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Development and evaluation of derivatives of Tyr^3-octreotate *in vitro* and *in vivo*

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Radiolabelled receptor-binding peptides have emerged as an important class of radiopharmaceutical that promises to dramatically change the field of nuclear medicine. Binding to the receptor on the target cell surface generally triggers the signal transduction mechanism of the target cell, and, thus, the biological effect of the ligand is transmitted to the target tissue. The specific receptor-binding property of the ligand can be exploited by labelling the ligand with a radionuclide and using the radiolabelled ligand as a radiopharmaceutical to image or treat tissues expressing a particular receptor. Theoretically, the high affinity of the ligand for the receptor facilitates retention of the radio-labelled ligand in receptor-expressing tissues, whereas its relative small size facilitates rapid clearance from the blood and other non-target tissues.

Somatostatin, a regulatory neuropeptide, widely exists in the central nervous system (CNS) and peripheral tissues, including pituitary, cerebral cortex, pancreas, adrenals, kidney and immune cells. The biological effects of SST are mediated by the five somatostatin receptor

subtypes (SSTR1-5). The subtype 2 (SSTR2) has been identified as the predominant expression in brain, neuroendocrine and kidney tumours as well as in human angiogenic blood vessels. Due to the short half-life of the native SST, the clinical application of SST was hampered. Thus, many SST analogs have been developed.

These SST derivatives are the prototype of receptor-binding peptides that allow the visualisation of receptor-expressing tissues non-invasively: SSTR-target radionuclide imaging and for the eradication of receptor-expressing tissues: SSTR-target radionuclide therapy (SRRT). Radionuclides labelled octreotide (OC) and its derivatives have been widely used to visualise SSTR-positive tumours (planar scintigraphy, SPECT and PET) and in SRRT. Studies have shown that radiolabelled OC derivatives are internalised by SSTR-positive tumour cells *in vitro*. This is the basis of SRRT with radionuclide labelled SST analogs. The substitutions of Phe with Tyr at the 3-position and Thr(ol) with Thr at the C-terminal of OC (now termed as Tyr^3-octreotate, TATE) have been shown to improve receptor affinities, enhance internalisation and uptake in SSTR-positive tissues and tumours. However, previous studies have shown that the major drawback of these analogs is the prolonged retention and increased uptake in the kidney. Much work was focused on the development of N-terminally modified SST analogs, however, little has been done to develop new analogs modified at the C-terminus of TATE.

In order to study the influence of different substituents on the receptor affinities, a series of novel homologous derivatives of TATE modified at the N-terminus or the C-terminus were investigated. Receptor affinities of these novel derivatives were determined by competition experiments with ^{125}I-Tyr^3-octreotide (^{125}I-TOC) on rat cortex membranes. Saturation binding experiments were conducted with one of the ^{125}I-labelled hydrophilic derivatives (compound 2, ^{125}I-2) compared to ^{125}I-TATE on rat cortex membranes and SSTR-positive rat pancreatic CA20948 tumour membranes. As indicated by the IC_{50} values on cortex membranes, neither lipophilisation nor hydrophilisation of the N-terminus of Tyr^3-octreotate seems to enhance the binding to the somatostatin receptors. Most of the C-terminally modified TATE derivatives showed unexpected receptor affinities. The Scatchard analysis showed that significantly increased B_{max} of compound 2 were observed in both membranes when compared to TATE. Compound 2 also showed improved receptor affinity (K_d) on CA20948 tumour membranes. This may be due to a different receptor subtype pattern in this tumour membranes as compared to the rat cortex membranes.

To investigate the influence of different substituents on the stability, the metabolic stability assays were performed in human serum or proteinase K at 37°C. The hydrophilic derivative

(compound 2) showed strikingly stable in both human serum and proteinase K even after 24 hours or 48 hours.

