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Labelling, molecular modelling and biological evaluation of vardenafil: a potential agent for diagnostic evaluation of erectile dysfunction

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ABSTRACT

^{99m}Tc–tricarbonyl–vardenafil was specifically radiosynthesized for diagnostic evaluation of erectile dysfunction with a radiochemical yield ~97.2%. It was stable in saline up to 15 h and in serum for more than 6 h. The radiocomplex was lipophilic with a partition coefficient ~1.32 and plasma protein binding 72–76%. Its structure was determined using molecular mechanics and confirmed by NMR. *In-silico* docking to its target PDE_5 enzyme was performed. The radiocomplex inhibitory activity was assessed and its IC_{50} was 0.7 nM. Biodistribution in normal rats and biological evaluation in rat models of erectile dysfunction were performed. The results strongly suggested that ^{99m}Tc–tricarbonyl–vardenafil is a good candidate to image erectile dysfunction in humans.

1. Introduction

Erectile dysfunction (ED) is the persistent inability to initiate or maintain penile erection adequate for satisfactory sexual performance (Andersson, 2011). The overall prevalence of ED among men is estimated to be approximately 5–20% worldwide (Kubin et al., 2003). The incidence of the disease is increasing and there are predicted 322 million cases by 2025 (Gajbhiye et al., 2015). ED can have profound impact on the quality of life and may lead to depression and loss of self-confidence.

Erection is a complex multifactorial process that depends on the integrity of hormonal, neurological, and vascular components (Lasker et al., 2010). Following sexual stimulation, anti-erectile neural input of the sympathetic efferents, limiting the blood flow to the penis, are withdrawn. At the same time, parasympathetic neural signals are sent leading to up-regulation of cholinergic and non-adrenergic activity and release of nitric oxide (NO) from the cavernous nerve terminals and vascular endothelium (Andersson, 2011, 2001). NO binds and activates guanylate cyclase enzyme leading to the production of cyclic guanosine monophosphate (cGMP). cGMP-dependent protein kinase activity triggers potassium efflux, calcium sequestration in the endoplasmic reticulum and blockage of calcium membrane channels leading to smooth muscle cell relaxation (Craven et al., 2004). This relaxation allows for increased blood flow and the copora become engorged compressing the emissary veins within the tunica albuginea limiting venous outflow and causing erection.

The raise in cGMP level stimulates its degradation. Produced cGMP, in the corpus cavernosum, binds to a specific allosteric site of the phosphodiesterase enzyme type 5 (PDE_5) altering its conformation and exposing a phosphorylation site. Phosphorylation of PDE_5 by protein kinase G (PKG) lead to its activation. The catalytic site of the activated PDE_5 breaks down the cGMP to GTP terminating the actions of cGMP and causing de-tumescence (Corbin, 2004). High affinity long-time binding inhibitors of PDE_5 compete with the cGMP arresting its breakdown. However, in pathologic conditions involving impaired NO synthesis, these inhibitors are ineffective. Clinical trials showed that 40% of diabetic patients and 50% of patients with post-prostatectomy have ED that is refractory to PDE_5 inhibitor treatment (Aversa, 2010; Morales et al., 1998).

Recently, soluble guanylyl cyclase (sGC) stimulators/activators, Rho-kinase inhibitors and novel NO donors have emerged as potential therapeutic for patients with impaired NO release and/or synthesis (Lasker et al., 2010). Accordingly, there is a need for a specific diagnostic method, which would evaluate ED and also would be capable of accurately monitoring the efficacy of a personalised treatment regimen.

2. Experimental

2.1. Materials

Vardenafil, (VRDN), 4-[2-Ethoxy-5-(4-ethylpiperazin-1-yl) sulfonyl-phenyl]-9-methyl-7-propyl-3,5,6,8-tetrazabicyclo [4.3.0] nona-3,7,9-trien-2-one was purchased from Aldrich, Germany. Unless

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otherwise mentioned, all other chemicals were of highest purity grade and purchased from Sigma Chemical Company, USA. Promega PDE-Glo™ Phosphodiesterase Assay Kit was used for the enzyme inhibition assessment. Male Wister rats were used for the quantitative biodistribution studies.

3. Methods

3.1. Preparation of $^{99m}Tc(CO)_3$-VRDN complex

The complex preparation followed a two steps approach where the precursor ^{99m}Tc-tricarbonyl was first synthesised. Technetium pertechnetate (1.5 mL, 370 MBq), from a commercial generator, was added to a closed 10 mL penicillin vial containing sodium carbonate (7.15 mg), sodium boranocarbonate (4.5 mg), sodium tetraborate (2.85 mg) and sodium tartarate (8.5 mg). The vial was purged with N_2 and heated in a boiling water bath for 30 min then the pH was adjusted to 6 using phosphate buffer. Thereafter, the radiolabelling of vardenafil (*VRDN)* with technetium-99m was performed using the $[^{99m}Tc(CO)_3(H2O)_3]^+$ precursor. 1 mL of the prepared precursor was added to 0.5 mL of *VRDN* solution (4 mg/mL in saline), at room temperature. Then, the reaction mixture was heated for 30 min in a boiling water bath. The radiochemical yield of the precursor was determined by instant thin layer chromatography (ITLC) method and high performance liquid chromatography (HPLC). While the radiochemical yield and the stability of ^{99m}Tc-tricarbonyl-VRDN were assessed using HPLC only.

