperfusion injury; however, the molecular mechanism is not very clear. The aim of this investigation was synthesizing a new adamantanyl-steroid derivative to evaluate their biological activity on ischemia/reperfusion injury. The steroid derivative was prepared using some tools chemical and the effect exerted by the adamantanyl-steroid derivative against ischemia/reperfusion injury was determined in an isolated heart model using noradrenaline, digoxine, milrinone, dobutamine and levosimedan as controls. Additionally, the biological activity induced by the adamantanyl-steroid derivative against left ventricular pressure was evaluated in the absence or presence of nifedipine drug. The results showed that 1) the adamantanil-steroid derivative significantly reduced ischemia-reperfusion injury, resulting in a reduction of the infarct area in a similar manner to levosimendan drug; 2) the adamantanyl-steroid derivative increases the left ventricular pressure and this effect was inhibited in the presence of nifedipine. All these data indicate that adamantanyl-steroid derivative decreases the area of infarction and increases left ventricle pressure via calcium channels activation; this phenomenon could constitute a new therapy for ischemia/reperfusion injury.

Keywords: Ischemia; reperfusion; steroid; adamantanyl; pressure.

INTRODUCTION

There are several data which indicate that myocardial ischemia can be one of the main causes to produce death (Kusmin et al., 2019; Luo et al., 2019 Pilz et al., 2019). In order to reduce acute myocardial infarction, some procedures can be performed that promote the return of blood flow to the ischemic area of the myocardium (Ferdinandy et al., 2014); however, these methods can induce reperfusion injury which can result in increased irreversible myocardial cell death. It is important to mention that some pharmacological alternatives to reduce reperfusion damage have used, such as amiloride (Na^+-H^+ exchange inhibitor) (Kusomoto et al., 2001), sanglifehrin-A [inhibits opening of the mitochondrial permeability transition pore] (Clarke et al., 2002), BW373U86 [δ-opioid agonist] (McPherson and Yao, 2001), Glibenclamide [ATP-regulated K+ channels inhibitor] (Mitani et al.,

1991); however, some these drugs can exert secondary effects such as hypoxic tolerance (Bofetiado et al., 1996) hypotension (Vanelli et al., 1995) and others. In the search of new drug for treatment of ischemia-reperfusion injury, several steroids have been evaluated; for example, a study showed that 17β-estradiol can decrease ischemia-reperfusion injury using an animal model (Hale et al., 1996). Another report indicates that 17β-estradiol induce a cardioprotective effect against myocardial arrhythmias exerted by ischemia/reperfusion injury (Kim et al., 1996). Additionally, progesterone has been used in conjunction with estrogen in an ischemia/reperfusion model, resulting in a significantly decreased myocardial injury (Dhote and Balaram, 2007). Other data suggest that testosterone could decrease some bioactive substances involved in the inflammation produced through ischemia/reperfusion injury using an isolated heart preparation (Wang et al., 2005).

In order to characterize the molecular mechanism involved in the biological activity of steroids against ischemia/reperfusion injury, several steroid derivatives have been evaluated; for example, a report showed that a progesterone derivative can exert cardioprotective effects against ischemia/reperfusion injury through via M_2 muscarinic receptor activation using an animal model (Figueroa-Valverde et al., 2012-A). In addition, one study shows that treatment with medroxyprogesterone acetate can inhibit the biological activity of estradiol in the ischemia/reperfusion injury, which can lead to changes in the concentration of neutrophils (Jeanes et al., 2006). Other data suggest that an estrogen derivative (succinic acid-estradiol) may modulate the ischemia/reperfusion injury in an isolated heart model through both estrogen receptor and nitric-oxide synthase activation (Figueroa-Valverde et al., 2012-B). All these data indicate that several steroids and their derivatives can exert effects against ischemia/reperfusion injury; however, the molecular mechanism involved in its biological activity is not very clear, perhaps this phenomenon is due to the different protocols used or to functional groups involved in the chemical structure of each steroid and its derivatives. To evaluate this hypothesis, in this study the main objective was to synthesize an adamantanyl-steroid derivative to determine its biological activity against ischemia/reperfusion injury in an isolated rat heart.

MATERIALS AND METHODS

All the reagents used in this study were purchased from Sigma-Aldrich Sigma-Aldrich Co., Ltd. The melting point for compounds was evaluated on an Electrothermal (900 model). Infrared spectra (IR) were determined using KBr pellets on a Perkin Elmer Lambda 40 spectrometer.^{1}H and ^{13}C NMR (nuclear magnetic resonance) spectra were recorded on a Varian VXR300/5 FT NMR spectrometer at 300 and 75.4 MHz (megahertz) in $CDCl_3$ (deuterated chloroform) using TMS (tetramethylsilane) as an internal standard. EIMS (electron impact mass spectroscopy) spectra were determined using a Finnigan Trace Gas Chromatography Polaris Q-Spectrometer. Elementary analysis data were determined from a Perkin Elmer Ser. II CHNS/02400 elemental analyzer.

Chemical Synthesis

Preparation of (2-[7-(adamantan-1-yl)-7-oxo-hept-5-yn-1-yl]isoindole-1,3-dione (2)

In a round bottom flask (10 ml), Adamantane-1-carbonyl bromide (200 mg, 1.15 mmol), 1-Phenyl-2-propyn-ol (100 µl 0.82 mmol), Copper(II) chloride (105 mg 0.78 mmol) and 5 ml of methanol were stirred to reflux for 12 h. The solution obtained was reduced pressure and purified through a crystallization using the methanol:water (3:1) system.

