Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 1397–1400

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis of Hybrid Distamycin–Cysteine Labeled with ^{99m}Tc: a Model for a Novel Class of Cancer Imaging Agents

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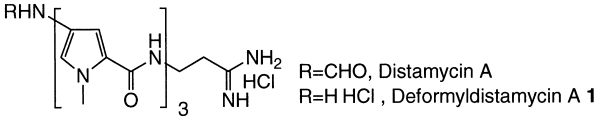
Received 2 March 2000; accepted 26 April 2000

Abstract—The synthesis of a hybrid constituted by distamycin A and cysteine labeled with the γ-emitting radionuclide ^{99m}Tc to afford the conjugate complex **5** is reported. This new radiopharmaceutical is of potential interest as tumor imaging agent in diagnostic nuclear medicine. The preparation of the hybrid distamycin A-cysteine **4** has been achieved by coupling deformyldistamycin A and Boc-Dmt-OH. Compound **4** was then successfully labeled with ^{99m}Tc by reaction with the novel, high-electrophilic, metal-containing fragment $[^{99m}Tc(N)(PP)]^{2+}$ (PP = diphosphine ligand) yielding the 1:1 complex **5**.

Introduction

The tripyrrole peptide distamycin A is a naturally occurring antibiotic agent isolated from the cultures of *Streptomyces distallicus*,[1] with interesting antibacterial and antiviral activities (but inactive as antitumor agent), which binds to the minor groove of DNA by hydrogen bonds, Van der Waals contacts and electrostatic interactions.[2] This oligopeptide shows a high selectivity for AT rich sequences, covers five base pairs and binds preferentially to 5′-AAATT-3′ sequences.[3] Distamycin A has been used as DNA sequence selective carrier of alkylating functions, leading to compounds which are substantially more cytotoxic than distamycin itself.[4]



Radiopharmaceuticals are radiolabeled substances containing a radioisotope, and designed to target specific sites of disease such as neoplastic tissues. In the last decades, there has been a growing interest in developing radiolabeled compounds for treatment of metastatic cancer.[5] In this paper, we report the first example of a radioactive complex incorporating distamycin A, which appears to be sufficiently stable for in vivo applications as tumor imaging agent using Single Photon Emission Tomography (SPET). This technique utilizes the emission characteristic of certain γ-emitting radioisotopes, which are incorporated into radiopharmaceuticals having the appropriate biodistribution properties to accumulate in selected target tissues.[5] Because of its low cost, wide availability and convenient half-life ($t_{1/2}$ = 6.02 h), ^{99m}Tc is the radionuclide of choice for SPET imaging.[6] In particular, we used here the ^{99m}Tc-containing fragment $[^{99m}Tc(N)(PP)]^{2+}$ to label distamycin A. This novel metal fragment comprises a $[Tc{\equiv}N]^{2+}$ core bound to a diphosphine ligand (PP) and exhibits a strong tendency to react with nucleophilic bidentate ligands having a set of two π-donor atoms as coordinating group.[7] Although it is well-demonstrated that the $[Tc{\equiv}N]^{2+}$ core forms complexes with a wide variety of chelators, and the nitrido complex $[^{99m}Tc(N)(noet)_2]$ (noet = *N*-ethoxy-*N*-ethyldithiocarbamato) has been recently developed as a new myocardial imaging agent,[8] the present work constitutes the first attempt to exploit the chemistry of nitrido Tc(V) complexes for the labeling of small biomolecules.

Chemistry

The hybrid cysteine–distamycin **4** was synthesized as reported in Scheme 1. The deformyl distamycin A **1**[1a] was condensed with *N*-L-(*tert*-butoxycarbonyl)-2,2-

