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^{111}In-labeled 1,4,7,10-Tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic Acid-Lys^{8}-Vasotocin: A New Powerful Radioligand for Oxytocin Receptor-expressing Tumors[1]

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ABSTRACT

We developed a radioactive ligand for tumors expressing oxytocin receptors (OTRs) by linking the chelating agent 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA) to Lys^{8}-vasotocin (LVT), an analogue of oxytocin with high affinity for OTRs. The new reagent (DOTA-LVT) retained high affinity for human OTRs, as proved by *in vitro* affinity binding to cells endogenously expressing OTRs, such as MCF7 breast carcinoma and MOG-U-V-W glioblastoma cells lines, as well as to transiently transfected COS7 cells. In *in vivo* experiments, DOTA-LVT carrying ^{111}In showed specific binding activity to OTR-positive TS/A mouse mammary tumors. The present study opens new perspectives for imaging and, possibly, therapy of OTR-positive human tumors such as breast and endometrial carcinomas, neuroblastomas, and glioblastomas.

INTRODUCTION

The hypothalamic nonapeptide OT[3] binds to several tumor types via specific G-protein-coupled membrane receptors. OTRs were first described in human breast cancer cell lines (1) and in a high percentage of primary breast cancers (2). Later, OTRs were detected in tumors arising in other organs, such as brain and endometrium, where OT is known to exert a physiological function (3, 4). Moreover, by *in vitro* and *in vivo* experiments, we proved that in breast and endometrial carcinomas, neuroblastomas, and glioblastomas, both OT and synthetic analogues play a biological role by inhibiting cell growth via OTRs and the cyclic AMP-dependent protein kinase pathway (5, 6). Recently, osteoblasts and osteosarcomas have been added to the list of normal and neoplastic cells where OT has been shown to play an OTR-mediated functional role (7, 8).

Given this scenario, we reasoned that a specific OTR radioactive ligand might represent a novel tool to detect (and possibly treat) OTR-positive tumors. A similar strategy already proved successful to detect and treat SSTR-positive tumors, using radioactive somatostatin or analogues. In SSTR-positive tumors, heavy deposition of radioactive metals, such as ^{111}In and ^{90}Y, leading to valuable radiodetection and radiotherapeutic effects, was achieved by the use of the chelating agent DOTA bound irreversibly to a free NH_2 group of somatostatin or analogues (9, 10).

Following this rationale, we planned to link DOTA to the α-NH_2 group of position 1 in OT. However, this peptide is very sensitive to substitutions in this position, often resulting in loss of binding activity to OTRs (11). We therefore focused our attention on LVT, a nonapeptide that retains a high affinity for OTRs and where the leucine in position 8 of OT is substituted with lysine (12). The ϵ-NH_2 group of this amino acid offers a safe binding site. In fact, it was used by others to produce a fluorescent tracer that selectively binds to OTR (13) and retains OT activity in *in vitro* and *in vivo* assays (13, 14).

The present study describes the experimental development and properties of DOTA-bound LVT and ^{111}In-labeled DOTA-LVT and the selective binding of this novel radioactive compound to OTR-positive tumor cells, both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reagents. DOTA (M_r 404) × $4H_2O$ was purchased from Macrocyclics (Richardson, TX), and LVT (M_r 1023.1) was from Neosystem (Strasbourg, France). OT and LVT were kind gifts of Dr. M. Manning (Medical College of Ohio, Toledo, OH). Somatostatin and the growth hormone secretagogue hexarelin were a gift of Prof. R. Deghenghi (Europeptides, Argenteuil, France). The somatostatin analogue DOTA-TOC was synthesized at the Institute of Radiological Chemistry of Basel University (Basel, Switzerland; Ref. 15) and used as a negative control. The other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO); the radioisotopes ^{111}In and ^{125}I were obtained from Mallinckrodt Medical (Petten, the Netherlands) and Amersham Sorin (Saluggia, Italy), respectively.

