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Small Animal Imaging in Development of New Generation Diagnostic and Therapeutic Agents

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1. Introduction

Imaging technologies form an inseparable part of molecular medicine and is a major research focus globally. Imaging of potential therapeutic or diagnostic agents in animal models in the early stage of research is an important step towards pre-clinical and clinical trials in humans. The resent achievements in molecular biology, virology and nanotechnology provide a totally new approaches to deliver therapeutic agents to the patient starting from conventional small molecules to virus based gene therapy. This creates a need for better tools for the pharmaceutical research. Small animal imaging provides excellent method for development of new generation diagnostic and therapeutic agents.

Modern transient medicine provides completely new approaches to the diagnosis and therapy of diseases. Aim of the research is to develop more specific and efficient agents with minimum side effects. Furthermore, early diagnosis of diseases and accurate follow-up is an important part of the therapy. These requirements have lead to the more complicated bioactive molecules and their carriers. Development and refinement of new bioactive agents like peptides, proteins, nanoparticles, cells or viruses to human drugs is challenged by the perplexities and instability of the complexes *in vivo*.

Due to the complexity of new diagnostic and therapeutic agents, their biodistribution and pharmacokinetic profiles *in vivo* is difficult to predict. Besides toxicity which is one of the main concerns to conventional small pharmaceutical compounds, new agents have to face defense mechanisms like reticuloendothelial system (RES), immunological response and liver as well. Larger size may also affect to the bioavailability of the agent. To overcome these problems comparative biodistribution studies *in vivo* with potential candidates should be started in early phase of the development.

Non-invasive imaging has become important part of the basic and applied research. It allows biodistribution studies within same animal in different time points and phases of the disease. This is important for accurate monitoring since variations between individuals should be minimized. In other words, using imaging applications more equal results may be achieved than with using traditional methods based on *post mortem* or dosing studies.

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Imaging is done with fewer animals which is cost-effective and also in accordance to 3R principle (Russell & Burch 1959). Appropriate dose is also easier to evaluate since behavior of the studied compound is immediately seen and changes can be done in relative short time interval compared to dosing studies which may last several months before any effects or results are obtained.

In pharmacological aspect several *in vivo* modalities for small animal imaging exist today. Magnetic resonance imaging (MRI) and resonance (MRS), single photon emission computed tomography (SPECT), positron emission tomography (PET) and optical imaging (OI) are widely used and several reviews (King et al. 2002, Gröhn & Pitkänen, 2007, Kagadis et al. 2010, Snoeks et al. 2011) have been published about these techniques, their strengths and faults. Choosing the most suitable imaging application for a certain study depends of the prioritization of features.

Our laboratory has experience to use wide range of targeting and carrier moieties in experimental animal imaging. In this review we discuss applications in different imaging moieties in development of novel diagnostic and therapeutic agents.

2. Small animal imaging

Small animal imaging provides a non-invasive method to study biodistribution and pharmacokinetics of novel bioactive agents in physiologically relevant environment. Dedicated imaging equipments for laboratory animals, mostly for rodents and rabbits, are available. Different imaging modalities produce information about anatomical structures and physiological processes. Using different modalities together and combining the information, the most accurate information of the function of studied agents with good anatomical reference is achieved.

2.1 SPECT

Single photon emission computed tomography (SPECT) is based on detection of gamma radiation from the studied object. Scanning of different projections from several angles enable three dimensional (3D) reconstruction and further analysis of the patient or animal from various planes and directions. Furthermore, using 2D planar imaging pharmacokinetics of the radiolabelled agents can be followed over the time in the same animal.

There are several radiotracers which can be used in SPECT imaging. The most used are Technetium, Indium and Iodine. Since different tracers have different physicochemical properties, labeling of the molecules or living particles for imaging purposes requires knowledge in biochemistry, traditional chemistry and radiochemistry.

Historically iodine radiolabels are the most used in biochemistry and cell biology. Over 30 isotopes of iodine have been reported of which around ten has been evaluated for biomedical applications (Welch & Redvanly 2002). Choose of the isotope depends on the purpose of the study. ^{123}Iodine decays with practical energy for imaging studies (159 and 127 keV), but its relative short half-life (13 hours) limits its usage to the transient biodistribution studies. The half life of ^{125}I is 60 days but emission energy is only 36 keV, which makes it impractical for human studies but adequate for animal experiments, especially in mice. Its long half-life enables imaging studies for several weeks with single administration of the studied agent.

