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A novel ^{99m}Tc-labeled testosterone derivative as a potential agent for targeting androgen receptors

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Abstract—With an insight that ligands possessing a N_2S_2 tetradentate array of donor atoms serve as ideal bifunctional chelating agents (BFCA) in the radiolabeling of target-specific agents, 5-hydroxy-3,7-diazanonan-1,9-dithiol (DAHPES) with a derivatizable substituent in the form of a hydroxyl group in the backbone was synthesized. The preparation of a steroid conjugate via coupling of this BFCA with testosterone-3-(*O*-carboxymethyl) oxime and the subsequent radiolabeling of the conjugate under optimized conditions with ^{99m}Tc, the ideal diagnostic radionuclide in nuclear medicine procedures, are reported. The immunoreactivity of the radiolabeled conjugate was demonstrated in a study using anti-testosterone antibodies, wherein the radiolabeled conjugate exhibited significant binding with antiserum to testosterone. Cell-uptake studies in DU145 prostate carcinoma cell line bearing androgen receptors (ARs) and comparison with AR non-bearing breast carcinoma cell line revealed the specific binding of the steroidal moiety with the testosterone receptor.

Several methods of radiolabeling steroidal substrates with radioisotopes of choice for targeting specific receptors over-expressed in target tissues in diseased states have been reported.[1–3] Testosterone is a steroidal hormone which binds to androgenic receptors which are over-expressed in cancerous regions of the prostate.[1] Agents to image the prostate or tumors therein, based on the androgen content, serve as a useful tool in staging of the disease and monitoring the course of therapy.[4] Various derivatives of androgens have been labeled with several γ-emitting radionuclides, such as, ^{77}Br, ^{82}Br, ^{125}I, ^{75}Se[5–8] as well as with the positron emitter ^{18}F,[4,9] in search of an ideal androgen receptor-based prostatic imaging agent. However, rapid metabolic cleavage, low receptor binding affinity, and inadequate specific activity are the major impediments toward use of these radiolabeled conjugates. The aforementioned inadequacies of currently available diagnostic methods for in vivo imaging of prostatic carcinoma have prompted us to envisage a strategy whereby testosterone could be radiolabeled with ^{99m}Tc in designing a potential agent for non-invasive imaging of androgen receptors. The desirable features of ^{99m}Tc as an isotope of choice in diagnostic nuclear medicine emerge from its manifold advantages attributable to its suitable nuclear decay characteristics (half-life 6 h, decay by isomeric transition with the emission of 140 keV gamma photon suitable for imaging procedures), easy availability from a ^{99}Mo–^{99m}Tc generator, and amenable chemistry. The present work reports an attempt to prepare a ^{99m}Tc-labeled testosterone conjugate for targeting androgenic receptors.

In our search for novel bifunctional chelating agents, a tetradentate ligand possessing an array of donor atoms identical with those in ethylene dicysteine (ECD) or diaminodithiols (DADT) was envisaged.[10–14] 5-Hydroxy-3,7-diazanonan-1,9-dithiol (DAHPES) with a N_2S_2 donor atom set and a derivatizable substituent in the form of a hydroxyl group in the backbone was synthesized by applying a single-step synthetic strategy (Fig. 1).[15–18] Successful radiolabeling of DAHPES with ^{99m}Tc at room temperature with high yield and a desirable biodistribution pattern of the labeled ligand with no major uptake in any organ and fast clearance via the renal route[16] provided impetus for its use as a BFCA