Plan of the Experiments. To prove an efficient binding of the radioactive OT analogue to OTR-positive tumor cells, a series of successive experimental steps was planned. We first had to link the chelating agent DOTA to LVT and prove that this compound (DOTA-LVT) still retained affinity for OTR. Therefore, we radiolabeled the compound with ^{111}In and then tested the specificity and intensity of the binding of the radioactive compound ^{111}In-labeled DOTA-LVT to OTR-positive cells and tumors, both *in vitro* and *in vivo*.

Conjugation of DOTA to LVT. The COOH group of DOTA was activated by means of a carbodiimide reagent (16). Briefly, DOTA was dissolved in anhydrous DMSO at 80°C, and the solution was allowed to cool under an argon atmosphere. A solution of *N*-hydroxy-2,5-pyrrolidinedione in DMSO was added dropwise to a stirred solution of DOTA, followed by the dropwise addition of *N*,*N*′-dicyclohexylcarbodiimide in DMSO. The molar ratio between DOTA:*N*-hydroxy-2,5-pyrrolidinedione:*N*,*N*′-dicyclohexylcarbodiimide was 1:1.4:0.8. The mixture was allowed to react overnight with stirring and then filtered to separate the by-product, dicyclohexylurea. The conjugation between DOTA and LVT was carried out at a molar ratio of 50:1 by adding an adequate volume of the DOTA-activated ester solution to the LVT dissolved in 0.1 M phosphate buffer (pH 8.0). After overnight reaction, the conjugate was purified by means of a reverse-phase column (Resource RPC, 1 ml; particle size, 15 μm; Amersham Pharmacia Biotech, Uppsala, Sweden) in a FPLC system (LCC-501 Plus controller; Pharmacia Biotech) coupled with a UV detector (LKB-UV-MII; Pharmacia Biotech) and a radiodetector (Flow scintillation analyzer, Radiomatic 150 TR; Packard, Meriden, CT). A linear gradient method was applied using a solution of distilled water with 0.1% trifluoroacetic acid (solvent A) and methanol (solvent B). The eluents were delivered at a flow of 4 ml/min, starting from 0% of solvent A to 100% of solvent B in 37 ml. Two peaks corresponding to the LVT conjugates with DOTA were evident in the UV profile (Fig. 1). The retention volume was 8.4 ml for the first compound (A) and 10.0 ml for the second (B), whereas unconjugated LVT eluted at 7.0 ml in the same conditions. An integrated fraction collector (Frac-100; Pharmacia Biotech) performed the recovery of

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[3] The abbreviations used are: OT, oxytocin; OTR, oxytocin receptor; LVT, Lys^{8}-vasotocin; DOTA, 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; SSTR, somatostatin receptor; MALDI-TOF, matrix-assisted desorption ionization-time of flight; FPLC, fast protein liquid chromatography; TOC, D-Phe^{1}-Tyr^{3}-octreotide.

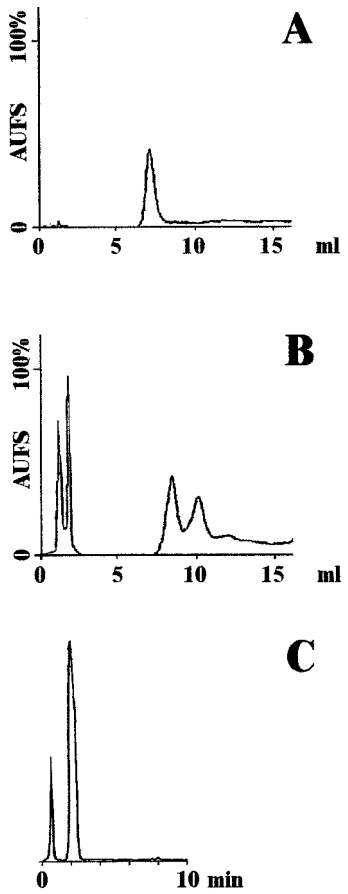

Fig. 1. Reversed-phase FPLC analysis. *A*, UV profile at 280 nm of LVT standard before conjugation showing that the peak eluted at 7.0 ml (1.7 min). *B*, UV profile at 280 nm of the conjugation mixture showing that two peaks eluted at 8.4 ml (2.1 min; compound A) and at 10.0 ml (2.5 min; compound B), respectively. The peak of unreacted LVT was not present. *C*, radioactivity profile of the isolated compound A labeled with ^{111}In showing a single peak at 2.1 min. The amount of the radiolabeled peptide is reported as percentage of the total radioactivity eluted.

each compound. Each peak was analyzed subsequently by MALDI-TOF mass spectrometry using a Reflex III instrument (Bruker, Germany).

