APPLICATIONS OF NANOPARTICLES IN NUCLEAR MEDICINE

PhD Thesis

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Summary

Detection of metastatic involvement of sentinel lymph nodes is one of the most common applications of colloid based radiotracers in nuclear medicine imaging. The first part of the dissertation focuses on lymphoscintigraphical investigations. Lymphoscintigraphical findings of 128 breast cancer patiens were compared with the results of intraoperative surgical gamma detection probe and blue dye mapping, and with results of 24 dogs having spontaneously occurred tumour, respectively. The injected radiocolloid was the ^{99m}Tc-labelled HSA-based Senti-Scint® product in the size range between 100 and 600 nm. Results confirmed that the proper particle size distribution of the injected radiocolloid has primary importance in the detection of sentinel lymph nodes. Results also showed that the parallel, combined detection methods, including gamma camera imaging, intraoperative radio-guided surgery and the blue dye method are superior for the localization of sentinel lymph nodes.

The thesis also presents different application of HSA based colloids. In the presented investigations a widely used antineoplastic agent, doxorubicin was loaded into different sized HSA based nanoparticles and microparticles and then particulate systems were labelled with ^{99m}Tc. Physicochemical, colloidal and radiochemical stability of ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 radiocolloids was examined with biological tests to determine the size-dependent different pharmacokinetic values. *In vitro* and *in vivo* examinations verified that colloid carriers have a high degree of stability and proper distributions according to their particle size. Therefore, our investigations verified that different and stable particle sizes make drug carrier

HSA nanoparticles possible to carry out different drug targeting in a potential clinical use together with a probably lower cardiotoxicity.

The next part of the dissertation presents the first SPECT and SPECT/CT try-out of a new folate-targeted, self-assembled nanoparticle product. In the experiments the novel nanosystem was first radiolabelled with ^{99m}Tc, and then we examined the stability of the compound, and the possibility of applying the ^{99m}Tc-BBS-NP product as a new potential imaging agent for imaging folate-receptor-overexpressing tumours. Investigations verified that the new targeted contrast agent shows higher tumour uptake, therefore the new targeted nanoparticles are able to detect folate-receptor-overexpressing tumours with appropriate sensitivity and selectivity.

Results showed that the properly selected and designed particle size distribution of the used radiocolloid has key importance in the detection of sentinel lymph node and in the preparation of new diagnostic and therapeutic particulate systems and theranostics. Besides, we were able to demonstrate the importance of companion animal models in the research of new and already used methods (e.g. SLN detection) of comparative oncology and pathology, as we used spontaneously diseased animals, as parallel to the human cases. Moreover, results validated that imaging methods of nuclear medicine could be important parts of the development of new nanotechnology based pharmaceuticals (such as novel theranostics and drug delivery systems) in their quality control and biological follow-up.

Objectives

We aimed to produce new potential diagnostic radiopharmaceutical compounds and verify the importance of suitable and stable particle size followed by examples of colloid based radiopharmaceuticals and drug delivery systems. Furthermore, we aimed to use the new colloid-based radiotracers in comparative oncology studies of spontaneously diseased animals. Besides, we aimed to assess that imaging procedures of nuclear medicine can be important parts of the development of new pharmaceuticals based on nanotechnology (theranostics and drug delivery systems). Detailed objectives of the dissertation were:

1. Preparations, radiolabeling

- to prepare a new self-assembled, folate-receptor targeted nanoparticles and radiolabel the new nanosystem with ^{99m}Tc isotope – production of the ^{99m}Tc-BBS-NP radiotracer.

- to prepare human serum albumin (HSA) based, doxorubicin-loaded nanoparticles and microparticles with different particle size and radiolabel the new compounds with ^{99m}Tc isotope – production of ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430, ^{99m} and Tc-DoxHSA1800 compounds.

- to radiolabel Senti-Scint® HSA nanocolloid with ^{99m}Tc isotope

2. Characterizations, *in vitro* follow-up

- to investigate doxorubucin-binding efficiency of ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 compounds.