For the labeling Iodine is oxidated. The target molecule typically contains benzene ring with ortho-substitution which in the most cases is OH-group, like tyrosine in the peptides or proteins. Oxidated iodine reacts to the positions 3 or 5 or both of the benzene ring. Iodine is oxidated by chloramine-T, Iodogen or lactoperoxidase in direct chemical methods (Hunter & Greenwood 1962, Fraker & Speck 1978, Marchalonis 1969). The most convenient method for iodination of biologically active molecules is commercially available Iodo-Gen tubes, which are coated with an oxidative agent 1,3,4,6-tetrachloro-3-6-diphenylglycouril. Due to the high hydrophobicity, this toxic oxidative compound is insoluble to water based buffers and remains in the walls of test tubes enabling solid phase oxidation of Sodium Iodine (NaI). Biomolecules are Iodinated in aquatic environment. The method is optimal for sensitive molecules and the toxic compound remains on the solid phase.

If the target molecule lacks a benzene ring, an additional radioiodinating reagent may be used. The most common and commercially available reagent is Bolton-Hunter reagent. This reagent is succinimidyl derivatized ortho-substituted benzene ring (*N*-succinimidyl-3-[4-hydroxyphenyl]propionate) (Bolton & Hunter 1973, Zalutsky & Narula 1987, Vaidyanathan et al. 1997, Gabel & Shapiro 1978). It reacts with primary amines, which are common in bioactive peptides, proteins, viruses and cells enabling iodination position to the target molecule.

Alternative methods for iodine tracers are ^{99m}Technetium (^{99m}Tc)and ^{111}Indium (^{111}In), which are the most used isotopes in nuclear medicine. However, these metals have to form complexes with donor ligands or chelates prior administration. If the molecule itself lacks chemical structures, which react with the metal as a ligand like sulphur fingers (Maret, 2004), the molecule has to be chelated. Diethylenetriaminepentaacetic acid (DTPA) and 1,4,7,10-tetraazacyclododecane-N,N',N,*N*''-tetraacetic acid (DOTA) are the most commonly used chelates in imaging. As with Bolton-Hunter reagent, these chelates are also available as bifunctional chelating agents (BCA) (Figure 1.) (Chakraborty & Liu 2010, Liu & Edwards 2001).

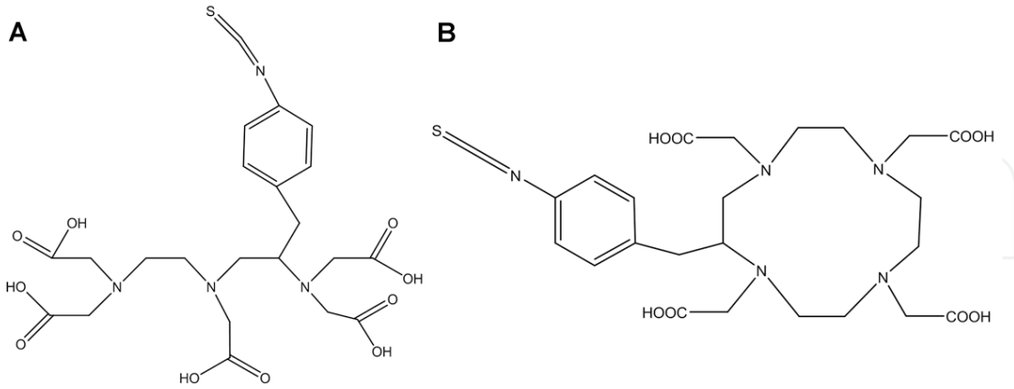

Fig. 1. Commonly used bifunctional chelating agents (BCA) in imaging. Isothiocyanate (S=CH=N-) reacts with primary amines in physiogical conditions allowing labeling of unstable peptides and proteins. A) Isothiocyanate DTPA and B) isothiocyanate DOTA.

Chelates increase the molecular weight of the target molecule and may also change the overall charge. Due to the molecular weight they may cause steric hindrances and change in total charge within small molecules. For larger molecules like peptides and proteins the use

of chelates is more common. In some cases the use of the linker between chelating part and reactive part may have effect to the pharmacokinetic properties (Garrison et al. 2008, Qu et al. 2001).

2.2 PET

In positron emission tomography (PET) imaging positron emitters and the following annihilation, is used to measure radioactive accumulation or consumption in the object. Annihilation produces two gamma quanta of 511 electron volts, which are emitted to the opposite directions (180^{o}). The gamma quanta are easily located by using a serie of stable gamma cameras around the animal. The advantage of the PET radiotracers is that radionuclides are incorporated in the molecule with minimal inference to the function of the pharmaceuticals. Also sensitivity in PET is superior to SPECT. One important limitation for PET resolution is that the localisation of positron emission is not the same that the place of annihilation. The distance between emission and annihilation depends of the energy of the particle and also the density of tissue, for example average range for ^{18}F in water is 0.064 cm (Cherry 2003).