Preparation of 1-(adamantan-1-yl)-3-{1,7-dihydroxy-11a-methyl-2H,3H,3aH,3bH,4H,5H,9bH, 10H,11H-cyclopenta[a]phenanthren-1-yl}prop-2-yn-1-one (3)

In a round bottom flask (10 ml), compound 1 (1,4,4a,8a-Tetrahydro-1,4-methano-naphthalene-5,8-dione (200 mg, 1.15 mmol), 1-Phenyl-2-propyn-ol (100 µl 0.82 mmol), Copper(II) chloride (105 mg 0.78 mmol) and 5 ml of methanol were stirred to reflux for 12 h. The solution obtained was reduced pressure and purified through a crystallization using the methanol:water(3:1) system.

Evaluation of biological activity.

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal care and use Committee of University Autonomous of Campeche (No. PI-420/12) and were in accordance with the guide for the care and use of laboratory animals (Figueroa et al., 2014). Male Wistar rats; weighing 200-250

g were obtained from University Autonomous of Campeche.

Reagents.

The drugs used in this study were dissolved in methanol and different dilutions were obtained using Krebs-Henseleit solution (≤ 0.01%, v/v) (Figueroa et al., 2011-A).

Langendorff method.

Briefly, the male rats (200-250 g) were anesthetized by injecting them with pentobarbital at a dose rate of 50 mg/Kg body weight. Then the chest was opened, and a loose ligature passed through the ascending aorta. The heart was then rapidly removed and immersed in ice-cold physiologic saline solution. The heart was trimmed of noncardiac tissue and retrograde perfused via a non-circulating perfusion system at a constant flow rate. It is important to mention that perfusion medium was the Krebs-Henseleit solution (pH 7.4, 37 ^{o}C) composed of (mM); 117.8 NaCl; 6 KCl; 1.75 $CaCl_2$; 1.2 NaH_2PO_4; 1.2 $MgSO_4$; 24.2 $NaHCO_3$; 5 glucose, and 5 sodium pyruvate. The solution was actively bubbled with a mixture of O_2/CO_2 (95:5). The coronary flow was adjusted with a variable-speed peristaltic pump. An initial perfusion rate of 15 ml/min for 5 min was followed by a 25 min equilibration period at a perfusion rate of 10 ml/min. All experimental measurements were done after this equilibration period.

Evaluation of left ventricle pressure.

To evaluate the biological activity of drugs involved in this study against left ventricle pressure, a latex balloon filled with saline solution (0.01 mm, diameter) was inserted into the left ventricle through the left atrium. It is important to mention that latex balloon was bound to pressure transducer which was connected to a computerized data capture system (MP-100). After, inotropic effect produced by compounds involved in this study was evaluated by determine left ventricular developed pressure (LV/dP) (Figueroa-Valverde et al., 2011-B).

Experimental design

First stage

***Effect exerted by adamantane-1-carbonyl bromide or the compounds 2 and 3 against ischemia/reperfusion injury*.**

After 15 minutes of equilibration time, the hearts were subjected to ischemia for 40 minutes by turning off the perfusion system. Then, the system was restarted, and the hearts were re-perfused by 40 minutes with Krebs-Henseleit solution. The hearts were randomly divided into 4 major treatment groups that involved the conditions control (without treatment), adamantane-1-carbonyl bromide (compound 1) and the compounds 2 or 3. (Table 1) with n = 9 as follows:

Group I. Hearts were subjected to ischemia/reperfusion but received vehicle only (Krebs-Henseleit solution).

Group II. Hearts were subjected to ischemia/reperfusion and treated with adamantane-1-carbonyl bromide (0.001 nM).

Group III. Hearts were subjected to ischemia/reperfusion and treated with Compound 2 (0.01 nM).

Group IV. Hearts were subjected to ischemia/reperfusion and treated with Compound 3 (0.01 nM).

Total area at risk was expressed as the percentage of the left ventricle. It is noteworthy that at the end of each experiment, the perfusion pump was stopped, and 0.5 ml of fluorescein solution (0.10%) was injected slowly through a sidearm port connected to the aortic cannula. The dye was passed through the heart for 10 sec to ensure its uniform tissue distribution. The presence of fluorescein was used to demarcate the tissue that was not subjected to regional ischemia, as opposed to the risk region. Then, the heart was removed from the perfusion apparatus and cut into two transverse sections at right angles to the vertical axis. The right ventricle, apex, and atrial tissue were discarded. The areas of the normal left ventricle non risk region, area at risk, and infarct region were determined using methods previously reported (Figueroa, et al., 2018). Total area at risk was expressed as the percentage of the left ventricle.

Second stage

Biological activity of the compound 3 on infarct area

The hearts perfused with the Krebs-Henseleith solution were subjected to ischemia for 40 minutes by turning off the perfusion system; then, the system was restarted. After, the hearts were randomly divided into 7 major treatment groups that involved the conditions-control (without treatment; Group VI), and the compounds to doses of 0.001 to 100 nM (n = 9) as follows:

Group VI. Hearts were subjected to ischemia/reperfusion but received vehicle only (Krebs-Henseleit solution).

Group VII. Hearts were subjected to ischemia/reperfusion and treated with compound 3 (0.001 nM).

