Calix[n]arene-based immunogens: A new non-proteic strategy for anti-cocaine vaccine

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HIGHLIGHTS

• The first total synthesis of the novel calix[n]arene-based immunogens V4N2 and V8N2 is reported.
• V4N2 and V8N2 promoted the production of cocaine antibodies and also modulated the biodistribution of [99mTc]TRODAT-1, a radiolabeled analogue of cocaine.
• V4N2 and/or V8N2 are potential candidates for the development of an immunogenic agent for the treatment of cocaine use disorder.

GRAPHICAL ABSTRACT

Two novel calix[n]arene-based immunogens were able to yield anti-cocaine antibodies and decrease the levels of [99mTc]TRODAT-1, a cocaine-based radionuclide, in the brain of the tested mice.

ABSTRACT

Introduction: Cocaine use disorder is a significant public health issue without a current specific approved treatment. Among different approaches to this disorder, it is possible to highlight a promising immunological strategy in which an immunogenic agent may reduce the reinforcing effects of the drug if they are able to yield sufficient specific antibodies capable to bind cocaine and/or its psychoactive metabolites before entering into the brain. Several carriers have been investigated in the anti-cocaine vaccine development; however, they generally present a very complex chemical structure, which potentially hampers the proper assessment of the coupling efficiency between the hapten units and the protein structure.

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Introduction

Cocaine use disorder (CUD) is a persistent public health problem with no single “best” pharmacological agent for its treatment [1-4]. Despite of the efforts to educate the population about the dangers of the use of cocaine, CUD remains a major social and health challenge [5-10]. Increment in the number of cocaine users from 14 million in 1998 to 18 million in 2017, was noted by the United Nations Office on Drugs and Crime, which corresponds to nearly 0.4% of the world population aged 15-64 [11]. Brazil occupies the first and second places in the rank of crack consumption (smoked form of cocaine) and cocaine traffic, respectively [12-15].

The international scientific community has been struggling to find an effective treatment for CUD. Currently, the available pharmacological treatments are only used to manage withdrawal syndrome and relapse prevention [16,17]. Given the range of therapies for CUD, the development of an immunologic strategy is a promising approach [18]. In fact, immunogenic agents may reduce the reinforcing effects of the drug if they are able to yield high titers of highly specific antibodies capable to bind cocaine and/or its psychoactive metabolites before entering into the brain. As this immune mechanism is similar to the one observed in antiviral or antibacterial vaccines, this strategy was popularly named “anti-drug vaccine”. This concept has been previously demonstrated to be successful in blunting the physiological effects of cocaine [18-25], methamphetamine [26-30], heroin [31-34], opioids [35-37], nicotine [38-41], etc.

Cocaine is barely not immunogenic, so, to become immunogenic, cocaine should be chemically modified into a derivative called hapten, which can subsequently be conjugated to a macromolecular carrier prior to the administration [18-25]. Both N-methyl and 2β methyl ester groups of cocaine have been extensively targeted to synthesize haptenes able to link to a carrier [9,42-48]. For instance, GNE (6-(1R,2R,3S,5S)-3-(benzoyloxy)-8-methyl-8-azabicycle[3.2.1]octane-2-carboxamide-hexanoic acid), a hydrolysis-tolerant hapten, stimulates the production of cocaine-specific antibodies when carried by high molecular weight proteins [25,46,49-51].

Several carriers have been investigated in the anti-cocaine vaccine development [48] such as viral-DNA fragments/proteins [25,49,52], peptide nanofiber [53] and proteins, like the subunit of cholera toxin [21], flagellin [54] and keyhole limpet hemocyanin (KLH) [43]. Peptide-based carriers have present a very complex chemical structure, which potentially hampers the assessment of the coupling efficiency between the hapten units and the protein structure [55]. Moreover, most synthetic methodologies to couple the hapten to the protein employ the use of a carrier in excess. Once in excess, the peptide-based carrier could increase the production of antibodies against the carrier itself and, consequently, decrease the desired immunogenicity of the exposed hapten [55-57]. In addition, protein-based vaccines require a complex system for sterilizations and a cold supply chain, considering the possible risk of denaturalization of proteins when exposed to heat.

Viruses and bacteria display a well-organized, repetitive array of antigens on surface, which is believed to successfully allow the clustering of antigen receptors on B cells. This phenomenon boosts the production of antibodies in comparison with the interaction between these antigens with soluble proteins [58-60]. These findings suggested that antigens’ subunit immunogenicity is improved when the antigens are rigidly ordered on surfaces, similarly to the observed for viral particles [58,60]. Therefore, this multivalency of antigen arrangement, together with the easing of immune cell recognition and antigen incorporation, has been explored as an approach to improve both humoral and cellular immunity [60].

Considering the difficulty in coupling efficiency verification between haptenes and biomacromolecules, the antibody production against the carriers due to excess in synthesis, and the importance of spatially organized antigens, we envisioned the use of calix[n]arene as a potential carrier for the development of cocaine immunogens. To the best of our knowledge, cocaine calix[n]arene-based immunogens are unprecedented, and such macrocycles were used as carriers only for the development of an anticancer vaccine, firstly reported by Geraci and collaborators in 2008, thus far [61]. According to these authors, the high antibody titer yield in their study, is a consequence of the three-dimensional arrangement adopted by the calix[4]arene platform. Subsequently, in another study, this same research group observed that a calix[8]arene-based anticancer vaccine candidate also induced a specific immune response, ratifying the promising applicability of calix[n]arenes in building vaccine constructs [62]. Thus, the present study reports the design, synthesis and preclinical evaluation of two novel calix[n]arene-based anti-cocaine immunogens (herein named as V4N2 and V8N2) by the tethering of the hydrolysis-tolerant hapten GNE (15) on calix[4]arene and calix[8]arene moieties.