Affinity Studies. To determine LVT and DOTA-LVT affinity constants, heterologous competition experiments were performed on membranes prepared from monkey kidney COS7 cells transiently transfected with the human OTR cDNA (17). Briefly, electroporated cells were homogenized in a Dounce glass potter, washed twice, and resuspended in binding buffer [50 mM Tris-HCl (pH 7.4) and 5 mM MgCl]. Five to 10 μg of membrane proteins were incubated with a fixed concentration of [^{3}H]OT (1–2 nM) for 30 min at 30°C in the presence of an increasing concentration of unlabeled peptides. Nonspecific binding was determined in the presence of 1 mM OT. Bound and free radioactivity were separated by filtration over Whatman GF/C filters presoaked in 10 mg/ml of BSA. Binding isotherms were analyzed with the iterative curve-fitting program ligand (18).

Radiolabeling of DOTA-LVT (Peak A) with ^{111}In. To prepare ^{111}In-labeled DOTA-LVT, 3.7 MBq of ^{111}In-labeled Cl_3, diluted in 0.1 M acetic acid-Na acetate buffer (pH 5.5), were added to 0.07 μmol of fraction obtained from peak A. The solution was heated for 25 min at 80°C, and a labeling yield was checked by FPLC as above.

Radiolabeling of DOTA-TOC with ^{125}I. Peptide iodination was performed with ^{125}I using the chloramine-T procedure according to previous descriptions (19). Purification was carried out by FPLC using the chromatographic conditions described above. The peak corresponding to ^{125}I-labeled DOTA-TOC was eluted at 14 ml and recovered by means of the fraction collector.

Binding Studies on OTR-positive and OTR-negative Tumor Cells. Human breast carcinoma (MCF7), glioblastoma (MOG-U-V-W), and colon carcinoma (HT29) cell lines were purchased from American Type Culture Collection (Manassas, VA). The mammary carcinoma cell line TS/A (20) growing in BALB/c mice was a gift of Prof. Guido Forni (University of Torino). All of the cells were grown as monolayers in RPMI 1640 (Life Technologies, Inc., Rockville, MD) with 10% FCS (Life Technologies, Inc.) in 25-cm^2 T flasks in a 5% CO_2-humidified atmosphere at 37°C. As proved in previous experiments (1, 3, 5), MCF7, MOG-U-V-W, and TS/A cells are OTR positive, whereas HT29 cells represent the OTR-negative control. Furthermore, lack of expression of SSTR2 in TS/A cells was tested using reverse transcription-PCR procedures similar to those used in our laboratory to study SSTR2 expression in human tumors (21).

Binding experiments were performed on intact cells. Briefly, 20×10^5 cells suspended in 100 μl of culture medium were incubated for 30 min at 4°C in the presence of 1 μM ^{111}In-labeled DOTA-LVT. The cell suspension was then centrifuged for 5 min at $2000 \times g$, and the pellet was washed and resuspended in fresh medium. Centrifugation and cell washing were repeated twice. The entity of the radiolabeling was evaluated measuring the radioactivity bound to cells (cpm/10^5 cells) by a Packard auto-gamma counter.

The specificity of the binding was determined by evaluating the radioactive displacement. Briefly, the cell suspension was incubated for 5 min in the presence of 100 μM and 1 mM of LVT or OT, or in the presence of unrelated peptides (such as 100 nM and 1 μM somatostatin and 1 μM hexarelin), followed by 20 min of incubation with 1 μM ^{111}In-labeled DOTA-LVT. Cells were then centrifuged and washed twice, as reported above. The activity of the two different agonists and of the unrelated peptides to compete with the radioligand was evaluated measuring the radioactivity bound to cells. All of the experiments were performed in triplicate. Statistical analysis was carried out by ANOVA. Cutoff for significance was 0.05.

