A FACILE AND IMPROVED SYNTHESIS OF 17α-{2-(E)-[^{125}I]-IODOVINYL}-19-NORTESTOSTERONE, A NO-CARRIER-ADDED LIGAND FOR PROGESTERONE RECEPTOR ANALYSES*

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SUMMARY

Strategies for human breast cancer therapy using ^{125}I-labeled steroid hormones are clinically attractive in light of the estrogen dependence of and progestogen receptor involvement in many cancers and the favorable microdosimetry resulting from ^{125}I decay. We have synthesized the no-carrier-added progesterone receptor ligand 17α-{2-(E)-[^{125}I]-iodovinyl}-19-nortestosterone (E-^{125}IVNNT) by a simple and high yielding method, and determined its uptake and specific progesterone receptor binding *in vitro* using T47D human breast carcinoma cells. The ligand was prepared by [^{125}I]-iododestannylation of 17α-[2-(E)-tri-n-butylstannylvinyl]-19-nortestosterone (E-TBSVNNT) by using the rare iodinating agent [^{125}I]-sodium iodide/ferric sulfate in mixed dichloromethane-water solvent. Cell binding assays demonstrated that E-^{125}IVNNT binding to T47D breast carcinoma was specific and saturable with an affinity for the progesterone receptor 10-fold greater than that of ^{3}H-R5020.

Key words: 17α-{2-(E)-[^{125}I]-iodovinyl}-19-nortestosterone, iododestannylation, [^{125}I]-sodium iodide/ferric sulfate, progesterone receptor ligand.

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INTRODUCTION

Strategies using ^{125}I-labeled steroid hormones and their analogues as tumor seeking carriers for systemic radiotherapy are particularly attractive in light of the estrogen dependence of and progestogen involvement in many human breast cancers [1]. Like the estrogen receptor (ER), the progesterone receptor (PgR) should be an

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excellent mechanism for selectively concentrating ^{125}I-labeled progestins within nuclei of target cells [2-4]. Progestins have a more limited biodistribution than estrogens and, hence, less non-specific radiotoxicity. Also, the combination of radiolabeled progestins and estrogens may be useful in ER/PgR+ tumors where PgR>>ER. ^{125}I is one of the more promising internally emitting radionuclides because of its high yield of low energy Auger electrons and its virtual lack of associated high energy β- and γ-radiations [3]. The exquisite radiotoxicity of ^{125}I in association with DNA is well-known [5].

We previously reported that 17α-{2-(E)-[^{125}I]-iodovinyl}-11β-methoxyestradiol is cytotoxic to exponentially growing MCF-7 cells and produces a survival curve typical of that observed for [^{125}I]-iododeoxyuridine and 16α-iodoestradiol [6]. Estrogen derivatives with alkyl substituents at the 17α position have increased target organ activity and retention because steric hindrance decreases catabolism [7]. Furthermore, the 17α-iodovinyl derivatives of estradiol appear to be more stable than the corresponding 17α-acetylenic derivatives [8,9]. This rationale prompted us to synthesize 17α-{2-(E)-[^{125}I]-iodovinyl}-19-nortestosterone and study its binding to PgR in human breast cancer cells.

E-^{125}IVNNT was first synthesized in its carrier-added form by Hochberg et al. [10] and studied as an alternative to the commercially available PgR ligand [^{3}H]R5020. Their results using rabbit uterine cytosol indicated that E-^{125}IVNNT has binding capacities equal to those of [^{3}H]R5020 and is a specific and useful probe of the PgR. While their synthesis is satisfactory for preparing the radioactive ligand in 1-2 mCi quantities, the amount of Na^{125}I used is relatively high, and the chemical reactions and HPLC purification methods are cumbersome and labor-intensive for ligand requirements on the order of 5-10 mCi, especially since the radiochemical yield is low (<30%). Other problematic aspects of the synthesis are the large amount of starting material (E-TBSVNNT) used [10], complex and time-consuming chemical procedures such as sodium borohydride treatment of Na^{125}I, preparation of dry Na^{125}I, and the use of a three-component solvent mixture (acetone-dichloromethane-THF) in the actual reaction. Chloramine-T may function poorly in the non-aqueous medium and can oxidize the sensitive steroid substrate [11]. Additionally, purification of the radiolabeled product required two separate HPLC procedures-reverse phase HPLC

using THF-water solvent system and normal phase HPLC using 2-propanol-dichloromethane as the solvent system.