Group VIII. Hearts were subjected to ischemia/reperfusion and treated with compound 3 (0.01 nM).

Group IX. Hearts were subjected to ischemia/reperfusion and treated with compound 3 (0.1 nM).

Group X. Hearts were subjected to ischemia/reperfusion and treated with compound 3 (1 nM).

Group XI. Hearts were subjected to ischemia/reperfusion and treated with compound 3 (10 nM).

Group XII. Hearts were subjected to ischemia/reperfusion and treated with compound 3 (100 nM).

Then, the areas of the normal left ventricle no-risk region, area at risk, and infarct region were determined as mentioned above.

Third stage

Effects induced by the noradrenaline, milrinone, dobutamine, levosimendan and the compound 3 against infarct area.

The hearts were randomly divided into 6 major treatment groups with n = 9, as follows:

Group XIII. Hearts were subjected to ischemia/reperfusion but received vehicle only (Krebs-Henseleit solution).

Group XIV. Hearts were subjected to ischemia/reperfusion and treated with noradrenaline (0.001 nM].

Group XV. Hearts were subjected to ischemia/reperfusion and treated with milrinone (0.001 nM].

Group XVI. Hearts were subjected to ischemia/reperfusion and treated with dobutamine (0.001 nM].

Group XVII. Hearts were subjected to ischemia/reperfusion and treated with levosimedan (0.001 nM].

Group XVIII. Hearts were subjected to ischemia/reperfusion and treated with compound **3** (0.001 nM].

Following, the areas of the normal left ventricle no-risk region, area at risk, and infarct region were determined. Here, it is important to mention that the doses administered of the drugs noradrenaline, milrinone, dobutamine, and levosimendan was based on some previously methods reported [20]. Therefore, in this study the biological activity exerted by these drugs on ischemia/reperfusion injury was used as a control to compare it with the effect exerted by compound 3 (amantadyl-steroid derivative) against ischemia/reperfusion injury.

Fourth stage

Histological Analysis

For histological evaluation, a previously method reported was used (Engelhardt et al., 1999). Cross sections from the heart were fixed with 10% paraformaldehyde for 8 h. Then, the samples are placed in a histocasette (Leica Mod. TP1020 SN: 042231418), for 12 h to be processed. After, the sections of heart were placed in a paraffin embedding Center (Leica EG1160 model) to form paraffin blocks which are cut into 2 µm slices using an apparatus Leica 50138178 model and following were introduced to bath water (Riossa-Rocha B7 SN: 070909 model). The samples were placed on a slide, which was dried at 60 ^{o}C for 30 minutes in a Binder-ED23 apparatus. Then, a solution of ethanol: xylol (1:1) was added to the slides for cell clearance. After 10 minutes the slides were washed with distilled water. To observe the tissue morphology, hematoxylin is added to the sample (for 1 minute), after which time it is washed again. Then eosin is added for 1 minute. Finally, ethanol/xylol (1:1) is added to the sample and the tissue is observed under the microscope. For morphometrical analysis, photographs of 20 ventricular sections were taken at 3320 magnifications (ZeissIM-35).

Fifth stage

Biological activity exerted by compound 3 or the Bay-k-8644 drug on left ventricular pressure.

Intracoronary boluses (50 µL) of the compound **3** or Bay-k-8644 drug at dose of 0.001 to 100 nM were administered and the corresponding effect on the left ventricular pressure was evaluated.

Sixth stage

Effects induced by the compound 3 on left ventricular pressure in absence or presence of nifedipine.

Intracoronary boluses (50 µL) of the compound 3 [0.001 to 100 nM] were administered and the corresponding effect on the left ventricular pressure was evaluated. The dose-response curve (control) was repeated in the presence of nifedipine* at a concentration of 1 nM (duration of the preincubation with nifedipine was for a period of 10 min). * The dose of nifedipine has been reported in a previously reported study (Figueroa et al., 2014).

Determination of calcium concentration

Calcium levels were determinate using a method previously reported (Boe and Khan, 1928). In this method, 5 ml of samples obtained of perfused (metabolic solution) were used to evaluate the intracellular calcium. It is important to mention that the samples were taken every 3 min (six times). After, 4 ml trifluoroacetic acid (10%) was added each to sample. This solution was mixed for 5 min at room temperature and after 1 ml of NaOH (25%) was added. To mixture 1 ml was trisodium phosphate added and the mixture was vortexed (1 min) and centrifuged to 4000 rpm (5 min). The supernatant was separated from the aqueous solution. After 5 ml of a buffer solution (pH = 10) and 3 ml of Eriochrome black was added to the aqueous solution. Finally, the mixture was titled with Ethylenediaminetetraacetic acid (f = 0.847).

Statistical analysis

The obtained values are expressed as average ± SE, using each heart as its own control. The data obtained were put under an analysis of variance (ANOVA) with the Bonferroni correction factor using the SPSS 12.0 software (Hotch et al., 1999). The differences were considered significant when ***p*** was equal or smaller than 0.05.