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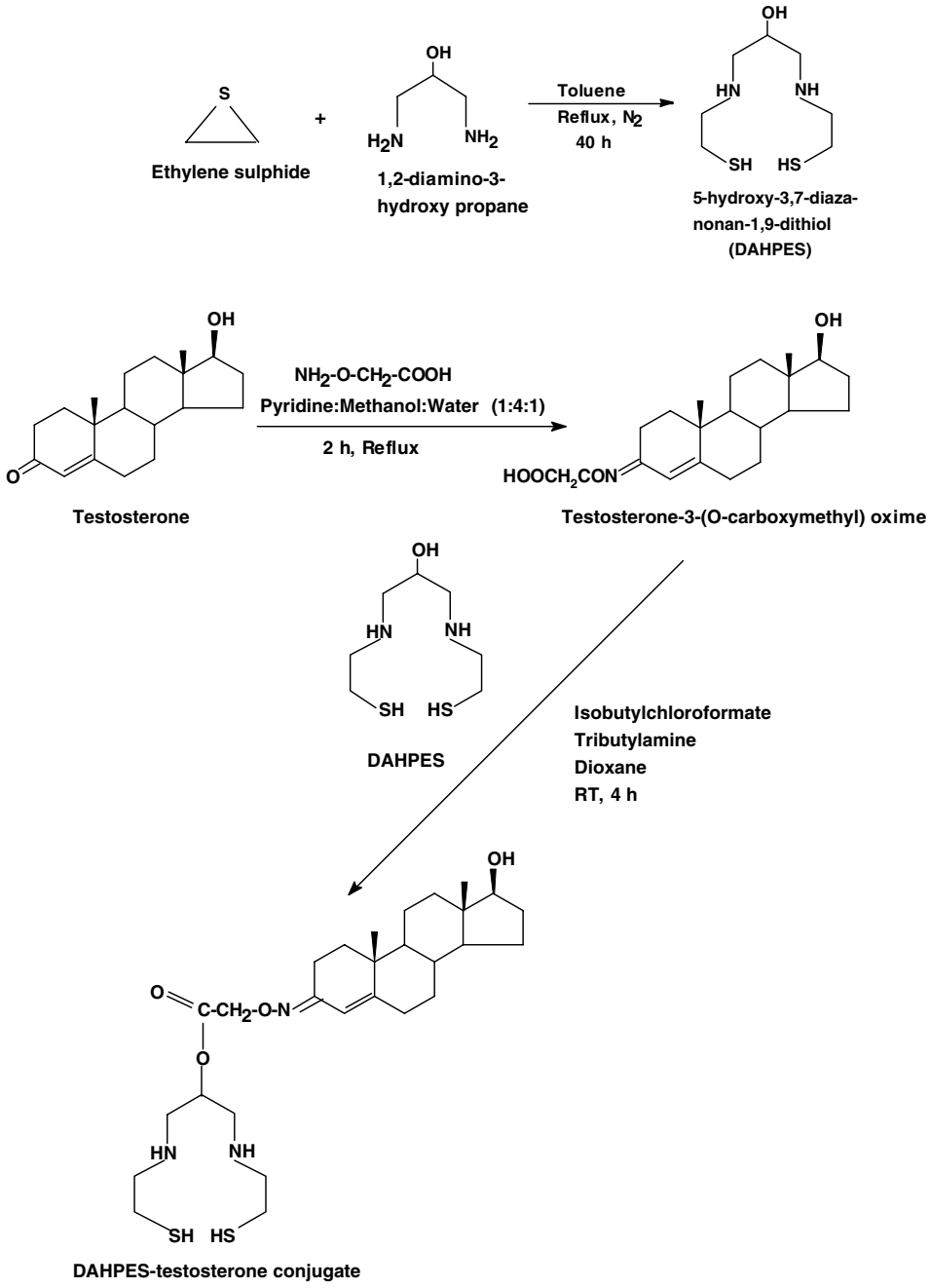

Figure 1. Synthesis of DAHPES–testosterone conjugate.

for the present study. It is well reported that testosterone when functionalized at the C_3 position retains its immunoreactivity, and hence a strategy toward preparation of a radiolabeled agent for androgen receptor targeting was envisaged using a C_3 functionalized testosterone.[19] In the present paper, we report the synthesis, purification, and ^{99m}Tc labeling of a testosterone–BFCA conjugate, and the preliminary biological evaluation of the developed radiolabeled conjugate in suitable in vitro systems.

The BFCA–testosterone conjugate was prepared via a two-step reaction (Fig. 1) involving the testosterone-3-(*O*-carboxymethyl)-oxime derivative as the precursor, which in turn was synthesized from testosterone.[20] The coupling of the testosterone derivative with the BFCA involved the activation of testosterone-3-(*O*-carboxymethyl)-oxime using isobutyl chloroformate and tributyl amine. The mixed anhydride thus obtained was allowed to react with the DAHPES in dry dioxane.[21] The product was characterized by ^{1}H NMR and mass spectra.[22] The peak integrations and multiplicities in the ^{1}H NMR spectrum observed were as expected and provided evidence in favor of the formation of the desired conjugate. The desired derivatization is indicated by the appearance of an 8-H multiplet between 3.20 and 3.36 corresponding to the C*H* protons of the DAHPES moiety. A shift in the δ values of the C*H*(O) proton from

3.50–3.52 to 3.72–3.78 at the site of derivatization provided confirmatory evidence toward formation of the steroid–BFCA conjugate. The appearance of two-proton singlet at 4.43 and 4.46 corresponding to the $=NOCH_2COO-$ proton in the ^1H NMR spectrum of the conjugate is indicative of the formation of geometrical isomers (60:40). The observation of the molecular ion peak at *m/z* 553 in the mass spectrum conclusively showed the formation of the DAHPES–testosterone conjugate.