This communication describes a new synthetic pathway for producing carrier-free E-^{125}IVNNT (Fig. 1) by a simple reaction using (a) ferric sulfate as the oxidant of Na^{125}I, (b) much less starting material (E-TBSVNNT), and (c) a simplified purification procedure. We also describe the binding characteristics of the ligand with PgR in T47D human breast cancer cells.

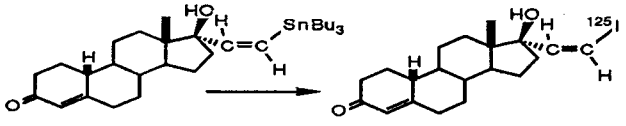

Fig. 1. Synthetic pathway for the synthesis of E-^{125}IVNNT from E-TBSVNNT.

RESULTS AND DISCUSSION

Radiochemical synthesis of E-^{125}IVNNT by [^{125}I]-iododestannylation of the steroid substrate E-TBSVNNT with aqueous Na^{125}I and aqueous ferric sulfate in dichloromethane solution consistently gave 50% radiochemical yield [14]. We used only 100-200 μg of the substrate E-TBSVNNT in conjunction with 10-20 mCi of Na^{125}I against the 437 μg used with 4 mCi of Na^{125}I reported previously [10]. Almost 90% of our starting material was recovered in the first stage of purification by simple column chromatography over silica gel or preparative TLC using ethyl acetate-hexane solvent mixture. Although the E-^{125}IVNNT from this purification was sufficiently pure for binding studies with PgR of T47D breast carcinoma cells, it was again purified by normal phase HPLC using ethyl acetate-hexane solvent system. TLC of this material on a silica gel plate using ethyl acetate-hexane solvent system showed a R_f value of 0.14 for the radioactive band and was free from any E-TBSVNNT which had a R_f value of 0.29.

A clue to the simplification of the [^{125}I]-iododestannylation of E-TBSVNNT was found in the procedure for the preparation of non-radioactive E-IVNNT by the same authors [12]. They obtained a good yield of E-IVNNT by reaction of the steroid substrate E-TBSVNNT with elemental iodine in dichloromethane solvent. We reasoned

that the E-^{125}IVNNT synthesis would be simplified if one could apply a similar procedure using [^{125}I]-iodine in dichloromethane. Since [^{125}I]-iodine solutions are not available, we prepared a solution of [^{125}I]-iodine in dichloromethane from aqueous Na^{125}I and dichloromethane solvent *in situ* at the exact time of reaction. Oxidizing Na^{125}I in aqueous medium with a suitable oxidant should liberate [^{125}I]-iodine which can be extracted into dichloromethane. In order to accomplish this, we wanted to avoid standard oxidants like $KMnO_4$, KIO_4, K_2CrO_4 and milder oxidants like H_2O_2, chloramine-T, and N-chlorosuccinimide, each of which can oxidize the stannyl steroid substrate at one or more reactive centers before and/or after the desired iododestannylation. Ferric sulfate is a mild oxidant and is non-reactive with the steroid substrate. It quantitatively liberates iodine in neutral or acidic solutions of sodium iodide by the reaction:

$$Fe_2(SO_4)_3 + 2NaI\text{------}> 2\ FeSO_4 + Na_2SO_4 + I_2$$

The iodine is almost completely extracted into added dichloromethane. Thus, ferric sulfate seemed to be a good candidate as the oxidant of Na^{125}I in the proposed [^{125}I] iododestannylation. It has indeed been used in the iodination of insulin to prepare ^{131}I-labeled insulin [13]. In our hands, ferric sulfate proved to be a mild, safe and efficient oxidant for generating elemental iodine from sodium iodide. Conducting the iododestannylation of E-TBSVNNT in dichloromethane with aqueous sodium iodide and excess aqueous ferric sulfate for only 30 min gave a quantitative yield of E-IVNNT which was identical in all respects (TLC, HPLC, NMR, MP, MS and IR) to the compound reported by Hoyte et al. [12]. In comparsion to the use of ferric sulfate, iododestannylation of E-TBSVNNT with sodium iodide and chloramine-T in acetone-dichloromethane-THF mixture for 30 min consumed only 50% of E-TBSVNNT and gave impure E-IVNNT.