***In Vitro* Effect of LVT and DOTA-LVT on Proliferation of MCF7 and TS/A Cells.** The biological effect of both LVT and DOTA-LVT was tested on the OTR-positive breast cancer cells. MCF7 and TS/A cells were plated in 24 multiwell dishes at a density ranging from 5 to 8 cells/ml and incubated 24 h after plating with different concentrations of LVT or DOTA-LVT (10 nM to 1 μM). Cell number was determined by cell counting using a hemocytometer at 48 and 96 h of culture by two independent investigators. Each experiment was done in triplicate and repeated three times. Statistical analysis was carried out by ANOVA. Cutoff for significance was 0.05.

***In Vivo* Studies.** To determine the entity and the specificity of receptor-mediated uptake of ^{111}In-labeled DOTA-LVT in tumors in comparison with the nonspecific compound ^{125}I-labeled DOTA-TOC, we used as an experimental model the OTR-positive and SSTR2-negative TS/A tumor growing in BALB/c mice. A total of eight BALB/c female mice weighing 25 g were used. TS/A mammary carcinoma cells (1×10^6 in 0.2 ml of medium) were injected s.c. Twenty days later, when the growing tumor reached a size of ~2 cm in diameter, the animals were injected i.p. with a mixture of 1.1 MBq of ^{111}In-labeled DOTA-LVT and 74 kBq of ^{125}I-labeled DOTA-TOC. The animals were divided into groups and sacrificed at 3 and 24 h after injection, respectively. Tumor, blood, liver, kidney, and brain were removed and weighed. The radioactivity was measured in a γ-ray detector with a well-counter geometry (Silena, Milan, Italy), together with standards of the injection mixture at two different time points to calculate the contribution of each isotope: (*a*) immediately after the removal of the tissues; and (*b*) after 2 weeks, corresponding to five physical half-lives of ^{111}In. Activity was expressed as a percentage of injected doses/mg of tissue, and ratios of uptake between tumor and brain *versus* blood were calculated. A statistical test (*t* test) was performed to determine the significant difference between the groups. Cutoff for significance was 0.05.

RESULTS

Radioligand Preparation. The conjugation between DOTA and LVT was complete as verified by the absence of unconjugated LVT in the reaction mixture. Radiolabeling yields were >95% for ^{111}In-labeled DOTA-LVT (Fig. 1*C*) and ~80% for ^{125}I-labeled DOTA-TOC after purification. Specific activities of 0.05 GBq/μM for each peptide were obtained.

The two peaks corresponding to the LVT conjugates with DOTA evident in the UV profile, as reported in "Materials and Methods,"

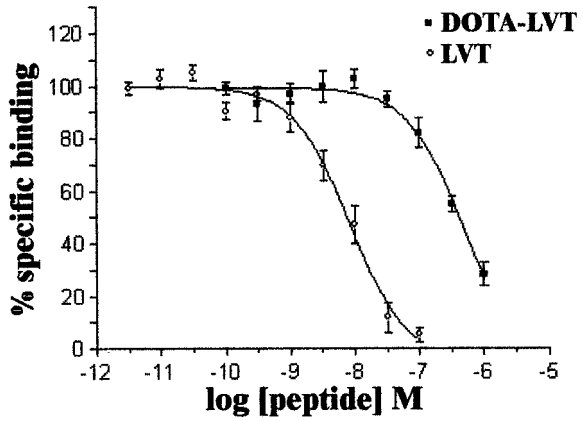

Fig. 2. Dose-dependent displacement of [^{3}H]OT binding to human OTR expressed in COS7 cells. Membranes were incubated in the presence of a constant amount of [^{3}H]OT (1–2 nM) and various amounts of unlabeled peptides. Data are expressed as the percentages of bound [^{3}H]OT in the absence of cold competitor and were analyzed with the nonlinear model fitting program ligand. Each curve is a mean of triplicate determinations of a single representative experiment; *bars,* SE.

were examined by MALDI-TOF mass spectrometry. The product corresponding to peak A presented 2 DOTA/molecule of peptide (1795 atomic mass units), whereas the mass spectra of the product corresponding to peak B showed a predominant peak at 1391 atomic mass units, attributable probably to an intramolecular bridge of DOTA between positions 8 and 1 of the peptide.