RESULTS

Chemical synthesis

The compound 2-[7-(adamantan-1-yl)-7-oxohept-5-yn-1-yl]isoindole-1,3-dione (**2**) showed in the Figure 1 was prepared via reaction of Adamantane-1-carbonyl bromide with *N*-(5-Hexynyl)phthalimide in presence of Copper(II) (Figure 1). The yielding of compound **2** was of 78 % with melting point of 88-90°C. In addition, the spectroscopic analyses show signals for IR (V_{max}, cm^{-1}) 3400, 3360, 3322 and 1712. The chemical shifts of the spectroscopic analysis of both ^{1}H NMR and ^{13}C NMR for compound **2** are displayed in Table 1. The results of mass spectroscopy (MS) (70 eV) showed an ion mass (*m/z) of* 389.19. Additionally, the elementary analysis data for the estradiol derivative ($C_{25}H_{27}NO_3$) were calculated (C, 77.09; H, 6.99; N, 3.60; O, 12.32) and found (C, 77.00; H, 6.90).

On the other hand, the adamantanyl-steroid (3) was synthesized via reaction of compound 2 with 17α-ethynylestradiol to form the compound 3 (Figure 1); with a yielding of 67 % and a melting point of 138-140 °C. In addition, the spectroscopic analyses show signals for IR (V_{max}, cm^{-1}) 3400, 3360, 3322 and 1712. In Table 2 are shown the data of both ^{1}H NMR and ^{13}C NMR spectra for compound 3. In addition, the results of mass spectroscopy (MS) (70 eV) showed an ion mass (*m/z) of* 458.28. Additionally, the elementary analysis data for the estradiol derivative ($C_{31}H_{38}O_3$) were calculated (C, 81.18; H, 8.35; O, 10.47) and found (C, 81.10; H, 8.28).

Biological activity

In this investigation, the biological activity of compounds 1 to 3 at a dose of 0.001 nM on myocardial injury was evaluated using an ischemia/reperfusion model. The results showed that only the compound 3 (adamantanyl-steroid derivative) significantly reduced infarct size compared with the compounds 1 or 2 and vehicle-treated hearts (control) (Figures 2 and 3). In addition, in Figure 4 are showed differences in the histological structure of the myocardium section in absence or presence of compound 3 [0.001 nM]. Other results showed that compound 3 [0.001 nM] decreases the infarct area in a dose-dependent manner (Figure 5).

On the other hand, other experimental results showed that the effect induced by compound 3 against infarct area was in a similar manner to the effect induced by levosimendan a dose of 0.001

nM (Figure 6). Additionally, other results (Figure 7) indicate that compound 3 increases the left ventricular pressure in a dose-dependent manner and this effect was in a similar manner to biological activity exerted by BAY K 8644 drug on left ventricular pressure; however, the effect induced by the steroid-derivative was significantly inhibited (p = 0.05) in the presence of nifedipine [1 nM]. Finally, other results (Table 3) showed that effect induced by the adamantly-steroid derivative [0.001 nM] against ischemia/reperfusion injury exert an increase in the intracellular calcium levels as a consequence of increases in the time (3-8 min); nevertheless, this effect is significantly reduced (p = 0.005) in presence of nifedipine [1 nM].

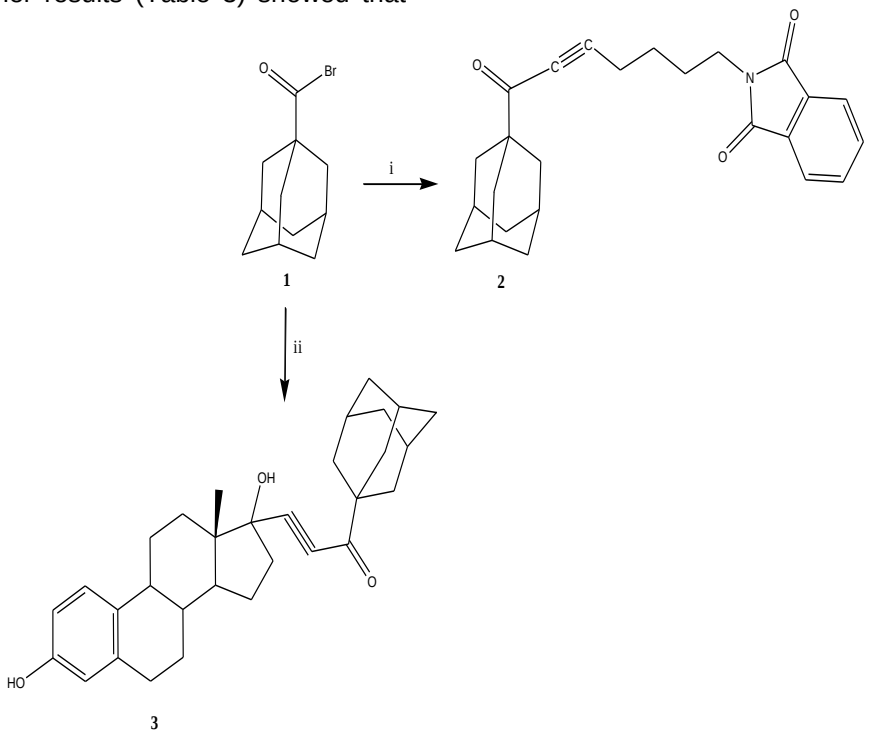

Figure 1. ***Preparation of an adamantanyl-steroid derivative*** (**3**). Reaction of Adamantane-1-carbonyl bromide (**1**) with *N*-(5-Hexynyl)phthalimide (i) to form an adamantanyl)-1,3-dione analog (**2**). Then **2** reacted with 17α-Ethynylestradiol for the synthesis of **3**. ii = CopperII.