Labeling of PET radiopharmaceuticals is complicated and requires on-site cyclotron and highly educated personnel. Due to the short half-lives of tracers the cyclotron facilities should be near the research laboratory. Only ^{18}F has adequate long half-life that the delivery time can be hours not minutes. It's also notable that even the half-lives of PET nuclides are shorter than SPECT nuclides the dose effect may be larger with PET nuclides since their emission energy of 511 electron volts is much higher than those of conventional SPECT nuclides.

In clinical PET studies ^{18}F in deoxyglucose (FDG) is the most commonly used diagnostic molecule for the functional studies in tissues. Others, such as sodium ^{18}fluoride, ^{18}fluorothymidine, ^{18}fluoromisonidazole, and ^{64}Cu-labeled diacetyl-bis N4-methylthiosemicarbazone are under evaluation for clinical use (Vallabhajosula et al. 2011).

2.3 CT

The oldest imaging modality is based on X-rays describe first by Wilhelm Röntgen already on 1895 (Röntgen, 1896). Ever since x-rays has been used to produce two dimensional images. Today X-rays are used in 3D topographic imaging. In principle, CT unit consist of high-voltage x-ray tube and oppositely located detector. Both x-ray source and detector rotate around the animal and a 3D reconstruction of the target can be made. Contrast is based on the ratio of the radiation which is passed through and absorbed in the patient. In contrast to SPECT and PET where radiation comes from the patient, in CT radiation is produced in the imaging equipment and the fraction of radiation passing through the target is measured. Since differences in linear attenuation coefficients for soft tissues are small (water = 0.21 cm^{-1}; lean tissue = 0.20 cm^{-1}; fat = 0.18 cm^{-1}; bone 0.38 cm^{-1}), contrast in the soft tissues is limited with x-ray based CT technique. Also increased resolution in CT raise significantly the radiation dose.

Although x-ray based CT is not optimal for small animal imaging this method facilitates the localization of labeleld molecules in biodistribution studies. Today there are few manufacturers, which provide combined SPECT/CT and PET/CT equipments for clinical

use but also dedicated animal devices are on the market (Picler et al. 2008, Golestani et al. 2010). The advantage of these multimodality systems is the ease of imaging with different modalities without moving the object and hence the co-localization of images can be performed easily (Figure 2).

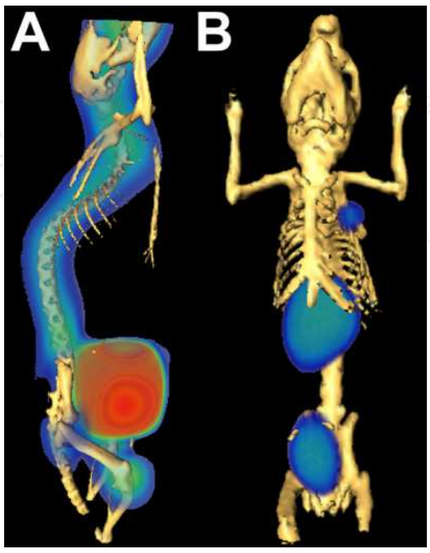

Fig. 2. Combined SPECT/CT images of mouse. A) 99mTc labeled commercially available bisphosphonate, Etidronate. Biodistribution profile studied in healthy mouse 30 min after i.v. injection. Etidronate accumulates mainly to the spine and joints of the hind limb. The image also visualizes the elimination of Etindronate through the kidneys and further excretion to the bladder (red accumulation). B) ^{111}In labeled monoclonal antibody mF4-31C1 against vascular endothelial growth factor receptor 3 (VEGFR-3) in ovarian carcinoma mouse model. Biodistribution profile studied 48 h after the single intra venous (i.v.) injection. Most of the antibodies are excreted through the liver. Signal in the lower part of the body indicates antibody's accumulation in the tumor area and the upper signal represents remote activation of VEGFR-3 in metastatic lymph nodes (Huhtala et al. 2010).

2.4 MRI/MRS

The best modality for high contrast soft tissue anatomical imaging is magnetic resonance imaging (MRI). It is based on nuclear magnetic resonance (NMR) and the nature of proton nucleus. Isotopes that contain an odd number of protons and/or neutrons and have an intrinsic magnetic moment and angular momentum, like ^{13}C, ^{2}D, ^{15}N and ^{31}P can be used for MRI. When an isotope with magnetic properties (usually a proton) is in a strong magnetic field, the nucleus of the isotope is aligned with the magnetic field. When using a short radiofrequency (RF) pulse, the nucleus will align itself with the magnetic field. After the pulse, the nucleus will return on its natural state at certain rate called relaxation time, emitting an RF signal which is recorded. The RF signal is analyzed and used to produce MR image. Since the environment of the proton affects strongly to the relaxation time, contrast is achieved between tissues. Furthermore, using Magnetic Resonance Spectroscopy (MRS) analyses the relative concentrations of molecules in the target tissue can be estimated (Liimatainen et al. 2006b, Liimatainen et al. 2006a).