Methods: The preclinical assessment corresponded to the immunogenicity and dose–response evaluation of V4N2 and V8N2. The potential of the produced antibodies to reduce the passage of cocaine analogue through the blood–brain-barrier (BBB), modifying its biodistribution was also investigated.

Results: Both calix[n]arene-based immunogens elicited high titers of cocaine antibodies that modified the biodistribution of a cocaine radiolabeled analogue (99mTc-TRODAT-1) and decreased cocaine-induced behavior, according to an animal model.

Conclusion: The present results demonstrate the potential of V4N2 and V8N2 as immunogens for the treatment of cocaine use disorder.

Materials and Methods

Synthesis procedures

5,11,17,23-tetra-tert-Butyl-25,26,27,28-tetrahydroxycalix[4]arene (2), 5,11,17,23-Tetra-tert-butyl-25,26,27,28-tetrabutoxycalix[4]arene (4), 5,11,17,23-Tetranitro-25,26,27,28-tetrabutoxycalix[4]arene (6) and 5,11,17,23-Tetraamino-25,26,27,28-tetrabutoxycalix[4]arene (8) were previously synthesized by our research
group and their spectroscopic data are in accordance with those published elsewhere [63]. IR, $^1$H- and $^{13}$C and HRMS for compounds 2, 4, 6 and 8 are available as a Supplementary Material.

5,11,17,23,29,35,41,47-Octa-tert-butyl-49,50,51,52,53,54,55,56-octahydroxy-calix[8]arene (3). A mixture containing $p$-tert-butylphenol (10.00 g; 66.5 mmol), paraformaldehyde (3.60 g; 120 mmol), 1.5 mL of a 10 mol L$^{-1}$ sodium hydroxide solution and 300 mL of xylene was additionally in a round-bottomed three-necked flask and, after, it was mechanically stirred for approximately 15 min. Subsequently was coupled to a Dean-Stark apparatus containing 100 mL of xylene. The mixture was refluxed at 115 °C by using a heating mantle, with continuous flow of nitrogen, for approximately 4 h. After 30 min of heating, the reaction became a homogeneous mixture, and after 1 h, a white precipitate begins to form. The mixture was cooled to room temperature and the precipitate formed was collected by filtration. The precipitate was washed successively with 200 mL of toluene, 200 mL of ethyl ether, 200 mL of acetone and finally with 200 mL of distilled water. After that, then dried under reduced pressure. The solid obtained was dissolved in 800 mL of chloroform and heated until its volume was reduced to 600 mL. The solution was then cooled to room temperature and the precipitate formed was then collected and dried. This methodology provides the desired product 3 as a white solid (10.50 g) in 52% yield. IR (ATR): 3225, 3052, 2955, 2902,2868,1602, 1486, 1452, 1427, 1391,1361, 1290, 1247, 1203, 1149, 1117 and 783 cm$^{-1}$. $^1$H NMR (300 MHz, CDCl$_3$): δ 9.62 (s, 8H, OH), 7.18 (s, 16H, H$_3$), 4.37 (d, 8H, J$_{7a,7b}$ = 12.0 Hz, H$_{7a}$), 3.50 (d, 8H, J$_{7b,7a}$ = 12.0 Hz, H$_{7b}$), 1.26 (s, 72H, H$_6$). $^{13}$C NMR (75 MHz, CDCl$_3$): δ 146.6 (C$_1$), 144.7 (C$_2$), 128.7 (C$_4$), 125.5 (C$_3$), 34.0 (C$_7$), 32.3 (C$_5$), 31.4 (C$_6$). HRMS (MALDI-TOF) calcd. for C$_{88}$H$_{113}$O$_8$ [M+H]$^+$1,298.8340; found 1,298.8239.

5,11,17,23,29,35,41,47-Octa-tert-butyl-49,50,51,52,53,54,55,56-octabutoxycalix[8]arene (5). In a round-bottomed flask (500 mL) with a magnetic bar, a mixture containing compound 3 (1.00 g, 0.77 mmol) and dry dimethylformamide (150 mL), under stirring, was heated to 70 °C until a translucent solution was obtained. After that, were added 6 g of dispersed NaH in 60% of oil solution. The mixture remained under stirring at 60 °C for 1 h. Subsequently, was added 50 mL of 99% 1-bromobutane (77 mmol) and potassium iodide (5.00 g; 30 mmol). The temperature of the mixture was lowered to 35 °C, and the reaction remained under stirring for 72 h. After this time, 100 mL of dichloromethane were added and the mixture was washed with 200 mL of 0.1 mol L$^{-1}$ HCl solution. The organic phase was concentrated in vacuo until half of the amount of solvent added was evaporated. After that, 200 mL of acetone were added, and the mixture was again concentrated in vacuo until a white precipitate was formed. The precipitate was recovered by filtration with a Büchner funnel and washed with
30 mL of cold acetone (10 °C). The product 5 is a white solid and was obtained in 80% yield (1.00 g). IR (ATR): 2955, 2900, 2868, 1460, 1413, 1381, 1360, 1286, 1242, 1204, 1190, 1111, 1063, 1017, 998 and 833 cm⁻¹. ¹H NMR (200 MHz, CDCl₃): δ 6.96 (s, 16H, H₃), 4.05 (s, 16H, H₇), 3.51 (t, 16H, J₈,₉ = 6.0 Hz, H₈), 1.45–1.60 (m, 16H, H₉), 1.19–1.45 (m, 16H, H₁₀), 1.09 (s, 72H, H₆), 0.99 (t, 24H, J₁₁,₁₀ = 6.0 Hz, H₁₁). ¹³C NMR (50 MHz, CDCl₃): δ 153.6 (C₁), 145.7 (C₂), 133.2 (C₄), 125.9 (C₃), 72.9 (C₈), 34.4 (C₅), 32.5 (CH₂), 31.6 (C₆), 30.1 (CH₂), 19.4 (C₁₀), 14.0 (C₁₁). HRMS (MALDI-TOF) calcd. for C₁₂₀H₁₇₆O₈Na [M+Na]⁺ 1,769.6664; found 1,769.275 and calcd. for C₁₂₀H₁₇₆O₈K [M+K]⁺ 1,785.7749; found 1,785.241.