For the labeling of DAHPES–testosterone conjugate, 0.2 mL of freshly eluted $^{99m}TcO_4^-$ (500 μCi) from a ^{99}Mo–^{99m}Tc generator was added to 1 mg (1.8 mM) of the conjugate in 0.4 mL phosphate buffer (0.05 M, pH 12) and 0.2 mL of normal saline. 0.2 mL of $SnCl_2 \cdot 2H_2O$ (0.2 mg) solution in dilute HCl was added to the reaction mixture. The mixture was purged with nitrogen for 2 min and then incubated for 30 min to 1 h at room temperature.

The extent of ^{99m}Tc labeling achieved was determined by paper chromatography (PC), thin-layer chromatography (TLC), and paper electrophoresis techniques following standard procedures reported elsewhere.[23,24] In PC, using saline as the solvent, it was observed that a considerable portion of the activity remained at the point of spotting ($R_f = 0$), which could be attributed to the presence of either the radiolabeled conjugate or reduced technetium. The presence of minor amount of activity at the solvent front indicated the presence of either pertechnetate or ^{99m}Tc-labeled DAHPES (as radiochemical impurity), both of which appeared at $R_f = 0.8$–1 under identical conditions. However, on TLC/saline, the major activity moved toward the solvent front, thereby differentiating the radiolabeled conjugate from the reduced technetium. Therefore, a combination of these two chromatographic methods enabled the preliminary determination of the extent of complexation which was further verified by paper electrophoresis studies. This was carried out using 0.025 M phosphate buffer of pH 7.5 at a potential gradient of 10 V/cm for a period of 75 min. It was observed that, while $^{99m}TcO_4^-$ moved 10 cm toward the anode, ^{99m}Tc-labeled conjugate remained at the point of spotting under identical conditions.

In optimization studies of the complexation parameters for achieving maximum yield, it was observed that 1 mg of the radiolabeled steroidal–BFCA conjugate at pH 12 yielded ~60% complexation when incubated at room temperature for 30 min to 1 h. Further increase in the ligand concentration did not show any appreciable effect on the complexation yield. Stannous chloride was used as the reducing agent and 0.2 mg of this compound was required to obtain maximum complexation. PC and TLC studies revealed that a maximum of 60% complexation was achieved when the complex was prepared under optimized conditions.

Radiochemical purification of the ^{99m}Tc-labeled DAHPES–testosterone conjugate was achieved by HPLC. This technique also provided an additional support in favor of the extent of complexation determined by using the combination of PC and TLC systems described above. A dual pump HPLC unit with a C_{18} reversed-phase column (25 cm × 0.46 cm) was used for the purification of the radiolabeled conjugate. The elution was monitored both by UV absorbance at 240 nm as well as by radioactivity signals. The flow rate was maintained at 1 mL/min. Acetonitrile (A) and water (B) mixtures containing 0.1% trifluoro acetic acid were used as the mobile phase and the following gradient elution technique was adopted for the separation (0–3 min 10% A to 90% A, 3–10 min 90% A to 10% A, and 10–20 min 10% A to 90% A). Typical HPLC patterns representing the radioactive chromatogram and UV profile are depicted in Figures 2a and b, respectively. It is evident from the figure that while ^{99m}Tc–DAHPES–testosterone complex exhibited a retention time of ~210 s, $^{99m}TcO_4^-$ eluted out from the column at ~280 s. ^{99m}Tc–DAHPES–testosterone complex thus purified was subsequently used for all biological evaluations.

Stability studies carried out using quality control techniques mentioned above showed that the radiolabeled conjugate retained its radiochemical purity when stored at room temperature up to 4 h after its preparation.

