INVITED LECTURE

RADIOLABELED PEPTIDES: EXPERIMENTAL AND CLINICAL APPLICATIONS

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Abstract

Radiolabeled receptor specific biomolecules hold unlimited potential in nuclear medicine. During the past few years much attention has been drawn to the development radiolabeled peptides for a variety of diagnostic applications, as well as for therapy of malignant tumors. Although only one peptide, In-111-DTPA-(D)-Phe^1-octreotide, is available commercially for oncologic imaging, many more have been examined in humans with hematological disorders, and the early results appear to be promising. Impetus generated by these results have prompted investigators to label peptides with such radionuclides as Tc-99m, I-123, F-18, Cu-64, and Y-90. This review is intended to highlight the qualities of peptides, summarize the clinical results, and address some important issues associated with radiolabeling of highly potent peptides. While doing so, various methods of radiolabeling have been described, and their strengths and weaknesses have also been discussed.

Introduction

During the past few years much attention has been given to the diagnostic applications of radiolabeled peptides and significant progress has been made. While one peptide is available commercially for routine oncologic imaging, several others are either in clinical trials or being developed for different indications. It is generally considered that the database with radiolabeled peptides is not yet sufficient enough that it warrants their use for therapeutic applications. However, a few groups have taken the lead and initiated studies, the early results of which appear to be promising.

Receptor specific peptides plays an important role not only in the diagnostic and therapeutic applications of neoplastic diseases, but also in the pathogenesis of other diseases. The receptor specificity of peptides has therefore generated excitement in the field of nuclear medicine and hopes are high that investigations will lead to the development of many clinically useful radiopharmaceuticals. It must be

kept in mind, however, that not only are novel peptides discovered at a rapid rate, but also new interactions of previously known peptides are being discovered. This wealth of information has made it difficult to keep the knowledge updated and to make a choice of peptide for use in a chosen application.

This article is intended to describe characteristics of peptides, their advantages and disadvantages for clinical applications, and the challenges met in their development as radiopharmaceuticals.

Peptides

By definition any compound consisting of 100 or fewer amino acids is considered as a peptide. However, "Peptides," one of the eleven journals with the word peptides in the title tends to prefer manuscripts about peptides containing fewer than 50 amino acids (1). Consensus is less on the definition of "polypeptides." By an arbitrary definition a polypeptide consists of 100-200 amino acids. For the purpose of the journal "Peptides" compounds with chains of 200 or more amino acids are generally referred to as "proteins" (11).

There are more than 6000 known peptides. A peptide is customarily named by the function by which it was originally discovered. Some of them have drawn a lot more attention than others. One review article published in 1994 stated that 298,105 papers were published on 42 peptides since 1962, of which 6840 were on insulin alone (2). A few of these peptides are listed in Table 1. As to the peptides in radiopharmaceutical chemistry, N-For-met-Leu-Phe, also known as FMLP, was labeled with In-111 in my laboratory in 1981 (3). However, it was not until 1994 that a peptide-based radiopharmaceutical, In-111-DTPA-(D)-Phe^1-octreotide, was approved by the FDA for imaging tumors. Although a few more have been radiolabeled and injected into patients, the interaction of the nuclear medicine community with radiolabeled peptides is relatively new.

Most of the peptides are non-polar, hydrophobic, or polar and hydrophilic. Only three (Arg, His, Lys) amino acids are cationic and two (Asp, Gly) are anionic. The hydrophobic and hydrophilic forces are especially important for peptide-protein interactions, making some peptides more toxic than others (4).

Advantages and disadvantages of peptide-based radiopharmaceuticals:

Like monoclonal antibodies, peptides are also receptor specific. As radiopharmaceuticals they have several advantages over murine monoclonal antibodies. Most peptides are endogenous and

bioactive analogs or key amino acid sequences of large protein molecules. These are less likely to produce any antigenic reaction. Peptides are much smaller in size and generally have rapid blood clearance than monoclonal antibodies. Yet they can often obtain high concentration in target tissues, leading to high target tissue/blood radioactivity ratios. Peptides can also withstand harsher chemical conditions of pH and temperature, making radiolabeling parameters more flexible and less damaging to the biological activity of a peptide. Peptides are relatively less expensive and can be easily synthesized in a short period of time using a conventional peptide synthesizer.