3.2. ITLC analysis

Silica gel impregnated ITLC strips (German Laboratories) were used for this study. Two developing system were used to determine the free pertechnetate, the colloids and the labelled precursor. Free $^{99m}TcO_4^-$ was determined using saline as developing solvent (R_f =0.8–1). Reduced hydrolysed technetium was determined using a mixture of ethanol: water: ammonium hydroxide (2: 5: 1) as developing solvent (R_f =0). The labelled precursor was calculated using to the following equation:

$$\%\ labelled\ precursor = 100 - (\%\ colloid + \%\ free\ pertechnetate) \quad (1)$$

3.3. Radio-HPLC characterisation

Reversed-phase HPLC analysis was performed. A Shimadzu HPLC unit interface with UV detector (operated at 230 nm), radioisotope detector and C-18 (4.6×150 mm) column was used. For tricarbonyl precursor assessment, the mobile phase consisted of solvent A (methanol) and solvent B (triethylammoniumphosphate buffer, 0.05 M, pH 2.25). A linear gradient of 100% A –0% B to 0% A –100% B for 15 min was applied. The flow rate was 1.5 mL/min and a 10 µL aliquot was injected into the column. For the characterisation of the labelled VDRN, the column was eluted at 1 mL/min with a mobile phase of acetonitrile-potassium dihydrogen phosphate (30:70 v/v). The run time was 20 min and ^{99m}Tc Tricarbonyl precursor would appear at retention time 2.4 min

3.4. Structure prediction, NMR analysis and docking to phosphodiesterase type 5

The energy optimised 3D structure of ^{99m}Tc-tricarbonyl-*VRDN* was predicted *via* molecular mechanics using ChemBioOffice Ultra V14 software (El-Kawy et al., 2015). Bruker High Performance Digital FT NMR Spectrometer Avance III 400 MHz (Bruker, UK) was used to determine the ^{1}HNMR of the complex rhenium analogue. Thereafter, NMR data analysis and structure correlation was performed using ACD/^{1}D NMR Processor V12 software (El-Kawy and Talaat, 2016). The affinity and binding energy of the complex to its target phosphodiesterase type 5 (PDE_5) enzyme was assessed using iGemDock 2.1 software (El-kawy et al., 2016). The structural format of the enzyme (PDB ID: 1XP0) was obtained from the protein data bank of the Research Collaboratory for Structural Bioinformatics (RCSB) (Card et al., 2004). The complex was subjected to very accurate docking. Then, post docking analysis was performed to evaluate the best docking pose and its energy values.

3.5. Physicochemical evaluation of the ^{99m}Tc-tricarbonyl-VRDN

3.5.1. Radiochemical stability in saline

In vitro radiochemical stability of the ^{99m}Tc-tricarbonyl-VDRN complex was assessed using the HPLC method previously mentioned. The labelled complex (1 mL) was incubated in saline (1 mL) for 20 h, at 25 °C. Samples (10 µL each) of the incubated mixture were withdrawn and tested for stability. Experiments were conducted in 3 replicates.

3.5.2. In serum stability

The complex (0.2 mL) was incubated with serum (1.8 mL) for 16 h at 37 °C. At different time intervals, samples in triplicate (10 µL each) were removed from the incubated mixture and tested for stability using HPLC as mentioned earlier. Then, the percent stability was calculated.

3.5.3. Plasma protein binding

In vitro precipitation method in heparinized blood was used to study the complex binding with plasma protein as reported (De et al., 2010). Briefly, blood cells were separated from plasma by centrifugation (5000*g*, 10 min) and the pellet was removed. An appropriately equal volume of trichloroacetic acid (TCA, 10%) was added to the plasma, followed by centrifugation (5000*g*, 10 min) and decantation of the supernatant. Thereafter, pellet was re-suspended in TCA (1 mL, 10%), centrifuged and the supernatant decanted. Using a γ-counter, radioactivity in the supernatant and pellet was determined. This count was expressed as percentage of count obtained with the same volume of unprocessed plasma.

3.6. Experiments were performed in triplicate

3.6.1. Lipophilicity

The lipophilicity of ^{99m}Tc-tricarbonyl-VRDN was studied by determining the apparent partition coefficient (P) between n-octanol and sodium phosphate buffer using previously reported procedure (El-Kawy and Farah, 2015). Briefly, in a centrifuge tube, the complex (0.2 mL), phosphate buffer (1.8 mL, 0.1 M, pH 7.4) and *n*-octanol (2 mL) were added. The mixture was then vortexed for 2 min followed by centrifugation (2800*g*, 5 min). Aliquots (0.1 mL) were withdrawn from each phase and activity was measured in a γ- counter. Experiments were performed in triplicate. Partition coefficient (P) was calculated as the mean value of each cpm/mL of *n*-octanol layer divided by that of the buffer. Subtraction was made for the background. Lipophilicity was expressed as log P.

3.7. Assessment of the labelled compound PDE enzyme inhibitory activity

The commercially available PDE-Glo™ Phosphodiesterase Assay Kit from Promega was used for enzyme inhibition studies (Staeben et al., 2010). Different amounts of labelled VRDN were mixed with the enzyme PDE 5 (30 units) and pre-incubated at room temperature for 5 min cGMP substrate (10 µM) was added and the reactions were incubated for 90 min at 25 °C. Luminescence was measured using the PHERAstar high-end plate reader (BMG Labtech, Germany). Data analysis was performed with GraphPad Prism software using a sigmoidal dose-response equation. Experiments were made in four replicates. IC_{50} value for PDE_5 inhibition was determined from the