In MRI fine structure investigation and spectroscopy of the tissues is performed without any tracers but biodistribution studies of active compounds are followed using contrast agents. It's notable that in small animal imaging, MRI is typically suitable to image certain part of the body and only local biodistribution e.g. the brain areas are imaged. Ferric, gadolinium or manganese are common contrast agents Ultra small superparamagnetic iron oxide (USPIO) particles, size range of 10 – 50 nm, are widely used for various applications like vascularity and macrophage content in atherosclerotic carotid plaques (Metz et al. 2011), lymph node metastasis (Lei et al. 2010), tumor vascular morphology and blood hemodynamics (Gambarota et al. 2010), diffusion in the brain disorders (Chin et al. 2009, Vellinga et al. 2009), cell number quantification (Cheung et al. 2006) and oncological studies (Gambarota et al. 2006, Baghi et al. 2005, Keller et al. 2004).

For the labeling several surface activated USPIO particles are available. The surface may contain chemically active groups like carboxylic acid, primary amines, aldehydes or isothiocyanates. Also biotinylated or avidin/streptavidin coated particles are available. Since biotin-avidin complex is one of the strongest found in nature, this phenomena can be used widely for various targeted applications or as a conjugation techniques. It should be noted that USPIO nanoparticles are several magnitudes larger than bioactive molecules and may cause sterical hindrances.

For MRI studies gadolinium and mangansese based contrast agents have also been used. They require, like Technetium and Indium, chelates for labelling. Gadolinium ion as a water soluble salt is also quite toxic to animals and chelating reduces significantly its toxicity. However, the sensitivity of these contrast agents in MRI or MRS is significantly lower than corresponding radioactive metals in SPECT or PET techniques. In MRI millimolar concentrations are needed whereas nano and even picomolar concentration of radionuclides gives reliable SPECT or PET imaging results.

Combination of PET/MRI is relatively new and rare hybrid scanning technique but very fascinating (Pichler et al. 2008, Bisdas et al. 2010, Antoch & Bockisch 2009). Especially in brain imaging combination of PET and MRI seems advantageous and promising (Heiss 2009). With combined PET and MRI imaging gives valuable information about function of the heart (PET) and also information about ventricular structure of the heart (MRI) (Nekolla et al. 2009). Combination of SPECT and MRI is available only for animal studies (Goetz et al. 2008).

2.5 Optical imaging

Compared to previously described methods advantaged of optical imaging (OI) include relatively ease usability, inexpensiveness and no need of radioactive tracers. In OI the detection is based on produced light from the tissues and monitored by common CCD camera. This method has been used for pharmacokinetic studies, angiogenesis, cancer, evaluating biodistribution or biological activity of potential therapeutic agents but also visualization of living embryos (Baker 2010, Dufort et al. 2010b, Penet et al. 2010, Eisenblatter et al. 2010, Canaria & Lansford 2010).

The OI modality uses either fluorescence of bioluminence as a tracer. The molecules are typically labelled with fluorescent molecules and their biodistribution is followed like in SPECT or PET modalities (Weissleder & Ntziachristos 2003, Napp et al. 2011). The labelling chemistry is similar as with chelates. Several fluorescence molecules like fluorescein or

cyanine based molecules (Cy3, Cy5 etc.) may contain either amino or carboxylic acid groups or are pre-activated with succinimides, maleimimides or isothiocynanates for the conjugation. Another fluorescence method is based on green fluorescence protein (GFP). Using cells transfected with GFP gene, the function of the cells can be studied (Chudakov et al. 2005). However using fluorescence the depth of imaging target, surface reflectance, absorption, scattering and autofluorescence limit the sensitivity in true 3D imaging (Dufort et al. 2010a, Welsh & Kay 2005, Bremer et al. 2003).

The light emission in bioluminescence is more sensitive, mainly because it is not interfered by autofluorescence since it is based on or oxygenation of Luciferin by Luciferase enzyme. During the oxygenation Luciferin substrate produces a photon, which is measured. As with GFP the function i.e. proliferation or cell death can be studied by using transfection with Luciferase gene. Transfected cells are inoculated to the experimental animal and followed over the time with Luficerin injections. After systemic injection, luciferin circulates and internalizes in to the cells. In Luciferase expressing cells the light is lit and can be imaged. This method has successfully use i.e. in imaging of the therapeutic effect of the viruses in cancer (Heikkilä et al. 2010).