5,11,17,23,29,35,41,47-Octanitro-49,50,51,52,53,54,55,56-octabutoxycalix[8]arene (7). In a round-bottomed flask (50 mL), with a magnetic bar, were added compound 5 (0.50 g, 0.285 mmol), NaNO₃ (2.60 g, 30.5 mmol) and trifluoroacetic acid (99%) (2.4 mL) dropwise under lowered stirring. The mixture was kept under stirring for approximately seven hours. After that, the mixture was poured into 200 mL of ice distilled water (5 °C), to precipitate the crude product (a yellow pale solid). The precipitate formed was filtered, washed with 30 mL of cold distilled water (5 °C) and 3 mL of cold methanol) and dried. Subsequently, the precipitate was solubilized in 10 mL of distilled ethyl acetate. After that, was added, 30 mL of cold (10 °C) methanol to provide the desired product 7, as a pink pale solid in 64% yield (0.50 g). IR (ATR): 3070, 2956, 2872, 1586, 1518, 1448, 1380, 1340, 1308, 1263, 1232, 1092 and 945 cm⁻¹. ¹H NMR (200 MHz, CDCl₃): δ 7.76 (s, 16H, H₃), 4.15 (s, 16H, H₇), 3.87 (t, 16H, J₈,₉ = 8.0 Hz, H₈), 1.49–1.80 (m, 16H, H₉), 1.24–1.49 (m, 16H, H₁₀), 1.09 (t, 24H, J₁₁,₁₀ = 8.0 Hz, H₁₁). ¹³C NMR (50 MHz, CDCl₃): δ 161.4 (C₄), 144.0 (C₁), 134.6 (C₂), 124.8 (C₃), 74.4 (C₈), 32.3 (CH₂), 19.3 (C₁₀), 14.0 (C₁₁). HRMS (MALDI-TOF) calcd. for C₈₈H₁₀₄N₈O₂₄Na [M+Na]⁺ 1,679.7061; found 1,679.610 and calcd. for C₈₈H₁₀₄N₈O₂₄K [M+K]⁺ 1,696.9049; found 1,696.592.

5,11,17,23,29,35,41,47-Octaamine-49,50,51,52,53,54,55,56-octabutoxycalix[8]arene (9). In a round-bottomed flask (250 mL) with a magnetic bar, were added compound 7 (0.1 g, 0.06 mmol), a mixture of ethanol/THF (50 mL, 1:1 v/v), hydrazine monohydrate (5 mL) and palladium on carbon 10% (catalytic amount). The reaction mixture was refluxed and stirred for 24 h. The catalyst was filtered off through Celite® filter and washed with 50 mL solution of 1 mol L⁻¹ of HCl in methanol. The mixture was concentrated in vacuo and the pure product 9 was obtained after washing with cold distilled water (30 mL) in 86% yield (85 mg). IR (ATR): 3358, 2955,
2930, 2868, 1606, 1458, 1379, 1210, 1124, 1082, 1064, 1019, 955 and 853 cm⁻¹. ¹H NMR (200 MHz, CDCl₃ and DMSO-d₆): δ 6.17 (s, 16H, H₃), 3.83 (s, 16H, H₇), 3.77 (t, 16H, J₈,₉ = 6.0 Hz, H₈), 3.51 (s, 24H, NH₂ and H₂O), 1.62–1.90 (m, 16H, H₉), 1.34–1.62 (m, 16H, H₁₀), 0.95 (t, 24H, J₁₁,₁₀ = 6.0 Hz, H₁₁). ¹³C NMR (50 MHz, CDCl₃ and DMSO-d₆): δ 147.5 (C₁), 142.0 (C₄), 134.8 (C₂), 115.2 (C₃), 73.5 (C₈), 32.2 (CH₂), 29.2 (CH₂), 19.1 (C₁₀), 13.8 (C₁₁). HRMS (MALDI-TOF) calcd. for C₈₈H₁₂₁N₈O₈ [M+H]⁺ 1,418.9511; found 1,418.9326.

6-Amino-hexanoic acid benzylester tolune-4-sulfonic acid (11). In a flask with 6-amino-hexanoic acid (1.32 g, 10.1 mmol) were added benzyl alcohol (3.82 g, 35.3 mmol), p-toluenesulfonic acid monohydrate (2.01 g, 10.6 mmol) and 25 mL of toluene. The reaction mixture was warmed and refluxed for 24 h with a Dean-Stark apparatus. After cooling to room temperature, the precipitate was filtered, washed with diethyl ether (2 × 25 mL) and dried to furnish 3.93 g of the desired product 11 (99% yield). IR (ATR): 3262, 3186, 3043, 2949, 2869, 1725, 1479, 1450, 1304, 1262, 1249, 1194, 1161, 1142, 1127, 1068, 1037, 1013, 960, 919, 824, 751 and 683 cm⁻¹. ¹H NMR (200 MHz, CDCl₃): δ 7.75–7.66 (m, 5H, H₁₃ and NH₃), 7.32 (brs, 5H, H₉, H₁₀ and H₁₁), 7.15 (d, 2H, J₁₄,₁₃ = 8.0 Hz, H₁₄), 5.07 (s, 2H, H₇), 2.75–2.73 (m, 2H, H₁₂), 2.31 (s, 3H, H₁₆), 2.20 (t, 2H, J₁₅,₁₄ = 8.0 Hz, H₁₅), 1.49–1.43 (m, 4H, H₂ and H₃), 1.20–1.16 (m, 2H, H₁₄). ¹³C NMR (50 MHz, CDCl₃): δ 173.3 (C₆), 141.4 (C₅), 141.0 (C₆), 136.2 (C₈), 129.2 (CH₂), 128.7 (CH₂), 128.3 (CH₂), 126.0 (CH₂), 66.3 (C₁), 39.9 (CH₂), 34.0 (CH₂), 27.2 (CH₂), 25.9 (CH₂), 24.3 (CH₂), 21.4 (CH₂).