Affinity of LVT and DOTA-LVT for OTRs. LVT and DOTA-LVT affinities for the human OTR were determined by means of heterologous competition experiments on membranes prepared from COS7 cells expressing 0.5–2 pmol of receptors/mg of proteins as determined by Scatchard analysis using [^{3}H]OT as the radioligand. As reported in Fig. 2, unlabeled LVT inhibited [^{3}H]OT binding with high affinity ($K_i = 1.87 \pm 0.418$ nM; $n = 3$); DOTA-LVT from peak A was also able to compete [^{3}H]OT binding with a calculated K_i of 238 ± 52.36 nM ($n = 3$). Displacement curves were parallel, and their slopes were not significantly different from the unit. No competition was observed when DOTA-LVT from peak B was used (not shown).

^{111}In-labeled DOTA-LVT Binding to OTR-positive and OTR-negative Tumor Cells. After 30 min of incubation with ^{111}In-labeled DOTA-LVT, the presence of a specific radiolabeling was observed in all of the OTR-positive cell lines (MCF7, TS/A, and MOG-U-V-W) and was negligible in HT29 (OTR-negative) cells. The amount of labeling, expressed as cpm/10^5cells, was 1711 ± 97 for MCF7 cells, 7252 ± 83 for TS/A cells, and 1811 ± 101 for MOG-U-V-W cells, whereas it was only 34 ± 6 for HT29 cells.

The specificity of binding was proved by displacement with cold radioligands. In OTR-positive cells, >90% of the specific ^{111}In-labeled DOTA-LVT binding was displaced by 5-min preincubation with 100 μM and 1 mM nonradioactive OT or LVT (Fig. 3). An additional confirmation of the specificity of binding was provided by the lack of radioligand displacement after incubation with two OT-unrelated peptides, somatostatin and hexarelin, used at 100 nM and 1 μM concentrations (not shown).

***In Vitro* Effect of LVT and DOTA-LVT on Cell Proliferation.** Incubation of MCF7 and TS/A cells with 100 nM and 1 μM of LVT determined a significant inhibition of cell growth in both cell lines. LVT at 1 μM and 100 nM, respectively, determined a 50 and 35% growth inhibition at 48 h of treatment and a 70 and 50% inhibition at 96 h ($P < 0.001$ at 48 and 96 h of treatment for both 100 nM and 1 μM LVT *versus* control). At a lower concentration, 10 nM, LVT was still inhibiting cell growth, although to a lesser extent ($P < 0.05$ at 96 h and not significant at 48 h). The entity of inhibition in these cell lines was similar and even higher than that reported previously after OT treatment (1, 6). On the contrary, treatment with DOTA-LVT did not affect the cell proliferation at any tested concentration (10 nM, 100 nM, and 1 μM).

***In Vivo* Tissue Distribution of the Radioligands.** The results of the biodistribution study in BALB/c mice bearing TS/A tumors showed that neither ^{111}In-labeled DOTA-LVT nor ^{125}I-labeled DOTA-TOC accumulated in the brain: $1.97 \times 10^{-5} \pm 1.44 \times 10^{-5}$ and $2.14 \times 10^{-3} \pm 1.80 \times 10^{-3}$ % of injected dose/mg, respectively, at 3 h; and $1.71 \times 10^{-6} \pm 1.09 \times 10^{-6}$ and $1.98 \times 10^{-4} \pm 1.84 \times 10^{-4}$, respectively, at 24 h. Tumor uptake was $1.69 \times 10^{-4} \pm 6.69 \times 10^{-5}$ *versus* $8.11 \times 10^{-4} \pm 4.61 \times 10^{-4}$, respectively, at 3 h and $4.33 \times 10^{-5} \pm 1.07 \times 10^{-5}$ *versus* $1.16 \times 10^{-4} \pm 2.04 \times 10^{-4}$ at 24 h. Liver uptake was also low at both time points: $4.20 \times 10^{-4} \pm 3.47 \times 10^{-4}$ *versus* $2.11 \times 10^{-3} \pm 1.3 \times 10^{-3}$ at 3 h; and $1.27 \times 10^{-4} \pm 9.28 \times 10^{-5}$ *versus* $3.72 \times 10^{-4} \pm 2.51 \times 10^{-4}$ at 24 h. As expected, kidney uptake was rather high, although lower for ^{111}In-labeled DOTA-LVT than for ^{125}I-labeled DOTA-TOC, especially at 24 h ($1.11 \times 10^{-3} \pm 2.59 \times 10^{-4}$ *versus* $1.49 \times 10^{-2} \pm 3.51 \times 10^{-3}$). The tumor:blood ratios and brain:blood ratios for both radiolabeled peptides are presented in Table 1.