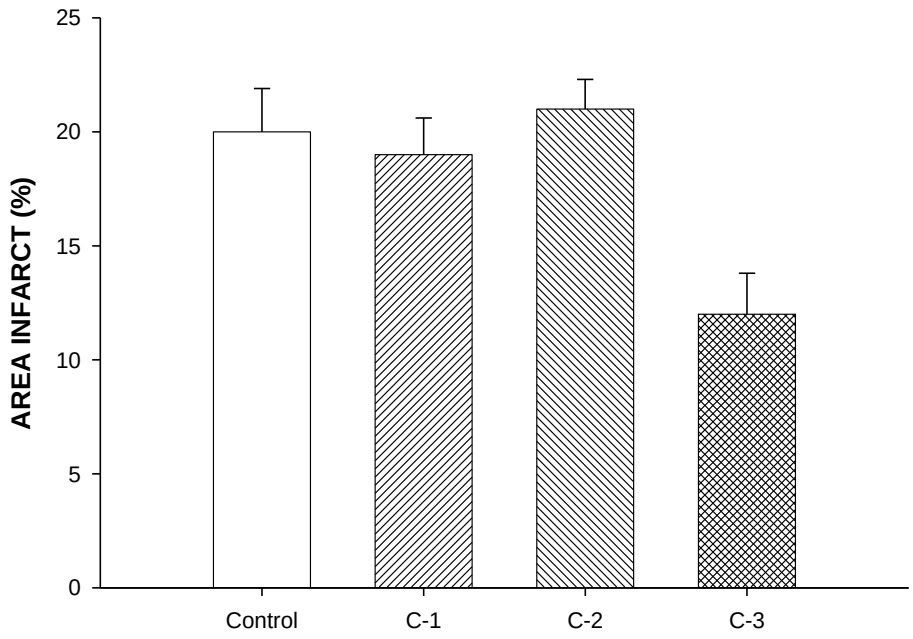

Figure 2. ***Effect exerted by the compounds 1, 2 and 3 against ischemia-reperfusion injury*****.** The hearts perfused with the Krebs-Henseleith solution were subjected to ischemia for 40 minutes by turning off the perfusion system; then, the system was restarted, and the hearts were re-perfused by 40 minutes in absence or presence of either compounds 1-3 at dose of 0.001 nM. The results showed that compound 3 (C-3) significantly reduced ($p = 0.05$) infarct size expressed as a percentage of the area at risk compared with compounds 1 (C-1) or 2 (C-2) and the conditions control. Each bar represents the mean ± S.E. of 9 experiments.

Table 1. Values of both ^{1}H NMR and ^{13}C NMR (500 MHz, Chloroform-*d*) spectra of compound 2.

δ_H: 1.19-1.67 (m, 12 H), 1.78-1.81 (m, 4H), 1.91-2.01 (m, 3H), 2.17 (m, 2H), 3.62-3.65 (m, 2H) ppm.
δ_C:18.92, 26.76, 27.84, 29.73, 36.64, 39.12, 40.12, 46.46, 81.40, 96.82, 123.14, 132.12, 133.88, 168.22, 194.20 ppm.

Table 2. **Values of both ^{1}H NMR and ^{13}C NMR (500 MHz, Chloroform-*d*) spectra of compound 3**

δ_H: 0.96 (s, 3H), 1.32-1.40 (m, 3H), 1.50 (m, 3H), 1.52 (m, 1H), 1.58-1.69 (m, 9H), 170-1.84 (m, 4H), 1.86-1.98 (m, 4H), 2.03-3.55 (m, 7H), 7.33 (broad, 2H), 8.33-8.38 (m, 3H) ppm.
δ_C: 12.34, 23.66, 26.85, 27.70, 27.80, 30.27, 34.94, 36.50, 36.66, 37.87,40.12, 44.92, 46.46, 49.54, 52.80, 81.34, 88.36, 95.62, 112.72, 115.34, 126.51, 132.24, 138.35, 153.02, 193.92 ppm.

Table 3. Effects induced by the adamantly-steroid derivative (compound 3) at a dose of 0.001 nM on the intracellular calcium levels and absence or presence of nifedipine [1 nM].

	Cai++ [nM]	
Time (min)	Compound 3	Nifedipine
3	1.28×10^{-4}	6.54×10^{-5}
6	2.06×10^{-4}	6.54×10^{-5}
9	1.28×10^{-4}	7.02×10^{-5}
12	2.06×10^{-4}	5.56×10^{-5}
15	2.06×10^{-4}	6.54×10^{-5}
18	4.85×10^{-4}	7.02×10^{-5}

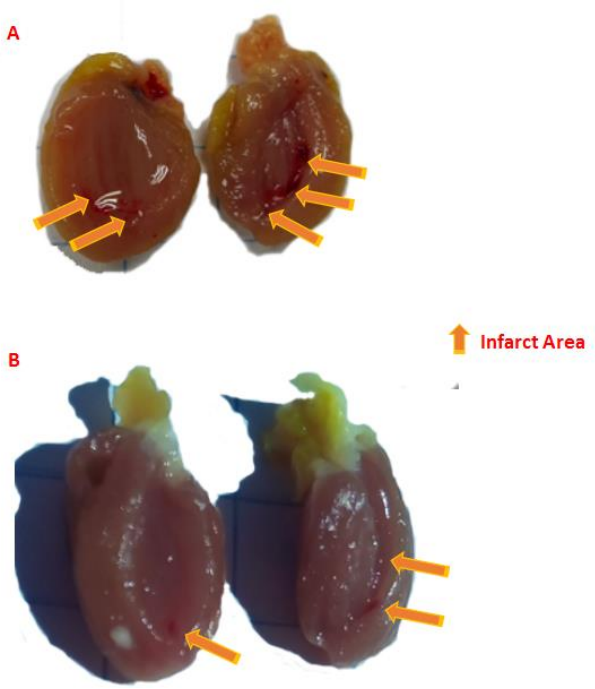

Figure 3. Comparison of cardioprotective effect of the adamantanyl-steroid derivative (compound 3) [B] at a dose of 1 nM with the control [A] on the functional recovery of rat hearts subjected to ischemia/reperfusion.