Benzoylecgonine (13). 1.17 g (3.87 mmol) of cocaine (12) was refluxed and stirred in water distilled (20 mL) for 24 h. The solvent was removed under reduced pressure and acetone (80 mL) was added into crude product. After stirred in an ice bath, the desired product was obtained as a white powder in 84% yield (0.94 g). IR (ATR): 3224, 1718, 1570, 1467, 1449, 1401, 1351, 1270, 1196, 1115, 1076, 1025, 996, 983, 810, 789 and 708 cm⁻¹. ¹H NMR (200 MHz, CD₃OD): δ 8.05–8.01 (m, 2H, H₁₁), 7.64–7.57 (m, 1H, H₁₃), 7.46 (t, 2H, J₁,₁₂ = 8.0 Hz, H₁₂), 5.48 (dt, 1H, J₁,₁₄ = 12.0 Hz, J₁₄,₁₃ = 6.0 Hz, H₁₄), 4.00–3.95 (m, 2H, H₁ and H₅), 3.10 (dd, 1H, J₁,₂ = 6.0 Hz, J₂,₁₂ = 2.0 Hz, H₂), 2.81 (s, 3H, H₁₆), 2.65–2.10 (m, 6H, H₄, H₆ and H₇). ¹³C NMR (50 MHz, CD₃OD): δ 177.0 (C₈), 167.2 (C₉), 134.6 (C₁₃), 131.3 (C₁₀), 131.0 (C₁₁), 129.6 (C₁₂), 66.7 (CH), 66.0 (CH), 63.7 (CH), 50.3 (CH), 38.3 (C₁₄), 34.4 (CH₂), 25.1 (CH₂), 24.7 (CH₂), 21.4 (CH₂).
(1R,2R,3S,5S)-2-((6-Benzoxyl)-6-oxohexyl)(carbamoyl)-8-methyl-8-azabicyclo[3.2.1]octan-3-yl Benzoate (14). To a solution of benzoylqueine 13 (1.16 g, 4.0 mmol), 6-amino-hexanoic acid benzyl ester toluene-4-sulfonic acid (1.57 g, 4.0 mmol) and 4-dimethylaminopyridine (0.50 g, 4.0 mmol) in dry dichloromethane (20 mL) were added EDC (0.86 g, 4.4 mmol) and triethylamine (0.56 mL, 4.0 mmol) at 0°C. The reaction mixture was warmed to room temperature and stirred for 96 h in a inert atmosphere (Ar). The organic layer was washed with 10% citric acid solution (3 x 15 mL), water (1 x 20 mL) and brine (1 x 20 mL), dried with anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The crude product was purified in a chromatography column using ethyl acetate/hexan/EtN (30:10:4. v/v) as mobile phase and the desired product was obtained in 71% yield (1.39 g). IR (ATR): 3260, 2934, 2864, 1716, 1651, 1600, 1539, 1467, 1451, 1452, 1267, 1216, 1176, 1113, 1069, 1027, 988, 748, 710 and 687 cm⁻¹. ¹H NMR (50 MHz, CD₃OD): δ 7.97 (brs, 1H, NH), 7.97 (d, J = 6.0 Hz, 8H, H₂9), 7.44–7.35 (m, 12H, H₃0, H₃1), 6.76 (brs, 4H, H₃), 6.75 (brs, 4H, H₃), 5.31 (dt, 4H, J₂₁,₂₂a = 10.2 Hz, J₂₁,₂₂b = 10.2 Hz, H₂₁), 2.93–2.87 (m, 1H), 2.37–1.88 (m, 9H), 2.73 (s, 3H, H₁₄), 2.52–2.01 (m, 8H), 1.58–1.20 (m, 7H). ¹³C NMR (100 MHz, CDCl₃): δ 173.1 (C₁₀), 171.5 (C₁₁), 166.1 (C₁₇), 153.5 (C₁₃), 135.2 (C₁₅), 133.1 (C₁₆), 132.0 (C₂₀), 130.4 (C₂₈), 128.9 (C₂₉), 128.5 (C₃₀), 124.1 (C₃₁), 75.1 (C₁₂), 66.2 (C₂₁), 63.4 (C₁₉), 60.6 (C₂₃), 51.3 (C₂₀), 40.6 (C₂₈), 38.9 (C₁₇), 37.0 (C₁₁), 36.0 (C₂₉), 32.3 (C₂₀), 31.2 (C₁₇), 29.6 (C₂₃), 26.6 (C₁₉), 26.0 (C₂₅), 25.3 (C₁₈), 25.0 (C₂₄), 19.5 (C₁₉), 14.2 (C₁₁). HRMS (MALDI-TOF) calcd. for C₁₃₂H₁₇₃N₁₂O₂₀ [M+H⁺] 2,247.2917; found 2,247.2908.