- to investigate labeling efficiency of ^{99m}Tc-labeled Senti-Scint®, ^{99m}Tc-BBS-NP, ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800.

- to examine the particles size distributions (and morphology) of the products.

- to investigate the cellular uptake of ^{99m}Tc-BBS-NP nanoparticles in folate-receptor overexpressing tumor cell line.

3. Applications*, in vivo* follow-up

- to investigate the labeled ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 compounds using healthy animal model.

- to investigate the labeled ^{99m}Tc-BBS-NP nanoparticles using folate-receptor overexpressing tumor transplanted animal model.

- to investigate the labeled ^{99m}Tc-BBS-NP nanoparticles in a dog with spontaneous occurred, folate-receptor overexpressing tumor.

- to perform sentinel lymph node investigations in spontaneously diseased dogs using ^{99m}Tc-labeled Senti-Scint® nanocolloid.

- to perform sentinel lymph node investigations in breast cancer patients using ^{99m}Tc-labeled Senti-Scint® nanocolloid.

Methods

1. Preparations, radiolabeling

To prepare ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 compounds, doxorubicin was adsorbed to human serum albumin **(**HSA) with stirring for 2h in aqueous media at different pH setting. For desolvation method ethanol was added to HSA with a calibrated laminar pump. Crosslinking method was accomplished with glutaraldehyde. Three different particle sized sample was separated for the further examinations and named to ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800. For labeling, tin-chloride ($SnCl_2$) was used as reducing agent. Preparation of "BBS-NP" nanoparticles was performed in laboratories of BBS Nanorechnology Ltd., Debrecen, HU. Stable self-assembled polyelectrolyte nanoparticles were developed via an ionotropic gelation process between the folated poly-gamma-glutamic acid (γ-PGA) conjugates and chitosan linear chains. Folic acid was bound to the γ-PGA previously. For labeling, $SnCl_2$ as reducing agent was added to the reaction mixtures, then generator-eluted $^{99m}TcO_4$- (aqueous pertechnetate solution) was added to the solvents. Labeling method of ^{99m}Tc-BBS-NP nanosystem was performed at room temperature. The Senti-Scint® nanocolloid was produced in the laboratories of Medi-Radiopharma Ltd., Budapest, HU. The labeling procedures were carried out according to the manufacturer's instructions. For labeling, generator-eluted ^{99m}Tc-pertechnetate between 600–1100 MBq activity was added to the freeze-dried form nanocolloids. Labeling was performed with 20 minutes incubation at room temperature.

2. Characterizations, *in vitro* follow-up

Non-adsorbed doxorubicin ratios of the ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 compounds were determined by HPLC after centrifugation in 16000 g of prepared particulates. Radiochemical purity (labeling efficiency) of ^{99m}Tc-labeled Senti-Scint®, ^{99m}Tc-BBS-NP, ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 was examined by means of thin layer chromatography (ITLC-SG) using ITLC-scanner. Plates were developed in methyl ethyl ketone (MEK). Particle sizes were measured with dynamic light scattering (DLS) using Dynapro instrument, (Proteinsolutions Inc., VA, USA) and Malvern Zetasizer Nano ZS instrument. Sizes and morphology of particles of sample ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 were validated by means of transmission electron-microscope (TEM) examinations. Cellular uptake of folate-targeted nanoparticles was observed in cultured rat hepatocellular carcinoma (HeDe) cells overexpressing folate-receptors by using confocal microscopy (Olympus FluoView 1000 confocal microscope).

3. Biological applications, *in vivo* follow-up

Laboratory animals were kept and treated in compliance with all applicable sections of the hungarian laws and regulations of the European Union. Biodistribution of different-sized, radiolabelled, doxorubicin-loaded ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 colloids were determined by means of scintigraphic imaging studies in healthy male Wistar rats using Nucline X-ring (Mediso) SPECT gamma camera. Images were