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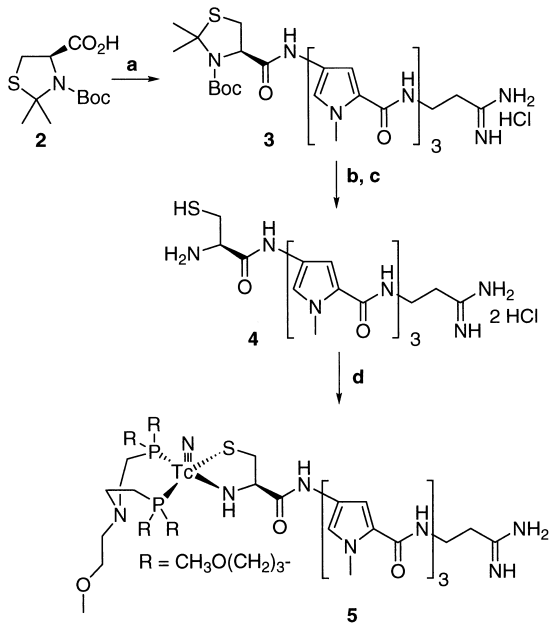

Scheme 1. Reagents: (a) deformul distamycin A **1** (1 eq.), EDCl, DIPEA, dry DMF, rt, 18 h; (b) CF_3CO_2H, DCM, rt, 2 h; (c) H_2O/EtOH (1:1, v/v); (d) $[^{99}mTcO_4]^-$, $SnCl_2$, SDH (DTC), PP, compound **4**.

dimethylthiazolidine-4-carboxylic acid (Boc-Dmt-OH) **2**, which was easily prepared in two steps from L-cysteine,[9] to afford product **3**. After removal of the Boc protecting group by trifluoroacetic acid (TFA) and subsequent hydrolysis with a water:ethanol mixture (1:1 v/v), **3** was finally transformed into **4**.

The distamycin–cysteine hybrid ligand **4**[10] was labeled with ^{99m}Tc using the following procedure. $[^{99m}TcO_4]^-$, $SnCl_2$ and succinic dihydrazide [SDH = H_2N-NH-C(=O)-$(CH_2)_2$-(O=)C-NH-NH_2] were mixed in a vial and, to the resulting solution, the diphosphine ligand [$((H_3CO(CH_2)_3)_2$-P-$(CH_2)_2$-N$(CH_2CH_2OCH_3)$-$(CH_2)_2$P-$((CH_2)_3OCH_3))_2$] (PP) and the hybrid **4** were successively added to give the labeled product **5**. To further assess the chemical identity of the new compound prepared at tracer level (typically, in the μM concentration range), exchange reactions were carried out starting from the complex $[^{99}Tc(N)(PP)Cl_2]$, which was prepared and characterized, in macroscopic amounts, with the long-lived isotope ^{99}Tc using previously reported methods.[7] Addition of an excess of **4** to an ethanolic solution of $[^{99}Tc(N)(PP)Cl_2]$ caused the two chloride groups to be replaced by **4** with the concomitant formation of the corresponding ^{99}Tc-analogue of **5**. This complex was characterized by elemental analysis, FT-IR spectroscopy and positive ion fast atom bombardment (FAB+) mass spectra. Results showed that it contains only one ligand **4** tethered to a $[^{99m}Tc(N)(PP)]^{2+}$ fragment, and coordinated to the Tc(V) center through the deprotonated thiol sulfur atom and the neutral nitrogen atom of the terminal cysteine group, thus producing a final cationic complex.[11] Chromatographic comparison between complex **5** and its ^{99}Tc-analogue revealed that these two compounds have essentially the same chemical identity. As shown by TLC analysis (Fig. 1), incorporation of the hybrid **4** into the Tc-99m complex was achieved approximately with 85% radiochemical yield. Stability of **5** was evaluated, both in physiological solution and in plasma, by measuring