Immunogenic compound V4N2. The hapten GNE (15) (0.98 g; 2.43 mmol) was dissolved in dry dichloromethane (8 mL) and reacted with PyBOP (1.58 g; 3.04 mmol) and DIPEA (0.31 g; 2.43 mmol). After stirring for 40 min at room temperature, a solution of calixarene 8 in dry dichloromethane (10 mL) was added dropwise in inert atmosphere (Ar). The mixture was kept under stirring for 26 h and then was added dichloromethane (80 mL). The organic layer was washed with sat. aq. NaHCO₃ (2 x 25 mL) and water (1 x 25 mL), dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The crude product was purified by flash chromatography (CHCl₃/MeOH/TEA, 93:6:1, v/v) to provide the desired product V4N2 (0.60 g; 59% yield) as a yellow solid. IR (ATR): 3260, 2934, 2864, 1716, 1651, 1600, 1539, 1467, 1451, 1452, 1267, 1216, 1176, 1113, 1069, 1027, 988, 867, 803, 748, 710 and 687 cm⁻¹. ¹H NMR (600 MHz, CDCl₃): δ 9.60 (brs, 4H, NH), 7.97 (d, J = 13.2 Hz, H₂₈), 3.48 (d, 4H, J = 13.2 Hz, H₂₈), 3.84 (brs, 8H, H₃), 3.35–3.27 (m, 16H, H₁₉, H₁₇, H₁₆ and H₂₃), 2.90 (d, 4H, J = 6.6 Hz, H₂₈), 2.25 (s, 12H, H₃₂), 2.21–2.06 (m, 20H, H₂₂a and H₂₂b), 1.99–1.87 (m, 52H, H₂₂b, H₁₄, H₁₆, H₂₄a, H₂₄b, H₁₃, H₂₃ and H₁₅), 1.00 (t, 12H, J = 7.2 Hz, H₁₂). ¹³C NMR (100 MHz, CDCl₃): δ 171.5 (C₁₂), 171.2 (C₁₈), 166.1 (C₁₇), 153.5 (C₁₃), 135.2 (C₁₅), 133.1 (C₁₆), 132.0 (C₂₀), 130.4 (C₂₈), 128.9 (C₂₉), 128.5 (C₃₀), 124.1 (C₃₁), 75.1 (C₁₂), 66.2 (C₂₁), 63.4 (C₁₉), 60.6 (C₂₃), 51.3 (C₂₀), 40.6 (C₂₈), 38.9 (C₁₇), 37.0 (C₁₁), 36.0 (C₂₉), 32.3 (C₂₀), 31.2 (C₁₇), 29.6 (C₂₃), 26.6 (C₁₉), 26.0 (C₂₅), 25.3 (C₁₈), 25.0 (C₂₄), 19.5 (C₁₉), 14.2 (C₁₁). HRMS (MALDI-TOF) calcd. for C₁₅₂H₁₇₃N₁₂O₂₀ [M+H⁺] 2,247.2917; found 2,247.2908.

Immunogenic compound V8N2. In a round-bottomed flask (100 mL), with a magnetic bar, was added the hapten GNE (0.710 g, 1.76 mmol), dry dichloromethane (10 mL), PyBOP (1.04 g, 2.0 mmol) and DIPEA (0.31 g, 2.43 mmol). The mixture was stirred for 40 min, at room temperature. After that, a solution of calixarene 9 (0.80 g, 0.056 mmol), in dry dichloromethane (10 mL), was added dropwise. The mixture was kept under hard stirring for 48 h in inert atmosphere. The mixture was subjected to two purifications: one with Sephadex LH-20 (in CH₂Cl₂ and another with silica-gel chromatography (CHCl₃/MeOH/TEA, 94:5:1, v/v), to provide the desired product V8N2, as a yellow pale solid, in 60% yield (200 mg). IR (ATR): 3261 and 3193 cm⁻¹ (CH of aromatic ring stretching), 2932 and 2863 cm⁻¹ (CH of methyl and methylene groups stretching), 1716 cm⁻¹ (carbonyl ester stretching), 1649 cm⁻¹ (carbonic amide stretching), 1542 cm⁻¹ (N-H amide angular deformation), 1267 cm⁻¹ (C=O stretching).
Animal studies

The Local Ethics Committee in Animal Experimentation approved the study protocol (CEUA-UFG Protocol N° 122/2016) and at all stages of the work, the recommendations of this commission were adopted.

Preparation of immunoconjugate hapten 15-KLH

2.0 mg of KLH protein were reconstituted with 200 μL of water ultra-purified and it was stored in an ice bath. Then, 2.0 mg of the GNE hapten (15) were diluted in 450 μL of the conjugation buffer (MES 0.1 M, 0.9 M NaCl, 0.02% sodium azide, pH 4.7), stirred until complete solubilization of the hapten and added to the aqueous solution containing the protein KLH. EDC coupling agent (10.0 mg) was reconstituted with 1000 μL of ultra-purified water
and quickly stirred. Immediately, 50 μL of this solution were transferred to the solution containing the hapten 15 and kept at room temperature for two hours. Then the brute of the reaction was purified on a desalination column (polypropylene resin) by centrifugation (1000 g) for 20 min. The emulsion at final concentration of 0.03, 0.3 or 300 μM of V4N2 or V8N2 was immediately stored on ice until time of administration.

Immunocoujugate hapten 15-KLH: In a 15 mL Falcon® tube containing 175 μL of the immunocoujugate hapten 15-KLH, 2500 μL of Freund’s Adjuvant (Complete or Incomplete) and 2500 μL of sterile saline solution, the system biphasic was then subjected to an immunoconjugate hapten 15-KLH stirring at 3000 rpm in Vortex® for 20 min. The emulsion was immediately stored on ice until the time of administration.