Peptides can, however, induce pharmacologic effects in much smaller quantities, and can also be more susceptible to *in vivo* proteolysis by endogenous proteases than monoclonal antibodies. Most useful peptides are therefore modified or molecularly engineered so that their enzymatic destruction could either be slow or inhibited. Common approaches toward this goal have been to cyclize peptides constructing one or more dicysteine bridges and to substitute D-amino acids for L-amino acids, or both. However, when labeled with radionuclides, often peptides can and do fail to follow the biodistribution of their C-14 incorporated counterparts.

Radionuclides for peptides:

The peptide, octreotide, that sparked the interest in radiolabeled peptides in nuclear medicine is labeled with In-111. This radionuclide is a cyclotron produced, has a half-life of 67 hours and decays with the emission of two gamma rays (173 KeV -89% and 247 KeV -94%) and by electron capture. Although used widely in nuclear medicine In-111 is expensive, contributes to the cost of the radiopharmaceutical and adds to the inconvenience as it must be ordered well ahead of time of its use.

The faster blood clearance of peptides justifies the use of shorter lived radionuclides such as Tc-99m. With a six hour half-life, 140 KeV (90%) gamma ray emission and universal availability on a generator, Tc-99m is the most commonly used radionuclide in diagnostic imaging. Efforts are therefore plentiful in developing Tc-99m labeled peptides for diagnostic imaging. In addition to this, peptides have been labeled with positron emitting radionuclides, as well as with radionuclides of therapeutic importance. These are given in Table 2 and some of the radiolabeled peptides for their proposed applications in Table 3.

Table 1

Some biologically active peptides with their primary function and receptor specificity

Peptide	Primary Function
ACTH (adrenocorticotrop (h) in)	Hormone of the pituitary gland (SH mutates glucocorticoid production)
Bombesin	Active in CNS GI tract. Suppresses feeding in rats. BN and GRP receptor specificity.
BNP (brain natriuretic peptides)	BNP receptors exist in human cardiac tissue.
Calcitocin	CRF receptors
CCK (cholecystokinin)	CCK receptors, homology to VIP and PACAP receptors. CCK receptors expressed on SCL and ovarian cancer.
GRP (gastrin releasing peptide)	GRP/neuromedin B (NMB) receptors
Glucogen	Specific for receptors expressed by endocrine pancreatic B cells.
α-MSH (α-melanocyte stimulating hormone)	Regulation of skin pigment, α-MSH receptors expressed on human melanoma cells.
Oxytocin	Uterus contracting and lactation stimulating hormone. Crosses BBB.
PACAP (pituitary adenylate cyclase activating peptide)	Includes secretin, glucogon, calcitonin, parathyroid hormone. PACAP I & II/VIP receptors are expressed on a variety of human tumors.
Somatostatin analogs	Somatostatin receptors I-V. Growth hormone release inhibiting factor. Receptors are expressed on a variety of tumors.
Vasopressin	Antidiuretic hormone. Hepatic V_1a receptor specific.
VIP (Vasoactive Intestinal Peptide)	Is involved in a wide range of biological activities. VIP I and VIP II receptors are expressed in high density on various types of tumors.
Neurotensin	Receptors are expressed on SCL carcinoma, H colon carcinoma and H-meningioma.

Table 2

Radionuclides labeled to peptides for SPECT, PET, and Therapeutic Applications

Planar or SPECT Imaging	Reference
In-111: t½ -67 hrs, γ-173 KeV (89%), 247 KeV (94%)	5-7
Tc-99m: t½ -6 hrs, γ-140 KeV (90%)	8-12
I-123: t½ 13.3 hrs, γ-159 KeV (83%)	13-15
Ga-67: t½ 78 hrs, γ-93 KeV (40%), 184 KeV (24%), 296 KeV(22%)	16
PET Imaging	
F-18: t½ 110 mins, β^+ -(97%)	16-17
Cu-64: t½ 12.8 hrs, β^+ -(19%)	18
Y-86, t½-14.6 hr, β^+ -(26%)	16
Therapeutic Applications	
Y-90: t½- 2.66 d, β^-, 2.27 MeV (100%)	19
Re-188: t½ -16.7 hrs, β^--2.12 MeV (100%),-155 KeV (10%)	20,21
In-111: t½ -67 hrs, 173 KeV (89%), 247 KeV (94%)	22
I-131: t½-8.3 d, β^- -806 KeV (max), γ-364 KeV (82%)	23

Radiolabeling Peptides:

Peptides have been labeled with radionuclides by using four different methods which are briefly discussed below. In general, peptides can be modified for radiolabeling without affecting their biological characteristics. However, precautions should be taken that the sequence of certain groups of functional amino acids involved in receptor specificity is neither altered, nor should the chelating agents added exert steric hindrance that would alter the biological activity of the peptide. Most peptides have been modified at the N terminus to facilitate chelation of radionuclide, but in certain peptides, such as VIP, modifications at N-terminal H^1 can significantly alter its biological activity (1,30,31). Since conjugation of most bifunctional chelating agents is facilitated by formation of an amide bond, ε amino groups where such conjugation is undesirable are protected during the synthetic procedure and then deprotected. Steric hindrance created by an added bulky group can also effect the biological activity of a peptide.

Table 3

Radiolabeled Peptides Evaluated for Various Applications

Tumor Imaging	Reference	Tumor Therapy	Reference
In-111-DTPA-(D)-Phe^1-Octreotide**	5	In-111-DTPA-(D)-Phe^1-Octreotide*	22
I-123-(3)-Tyr-Octreotide*	14	Y-90-DOTA-(D)-Phe-Octreotide*	19,22
I-123-VIP*	13	Re-188-RC-160	20,21
Tc-99m-P-829*	10		
Tc-99m-RC-160	11		
F-18-Octreotide	17		
Cu-64-Octreotide	18		
Imaging Vascular Thrombosis		**Imaging Infection**	
Tc-99m-P280*	24	In-N-for-MLF (FMLP)	3
Tc-99m-Bitistatin	25	Tc-99m-Nor-for-MLFK	28
Tc-99m-DMP-444*	26	Tc-99m-Tuftsin*	29
Tc-99m-CSVTCG	27		

** Approved by FDA in 1994
* Evaluated in humans

Subsequent to such a modification and radiolabeling net charge on peptide can also alter. In general, lipophilic peptides are sequestered by the liver and can clear by hepatobiliary excretion whereas hydrophilic peptides are usually removed rapidly from plasma by glomerular filtration in the kidneys Indium-111-DTPA-Phe^1-Octreotide clears primarily through the kidneys whereas I-123-Tyr^3-Octreotide has predominant hepatobiliary excretion (14). Furthermore, Breeman et al (32) have reported that the uptake of radioactivity in somatostatin receptor positive tissues is a bell-shaped function of the injected mass of the peptide. Tissue distribution of a given peptide may vary depending upon the nature of a radionuclide used for radiolabeling and upon the animal species used. While approaching for radiolabeling and biological evaluation of a peptide, all these parameters must be carefully considered.

Radioiodination of peptides:

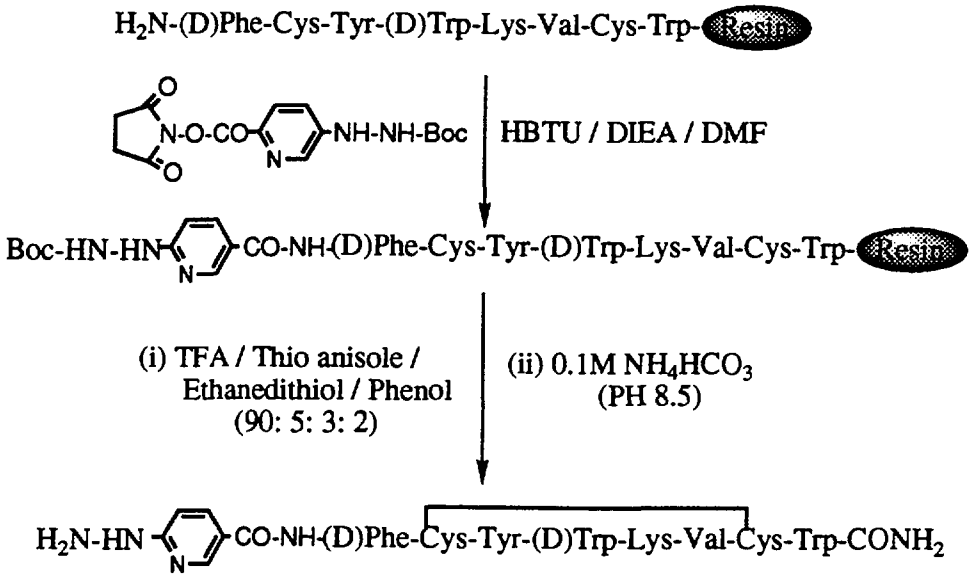
Radioiodination of a peptide is exclusively carried out at the aromatic amino acid Tyr. Peptides such as VIP (Tyr^{10} and Tyr^{22}) and RC-160 (Tyr^{3}) which contain Tyr have been radioiodinated without any structural modifications of the native peptide. In order to facilitate radioiodination in other peptides such as Sandostatin, tyrosine has been added in place of Phe^{3} which is considered to be the amino acid not important for its biological activity. For radioiodination most investigators use iodogen as oxidizing agent (13) although chloramine-T has also been successfully used (11). In order to prevent oxidation of other amino acids, long incubation times of the reaction mixtures containing oxidizing agents have been avoided and mono and diiodo species formed have been separated using reverse phase HPLC.