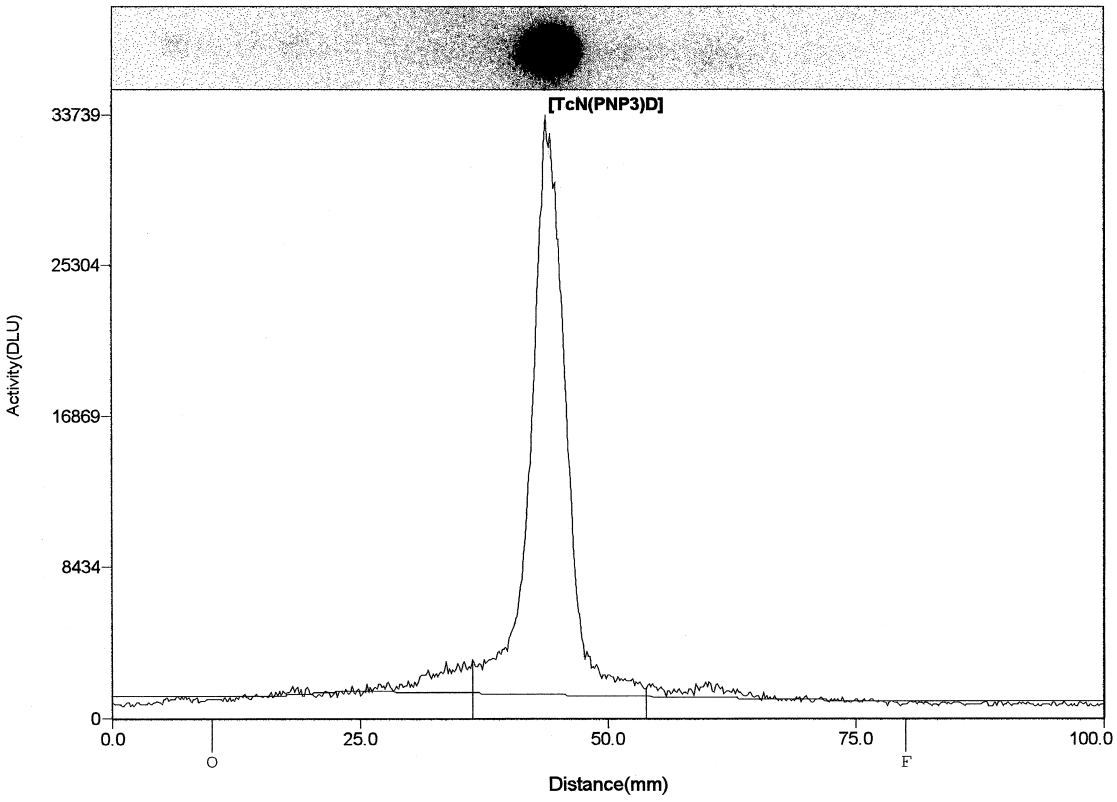

Figure 1. TLC analysis related to the incorporation of Tc-99m in the hybrid **4**.

its radiochemical purity (RCP) by TLC chromatography over a period of 6 h. No significant alteration of RCP was observed under these conditions.

Results and Discussion

Introduction of L-cysteine on the N-terminus of deformyl distamycin A (**1**) gives compound **4** without affecting its cytotoxicity, which is comparable to that of the parent compound distamycin A. In fact, the hybrid **4** was found to be cytotoxic against L1210 murine leukaemia cell line[12] (NCI, Bethesda, USA) ($IC_{50} = 7.962$ μM after 48 h of drug exposure) showing almost the same behavior as reported for distamycin A ($IC_{50} = 9.64$ μM). This observation was confirmed by further experiments showing that distamycin (**1**) and the hybrid **4** retain similar inhibitory activity on cell growth of human leukemic K562 cells (10 and 12 μM, respectively). In addition, distamycin A **1** and the hybrid **4** retain very similar efficiency in arrested polymerase-chain reaction (PCR) experiments[13] (Fig. 2). In these experiments we tested the ability of these two compounds to inhibit the PCR mediated amplification of A+T rich sequences of an upstream region of the human estrogen receptor (ER) gene.[14] We published elsewhere that this approach is useful to study sequence selectivity[13] as well as binding efficiency of DNA-binding drugs.[15] The results shown in Figure 2 demonstrate that in PCR experiments using primers amplifying the human ER gene IC_{50} occurrs at very similar micromolar concentrations of distamycin A and distamycin–cysteine hybrid **4**, suggesting a very similar efficiency of these two related compounds in interacting with target DNA. The results shown in Figure 2 are in agreement with elsewhere published reports demonstrating that changes of the formyl group of the distamycin only lead to minor changes in sequence-recognition and biological activity.[16] Both distamycin and the hybrid **4** exhibited low activity in inhibiting PCR-mediated amplification of G+C rich human genomic sequences, including a region on the Ha-ras oncogene containing the exon 62 (data not shown). Taken together, these observations suggest that distamycin A **1** and the hybrid **4** display similar DNA binding activity and sequence-selectivity.