The vehicle for the immunogen’s formulation was prepared as following: In a 15 mL Falcon® tube containing 125 μL of DMSO were added 2435 μL of Freund’s Adjuvant (Complete or Incomplete) and 2435 μL of sterile saline solution. The two-phase system was then transferred to a 3000 rpm agitation in Vortex® for 20 min. The emulsion was immediately stored on ice until the time of administration.

Animals and blood sample collections

Male BALB/c mice aged 4 to 5 weeks were used. The animals were kept on a 12-h light–dark cycle with Chow and water provided ad libitum. All experiments were carried out in a room with an ambient temperature of 23 ± 1 °C. Blood samples were collected by the puncture method of the submandibular vein into 1.5 mL Eppendorf® tubes (0.5 mL) and stored in a freezer at −20°C. After 40 min of incubation at room temperature (RT) in the dark, the reaction was stopped with 50 μL of 3 N NaOH (STOP solution) in each well. The optical density (OD) was determined at 405 nm using a VICTOR X4 multilabel plate reader (PerkinElmer®) (Fig. 1. A-B, Fig. 2. A-B).

Radiochemical assay

99mTc-TRODAT-1 was both prepared and the radiochemical purification was evaluated as recommended by the manufacturer (RPH Pharma, Porto Alegre, Brasil). Male Balb/c mice immunized with calix[n]arene-based immunocoujugates V4N2 or V8N2 or placebo on days 0, 7 and 21 at the dose of 30 nM. Antibodies IgG anti-cancer levels was assessed by ELISA method as described before. At 40th day, animals were submitted at radiochemical assay. 0.1 mL of the 99mTc-TRODAT-1 was administered in the tail vein of each animal. After 90 min, we acquired scintigraphic images of the brain region of each animal in a gamma camera scan (Nuclide TM TH 22, Mediso, Hungria) (Fig. 4A). Animals were euthanized immediately after image acquisition and the radioactivity levels were determined in blood, brain (Fig. 4B), spleen, liver, lung, kidney, heart and stomach (Fig. 4C).

Statistical analysis

Statistical analyzes were performed using the GraphPad program Prism, version 5.01 (San Diego, CA, USA). The results were evaluated by the Kolmogorov–Smirnov normality test and the presence of outliers was detected by the Grubbs test. The results regarding the biodistribution of the [99mTc]TRODAT-1 were analyzed by Student’s t test and presented by mean and standard error. In evaluating the production of antibodies between the groups, data were analyzed using simple analysis of variance, followed by of the Bonferroni test. Values of p ≤ 0.05 were considered as a difference statistically significant.

Results and discussion

Our initial efforts to obtain cacine calix[n]arene-based immunogens focused on the preparation of calix[n]arenes 8 and 9 (Scheme 1). Firstly, the treatment of p-tert-butylphenol (1) under basic conditions at appropriate temperatures and solvents [65-67] provided the corresponding p-tert-butyl-calix[4]arene (2) and p-tert-butyl-calix[8]arene (3) in 51% and 52% yield, respectively. Then, alkylation reactions of the phenolic hydroxyl groups of 2 [63,68] and 3 [69] were performed using butyl bromide in the presence of sodium. However, in the case of calix[8]arene 3, the per-O-alkylation was only observed when potassium iodide (KI) was used as an additive. The addition of KI to promote a per-O-alkylation of 3 with propyl bromide was also reported by Yi et al (2008) [69]. Thus, compounds 4 and 5 were synthesized in 77% and 80% yields, respectively. [69]. The ipso-nitration of 4 to furnish 6 in 91% yield was performed using a mixture of fuming nitric acid (HNO3) and trifluoroacetic acid (TFA) in dichloromethane (DCM) [70]. Nonetheless, these conditions provided the corresponding octa-nitro calix[n]arene 7 only with yields lower than 20%. Fortunately, under the Dudic’s conditions, which use sodium nitrate (NaNO3) instead of fuming HNO3, with some modifications, we obtained 7 in 64% yield [71]. Finally, the reduction of the aromatic nitro groups of 6 and 7 to their corresponding amines was accomplished upon treatment with hydrazine hydrate in the presence of Pd/C [67,72,73]. At this point, calix[n]arenes 8 and 9 were synthe-
was then reductively cleaved by treatment with H\textsubscript{2} and Pd/C to 71% yield [44] (Scheme 2 - panel A). The benzyl ester group of cocaine was prepared in 98% yield by using PyBOP\textsuperscript{10} as the coupling agent afforded the desired calix[4]arene-based immunoconjugates V4N2 and V8N2 (59% and 60% yields, respectively) (Scheme 3). The mass spectra of compounds V4N2 and V8N2 showed the [M+H]\textsuperscript{+} ion \(m/z\) 2,251.2278 (\(m/z\) calc for \(C_{132}H_{168}N_8O_{24}\) [M+H]\textsuperscript{+}: 2,251.2373) and \(m/z\) 4,494.7009 (\(m/z\) calc for \(C_{264}H_{343}N_{24}O_{40}\) [M+H]\textsuperscript{+}: 4,494.6128), respectively, which confirmed the expected structures. The structures of V4N2 and V8N2 were also validated by IR (see Supporting information for reference).

In addition to the calix[4]arene-based immunoconjugates V4N2 and V8N2, we also prepared the immunconjugate hapten KLH-GNE, which presents a high hydrolitic stability profile and is recog-
sized from \(p\)-tert-butyphenol (1) in 31% and 22% yields, respectively (Scheme 1).