The consistent 50% radiochemical yield of E-^{125}IVNNT in contrast to the quantitative yield of E-IVNNT in the non-labeled reaction of E-TBSVNNT with sodium iodide/ferric sulfate could be explained on the basis of a probable mechanism operative in the iododestannylation. In the two phase reaction mixture, aqueous ferric sulfate oxidizes sodium iodide liberating iodine which is extracted into dichloromethane. Mechanistically, it can reasonably be assumed that the carbon to tin bond is polarized as C^--Sn^+ and iodine molecule as I^+-I^-. It is now easy to visualize a four membered transition state in which the iodine atoms are shared by the reactive

carbon and tin centers leading to the iodovinyl steroid [steroid-CH=CH-I] and an iodostannyl compound I-$SnBu_3$. In the non-labeled reaction E-TBSVNNT is reacted with an excess of iodine. Thus enough iodine is present to transform all of the substrate quantitatively to E-IVNNT and an iodostannyl compound which is not isolated. On the other hand, in the radiochemical synthesis a large excess of substrate (E-TBSVNNT) is present, and sharing of the iodine-125 by the reactive carbon and tin atoms causes the radiochemical yield of E-^{125}IVNNT to be limited to 50%. In the labeling experiments, we have observed that all of the ^{125}I is found in the organic layer. The unknown ^{125}I-iodostannyl compound is presumably lost during evaporation and/or during silica gel chromatography, thus lowering the label recovery to about 50%.

Realization of 30-90% radiochemical yields in ^{125}I-iododestannylations [10,14] where other oxidants such as chloramine-T and hydrogen peroxide are used can be explained as follows. Mechanistically, reaction of chloramine-T or H_2O_2 with sodium iodide produces *in situ* the reactive species HO-I which is polarized as HO^--I^+, and no I_2 molecule is formed. Thus it can be visualized that reaction of this reactive species with the reactive carbon and tin centers of the substrate will give rise to product "C-I" and by-product "HO-Sn" and presumably little or no iodine is captured by tin.

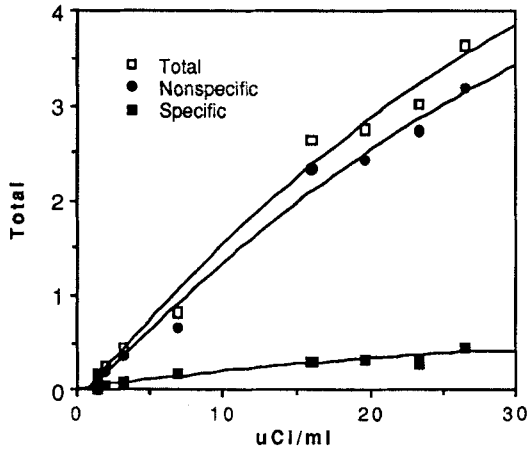

Figure 2. Specific binding of E-^{125}IVNNT was determined by measuring the binding of varying doses of E-^{125}IVNNT to T47D cells in the presence (nonspecific binding) or absence (total binding) of 1 x 10^{-6} M unlabeled progesterone. Specific binding is represented by the difference between the total and nonspecific binding.

Specific and saturable binding was observed at 13 μCi/ml for E-^{125}IVNNT (Fig. 2). This represents binding to 1.01 X 10^5 progesterone receptors per cell compared to 3.67 X 10^5 receptors for [^{3}H]R5020.

However, E-^{125}IVNNT has a greater affinity for the progesterone receptor as seen in Figure 3 since a higher concentration of cold progesterone was required to inhibit 50% of the binding of E-^{125}IVNNT (5.0 X 10^{-7} M) compared to [^{3}H]R5020 (5.0 X 10^{-8} M). E-^{125}IVNNT should be useful for determining PgR+ tumors and measuring the number of progesterone receptors in these tumors.

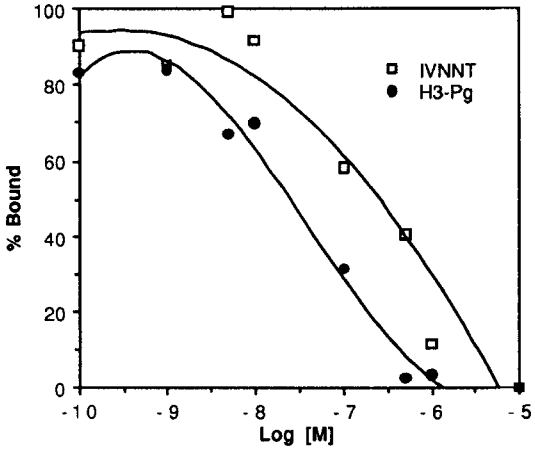

Figure 3. The affinity of the binding of E-^{125}IVNNT and ^{3}H-R5020 was determined by measuring the binding to T47D cells in the presence of increasing concentrations of unlabeled progesterone ranging from 1 x 10^{-10} to 1 x 10^{-5} M.