Another fascinated optical imaging application is splice correction method developed by Kole and his colleagues in 1998 (Kang et al. 1998). This method is based on transfection of a plasmid containing mutated Luciferase gene. This mutation causes an aberrant splicing of the pre-mRNA resulting non-functional mRNA. Upon the treatment with slice correcting oligonucleotide , which has complementary structure to the mutation site, the aberrant splicing is corrected and active Luciferase enzyme is expressed. This method has successfully used in cell cultures to study oligonucleotide internalisation in to the nucleus using cell penetrating peptides (CPP) (Mäe et al. 2009), but in the future it may have several applications in *in vivo* optical imaging.

3. Therapeutic and diagnostic agents in imaging

Although most of the pharmaceutical compounds are small and relatively simple structures, there are a growing number of other types of molecules for therapy and diagnostics. Exactly speaking it's inaccurate to speak novel therapeutic molecules, since there are also other solutions to deliver the therapeutic agents and affect the target tissue or cells. In recent years nanoparticles (NP), viruses and stem cells has been in focus.

What is common aim in developing novel therapeutic or diagnostic agents? The first aim is to develop specific targeting to the pathological alterations in tissues or cells. Secondly, they should be multifunctional containing several biological or chemical structures like targeting, drug, carrier and tracer moieties. Thirdly, the side effects should be minimized. Gene technology provides totally new approach to therapeutic field by delivering genes to the host cell, which transcription and translation machinery is used as "drug factory".

Small animal imaging methods are ideal to study biodistribution of various types of molecules or even viruses and cells. Since nano- and picomolar concentration of radiolabel gives adequate signal, small amounts of label is needed to preliminary results of the biodistribution, accumulation, pharmacokinetics and metabolic routes of the studied compound. Other advantages in imaging include smaller animal groups than traditional

pharmacological studies since whole body results can be achieved over the time *in vivo* without sacrificing the animals.

3.1 Conventional pharmaceutical and imaging compounds

Most of the commercially available pharmaceutical compounds are small molecules below 500 Da and they typically lack homing properties but the effect is based on specific binding as an agonist or antagonist in the target tissue or cell. If small pharmaceutical compounds are used in imaging studies, in the most cases the tracer should be directly incorporated to the molecule structure like chemical labelling of Iodine for SPECT, with cyclotrone for PET or the molecule should have chelating properties, like bisphosphonates, if a radioactive metal is used.

There are several small molecules used in diagnostic imaging. One widely used imaging agent is (-)-2β-carbomethoxy-3β-(4-iodophenyl)tropane (β-CIT or RTI-55). In SPECT and PET imaging it has been used as ^{123}I labeled or ^{18}F (FP-CIT) labeled to map distribution of dopamine transporters and serotonin transporters in the brain e.g. in Parkinson's disease and supranuclear palsy (Zubal et al. 2007, Shaya et al. 1992, Shang et al. 2007, Staffen et al. 2000, Seppi et al. 2006).

For PET the small organic imaging agent ^{18}FGD, a glucose derivate, which accumulation through the body is related to tissue glucose consumption. This phenomenon is utilized in several applications of brain, tumor and myocardial metabolism (Berti et al. 2010, Miletich 2009, Chen & Chen 2011, Kopka et al. 2008). ^{18}FGD is widely used especially in neurosciences including drug research and development. With ^{18}FDG it's possible to determine activation of certain brain areas and hence applications are numerous, e.g. the sensitivity of brain areas to drugs as well as behavioral and therapeutic effects of the drug (Welch & Redvanly 2002).

^{99m}Tc is the most commonly used isotope in nuclear medicine. When it is conjugated with DTPA, it is used to measure functionality of the kidneys (Eckelman & Richards 1970), as a pyrophosphate or bisphosphonate for skeletal imaging (Thrall 1976) and with hexamethylpropyleneamine oxine (HMPAO, Ceretec™) for brain perfusion (Leonard et al. 1986a). ^{111}Indium, chelated to oxine is used in clinics to label white blood cells or platelets to study sites of acute inflammation and infection but also thrombocytopenia *in vivo* (Leonard et al. 1986b, Thakur et al. 1977, Thakur 1977, Rodrigues et al. 1999, Louwes et al. 1999).

3.2 Peptides

Although peptides and polypeptides have been used for therapeutic purposes already for over 80 years when insulin was taken in clinical use, only few novel peptide based drugs have been approved by FDA or EMEA. Most of the drugs are direct copies from nature like follitropin beta, which is a synthetic copy of follicle stimulating hormone (FSH) (Fares et al. 1992, Shome et al. 1988). Second generation peptide drugs are modified from the original molecule or are part of the larger proteins. Octreotide is a long-acting octapeptide with pharmacologic properties mimicking those of the natural hormone somatostatin (Bornschein et al. 2009, Anthony & Freda 2009, Stajich & Ashworth 2006). Fuzeon (Enfuvirtide) is a 36 residue synthetic peptide that inhibits HIV-1 fusion with CD4 cells. Enfuvirtide binds to the first heptad-repeat (HR1) in the gp41 subunit of viral envelope glycoprotein and prevents

the conformational changes required for the fusion of viral and cellular membranes. It interferes the HIV-1 molecular machinery at the final stage of fusion with the target cell. Enfuvirtide is a biomimetic peptide that was rationally designed to mimic components of the HIV-1 fusion machinery and displace them, preventing normal fusion. (Joly et al. 2010, McKinnell & Saag 2009, Makinson & Reynes 2009)