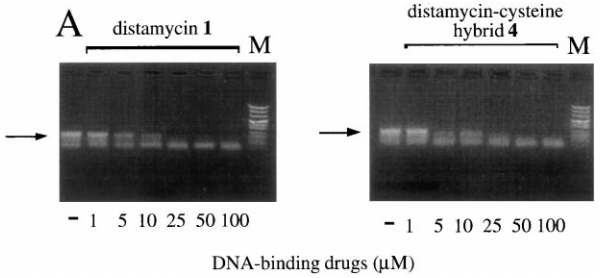

Figure. 2. Effects of distamycin **1** (A) and distamycin–cysteine hybrid **4** (B) on PCR-mediated amplification of estrogen receptor (ER) gene sequences. Target DNA was 20 ng of genomic human DNA. Before PCR, target DNA was incubated in the absence or in the presence of the indicated mM concentrations of DNA-binding drugs. After PCR (20 cycles), 10 mL of each PCR mixture were analysed by agarose gel electrophoresis. M = molecular weight marker (pUC mix, MBI, Fermentas, Vilnius, Lithuania). ER PCR products are arrowed.

Further experiments (including DNase I footpinting assays and gel-shift studies) are required on a variety of eukaryotic and viral genes sequences in order to identify the most frequently bound nucleotide sequences and the effects of these two compounds on protein-DNA interactions.

The hybrid **4** was efficiently labeled with ^{99m}Tc through an unprecedent procedure based on the reaction of the novel nitrido Tc(V) fragment $[^{99m}Tc(N)(PP)]^{2+}$ to give the monocationic, lipophilic complex **5** comprising the hybrid **4** coordinated to the metal ion through the terminal deprotonated cysteine group. Complex **5** was found to be stable both in physiological solution and in plasma. This is an essential requirement for a radiopharmaceutical being used as diagnostic or therapeutic agent.[17] Further studies, carried out both in vitro and in vivo using animal models, are underway to establish the ability of complex **5** to target specific tumor cell lines. Due to the chemical similarities between technetium and rhenium, complex **5** may be viewed as a suitable model for the design of ^{99m}Tc- and $^{186/188}Re$-radiopharmaceuticals potentially useful for the diagnosis and treatment of various types of cancerous diseases.

Acknowledgements

We wish to thank Pharmacia & Upjohn for providing distamycin A and Ministero Università e Ricerca Scientifica (MURST) (60%) for generous financial support of this work. R.G. is supported by CNR Target Project Biotecnologie, by ISS (AIDS-1998) and by MURST Co-fin 98.