EXPERIMENTAL PROCEDURES

Chemicals and solvents were analytical grade and were used without further purification. Melting points were taken on a MEL-TEMP II apparatus (Laboratory Devices, Holliston, MA) and were uncorrected. Proton (^{1}H)NMR spectra were obtained using a Bruker Model Ac 300 (300 MHz) spectrometer with $CDCl_3$ solutions using Me_4Si as an internal standard. Mass spectra were obtained on a VG Analytical Model 70-G mass spectrometer; and IR spectra were recorded on a Perkin-Elmer Model 1310 infrared spectrophotometer. Column chromatography was carried out on 60-200 mesh silica gel. TLC analyses of unlabeled and labeled compounds were done on silica

gel GF glass plates (2.5 x 10 cm or 5 x 20 cm with 250 μm layer) and preparative TLC were run on silica gel glass plates (20 x 20 cm, 1000 μm, with 250 nm phosphor) both obtained from Analtech, Newark, DE. HPLC analyses and purifications were performed on a Waters Associates HPLC system equipped with a Model 6000A solvent delivery system employing a Beckman ultrasphere 5μ silica column (25 cm, 4.6 mm I. D.) and a silica semi-prep column (25 cm, 10 mm I. D.). Radioactivity was measured with a CAPINTEC radioisotope calibrator CRC-7, Packard Auto Gamma 5650 gamma counter and Packard Model 1900 CA TRI-CARB liquid scintillation analyzer.

17α-[2-(E)-iodovinyl]-4-estren-17β-ol-3-one (E-IVNNT)

Aqueous ferric sulfate (120 mg, 1 ml) was added to a mixture of 17α-[2-(E)-tri-n-butylstannylvinyl]-4-estren-17β-ol-3-one (E-TBSVNNT) (30 mg) in dichloromethane (1 ml) and aqueous sodium iodide (18 mg, 1 ml), and the mixture stirred at room temperature for 30 minutes. The reaction mixture was diluted with water and the organic material extracted with ethyl acetate. The organic extract was washed with water, 10% aqueous sodium bisulfite, water and brine. The extract was dried over anhydrous sodium sulfate and the solvent distilled off *in vacuo* to give a pale yellow gummy material. TLC of this material indicated it to be almost pure new compound [ethyl acetate-hexane (35:65), R_f=0.19; no starting material at R_f=0.42]. Preparative TLC of this material on silica gel plate using the same solvent system gave a crystalline product (20 mg) which was recrystallized from ethyl acetate-hexane: mp: 110-113° C dec [Lit. 111-114° C dec]; NMR: δ 6.7 and 6.28 (AB pattern, 2, E-CH=CH-, J=15.2 Hz), 5.82 (s, 1, H-4) and 0.94 (s, 3, H-18). HPLC: 25 cm X 10 mm silica column, 0.8% 2-propanol in dichloromethane solvent system, 5 ml/min, R_T=20.87min; (E-TBSVNNT eluted at R_T=9.93 min); ethyl acetate-hexane (40:60) solvent system, 2 ml/min, R_T=24 min (E-TBSVNNT eluted at 15.51 min). Mass spectrum: m/z 426 (parent, 10.25%, M), 299 (base, M-I), 281 (22.25%, M-I-H_2O), 231 (90%) and 196 (35%); high resolution M_r for $C_{20}H_{27}O_2I$, calcd: 426.1020; found: 426.1104. IR: ($CHCl_3$): 3430, 1660, 1615, and 962 cm^{-1}.

17α-{2-(E)-[^{125}I]-iodovinyl}-4-estren-17β-ol-3-one (E-^{125}IVNNT)

Aqueous ferric sulfate (100 μl, 50 mg/ml) was added to a mixture of E-TBSVNNT (200 μg) in dichloromethane (250 μl) and Na^{125}I (Du Pont -NEN,

Boston, MA) (18 mCi, 80 µl) in a 4.5 ml screw-cap glass vial which was subsequently capped. The reaction mixture was stirred vigorously at room temperature for 30 min and the contents diluted with water. The organic material was extracted with ethyl acetate followed by washing with water, 10% aqueous sodium bisulfite, water and brine. After evaporation of the solvent, the radioactive organic material (10 mCi) was purified by preparative TLC using ethyl acetate-hexane (35:65) solvent system (R_f of radioactive band: 0.14 and R_f of starting material band: 0.29) to give practically pure E-^{125}IVNNT (9.2 mCi). The material was further purified by HPLC: 25 cm x 10 mm silica column, ethyl acetate hexane (40:60) solvent system, 2 ml/min, R_T=22 min (8.9 mCi). The theoretical specific activity of E-^{125}IVNNT is 2200 Ci/mmol.