Direct labeling:

This method is limited to labeling peptides only with Tc-99m or Rhenium radionuclides and is applicable to radiolabeling of only those peptides which are cyclic (11,12,20,21). In this method, the dicystein bond is reduced either with sodium ascorbate or stannous chloride. The sulfhydryls resulted by the reduction serve as a strong chelating groups for reduced Tc-99m, or rhenium radionuclides. The method is simple, generally efficient, does not require conjugation of bifunctional chelating agents, blocking or deblocking of functional groups and the subsequent purification and characterization of the required product. Biological activity of the peptides labeled by this method is not altered (11,12) and the spatial topography of the side chains essential for somatostatin receptor binding is maintained (33). This method, however, cannot be used for peptides that are not cyclic.

Bifunctional chelating agent (BFCA) method:

This is the most common method applied for radiolabeling of peptides. It has been used to label peptides with all radionuclides given in Table 2, except F-18 and Iodine-123. A large number of chelating agents have been synthesized and covalently linked to the N terminus of a chosen peptide. The conjugation reaction can be performed in liquid or in solid phase using resin on which the peptide is synthesized. In solution phase the length of the process is compounded by protecting side chain groups either by Boc (t-butyloxycarbonyl) or Fmoc (9-fluorenylmethyloxycarbonyl). Conjugation of the BFCA on resin on which a given peptide synthesized is facilitated by pre-protected side chain groups. Furthermore, by using this procedure, deblocking of the peptide side chains and removal of the peptide



HYNIC-RC-160

Fig. 1 Schematic presentation for the preparation of HYNIC-RC-160 (11,12).

from the resin is achieved simultaneously. In solid phase synthesis removal of solvents and impurities also becomes less cumbersome. The process on resin is generally considered to be more efficient than that in the liquid phase. A typical procedure in which RC-160, a somatostatin analog (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp) also known as vapreotide, was conjugated with Hynic (hydrozinonicotinamide) is briefly described below and schematically presented in Fig. 1.

RC-160 was synthesized commercially and obtained as its carboxamide on 5-[4-(9-fluorenylmethoxy carbonyl) (amino methyl)-3,4 dimethoxy phenyl] valeric acid (PAL) resin (Lysine residues blocked). A five fold molar excess BFCA, BOCHYNIC (6-Boc- hydrazinopyridine-3-carboxylate) was activated with 2 (1H-benzotriazol-1-yl) 1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU) for 20 minutes, added to the resin in dimethylformomide (DMF) and allowed to react at 22°C for three hours. Using a sintered funnel, the resin was washed with DMF, followed by dicholoromethane and vacuum dried. The peptide was cleaved from the resin and deblocked with 95% TFA, diethylether was added and the mixture was cooled at –20°C for 4-5 hrs. The resultant precipitate was dissolved in 0.1M ammonium bicarbonate solution (pH-8.5) and stored overnight for cyclization. The reaction mixture was then separated on a preparative HPLC using Rainin's Dynamax

(2.14 x 41.4 cm) column and a gradient solvent consisting of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B). The flow rate was 6 ml/min and Solvent B increased from 50% at 1 minute to 90% at 45 min. Fractions were collected, lyophilized and analyzed using matrix assisted laser desorption ionization (MALDI) mass spectrometer. The fraction with the expected molecular weight was then chosen for radiolabeling. Thus, even in solid phase this method is long, laborious, and can be result in poor yield.