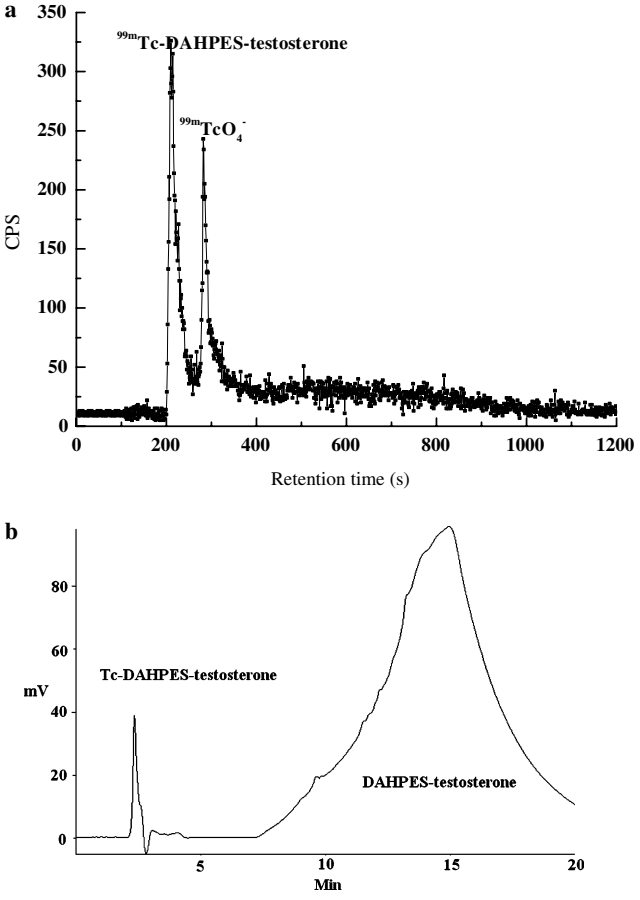

Figure 2. (a) HPLC pattern (radioactive chromatogram) of ^{99m}Tc-labeled DAHPES–testosterone conjugate. (b) HPLC pattern (UV profile) of ^{99m}Tc-labeled DAHPES–testosterone conjugate.

Biological activity of the resultant ^{99m}Tc–DAHPES–testosterone complex was studied by carrying out preliminary in vitro cell-uptake studies with DU145, a human prostate carcinoma cell line known to express testosterone receptors, as well as by binding studies with anti-testosterone antibodies.

Antibody binding studies[25] were carried out to preliminarily ascertain the retention of immunological activity which could provide a clue toward structural integrity of the steroid after introduction of the BFCA. It was observed that the radiolabeled conjugate exhibited a binding of ~50% with 1:5 diluted antiserum, while binding was ~43% with a 1:10 diluted antiserum. The decrease in binding with increased dilution of the testosterone-specific antiserum indicated that the radiolabeled conjugate retained its binding capacity to the antiserum. The binding was comparable to the observation made in earlier studies carried out in our laboratory wherein 100 pg of a radioiodinated (1000 μCi/μg) testosterone derivative showed a binding of 35–45% with a 1:150,000 diluted antiserum.[26] It was also observed in the present studies with ^{99m}Tc-labeled DAHPES–testosterone conjugate that, as the amount of conjugate was increased from 3 to 6 μg, the tracer showed a decrease in binding from ~50% to ~40%. Blank studies where the antiserum was incubated with either only TcO_4^- or with ^{99m}Tc–DAHPES gave a binding of 2–5%. These results indicated that the binding of testosterone toward the specific antiserum was retained after radiolabeling.

Prostate cancer is one of the most common forms of malignant growth in human males. Prostatic carcinoma cells have been reported to bear androgen receptors not only intracellularly, but also on the membrane surface. The latter are hypothesized to be responsible for rapid-acting non-genomic effects.[27] The DU145 prostatic carcinoma cell line is androgen-independent, but possesses androgen receptors[28] and therefore could serve as a target for radiolabeled steroidal substrates possessing affinity for androgenic receptors. Blank experiments were also performed by incubating the DU145 cells with $^{99m}TcO_4^-$ and a negative control was set up by incubating the radiolabeled BFCA–testosterone conjugate with an androgen receptor negative cell line, namely, MDA-MB-468, a human breast carcinoma cell line, keeping other experimental conditions identical.[29] About ~24–31% cell uptake was observed when 0.5–1 μg of the ^{99m}Tc-labeled DAHPES–testosterone conjugate was incubated with 4×10^4 cells. When the amount of tracer was increased 10-fold (10 μg), the cell uptake decreased from an average of 31% to 22%, whereas with a 100-fold (100 μg) increase in the amount of tracer, the uptake decreased from 31% to <5%. Blank experiments carried out using TcO_4^- in place of the radiolabeled conjugate and negative controls with MDA-MB-468 breast carcinoma cell line in place of DU145 showed <5% cell uptake. These results appear to indicate that the radiolabeled conjugate has specific affinity toward testosterone receptors on the prostate cancer cell line.

The present study describes the preparation of a novel BFCA–testosterone conjugate and its successful radiolabeling with ^{99m}Tc. Retention of immunological activity and thereby a gross indication of structural integrity of testosterone in the radiolabeled conjugate was demonstrated in serum antibody binding studies. While in vitro cell binding studies showed significant uptake in the human prostatic carcinoma cell line, negligible uptake observed in the androgen receptor negative breast carcinoma cell line is indicative of the androgen receptor specificity of the developed agent. These studies showed that the radiolabeled agent ^{99m}Tc–DAHPES–testosterone possesses considerable promise toward use as a potential targeting agent for androgen receptors.