DISCUSSION

The present study reports on the development of a radioactive analogue of OT and demonstrates its potential value to bind selectively to OTR-positive tumors.

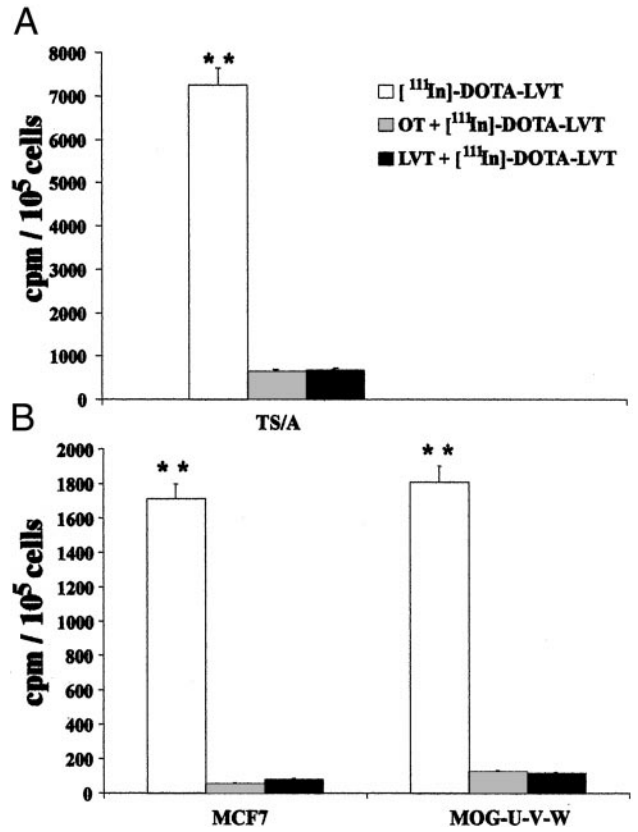

Fig. 3. Presence and specificity of ^{111}In-labeled DOTA-LVT binding to neoplastic OTR-positive cells. Binding of ^{111}In-labeled DOTA-LVT was assessed in TS/A mouse mammary carcinoma cells (*A*) and in two human cancer cell lines of breast (*MCF7*) and glial (*MOG-U-V-W*; *B*) origins. The specificity of binding was evaluated by radioligand displacement when a 5-min incubation with OT analogues preceded that with ^{111}In-labeled DOTA-LVT. In all of the cell lines, an intense binding was observed, and both analogues (OT and LVT) displaced the radiolabeled compound. **, $P < 0.0001$ (analogues + ^{111}In-labeled DOTA-LVT *versus* ^{111}In-labeled DOTA-LVT). No displacement was observed in the presence of OTR-unrelated peptides (somatostatin and hexarelin, 100 nM and 1 μM, not shown).