Hybrid peptide method:

Hybrid peptide procedure, developed in our laboratory for chelation of Tc-99m and possibly for other radionuclides such as Re-186, Re-188, and Y-90 is a simple and yet efficient one step method. In this method, the chelating moiety consists of a group of peptides itself which is synthesized along with the 0synthesis of the primary peptide. This eliminates the number of secondary synthetic steps required for synthesis and purification of a BFCA and its conjugation to the peptide. For validation of this concept we have synthesized several peptides ranging from 4 to 28 amino acids using Gly-(D)-Ala-Gly-Gly as a chelating moiety which provides N_4 configuration for chelation of radionuclides. The primary peptide can be extended with the required chelating moiety either at N or C terminus. Although we have not yet studied the stability constants of these metal chelates, our experimental data indicate that the complex is stable when challenged with such strong metal chelating agents as human serum albumin, cysteine, and DTPA (34). In order to avoid steric hindrance due to the bulky metal chelate, we have inserted a spacer molecule, α-amino butyric acid, between the primary peptide and the chelating moiety. Our data, *in vitro* and in experimental animals, have indicated that the biological activity of hybrid peptides resembles that of the primary peptide.

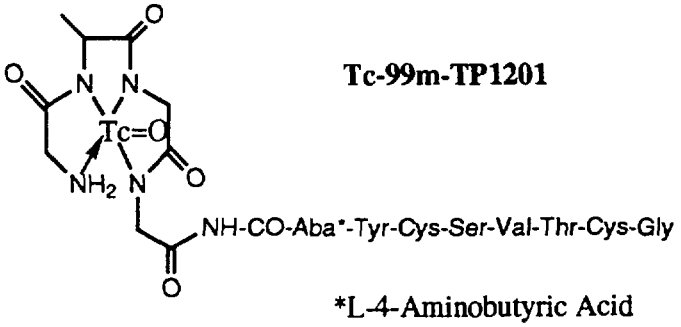
The advantage of this method is that it eliminates the multiple steps for synthesizing chelating agents and their conjugation to the peptide which, as described previously, is time consuming and inefficient. With the hybrid method, a usable final product is achieved without additional efforts, cost and compromising the yield. This method also allows one to choose a group of amino acids as a chelating agent as may be required for a chelation of a given radionuclide. This also allows one to add peptides of choice that may alter net charges on the peptide, which may change their pharmacokinetics, as shown by Lister-James et al (10). Additionally, this method is applicable to label straight chain or cyclic peptides.

In our laboratory the hybrid peptides have been formulated into a simple kit for instant labeling with Tc-99m. A small (μg) quantity of a peptide is lyophilized with a required quantity of $SnCl_2$ in phosphate buffer solution at pH-12 and stored at -20°C. At the time of preparation sodium pertechnetate (Tc-99m-O_4) solution is added to the vial and after incubation at 22°C or 100°C as the case may be, pH of the solution is adjusted to pH-6.5 with the addition of precalculated quantity of phosphate buffer, pH-4. The radiolabeling yield is quantitative and radiochemical purity of the preparation is confirmed by analytical HPLC.

A proposed structure of one such hybrid peptide, Tc-99m-TP1201, is given in Fig. 2. This peptide is being developed in our laboratory for imaging vascular thrombosis.

Experimental and Diagnostic Applications:

Routine applications of radiolabeled peptides are centered around oncologic imaging with In-111-DTPA-(D)-Phe[1]-octreotide (5). Given in Table 4 are some early results of the efficacy of In-111-DTPA-(D)-Phe[1] scintigraphy as compared to the somatostatin receptor expression determined by autoradiography (36). The high sensitivity of detection and the excellent correlation with the *in vitro* data for most tumors is impressive. However, not so well detected with In-111-DTPA-(D)-Phe[1]-octreotide scintigraphy appear to be the tumors of the breast, pituitary glands, thyroid, and pancreas and astrocytoma. In Table 5 are compared the scintigraphic results of I-123-MIBG and In-111-DTPA-(D)-Phe[1]-octreotide (36), which depict the excellent results of In-111-(D)-Phe[1]-octreotide. In addition to In-111-DTPA-(D)-Phe[1]-octreotide, a large number of other radiolabeled peptides have also been evaluated



Tc-99m-TP1201

*L-4-Aminobutyric Acid

Fig. 2 Proposed structure of Tc-99m labeled Gly-(D)-Ala-Gly-Gly-Aba-Tyr-Cys-Ser-Val-Thr-Cs-Gly; (TP-1201) (27)

Table 4

Results of In-111-DTPA-(D)-Phe^1-Octreotide scintigraphy in patients with malignant tumors compared to the expression of somatostatin receptors as determined by autoradiography (36)