Cell Cultures

Stock monolayer cultures of T47D human breast ductal carcinoma (American Type Culture Collection, Rockville, MD) were maintained by weekly passage in RPMI-1640 medium (Mediatech, Washington, DC) supplemented with 5 ml of a 200 mM solution of glutamine, 10 µg of penicillin/ml, 10 µg of streptomycin/ml, 1% nonessential amino acids, 10 mM HEPES buffer, 1 mM sodium pyruvate and 10% fetal calf serum (Hyclone, Logan,UT). Cell cultures were harvested using trypsin (Sigma, St. Louis, MO), collected in 50 ml centrifuge tubes, and washed in media by centrifugation at 450 x g for 10 min. The cells were resuspended in 5 ml of media with the cell aggregates being disrupted by passing the suspension through a 10 ml syringe with a 21 gauge needle 5 times. Single-cell suspensions prepared in this manner were used in cell binding studies. The cells were allowed to incubate for 3 hr at 37° C to allow recovery from the harvesting procedure.

Cell Binding Assays

To determine whether E-^{125}IVNNT binding was specific and saturable, 2 X 10^5 T47D cells for studies involving E-^{125}IVNNT or 1.2 X 10^6 cells for [^{3}H]R5020 (DuPont-NEN, Boston, MA) were placed in 0.5 ml of medium containing 0.5 to 10 µCi of E-^{125}IVNNT or 0.02 to 0.25 µCi of [^{3}H]R5020 with or without non-radioactive progesterone ($1x10^{-6}$M). The cells were incubated for 2 hr at 37 °C, placed on ice and assayed by the oil-microcentrifuge method [15]. Subtraction of nonspecific binding from total binding yields the specific binding. Using the back extrapolate at the plateau to the ordinate and the known specific activity of the radionuclides, the number of progesterone receptors/cell was calculated (6, 15).

The affinity of E-^{125}IVNNT or [^{3}H]R5020 was determined by measuring the cell binding in the presence of an increasing concentration of unlabeled progesterone. The affinity was the progesterone concentration at which 50% of the specific binding of the labeled progesterone was inhibited.

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REFERENCES

1. Seevers R. H. Jr.-Radionuclides in Therapy (edited by Spencer R. P., Seevers R. H. Jr, and Friedman A. M.), CRC Press, Boca Raton, FL (1987), pp 145-166
2. King W. J., DeSombre E. R., Jensen E. V., and Greene G. L.-Cancer Res. 45: 293 (1985)
3. Rao D. V., Narra V. R., Howell R. W., Govelitz G. F., and Sastry K. S.-Lancet 2: 650 (1989)
4. Welshons W. V., Lieberman M. E., and Gorski J.-Nature 307: 747 (1984)
5. Halpern A., and Stocklin G.-Radiat. Envir. Biophys. 14: 167 and 257 (1977)
6. Epperly M. W., Damodaran K. M., McLaughlin W. H., Pillai K. M. R., and Bloomer W. D.-J. Steroid Biochem. Molec. Biol. 39: 729 (1991)
7. Raynaud J. P., Bouton M. M., Gallet-Bourguin D., Philibert D., Tournamine C., and Azadian-Boulanger G.-Molec. Pharmac. 9: 520 (1973)
8. Hanson R. D., Seitz D. E., and Botarro J. C.-J. Nucl. Med. 23: 431 (1982)
9. Nakatsuka I., Ferreira N. L., Eckelman W. C., Francis B. E., Rzeszotarsky W. J., Gibson R. E., Jagoda E. M., and Reba R. C.-J. Med. Chem. 27: 1287 (1984)
10. Hochberg R. B., Hoyte R. M., and Rosner W.-Endocrinology 117: 2550 (1985)
11. Bremner D. H. -Synthetic Reagents, Vol. 6 (edited by Pizey J. S.), Ellis Horwood Ltd., Chichester, U. K. (1985), pp 9-59
12. Hoyte R. M., Rosner W., Johnson I. S., Zielinski J., and Hochberg R. B.-J. Med. Chem. 28: 1695 (1985)
13. Stadie W. C., Haugaard N., and Vaughn M.-J. Biol. Chem. 199: 729 (1952)

14. The exact mechanism of iododestannylation is not known. Radiochemical yields vary from 30 to 90% depending upon the oxidant, solvent and reaction conditions employed; e. g. Damodaran K. M., Epperly M. W.,and Bloomer W. D.-unpublished work; Fang Y-B., Mukherjee J., Yang Z-Y., and Cooper M,-J. Nucl.Med. 33: 982 (1992)

15. McLaughlin W. H., Milius R. A., and Gill L. M.-J. Steroid Biochem. 20: 1129 (1984)