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17. *Synthesis of 5-hydroxy-3,7-diazanonan-1,9-dithiol (DAHPES)*. A mixture of ethylene sulfide (5.87 g, 97 mM) in 20 mL of dry toluene was added to a stirred solution of 1,3-diamino-2-hydroxypropane (4 g, 44 mM) in 30 mL dry toluene under reflux and with nitrogen flushing over a

period of 3 h. The refluxing was continued for 40 h after which toluene was removed under vacuum distillation, whereby a white residue was obtained. The residue was extracted several times with a mixture of methanol and dichloromethane (1: 1 v/v, 5× 10 mL). The pooled organic extracts were concentrated to yield a viscous liquid, which subsequently solidified to a pale white solid (5.2 g, yield 57%) on storage. The crude product thus obtained was purified by column chromatography over silica gel using 10% ammonium hydroxide in methanol as the eluant.

18. *Characterization of 5-hydroxy-3,7-diazanonan-1,9-dithiol (DAHPES)*. FT-IR (KBr, ν cm^{-1}): 3364 (–NH), 2960 (–SH). ^1H NMR, CD_3OD (δ ppm): 2.55–2.58 (2H, m) CH_AH_BNH, 2.64–2.71 (2H, dd) $NHCH_AH_B(CHOH)$, 2.67–2.69 (2H, m) CH_AH_BNH, 2.79–2.93 (6H, m) $–NHCH_AH_B(CHOH)$ and $–NHCH_2CH_2SH$, 3.16 (4H, t, J = 5.4 Hz) $–CH_2SH$, 3.50–3.52 (1H, m) –CHOH. Mass spectra (EI) *m/z*: 210 (M^+).
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21. *Coupling of DAHPES with testosterone-3-(O-carboxymethyl)-oxime*. In a typical reaction, 20 mg of testosterone-3-(*O*-carboxymethyl)-oxime (0.055 mM) in 1 mL of dry dioxane was incubated at 4 °C for 30 min with 10 μL of isobutyl chloroformate and 20 μL of tributyl amine. A solution of 12 mg DAHPES (0.057 mM) in 2 mL dry dioxane was added to the mixture and stirred at room temperature for 4 h at pH 8. The desired coupled product (DAHPES–testosterone conjugate) thus obtained was purified by preparative thin-layer chromatography (TLC) on pre-coated silica gel plates using 15% methanol in ethyl acetate as the eluting solvent.
22. *Spectral data for DAHPES–testosterone conjugate*. ^1H NMR, CD_3OD (δ ppm): 0.78 (3H, s) $C_{18}–H_3$, 1.11 (3H, s) $–C_{19}–H_3$, 2.22–2.38 (4H, m) $–CH_AH_BNH$, 3.0–3.1 (2H, m) $C_6–H_AH_B$ of testosterone, 3.20–3.25 and 3.34–3.36 (8H, m) $C_1–H$ $C_2–H$ $C_8–H$ $C_9–H$ of DAHPES, 3.57 (1H, t, J = 9 Hz) $C_{17}–H$, 3.72–3.78 (1H, m), DAHPES–CH(O)-$COCH_2–$, 4.43 and 4.46 (2H, s) $=N–O–CH_2COO$ (geometrical isomers), 5.71 (1H, s) $C_4–H$ of testosterone. Mass spectra (EI) *m/z*: 553 (M^+).
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25. *Protocol for antibody binding studies*. Antiserum was raised in rabbit against testosterone-3-(*O*-carboxymethyl)-oxime–bovine serum albumin (BSA) conjugate in our laboratory. When the specific binding of the antiserum with testosterone was taken as 100%, <10% cross-reactivity with dihydrotestosterone and <1% cross-reactivity with all other structurally related compounds were observed. 0.1 mL of the purified diluted radiolabeled conjugate (~3 μg) was incubated with diluted antiserum in 0.1 M Tris buffer of pH 8.5 containing 0.1% gelatin. 0.1 mL of 3% γ-globulin was added to facilitate precipitation. At the end of the 1 h incubation at room temperature, 1 mL of 22% polyethylene glycol in normal saline was added to precipitate the ^{99m}Tc-labeled DAHPES–testosterone conjugate bound to antibody. The precipitate, which carried the radioactivity bound to antibody, was separated by centrifugation and the radioactivity in the precipitate was measured. Blank studies were also performed simultaneously with $^{99m}TcO_4^-$ as well as with the ^{99m}Tc–DAHPES complex in place of the radiolabeled conjugate.
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