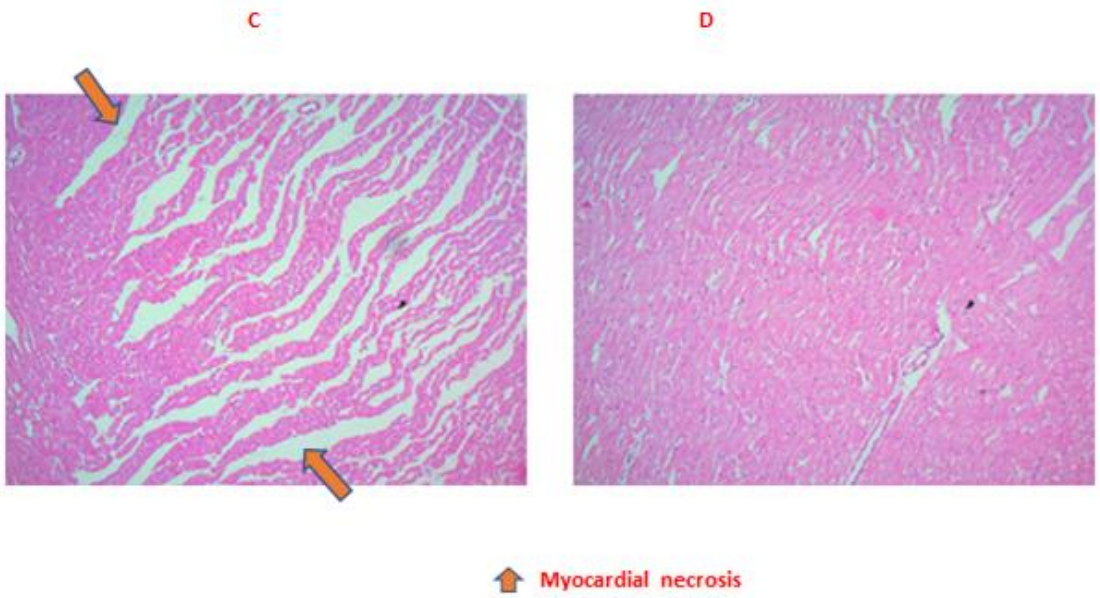

Figure 4. ***Histological evaluation of effect exerted by the adamantanyl-steroid derivative against ischemia-reperfusion using the technique modifies reported by Engelhardt [28***]. T
he scheme C (control) showed a marked alteration of the structure of the myocardium section characterized by the appearance of extensive necrosis: In addition, several bands of contraction and thinning of myofibrils were observed. The scheme D show a decreased of myocardial necrosis by presence of the adamantanyl-steroid derivative (0.001 nM).

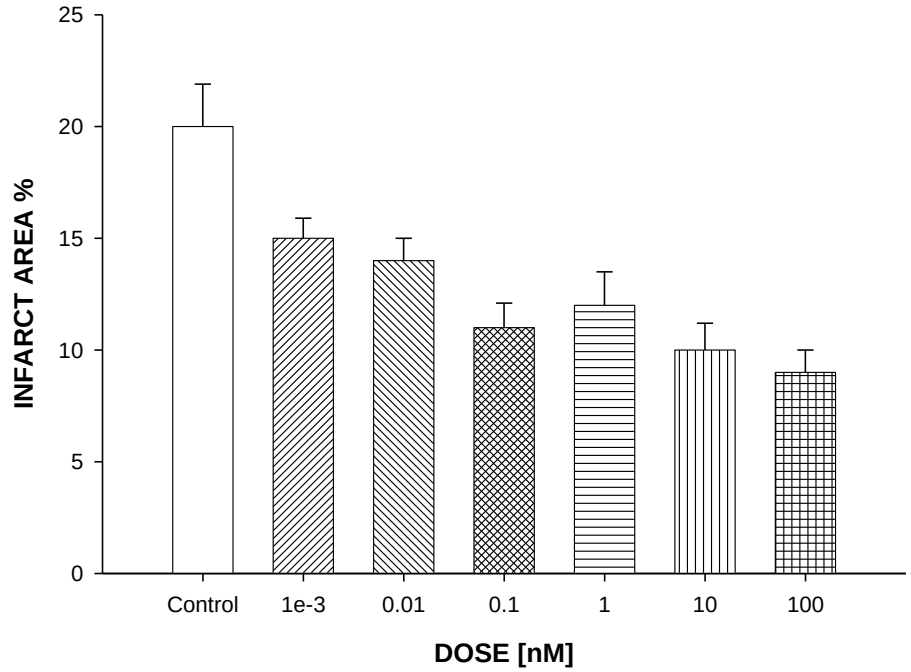

Figure 5. ***Biological activity induced by the adamantanyl-steroid derivative (compound 3) derivative on infarct area***.