Next, our efforts were directed to obtain the hapten GNE. Initially, we synthesized benzoylcegonine (13; Scheme 2 - panels A and B), the corresponding carboxylic acid of cocaine, and amine 11 (Scheme 2 - panel A). Amine 11 was prepared in 98% yield by the protection of the commercially available 6-aminohexanoic acid (10) with benzyl alcohol [74], while the benzoylcegonine (13) was furnished in its salt form (Scheme 2 - panel B) in 84% yield from the chemoselectivity hydrolysis of the methyl ester of cocaine (12) [75] (Scheme 2 - panel A). Subsequently, the carboxylic acid group of 13 was coupled with amine 11, in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), furnishing amide 14 in 71% yield [44] (Scheme 2 - panel A). The benzyl ester group of 14 was then reductively cleaved by treatment with \(H_2\) and Pd/C to provide the hapten GNE in 52% overall yield (3 steps) from cocaine (12) [51] (Scheme 2 - panel A).

The conjugation of GNE to calix[n]arenes 8 and 9 using PyBOP\textsuperscript{10} as the coupling agent afforded the desired calix[n]arene-based immunoconjugates V4N2 and V8N2 (59% and 60% yields, respectively) (Scheme 3). The mass spectra of compounds V4N2 and V8N2 showed the [M+H]\textsuperscript{+} ion \(m/z\) 2,251.2278 (\(m/z\) calc for \(C_{132}H_{168}N_8O_{24}\) [M+H]\textsuperscript{+}: 2,251.2373) and \(m/z\) 4,494.7009 (\(m/z\) calc for \(C_{264}H_{343}N_{24}O_{40}\) [M+H]\textsuperscript{+}: 4,494.6128), respectively, one- and two-dimensional NMR spectra confirmed the expected structures. The structures of V4N2 and V8N2 were also validated by IR (see Supporting information for reference).

In addition to the calix[n]arene-based immunoconjugates V4N2 and V8N2, we also prepared the immunconjugate hapten KLH-GNE, which presents a high hydrolitic stability profile and is recog-
nized to display a potent eliciting titers of anti-cocaine antibodies with high affinity and specificity for cocaine [51]. Immunoconjugate hapten KLH-GNE was prepared following the methodology described by Cai et al. (2013) [51] and, herein, used as the positive control in our preclinical studies.

The efficacy of calix[n]arene-based immunoconjugates V4N2 and V8N2 at three concentrations (30 nM; 3 μM and 300 μM) was evaluated by ELISA to assess anti-cocaine antibody titers. The effect of both immunogens was also evaluated on the in vivo biodistribution by radiochemical studies using [99mTc]TRODAT-1, a radiolabeled analogue of cocaine. [99mTc]TRODAT-1 was employed because, similarly to cocaine, it also acts on presynaptic dopaminergic transporters and is able to cross the blood–brain barrier, maintaining a pharmacokinetic profile similar to the one expected for cocaine [76].

Compound V4N2 administered on days 0, 7 and 21 at the dose of 30 nM induced the production of IgG-type anti-cocaine antibodies along the whole experimental period (Fig. 1A). On the other hand, the effect produced by the doses of 3 μM or 300 μM was observed only on day 7 (Fig. 1B). Moreover, the two highest doses (3 μM and 300 μM) did not display a dose–response effect. This apparent lack of dose–response can partially be explained by the high viscosity of the formulation, which could induce a slow molecule dispersion and activation of the immune response until the day 42. Thus, in order to evaluate the distribution of [99mTc]TRODAT-1 (Fig. 4) in animals previously immunized with V4N2,
we adopted the lower dose (30 nM) that exhibited activity around day 42.

Compound V8N2 administered on days 0, 7 and 21 at the doses of 30 nM, 3 μM or 300 μM induced the production of IgG-type anti-cocaine antibodies at different times (Fig. 2A). On day 7, all the doses (30 nM, 3 μM or 300 μM) demonstrated an immune response. However, on day 21, only the lowest dose (30 nM) showed activity significantly higher compared to the vehicle group control. Finally, the highest dose (300 μM) induced the highest levels of IgG antibodies only on day 42 (Fig. 2B). The high viscosity of the formulation of the doses 3 μM and 300 μM may have accounted, at least partially, for the lack of dose–response, possibly inducing a slow molecule dispersion and activation of the immune response until the last day of the evaluation. The data suggested that these doses (3 μM or 300 μM) should be evaluated over a longer period. Since the lower dose (30 nM) did not exhibit a significant difference with the highest dose (300 μM) around day 42, we selected the lower dose (30 nM) to evaluate the distribution of [99mTc]-TRODAT-1 in the bloodstream (Fig. 4) in animals previously immunized with substance V8N2.

In addition, to validate the results observed on the production of IgG-type anti-cocaine antibodies, we also investigated the effect induced by the immunoconjugate KLH-GNE (positive control), used as a reference for the antibody production, during all the times of the experiment. The immune response effect of the KLH-GNE was observed on days 7, 21 and 42 (Fig. 3A). Considering the area under the curve (AUC) along the whole experimental period, KLH-GNE and both compounds V4N2 (30 nM) and V8N2 (30 nM) exhibited immunological activity (Fig. 3B). Brain scintigraphy (Fig. 4A) using [99mTc]TRODAT in animals previously immunized with the vehicle, V4N2 (30 nM) or V8N2 (30 nM) showed higher levels of radioactivity in the vehicle control group compared to the other groups (V4N2 or V8N2). High levels of [99mTc]TRODAT-1 were observed in the blood for the treatments with V4N2 or V8N2, but, at the same time, a reduced concentration of the radiotracer was noticed in the brain. On the other hand, the vehicle group control exhibited higher [99mTc]TRODAT-1 levels in the brain compared to other groups (V4N2 or V8N2; Fig. 4B). The distribution of [99mTc]-TRODAT-1 in different organs also showed that immunization with V4N2 or V8N2 did not influence the presence of this radiotracer in a specific organ. No differences between the groups (V4N2 or V8N2) compared to vehicle group control of each organ were observed; however, for the same group it was observed a high level of [99mTc]-TRODAT-1 in the liver compared with the other organs. The high level in the liver is expected since this organ plays a major role in xenobiotic metabolism and elevated levels of different exogenous compounds are located in this organ (Fig. 4C). Thus, the reduction of [99mTc]TRODAT-1 uptake in the brain, in animals previously immunized with V4N2 or V8N2, can indicate that the antibodies induced by V4N2 or V8N2 were able to bind to [99mTc]TRODAT-1 in the bloodstream (Fig. 4A and B).