The number of bioactive peptides , with potential therapeutic or diagnostic properties, will be increased due to new screening methods for novel peptides. Epitope scanning (Reece et al. 1994, Frank, 2002) and phage display libraries produce novel biologically active peptides with specific binding properties to target proteins such as receptors and proteases (Nilsson et al. 2000). Some of the identified peptides are highly specific to the receptors of the specialized tissues providing a possibility to use peptides for targeting (Laakkonen et al. 2002). These peptides serve as lead molecules for development of molecules for tumour imaging and therapy.

Both natural peptides and peptides characterized by phage display are sensitive to metabolic processes like protease activity. This limits their usefulness as diagnostic and therapeutic agents. Rationale design of chemical modifications to maximize enzymatic bioavailability while preserving the potency and specificity of the peptide is needed (Adessi & Soto 2002). Typically peptides are cyclised or the amino acid side chains or bridge structures are modulated by using unnatural structures called peptidomimetics (Pakkala et al. 2007, Pakkala et al. 2010).

Peptides and their modifications are typically produced by using solid phase peptide synthesis method (SPPS). Today synthesis is made with automated synthesiser and the time to produce a peptide is relatively short and several companies provide synthesis services for reasonable price. For labelling an additional reactive amino acid like tyrosine or cysteine are easy to add to the sequence for further labelling or conjugation purposes.

3.3 Proteins

Unlike peptides proteins are large and contain secondary, tertiary and some cases even quaternary structures on which the biological activity is based. Due to their defined tertiary structure and size, they may be sensitive to the labelling and purification methods. Furthermore, the administration route, which is mainly the systemic injection, and immunological response limits the usefulness of the proteins as drug candidates.

For the biodistribution studies the surface of the proteins contains several different chemically active amino acid side chains or polysaccharides, which can be used for labelling purposes. Typically proteins are labelled via the ortho-hydroxy benzene ring of tyrosine or via primary amino groups of either the amino terminus or the side chain of lysine. For the imaging purposes proteins are labelled with iodine or conjugated with chelates as previously described. After the conjugation proteins can be purified with conventional size-exclusion chromatography, dialysis or ultrafiltration using physiological conditions. In addition, chelate conjugated proteins can be labelled with ^{99m}Tc or ^{111}In without further purification steps (Helppolainen et al. 2007).

One of the most used group of proteins for diagnostic and therapeutic purposes are monoclonal antibodies. Already 50 products have been passed the long and very expensive

way from the primary finding to the licensed drug (Biopharma, 2011). The limiting factors of antibodies are large size (150 000 Da) which may interfere penetration of the molecule to the target tissue and possible squeamishness. Large size can be bypassed with Fab1 or Fab2 fragments of the antibodies or as in human therapy using humanized monoclonal antibodies. The advantage of antibodies is high affinity compared to other protein-ligand interactions but also relatively easiness and diversity of modification chemistry without losing the binding activity.

Antibodies have been successfully used in cancer therapy. Cetuximab (Erbitux) is a humanized monoclonal antibody against epidermal growth factor receptor (EGFR) which is over-expressed in various cancers (Vincenzi et al. 2008, Rivera et al. 2008). It has been successfully used in the treatment of colon carcinoma in humans. Same antibody has been studied as versatile SPECT and PET imaging agent in several cancer models, e.g. malignant mesothelioma, prostate cancer, head-and-neck squamous cell carcinoma, ovarian carcinoma (Figure 2.), colon cancer and universally EGFR positive tumors (Nayak et al. 2011, Malmberg et al. 2011, Hoeben et al. 2011, Huhtala et al. 2010, Cho et al. 2010, Ping Li et al. 2008).

3.4 Viruses

This very exiting approach is based on natures own gene delivery method. After delivery, modified virus in target cell begins to use cell's natural amplification techniques to produce therapeutic molecules. Today there are both transient (Adenoviruses) and stable (Lentiviruses) viral delivery systems (Rissanen & Yla-Herttuala 2007, Mahonen et al. 2010, Lesch et al. 2009).

Biodistribution studies using non-invasive imaging is an important part of the development of virus based therapeutic agents. Viruses for the therapy are modified, they are unable to multiply and additional therapeutic and/or reporter genes are added to the viral genome. Expressed reporter genes can be imaged by using radiolabelled ligands. Using sodiumiodine symporter (hNIS) gene together with cancer-specific human telomerase promoter, human colocarcinoma xengraft has been imaged using radiolabelled iodine with SPECT/CT in animal model (Merron et al. 2007).