The hearts perfused with the Krebs-Henseleith solution were subjected to ischemia for 40 minutes by turning off the perfusion system; then, the system was restarted, and the hearts were re-perfused by 40 minutes in absence or presence of the compound 3 at dose of 0.001 to 100 nM. The results showed that compound 3 significantly reduced ($p = 0.05$) infarct (size expressed as a percentage of the area at risk) in a dose-dependent manner compared with the conditions control. Each bar represents the mean ± S.E. of 9 experiments.

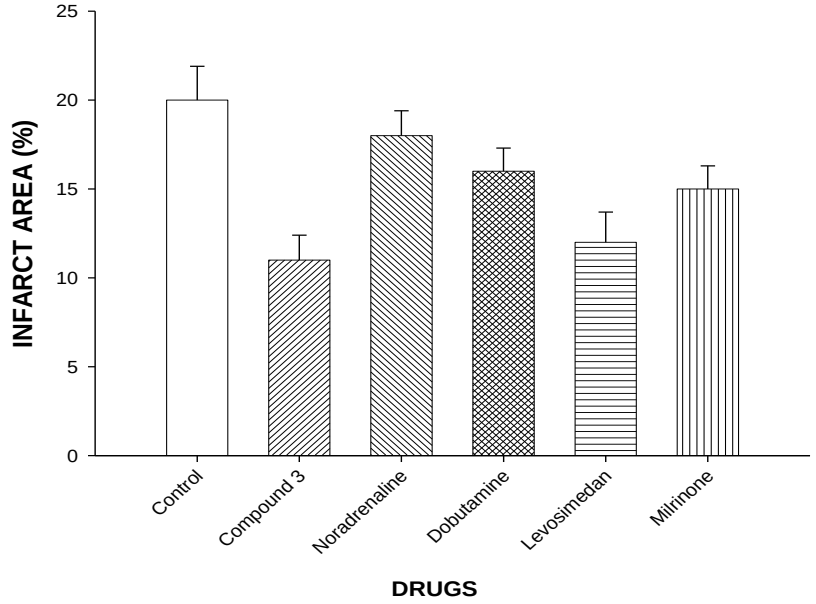

Figure 6. ***Biological activity induced by noradrenaline, milrinone, dobutamine, levosimedan and the adamantanyl-steroid derivative (Compound 3) against the ischemia-reperfusion injury***.
The hearts perfused with the Krebs-Henseleith solution were subjected to ischemia for 40 minutes by turning off the perfusion system; then, the system was restarted, and the hearts were re-perfused by 40 minutes in absence or presence of noradrenaline or milrinone or dobutamine or levosimedan or the compound **3** at dose of 0.001 nM. The results showed that compound **3** significantly reduced (p = 0.05) infarct size expressed as a percentage of the area at risk in a similar form that levosimedan drug. Each bar represents the mean ± S.E. of 9 experiments.

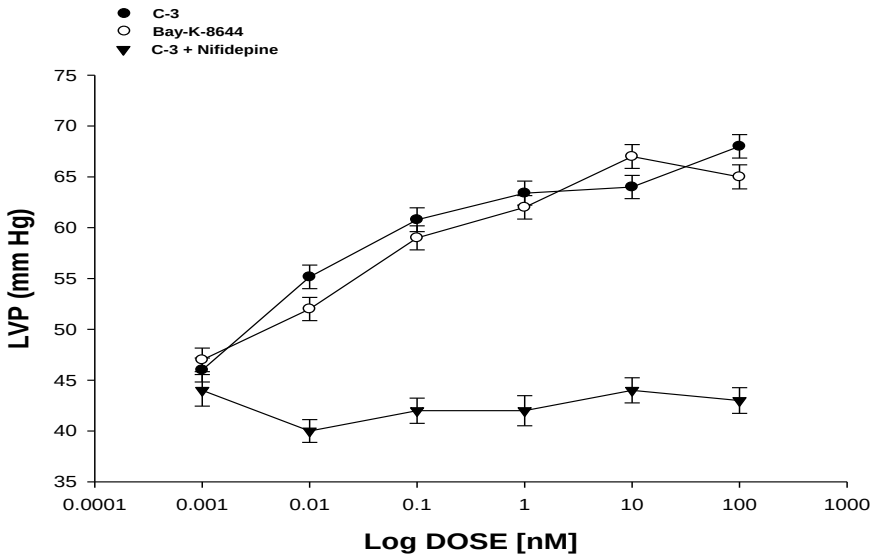

Figure 7. ***Effect exerted by induced by BAY-K-8644, nifedipine and compound 3 against left ventricular pressure (LVP)***.
Intracoronary boluses (50 µl) of compound **3** or BAY-K-8644 at a dose of 0.001 to 100 nM were administered and the effects on LVP were determinate. Then, the biological activity of **3** was repeated in the presence of nifedipine. The scheme showed that biological activity exerted by **3** against LVP was in a similar manner that BAY-K-8644; however, this effect inhibited in presence of nifedipine. The effect it is expressed as the area under the curve, and each bar represents the mean ± SE of 9 experiments.