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10. Selected data for the compound **4**: Pale yellow solid; mp (uncorrected): 245–248 °C; $[a]_D^{25}$ (0.1 N HCl in water): +109.8 ($c=0.275$). ^{1}H NMR (DMSO-d_6) d: 2.62 (m, 2H); 2.87 (m 1H); 3.03 (m, 2H); 3.52 (m, 2H); 3.81 (s, 3H); 3.84 (s, 3H); 3.86 (s, 3H); 4.16 (m, 1H); 6.95 (s, 1H); 6.98 (s, 1H); 7.06 (s, 1H); 7.19 (s, 1H); 7.24 (s, 2H); 8.26 (t, $J=7.3$ Hz, 1H); 8.53 (m, 3H), 8.70 (s, 2H); 9.03 (s, 2H); 9.95 (s, 1H); 10.05 (s, 1H), 10.88 (s, 1H). IR (KBr) cm^{-1}: 3434, 1647, 1458, 1378, 1225, 1174, 828. UV (0.1 N HCl in water): λ_{min} (ε): 212 (239), 264 (231); $\lambda_{max:}$ 239 (402), 303 (487). Elemental analysis for $C_{24}H_{34}Cl_2N_{10}O_4S$; (calcd): C, 45.79; H, 5.44; Cl, 11.26; N, 22.25; S, 5.09; (found): C, 45.67; H, 5.21; Cl, 11.10; N, 22.02; S, 4.86. Mass (Maldi-Tof): 558.0 (M).
11. Selected data for complex **5**. 250 μL of generator-eluted $[^{99m}TcO_4]^-$ were added to a vial containing 5.0 mg of SDH, 0.1 mg of $SnCl_2$ and 1.0 mL of EtOH. The vial was kept at room temperature for 30 min. Then, 5.0 mg of **4**, dissolved in 250 mL of H_2O, and 3.0 mg of PP, dissolved in 250 μL of EtOH, were added, and the reaction was further kept at room temperature for 1 h. Radiochemical yield, 70–85%. Thin-layer chromatography (TLC) on SiO_2 plates: (a) mobile phase, EtOH/$CHCl_3$/toluene/[NH_4][CH_3COO] (0.5 M) (6:4:3:1.5), $R_f=0.64$; (b) mobile phase, EtOH/$CHCl_3$/toluene/[NH_4][CH_3COO] (0.5 M) (6:3:3:1.5), $R_f=0.27$. Activity on the plates was analyzed with a Cyclone instrument (Packard) using a phosphor imaging screen (Fig. 1). Stability was evaluated by incubating, at 37 °C, 100 L of the reaction solution of **5** with 900 L of (a) saline, and (b) human plasma, and by measuring the variation of its radiochemical purity (RCP) by TLC analysis at 1, 3 and 6 h after preparation. Synthesis of the complex with the long-lived isotope ^{99}Tc was carried out as follows. [^{99}Tc(N)(PPh_3)$_2Cl_2$] (0.11 g, 0.15 mmol) was mixed in acetonitrile (20 mL) with the ligand PP (0.10 g, 0.20 mmol), and the resulting yellow solution was refluxed for 30 min under argon stream. After cooling to room temperature, the ligand **4** (0.14 g, 0.25 mmol) was then added, and the reaction solution was vigorously stirred under argon for 2 h. The final, yellow-orange product was recovered as hexafluorophosphate salt by addition of excess $NaPF_6$ followed by slow evaporation of the reaction solution under a weak argon flow. (Yield, 54%). FT IR (KBr) cm^{-1}: 1040 [(TcN)]. Elemental analysis for $C_{47}H_{82}N_{12}O_9P_3SF_6Tc$; (calcd): C, 43.52; H, 6.33; N, 12.96; S, 2.47; Tc, 7.64; (found): C. 43,88; H. 6.63; N, 12.77; S, 2.00; Tc, 7.14. FAB MS (NBA matrix): m/z 1152 [MH^+].
12. The cytotoxicity was evaluated following the procedure reported in the article: Geroni, C.; Pesenti, E.; Tagliabue, G.; Ballinari, D.; Mongelli, N.; Broggini, M.; Erba, E.; D'Incalci, M.; Spreafico, F.; Grandi, M. *Int. J. Canc*. **1993**, *68*, 916.
13. The sequences of the primers used for polymerase chain reaction were the following: ER-forward, 5′-GACGCATGATATACTTCACC-3′, ER-reverse, 5′-GCAGAATCAAATATCCAGATG-3′. Taq DNA polymerase (Perkin–Elmer, Cetus, USA) was added at 2.5 U/25 μL final concentration. The nucleotide sequence of a 3.2 Kb genomic region located upstream of the human estrogen receptor (ER) gene sequence originally designated exon 1 was investigated and described in our laboratory. PCR buffer, Taq DNA polymerase and the four dNTPs were added as elsewhere described.[14,15] Conditions of PCRs were: denaturation, 92 °C, 1 min; annealing, 55 °C, 1 min; elongation, 72 °C, 1 min (20 cycles). The effects of DNA-binding drugs were analysed after incubating target DNA at room temperature, for 5 min, with increasing amounts of the compounds, as reported in the text, followed by polymerase chain reaction. Amplified DNA was analysed by electrophoresis on 2.5% agarose, in TAE (0.04 M Tris-acetate, 0.001 EDTA), 0.5 μg/mL ethidium bromide.
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