taken by gamma camera at several times (5, 15, 30, 60 minutes and 2, 8, 22 hours post inj.) and critical organ uptakes were estimated by quantitative ROI analysis. For *in vivo* studies of ^{99m}Tc-BBS-NP, folate-receptor overexpressing rat hepatocellular carcinoma (HeDe) was used as model system. HeDe tumor-transplanted animals were adult male Fischer 344 rats using subrenal capsule assay (SRCA). ^{99m}Tc-DMSA radiopharmaceutical was injected to the control group of animals. A spontaneous diseased dog, a male Dachshund with a known (visible, palpable) oral tumor was studied with the new ^{99m}Tc-BBS-NP nanoparticles. We administered ^{99m}Tc-BBS-NP by intravenous injection into the animal patient. Ventrodorsal and right lateral whole body scans were taken and 2 hours after injection a 3D SPECT examination. Sentinel lymph node detection was investigated in 24 client-owned dogs with spontaneously occurring tumors. A multiple method was used with a nuclear medicine technique (injection of low dose of ^{99m}Tc-HSA colloid, Senti-Scint®) with scintigraphy and intraoperative guidance, and blue dye injection. Detection rate of sentinel lymph nodes in breast cancer was evaluated prospectively in 128 consecutive breast cancer patients without palpable lymph nodes. Pre-operative static lymphoscintigraphic mapping of the breast was performed after subcutaneous injection of 15 MBq of the large (100–600 nm) HSA-based radiocolloid (Senti-Scint®). Lymphoscintigrahic results were compared with intraoperative surgical gamma detection probe and blue dye mapping data.

Results

1. Preparations, radiolabeling

- Preparation of ^{99m}Tc-BBS-NP radiotracer: a new, folate-receptor overexpressing tumor targeted, stable nanosystem were prepared. Nanoparticles with a hydrodynamic size of 124 nm were prepared by self-assembly of chitosan and folated poly-γ-glutamic acid, and then first time radiolabeled ^{99m}Tc successfully.

- Preparation of ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 compounds: preparation methods and investigations of human serum albumin (HSA) based, doxorubicin-loaded nanoparticles and microparticles with different particle size was presented in the dissertation and ^{99m}Tc-radiolabeling methods of new compounds was also presented.

- Radiolabeling of Senti-Scint®: successful radiolabeling method with ^{99m}Tc isotope was presented.

2. Results of characterizations, *in vitro* follow-up

- Non-adsorbed doxorubicin ratios of the ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 compounds were determined by HPLC after centrifugation in 16000 g of prepared particulates. Measurements validated that more than 95% of doxorubicin proportion was permanently adsorbed to human serum albumin matrix.

- Radiochemical purity (labeling efficiency) of compounds was investigated by means of thin layer chromatography (ITLC-SG) using ITLC-scanner. Examinations validated that labeling efficiency of ^{99m}Tc-labeled Senti-Scint® was appropriate before the i.v. applications. Measurements also validated that the labeling of ^{99m}Tc-BBS-NP, ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 compounds was successful and radiocolloids have high-degree and durable labeling efficiency.

- Determined particle size distributions validated that each product have a high degree of colloid size stability. Results proved that labeling method and storing at room temperature did not produce a remarkable difference in mean values of size distributions.

- Transmission electron-microscope (TEM) examinations of sample ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 were reinforced and validated DLS particle size results and assess spherical morphology of particles.

- *In vitro* confocal microscope cellular uptake examinations of ^{99m}Tc-BBS-NP coumpounds validated that results showed that the radiolabeled nanosystem was efficiently internalized by the hepatocellular carcinoma (HeDe) tumor cells overexpressing folate-receptors.

3. Results of biological applications, *in vivo* follow-up

- Investigations of the labeled ^{99m}Tc-DoxHSA180, ^{99m}Tc-DoxHSA430 and ^{99m}Tc-DoxHSA1800 compounds in healthy Wistar rats validated that

biodistribution results has close correlation to earlier described results of radiocolloids in similar particle size ranges. Ventrodorsal and left-lateral images were taken with a digital SPECT gamma camera at different times till 22 h post injection and organ uptakes were estimated by quantitative ROI analysis. *In vivo* examinations verified that colloid carriers have insignificant size fluctuations after an intravenous application and they show the proper distribution according to their particle size. Therefore, our investigations verified that different and stable particle sizes make drug carrier HSA nanoparticles possible to apply different drug targeting in a potential clinical use.