Fusion proteins composed of avidin and either macrophage scavenger or low-density lipoprotein receptors (LDLR) have been constructed in order to target biotinylated molecules to cells of desired tissues. Using adenovirus mediated gene transfer transient expression of the fusion protein on cell membrane was achieved (Lehtolainen et al. 2002, Lehtolainen et al. 2003). When biotinylated molecule binds to the fusion receptor, it is internalized into the cell. Local gene transfer to target tissues could be used as a universal tool to deliver therapeutic agents at systemic low concentrations. Using biotinylated tracers like biotin-DTPA or biotin-DOTA compelexes these cells can be imaged *in vivo* (Turhanen et al. 2011).

An alternative method to study the biodistribution of the viruses is avidin expression on the surface of the viral particle. Their homing properties to the target tissue may be enhanced using biotinylated moieties like antibodies or peptides. For imaging purposes biotinylated radiotracer is conjugated on the virus surface and biodistribution of the labelled virus is followed by SPECT (Raty et al. 2007, Raty et al. 2006, Kaikkonen et al. 2009).

Homing properties of viruses can be modified with biochemical methods using hybrid peptide with poly-lysine spacer together with cyclic peptide HWGF (His-Tyr-Gly-Phe) which binds to membrane metalloprotein receptors, MMP-2 and MMP-9 (Koivunen et al. 1999). This peptide has been conjugated with trasnglutaminase enzyme on the surface of Adenovirus. The use of enzyme for conjugation is gentle and do not decrease the infectivity of the virus. Conjugated receptor specific peptide enhanced the tropism of the virus *in vivo* in rabbits (Turunen et al. 2002).

3.5 Living cells

Nuclear medicine has been used to image leucocytes in infectious or inflammatory processes *in vivo* already over four decades. The techniques detect inflammatory processes to which leukocytes migrate, such as those associated with abscesses or other infection. During 1970's a new cell membrane tropic radioactive compound was developed. ^{111}In-oxine is a lipophilic complex and penetrates through cell membrane without interference of the membrane bound molecules like receptors. Penetration is unspecific and all cell types can be labelled (Thakur et al. 1977, Becker & Meller 2001).

Stem cells are immature cells, which have regenerative potential in various diseases. Especially neurodegradative disorders have been in the focus due to the poor regenerative properties on neuronal cells. The regenerative properties of the stems cells have been studied in Parkinson disease (PS), amyotrophic lateral sclerosis (ALS), Huntington's disease and stroke (Lindvall et al. 2004). For in vivo biodistribution studies stem cells have been labelled either with paramagnetic nanoparticles and followed with MRI (Arbab et al. 2003, Frank et al. 2003) or with ^{111}In-oxine (Figure 3.) for SPECT imaging (Lappalainen et al. 2008, Makinen et al. 2006).

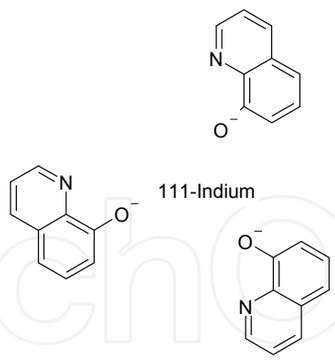

Fig. 3. ^{111}Indium oxine i.e. Indium 111 oxyquinoline. Indium is coordinating three oxyquinoline molecules. Due to the relative high hydrophobicity Indium oxine penetrates directly but unspecifically into the cytoplasm of the target cells and do not bind to the surface proteins.

Radiolabeling of living cells is probably the most challenging labeling process since several issues has to be considered. Firstly, labeling conditions have to be effective, mild, temperate, fast and without complicated purification steps. Secondly, aseptic techniques have to be followed and last, appropriate dose for the cell batch has to be evaluated avoiding too high dose for the cells. For these reasons labeling conditions must always plan carefully for each

different cell type according their usual cultivation techniques. If longer (i.e. over 24 h) biodistribution studies are measured, effect of the labeling to the viability of the cells *in vitro* during timescale is worth to analyze. This is important since only the nuclide is seen in *in vivo* imaging but no information is achieved about the absolute condition of the cells viability.

3.6 Nanoparticles

There have been invasion of basic nanoparticle research in biomedicine. Many therapeutic agents like small organic compounds, nucleic acids, peptides and proteins are unstable *in vivo* and novel delivery technologies should be developed to improve their pharmacokinetic properties. Development of nanoparticle based delivery could enable sustained and hence regular release of drug. If NPs are also targeted, in the ideal case they would concentrate to the desired area and allow sustained release of the drug to the circulation or locally if needed. This would be beneficial for the patient as fewer drug intakes, steadier effect of the drug and hence milder side-effects but maybe also economically cost-effective.