Conclusions

In summary, we reported the first total synthesis of the novel calix[n]arene-based immunogens V4N2 and V8N2 by the tethering of hydrolysis-tolerant hapten GNE on calix[4]arene and calix[8]arene moieties. Both calix[n]arene-based immunogens promoted the production of cocaine antibodies and also modulated the biodistribution of [99mTc]TRODAT-1, a radiolabeled analogue of cocaine. These results highlights the immunological potential of V4N2 and/or V8N2 as an additional strategy for the development of an immunogenic agent for the treatment of CUD.

Compliance with ethics requirements

The Local Ethics Committee in Animal Experimentation approved the study protocol (CEUA-UFMG Protocol N°. 122/2016) and at all stages of the work, the recommendations of this commission were adopted.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2021.09.003.

References

[1] Tutton CS, Crayton JW. Current pharmacotherapies for cocaine abuse: a review. J Addict Dis 1993;12(2):109–27.
[2] de Lima MS, Soares BGO, Reisser AAP, Farrell M. Pharmacological treatment of cocaine dependence: a systematic review. Addiction 2002;97(8):931–49.
[3] Karila L, Gorelick D, Weinstein A, Noble F, Benyamina A, Cósca S, et al. New treatments for cocaine dependence: a focused review. Int J Neuropsychoph 2008;11(03). doi: https://doi.org/10.1017/S1461145707008097.
[4] Penberthy J, Ait-Daoud N, Vaughan M, Fanning T. Review of treatment for cocaine dependence. Curr Drug Abuse Rev 2010;3(1):49–62.
[63] da Silva CM, da Silva DL, Magalhães TFF, Alves RB, de Resende-Stoianoff MA, Martins FT, et al. Iminecalix[4]arenes: microwave-assisted synthesis, X-ray crystal structures, and antifungal activity. Arab J Chem 2019;12(8):4365–76.

[64] Fetissov SO. Neuropeptide autoantibodies assay. Methods Mol Biol 2011;789:295–302.

[65] Gutsche CD, Dhawan B, No KH, Muthukrishnan R. A. The synthesis, characterization, and properties of the calixarenes from p-tert-butylphenol. J Am Chem Soc 1981;103(13):3782–92.

[66] Gutsche CD, Iqbal M. p-tert-Butylcalix[4]arene. Org Synth 1990;68:234–6.

[67] de Oliveira MC, Reis FS, de Fátima Â, Magalhães TFF, da Silva DL, Porto RR, et al. Synthesis and anti-paracoccidioides activity of calix[4]arenes. Lett Drug Des Discov 2012;9:30–6.

[68] Kenis PJA, Noordman OFJ, Schönherr H, Kerver EG, Snellink-Ruël BHM, van Hummel GJ, et al. Supramolecular materials: Molecular packing of tetranitrotetrapropoxycalix[4]arene in highly stable films with second-order nonlinear optical properties. Chem Eur J 1998;4(7):1225–34.

[69] Yi J, Tang K, Huang S, Huang K. Synthesis of p-tert-butylcalix[8]arene ether derivatives. Indian J Chem 2008;47B(09):1435–7.

[70] Verboom W, Durie A, Egberink RJM, Asfari Z, Reinheudt DN. Ipso nitration of p-tert-butylcalix[4] arenes. J Org Chem 1992;57(4):1313–6.

[71] Dudic M, Colombo A, Sansone F, Casnati A, Donofrio G, Ungaro R. A general synthesis of water soluble upper rim calix[n]arene guanidinium derivatives which bind to plasmid DNA. Tetrahedron 2004;60(50):11613–8.

[72] Sansone F, Dudic M, Donofrio G, Rivetti C, Baldini L, Casnati A, et al. DNA Condensation and cell transfection properties of guanidinium calixarenes: dependence on macrocycle lipophilicity, size, and conformation. J Am Chem Soc 2006;128(45):14528–36.

[73] Podoprygorina G, Zhang J, Bruko V, Bolte M, Janshoff A, Böhmer V. Supramolecular structures formed by calix[8]arene derivatives. Org Lett 2003;5(26):5071–4.

[74] Machida S, Kato N, Harada K, Ohkanda J. Bivalent inhibitors for disrupting protein surface-substrate interactions and for dual inhibition of protein prenyltransferases. J Am Chem Soc 2011;133(4):958–63.

[75] Findlay SP. The three-dimensional structure of the cocaines. Part I. Cocaine and pseudococaine. J Am Chem Soc 1954;76(11):2855–62.

[76] Mozley PD, Stubbs JB, Pössl K, Dresel SH, Barracough ED, Alavi A, et al. Biodistribution and dosimetry of TRODAT-1: a technetium-99m tropane for imaging dopamine transporters. J Nucl Med 1996;39(12):2069–76.