- Cell specificity and tumor targeting efficacy of ^{99m}Tc-BBS-NP were *in vivo* using SPECT and fusion nanoSPECT/CT imaging. *In vivo* studies of ^{99m}Tc-BBS-NP compound verified specific binding to folate-receptor overexpressing rat hepatocellular carcinoma (HeDe). Tumor-transplanted animals were Fischer 344 rats and was the injected control radiopharmaceutical was ^{99m}Tc-DMSA. Whole-body biodistribution of the new radiotracer revealed higher uptake in the tumorous kidney compared to the non-tumorous contralateral side and compared to the tumor-uptake of control radiotracer. Activities of the lungs and thyroids were negligible, which confirmed the *in vivo* stability of the nanoparticles.

- Investigations the labeled ^{99m}Tc-BBS-NP nanoparticles in a spontaneous diseased dog with a known (visible, palpable) oral tumor verified that labeled nanoparticles target folate-receptors of the oral tumor cells and internalize specifically into them to realize early tumor diagnosis detected by SPECT imaging. Accumulation of the nanoparticles in oral tumor proved a relative lower 3.1% of injected activity. Results verified that the rapid, simple,

reproducible labeling method, *in vivo* size stability and non-toxicity is allow the possibility of a relative rapid manufacture and clinical use of new ^{99m}Tc-BBS-NP product in SPECT and SPECT/CT imaging. Our examinations also verified that a spontaneous diseased dog is a suitable animal model for the development of a new folate-targeting radiotracer.

- Detection rate of sentinel lymph nodes in breast cancer was evaluated prospectively in 128 consecutive breast cancer patients without palpable lymph nodes using ^{99m}Tc-labeled Senti-Scint® nanocolloid, as radiotracer. Preoperative lymphoscintigraphy and surgical gamma detection probe both detected 203 sentinel lymph nodes in 122 patients (95%). It is concluded that lymphoscintigraphy with 175 nm mean diameter radiocolloid particles is a reliable procedure that assists in the surgical management of breast cancer.

- Sentinel lymph node detection was investigated in 24 client-owned dogs with spontaneously occurring tumors using ^{99m}Tc-labeled Senti-Scint® nanocolloid. Results showed that 97% of sentinel lymph nodes were found by radioguided surgery, 89% of nodes were clearly visualized in the gamma camera images, and only 77% of nodes were blue-stained by vital dye. Our examinations verified that Senti-Scint® proved to be suitable for sentinel lymph node detection in dogs with a 187 nm mean diameter particle size. The relatively large radiocolloid particles clearly visualized only the sentinel, and not all the regional lymph nodes, with accompanying low body background activity. Our comparative oncologic studies assessed that the sentinel lymph node concept is well applicable in the veterinary clinic and spontaneous diseased dogs are reliable animal model for research studies of SLN detection.

Conclusions

We reported the new potential diagnostic radiopharmaceutical compound ^{99m}Tc-BBS-NP, a new, folate-receptor overexpressing tumor targeted, stable nanosystem. Nanoparticles were prepared by self-assembly of chitosan and folated poly-γ-glutamic acid, and then first time radiolabeled ^{99m}Tc successfully. Our examinations validated that the new nanoparticles as a targeted contrast agent improve tumor targeting and are able to detect folate-receptor-overexpressing tumors in animal models with enhanced contrast.

Furthermore, our investigations verified the key importance of suitable and stable particle size distribution followed by examples of colloid based radiopharmaceuticals for SLN detection or SPECT imaging and new drug delivery systems in colloid size range.

We reported comparative oncology studies of spontaneously diseased tumorous dogs with biological trials of the human diagnostic agent Senti-Scint® and the new folate-receptor targeting radiotracer ^{99m}Tc-BBS-NP.

Besides, our findings suggest that SPECT and SPECT/CT imaging procedures of nuclear medicine can be important parts of the development of new pharmaceuticals based on nanotechnology (theranostics and drug delivery systems).

List of publications

Publications related to the doctoral thesis

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