Table 1 *Tumor:blood and brain:blood uptake ratios*

Tumor:blood	Brain:blood
3-h ^{111}In-labeled DOTA-LVT[a]	
2.70	$1.16\ 10^{-1}$
4.10	$1.28\ 10^{-1}$
1.20	$7.89\ 10^{-2}$
NA[b]	$3.50\ 10^{-2}$
Mean ± SD, 2.67 ± 1.45	Mean ± SD, $8.95\ 10^{-2} \pm 4.19\ 10^{-2}$
24-h ^{111}In-labeled DOTA-LVT[a]	
2.84	$1.66\ 10^{-1}$
4.41	$4.41\ 10^{-2}$
9.10	$6.72\ 10^{-1}$
$1.26\ 10^{-1}$	$4.62\ 10^{-1}$
Mean ± SD, 7.24 ± 4.46	Mean ± SD, $4.35\ 10^{-1} \pm 2.08\ 10^{-1}$
3-h ^{125}I-labeled DOTATOC[a]	
$9.60\ 10^{-1}$	$4.76\ 10^{-2}$
1.85	$6.43\ 10^{-2}$
$8.10\ 10^{-1}$	$3.91\ 10^{-3}$
$4.90\ 10^{-1}$	$7.04\ 10^{-4}$
Mean ± SD, 1.03 ± 0.58	Mean ± SD, $2.91\ 10^{-2} \pm 3.17\ 10^{-2}$
24-h ^{125}I-labeled DOTATOC[a]	
4.86	$1.04\ 10^{-1}$
2.07	9.03
2.13	$2.10\ 10^{-1}$
1.88	$1.92\ 10^{-2}$
Mean ± SD, 2.74 ± 1.42	Mean ± SD, 2.34 ± 4.46

[a] $P < 0.05$ between the two sets of data.
[b] NA, not applicable.

Previous investigations already proved the high affinity of LVT for OTR (13, 14). We have confirmed such high affinity using the well-established experimental model of transfected kidney COS7 cells expressing human OTRs (22). Moreover, we demonstrated that LVT shares the same biological activity as OT on human and mouse breast cancer cells. In fact, LVT inhibited both MCF7 and TS/A cell proliferation. Such activity was not related to a toxic effect because it was not observed in OTR-negative HT29 colon cancer cells. Yibchok-Anun *et al.* (13) showed recently that substitution of the ϵ-NH_2 group of LVT with fluorescein did not affect its selectivity for pancreatic OTRs. Such findings are in line with molecular modeling and site-directed mutagenesis studies showing that the amino acid in position 8 plays a minor role in determining the affinity of OT to its receptor (22, 23).

Therefore, we pursued the conjugation of a chelating agent following the same protocol used successfully to produce a radioactive ligand for imaging and treatment of SSTR-positive tumors. Scintigraphy with the octapeptide analogue octreotide radiolabeled with ^{111}In by the chelation with diethylenetriaminepentaacetic acid is commercially available (OctreoScan) and widely used as a diagnostic tool to detect neuroendocrine and other SSTR-positive tumors (24, 25). However, diethylenetriaminepentaacetic acid-octreotide is not suitable for labeling with the therapeutic radionuclide ^{90}Y because of the poor *in vivo* stability of the complex (26). The high-energy and long-range β-particles emitted by ^{90}Y ($E_{\beta max} = 2.28$ MeV; range, 1.1×10^{-2} m) require a stable binding to reduce hematopoietic toxicity attributable to bone marrow irradiation caused by free ^{90}Y accumulating in the bone (27). Therefore, the macrocyclic chelator DOTA has been linked to the somatostatin analogue TOC. The new radiolabeled complex, ^{90}Y-labeled DOTA-TOC, has been used in several therapy trials for the treatment of patients with different SSTR-positive tumors, who have obtained symptomatic relief and objective therapeutic responses (28, 29).

Binding of DOTA to free NH_2 groups of LVT, located respectively at positions 1 and 8, generated two products that could be separated by chromatography. MALDI-TOF mass spectrometry of the two compounds showed that the product corresponding to peak A presented two DOTA/molecule of peptide, whereas peak B was determined by a product characterized by an intramolecular bridge of DOTA between positions 8 and 1 of the peptide. This intramolecular bridge is probably responsible for a major change in the conformation of the peptide ring, resulting in the complete loss of binding and biological activity. On the contrary, the presence of the double substitution in peak A at positions 1 and 8 may account for the 100-fold decreased affinity of peak A as well as for the loss of antiproliferative activity. In fact, whereas substitution in position 8 does not affect LVT affinity for OTR (12), a number of studies (reviewed in Ref. 11) indicated that substitutions of the β-carbon at position 1 of OT may severely impair its biological activity; in particular, a $d(CH_2)_5$ substitution at this position of OT resulted in a leading antagonist being used for the developments of highly potent and selective OTR antagonists (reviewed in Ref. 30).