To assay the possibilities of these new derivatives into SSTR-positive tumour cells, the internalisation and externalisation experiments were conducted in AR42J cells at 37°C. SSTR-negative rat Morris Hepatic tumour MH3924A cells were used as negative control. All of the tested derivatives showed specific internalisation in AR42J cells at 37°C. The modification at the N-terminus and the C-terminus significantly influenced the externalisation rates. Compound 2 showed the highest internalisation rate, however, it showed more rapid externalisation than the other radioligands.

In order to study the potential of the intercalating (Hoechst) derivatives to bind the DNA, fluorescence microcopy experiments were performed in AR42J cells and MH3924A cells with compounds of Hoechst-coupled to TATE (compounds 42 and 44). The fluorescent TATE derived probe (BB-125) was used as positive control. DAPI, propidium iodide (PI) and Hoechst 33528 were used as positive controls for DNA staining of the cells. All of the tested compounds showed blue fluorescence labelling in the periphery of AR42J cells. No fluorescence labelling was observed in MH3924A cells. These findings further demonstrate that the TATE derivatives are specifically internalised into SSTR-positive tumour cells and the internalisation was mediated by the SSTRs.

To investigate the improvement of tissue uptake and the elimination *in vivo*, a pharmacokinetic study of ^{125}I-2 was conducted in normal COP rats. ^{125}I-2 showed excellent pharmacokinetic properties. The elimination half-life in circulation was 137 min. High uptake in SSTR-rich tissues, e.g. pancreas, adrenals and GI was observed, and low uptake in the kidney and liver.

Previous studies had proven that radioiodine labelled HIPDM (N,N,N'-trimethyl-N'-(2-hydroxy-3-methyl-5-iodobenzyl)-1,3-propanediamine) showed a clinical potential as a pancreas imaging agent. In order to compare the uptake of the tested compounds in the pancreas, the comparison study was performed with ^{125}I-2 and ^{131}I-HIPDM in COP rats 1 hour after *i.v.* injection. ^{125}I-2 showed strikingly higher uptake in pancreas and significantly lower uptake in liver, kidney and lung than ^{131}I-HIPDM. A higher target-to-non-target ratios were also observed when compared to ^{131}I-HIPDM.

The tumour uptake experiments were performed in Lewis rats bearing CA20948 tumours with ^{125}I-labelled selected derivatives. ^{125}I-TOC and ^{125}I-TATE were used as positive controls. All of the tested N-terminal derivatives showed a high uptake in the pancreas and tumours. A low uptake in the liver and kidney of these derivatives was also observed. However, the ^{125}I-

labelled C-terminal derivatives showed significantly lower uptake in SSTR-positive tissues and tumours than ^{125}I-TOC and ^{125}I-TATE. The competition tumour uptake experiment indicates that the uptake of these ^{125}I-labelled selected derivatives was specific in CA20948 tumour *in vivo*.

PET imaging studies were conducted in the same tumour model with ^{68}Ga-labelled DOTA-coupled TATE derivatives: compounds 32 (C-terminally modified derivative) and compound 44 (N-terminally modified derivative). ^{68}Ga-DOTATOC was used as control. High tumour uptake was observed with both compounds. Comparatively uptake of the tested derivatives was observed in liver and kidney.

In conclusion, the tumour uptake is not correlated with the receptor affinity. These findings reveal that the receptor affinity is not the predominant factor controlling accumulation in the tumour. The tumour uptake of the tested compounds are positioned within the range of TATE and TOC. However, the receptor affinity does not strongly vary within this group of derivatives investigated. The results simply reflect that the organ distribution is dominated by the nature of the substituent with respect to pharmacokinetic behaviour rather than to receptor affinity.

It can also be concluded that the N-terminus substituents do not significantly affect the pharmacological properties of the parent TATE, while the C-terminus substituents seems to significantly influence the pharmacological properties. These findings imply that the nature of the C-terminal residue is the key determinant for the receptor binding affinity as well as the internalisation *in vitro* and uptake in SSTR-positive tissues *in vivo*. Therefore, the N-terminal modification of peptides might be a better site for the improvement of drug targeting agents based on receptor-affine peptide carriers.