The size range of nanoparticles is comparable to the viruses. Conventionally nanosized materials like polymeric nanoparticles, liposomes and micelles are prepared from organic materials although they have limited chemical and mechanical stability and inadequate control over the drug release rate (Arruebo et al. 2006). Today there are NPs made of inorganic materials like silica or silicon (Haley & Frenkel 2008, Salonen et al. 2008). Inorganic material allows the production of porous or mesoporous nanoparticles with particle size in range of 50 – 300 nm and the pore diameter in the range 5 – 50 nm. The porous structure allows high loading capacity for the therapeutic agents and/or tracers, like fluorescein, radioactive compounds or paramagnetic iron (Wiekhorst et al. 2006, Alexiou et al. 2006a, Alexiou et al. 2006b).

Furthrmore, the transportation and release of the molecules can be controlled. Mesoporous silicon nanoparticles have also shown to be non-toxic and stable (Salonen et al. 2008, Brigger et al. 2002, Limnell et al. 2007, Salonen et al. 2004). The surface of the nanoparticles can be derivatized with chemically active groups like primary amines or carboxylic acids and conjugated with several biologically and chemically active molecules (Figure 4). Large surface area allows conjugation of several different molecules on the same particle. Using targeting moieties the tropism of NPs can be modulated (Kukowska-Latallo et al. 2005, Costantino et al. 2005).

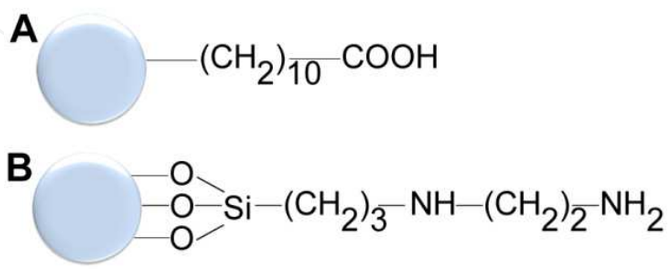

Fig. 4. Chemically modified surfcaes of the silicon based mesoporous nanoparticles for the conjugation of bioactive molecules. A) carboxylic acid derivatized nanoparticles and B) primary amino derivatized nanoparticles with alkane spacers.

4. Conclusions

Several imaging modalities for small animal pre-clinical studies have been developed. Various modalities provide different information about biodistribution, pharmacokinetics and effect of potential therapeutic agents to the target tissues and cells. Using SPECT or PET, biodistribution of the labelled agents can be easily followed over the time in animals with high sensitivity. Due to high spatial resolution, chances in fine structure and furthermore chemical chances of the target tissue can be studied using MRI and MRS. Contrast of CT is not optimal for soft tissue studies in small animals *in vivo* but using combined images with SPECT and PET it facilitates the localisation of the labelled bioactive agents. Optical imaging provides an excellent tool for the viability studies of cells and tissues. Luciferase expression based on transfected cells or whole transgenic animal gives direct information of the gene activation, growth and the death of the cells *in vivo*.

Today several new therapeutic and diagnostic agents are large and/or complexed structures especially viruses, stem cells and nanoparticles. Due to high variety of the structures in new agents, requirement of interdiscipline skills and collaboration starting from basic organic chemistry to virology and cell biology is required. Accurate information of the biodistribution and pharmacokinetics before clinical trials is needed. Using different imaging modalities and combining the information, excessive preliminary knowledge of behaviour and effect of the studied complexes *in vivo* can be achieved.

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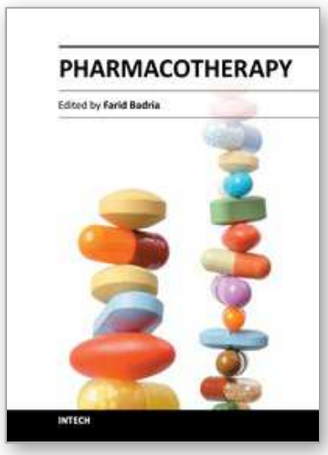

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The intent of this book is to provide an overview of current conceptualizations of Pharmacotherapy. The book focuses on three major areas; diagnosis, treatment, and prevention for a wide array of diseases; Cognitive and Psychological disorders (Schizophrenia and Nicotine addiction), Inflammatory disorders (New Chemical anti-inflammatory and Immunotherapy), updated antihypertensive therapy and healing of ulcers with venous origin. A separate chapter is dedicated to the rationality of drug use in earthquake injuries. The last chapter deals with Imaging of potential therapeutic or diagnostic agents in animal models in the early stage of research. We hope this book is useful to a wide range of people, from students first learning about Pharmacotherapy, to advanced clinicians and researchers.

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