Both of these two new reagents could be labeled efficiently with ^{111}In. However, as demonstrated by *in vitro* affinity tests on COS7 cells, only one of the two reagents (peak A) maintained a good affinity for the human OTR. This new reagent, thanks to the high chelating activity of DOTA, linked radioactive indium in a highly effective and stable way. Although *in vitro* and *in vivo* experiments demonstrated that ^{111}In-labeled DOTA-LVT binds selectively to OTR-positive cells and tumors, more experiments will be necessary to fully characterize the pharmacological properties of DOTA-LVT.

Expression of OTR and selective biological response to OT in MCF7 breast cancer cells had been demonstrated previously in our laboratory by immunocytochemical localization of the NH_2-terminal fraction of the receptor, by molecular biological demonstration of the specific mRNA, and by the *in vitro* evidence of cell growth inhibition (1). The TS/A mouse mammary carcinoma model was selected because, in addition to the above evidence, it offered the possibility of an *in vivo* study using BALB/c mice transplanted with the tumor growing s.c. In previous experiments, we proved that TS/A tumor growth is selectively inhibited by OT administration *in vivo* (5). The MOG-U-V-W glioblastoma cell line represents an interesting alternative model because it also shows OTR expression and OT-induced growth inhibition, as shown in previous experiments (3).

In *in vitro* experiments, ^{111}In-labeled DOTA-LVT specifically linked OTR-positive breast cancer and glioblastoma cells (TS/A, MCF7, and MOG-U-V-W cells) but not OTR-negative-HT29 colon cancer cells. The specificity of the ^{111}In-labeled DOTA-LVT binding was proved by displacement by both OT and LVT.

The DOTA-LVT compound lacked the antiproliferative effect of unbound LVT *in vitro*, suggesting that although the binding selectivity to OTR was maintained, its biological effect was lost. However, because DOTA optimally links ^{90}Y, future therapeutic applications can be envisaged using the DOTA-LVT compound as a specific carrier of the therapeutic agents to OTR-positive tumors.

In the *in vivo* experiments in BALB/c mice bearing the OTR-positive and SSTR2-negative TS/A tumors, we checked the selective binding distribution of ^{111}In-labeled DOTA-LVT. As an internal control, animals were injected simultaneously with a similar reagent, DOTA-TOC, a DOTA-conjugated analogue of somatostatin binding selectively to SSTR2. This latter reagent was labeled with ^{125}I. Using such double-labeling procedure, we were able to test both the distribution of our new reagent, ^{111}In-labeled DOTA-LVT, and its specificity for the OTR-positive tumors. The results indicate that ^{111}In radioactivity uptake, expressed as TS/A tumor:blood ratio, is significantly higher in comparison to the brain:blood ratios either at 3 or 24 h after injection. The higher uptake in TS/A tumor compared with brain, where OTRs are known to be expressed in several areas (31), is

probably attributable to the OTR overexpression in neoplastic cells. The numerical difference between tumor:blood ratios for ^{111}In and ^{125}I peptides at 24 h (7.24 ± 4.46 *versus* 2.74 ± 1.42) was not statistically significant, which was attributable either to the limited available data ($n = 5$/group) and/or to the high SD of the mean value of tumor:blood ratios of ^{111}In peptide.

In conclusion, the present study describes the development of ^{111}In-labeled DOTA-LVT, a potentially powerful reagent designed specifically to transfer radioactive agents to OTR-positive tumors. Although additional improvements in the conjugation step are warranted, our present results indicate that this new radiolabeled peptide shows prospects for imaging and biodistribution investigations (using gamma cameras and dose evaluations) on human malignant neoplasms expressing OTRs, such as breast and endometrial adenocarcinomas, glioblastomas, neuroblastomas, osteosarcomas, and choriocarcinomas (1–6, 8, 32).

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Note Added in Proof

To fully prevent DOTA binding to the $-NH_2$ residue in position 1, we are now employing a peptide having this residue protected. The alloc-blocking can be removed after DOTA labeling in position 8. Synthesis of a DOTA-LVT complex with a single DOTA linked to lysine in position 8 and keeping free the $-NH_2$ residue in position 1 will produce a complex having the highly sensitive 1–7 peptide sequence ready to bind with high affinity to the OTR docking site.

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