	In vivo	Scintigraphy	In vitro	Receptor status
GH-producing pituitary tumor	7/10	70%	45/46	98%
TSH-producing pituitary tumor	2/2	100%	—	—
Non-functioning pituitary tumor	12/16	75%	12/22	55%
Gastrinoma	12/12	100%	6/6	100%
Insulinoma	14/23	61%	8/11	72%
Glucogonoma	3/3	100%	2/2	100%
Unclassified APUDoma	16/18	89%	4/4	100%
Paraganglioma	33/33	100%	11/12	92%
Medullary thyroid carcinoma	20/28	71%	10/26	38%
Neuroblastoma	8/9	89%	15/23	65%
Phaeochromocytoma	12/14	86%	38/52	73%
Carcinoid	69/72	96%	54/62	88%
Small cell lung cancer	34/34	100%	4/7	57%
Non-small cell lung cancer	36/36	100%	0/17	9%
Meningiomas	14/14	100%	54/55	98%
Breast cancer	37/50	74%	33/72	46%
Exocrine pancreatic tumors	0/24	9%	0/12	0%
Astrocytoma	4/6	67%	14/17	82%
Non-Hodgkin's lymphoma	59/74	80%	26/30	87%
Hodgkin's disease	23/24	96%	2/2	100%
Sarcoidosis	23/23	100%	3/3	100%
Wegener's granulomatosis	4/4	100%	—	—
Tuberculosis	6/6	100%	2/2	100%
Grave's disease: thyroid	9	[a]	1	—
Grave's opthalmopathy	25	[b]	—	—

Table 5

Comparison of results with In-111-DTPA-(D)-Phe^1-Octreotide and I-123-MIBG scintigraphy in patients with comparable tumors (36)

Tumor type	Percentage of positive scans (number)	
	In-111-Octreotide	I-123-MIBG
Phaeochromocytoma	86% (14)	88% (> 1000)
Neuroblastoma	89% (9)	91% (841)
Paraganglioma	100% (33)	52% (25)
Medullary thyroid carcinoma	71% (28)	35% (178)
Carcinoid	96% (72)	70% (237)
Endocrine pancreatic tumor	80% (56)	25% (4)

in humans. These include I-123-VIP for imaging VIP receptor specific tumors (13), Tc-99m-DMP-444 and Tc-99m-P-280 for imaging DVT (24,26), Tc-99m-P-748, and I-123(Tyr^3)-octreotide for imaging tumors (14,35)and Y-90-DTPA-octreotide for treating tumors (19,22). Generally the absolute uptake of radiolabeled peptides in a given lesion is small, but the rapid blood clearance of agents renders target tissue/blood ratio high, leading to a high degree of success in clinical results. Impetus generated by these observations has led many investigators to label a variety of peptides with Tc-99m and evaluate them in experimental animals. Many of these are labeled with Tc-99m and also include agents for imaging infection and vascular thrombosis as shown in Table 3.

Therapeutic Applications:

In-111-DTPA-(D)-Phe^1-octreotide and Y-90-DOTA-octreotide, are in the early stages of their use in tumor therapy. Although In-111 is a gamma emitting radionuclide (Table 2), it decays with the emission of conversion electrons and x-rays that form the basis for its application in therapy. It has been estimated that the radiation dose to a cell with 10 μm diameter with a decay of a single In-111 atom is 0.135 rad (37). Up to 500 mCi of In-111-DTPA-Phe^1-Octreotide has therefore been used as a single therapeutic dose. Results of any systematic clinical trials are unknown at the time of this writing but general comments on the outcome of results are encouraging.

Lately Otte et al and Krenning et al (19,22) have used the β^- emitting Y-90 (Table 3) as a tracer for similar applications. Octreotide is also labeled with Y-90 using DOTA (1,4,7,10-tetraazacyclododecane-N,N', N', N", N"'-tetra acetic acid) as a chelating agent conjugated at D-Phe[1] and the early results in the first few patients are encouraging (38).

In summary, in a relatively short period of time, the progress made with radiolabeled peptides is impressive. It ranges from the development of a wide variety of techniques to radiolabel peptides to evaluate them for their potential in significant clinical and experimental applications. The glimpses of the subject provided here may neither do justice to the capacious volume of literature existing on the subject, nor may it provide a vision to its reader. We wish to remind the readers, however, that with the space limitation, this article was intended only to serve as a brief introductory review and we hope that it serves the purpose.

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