DISCUSSION

Chemical synthesis

The aim of this study was synthesizing an adamantanyl-steroid derivative (compound 3) using some strategies chemical as follows: The first stage was achieved by synthesis of 2-[7-(adamantan-1-yl)-7-oxohept-5-yn-1-yl]isoindole-1,3-dione (2), it is noteworthy that several dione-derivatives have prepared using some reagents such as glycol (Smith and Newman, 1968), lithium aluminum (Ping et al., 2009), CrO_3 (Hunter and Priest), 2-acetylthiophene (Flores et al., 2002) and others; however, some of these reagents are expensive and require special conditions. In this study, compound 2 was synthesized by reaction of adamantane-1-carbonyl bromide with *N*-(5-Hexynyl)phthalimide using cupric chloride (CopperII) as catalyst. Then 2 reacted with 17α-ethynylestradiol in presence of CopperII to formation of 3.

Biological activity

Several studies suggest that steroid derivatives exert effects against ischemia/reperfusion injury (Hale et al., 1996); however, the biological activity is very confusing, this phenomenon could be due to different molecular mechanisms involved, through of activation of estrogen-receptor (Gabel et al., 2005), β_1-adrenergic receptor (Kam et al., 2004, potassium channels (Das and Sarkar, 2006) or connexin43 protein (Lee et al., 2004) and others. Analyzing these data, in this investigation the biological activity of an adamantanyl-steroid derivative **(**compound 3) against ischemia/reperfusion injury (translated as infarct size) was determinate using the compounds 1 or 2 as controls. The results showed that compound 3 significantly decreases infarct size (expressed as the percentage of the area at risk) compared with vehicle-treated hearts, and compounds 1 or 2. In addition, the histological evaluation showed a marked alteration of the structure of the myocardium section characterized by the appearance of extensive necrosis in absence of compound 3; this phenomenon suggests that 3 could produce an effect cardioprotective against ischemia/reperfusion injury. In addition, these data indicate that biological activity exerted by the compound 3 against ischemia/reperfusion injury which is translated as the infarct area depends on the chemical characteristics of compound 3. On the other hand, analyzing these results, other experiments were carried out at different doses in order to evaluate the different changes in biological activity exerted by compound 3 on ischemia/reperfusion injury; the results showed that compound 3 decrease the myocardial injury in a dose-dependent manner.

In the search of molecular mechanism involved in the biological activity exerted by compound 3 against ischemia/reperfusion injury, several reports were analyzed; it is noteworthy that these studies indicate that some drugs such as noradrenaline, dobutamine, levosimendan, and milrinone can exert effects on ischemia/reperfusion injury (Richardt et al., 1987; Lilleberg et al., 1995; Wang J et al., 2013). Therefore, in this investigation, the biological activity induced by these drugs against ischemia/reperfusion injury was evaluated to compare with the effect exerted by compound 3 on this clinical pathology. The results showed that compound 3 significantly decreases the infarct area in a similar manner compared to levosimendan [calcium sensitizer] (Du-Toit et al., 1999). Nevertheless, this effect was different to effect exerted by noradrenaline, dobutamine, milrinone against ischemia/reperfusion injury; this phenomenon suggested that compound 3 could exert changes on the concentration of calcium intracellular and left ventricular pressure just as levosimendan does (Pap et al., 2006). To evaluate this hypothesis, the biological activity exerted by BAY K 8644 drug [calcium channel agonist] (Garcia et al., 1984) against left ventricular pressure was evaluated, for comparing the results with the effect exerted by compound 3. The results showed that compound 3 increases the left ventricular pressure in a dose-dependent manner and this effect was in a similar manner at effect exerted by Bay K 8644 drug; these data suggest that biological activity exerted by compound 3 could be through of calcium channel activation. Analyzing this data, in this study the effect induced by 3 on left ventricular pressure was evaluated in the absence or presence of nifedipine [calcium channel antagonist] (Tsukuda et al., 2008). The results showed that compound 3 increases the left ventricular pressure in a dose-dependent manner; however, this effect was inhibited by nifedipine. These data suggest that the effect exerted by compound 3 on ischemia/reperfusion injury could induce changes on intracellular calcium levels. To evaluate this hypothesis and analyzing other reports which suggest that the effect induced by some steroid derivatives on left ventricular pressure involving

an increase in intracellular calcium (Figueroa-Valverde et al., 2011-B). In this study, the activities induced by compound 3 on intracellular calcium levels were evaluated in the absence or presence of nifedipine using a previous method reported. The results showed that the effect exerted by compound 3 exerts increases in intracellular calcium levels in a time-manner dependent; however, this effect is significantly reduced in the presence of nifedipine. All, these data suggest that the biological activity exerted by compound 3 against left ventricular pressure was via *type-L* calcium channel activation. However, it is important to mention that calcium channel activation could depend on the site of interaction of compound 3 with the protein-surface to produce changes in left ventricular pressure which may be translated as a decrease in ischemia-reperfusion injury.

CONCLUSION

The biological activity of the adamantanyl-steroid derivative is particularly interesting, due to its cardioprotective effect exerted on the ischemia/reperfusion injury, which is translated by a decrease in the area of infarction and increase in pressure of the left ventricle through the activation of *type-L* calcium channels; This phenomenon could constitute a new therapy for ischemia/reperfusion injury; however, it is necessary to carry out some toxicity studies to evaluate if it could be considered for this type of clinical pathology.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

FVL, DCF and RNM designed, synthesized the steroidal derivatives, performed the biological evaluation and also wrote the manuscript. MAV, HVP, GEA, BCL, PGE, HHL, LRM and BBY performed animal treatments, Histological evaluation, and data analysis. All authors read and approved the final version.

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