Targeting of lanthanide(III) chelates of DOTA-type glycoconjugates to the hepatic asyaloglycoprotein receptor: cell internalization and animal imaging studies

M.I. M. Prata[1], A.C. Santos[1], S. Torres[2], J.P. André[2], J.A. Martins[2], M.Neves[3], M.L.García-Martín[4], T.B. Rodrigues[4,5], P.López-Larrubia [4], S.Cerdán [4] and C.F.G.C. Geraldes[5]*

[1]*Instituto de Biofísica e Biomatemática, Faculdade de Medicina, Universidade de Coimbra, Coimbra, Portugal*

[2]*Centro de Química, Campus de Gualtar, Universidade do Minho, 4710-057 Braga, Portugal*

[3]*Instituto Tecnológico e Nuclear, Sacavém, Portugal*

[4]*Instituto de Investigaciones Biomédicas "Alberto Sols", CSIC-UAM, Madrid, Spain*

[5]*Departamento de Bioquímica, Centro de RMN e Centro de Neurociências e Biologia Celular, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, Coimbra, Portugal.*

Correspondence to: C.F.G.C. Geraldes, Departamento de Bioquímica, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, Apartado 3126, 3001-401 Coimbra, Portugal.

Contract/grant sponsor: Foundation of Science and Technology, Portugal; *contract/grant number*: POCTI/QUI/47005/2002.

Contract/grant sponsor: Institute of Health Carlos III, Spain; *contract/grant number*: PIO051845.

Contract/grant sponsor: FEDER.

Contract/grant sponsor: III Instituto de Investigação Interdisciplinar University of Coimbra, Portugal; *contract/grant number*:III/BIO/45/2005.

Abbreviations used:

AG, arabinogalactan; ASF, asialofetuin; ASGP-R, asialoglycoprotein receptor; BW, body weight; CA, contrast agent; DCE, dynamic contrast enhanced; MEM, minimum essential medium; DTPA, diethylene triamine pentaacetic acid, a linear ligand; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, a macrocyclic ligand; DOTAGal, $DOTAGal_2$, $DOTAGal_4$, are DOTA monoamide derivatives with one, two and four terminal galactosyl groups; DOTALac and $DOTALac_2$, the same with one and two terminal lactosyl groups; DOTAGlc and $DOTAGlc_2$, the same with one and two terminal glycosyl groups; $DO2AGal_2$, DOTA cis-bisamide derivative with one terminal galactosyl group at each amide substituent; FBS, fetal bovine serum; FOV, field of view; GSA, galactosylated serum albumin; ID, injected dose; MION, monocrystalline iron oxide nanoparticles; MRI, magnetic resonance imaging; PBS, modified phosphate-buffered saline; PL, polylysine RARE, rapid acquisition and relaxation enhancement, fast spin echo MRI method; SPECT, single photon emission computed tomography; T_1, spin-lattice relaxation time in MR; *TE*, echo time; *TR*, repetition time; TLC, thin layer chromatography; USPIO, ultra-small superparamagnetic iron oxide particles.

ABSTRACT

The characterization of a new class of hydrophilic liver-targeted agents for gamma-scintigraphy and MRI, consisting, respectively, of $[^{153}Sm]^{3+}$ or Gd^{3+} complexes of DOTA monoamide or bisamide linked glycoconjugates (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), is reported. *In vitro* studies show high uptake of radiolabeled $^{153}SmDOTAGal_2$ by the human hepatocyte carcinoma cell line Hep G2 containing the asialoglycoprotein receptor (ASGP-R), which is decreased to less than 50 % by the presence of its high affinity ligand asialofetuin (ASF). *In vivo* biodistribution, gamma-imaging and pharmaco-kinetic studies on Wistar rats using the $[^{153}Sm]^{3+}$-radiolabeled conjugates show an high uptake in the receptor-rich organ liver of the compounds containing terminal galactosyl groups, but very little uptake for those with terminal glycosyl groups. Blocking the receptor *in vivo* reduced liver uptake by 90%, strongly suggesting that the liver uptake of these compounds is mediated by their binding to the asyaloglycoprotein receptor (ASGP-R). This study also demonstrated that the valency increase improves the targeting capability of the glycoconjugates, which is also affected by their topology. However despite the specific liver uptake of the radiolabeled galactose-bearing multivalent compounds, the animal MRI assessment of the corresponding Gd^{3+} chelates shows liver-to-kidney contrast effects which are not significantly better than those shown by GdDTPA. The maximum concentration/dose ratio attained in the kidneys by the Gd^{3+}- based CAs is considerably lower than for GdDTPA, indicating that part of the injected CAs is T_1 contrast-silent, presumably when taken up into the liver. The T_1 shortening effect of the Gd^{3+}-labeled Gal-containing compounds is probably strongly diminished upon hepatocyte internalization into an endossomic vesicle via receptor-mediated endocytosis of the ASGP-R.

Keywords: magnetic resonance imaging; contrast agents; gadolinium; glycoconjugates, liver targeting, asialoglycoprotein receptor, gamma scintigraphy

INTRODUCTION

In humans and animals, oligosacharides are covalently bound to proteins (glycoproteins) and lipids (glycolipids) and control a wide variety of cellular processes. These functions are mediated by a class of membrane receptor proteins belonging to the general family of lectins. Lectins are monomeric or (homo/hetero)-oligomeric proteins, either soluble or membrane-bound, bearing a single or multiple carbohydrate recognizing domain(s) (CRD) per subunit (1,2). The CRDs interact specifically with sugars (glucose, mannose, galactose, etc) with affinity constants (lectin-monomeric sugar) (K_d) in the milimolar range (3). Assembly of monomeric units produces oligomeric lectins displaying an array of CRDs with a variety of topologies (4). Often one or both of the interacting sugar/lectin partners are membrane-bound. A membrane-bound lectin (receptor) may recognize a soluble or a membrane-bound oligosaccharide (5). Both types of interactions are appealing for pharmacologic intervention as long as the intrinsically low monomeric sugar-lectin affinity can be overcome (6). The use of soluble multivalent glycoconjugate constructs displaying a *cluster glycosid effect* is mandatory for the targeting of cell/organ-specific lectins for drug delivery purposes and for the inhibition of cell-cell or cell-foreign body (bacteria/virus) interactions (7, 8). The hepatic asialoglycoprotein receptor (ASGP-R) is an organ-specific lectin, not found anywhere else in the body except on the surface of hepatocytes. This hetero-oligomeric lectin has affinity for terminal β-galactoside and β-N-acetyl-galactosaminyl residues on glycoproteins. Although its precise function *in vivo* is still unclear, it is involved in selectively removing de-sialylated glycoproteins, with exposed terminal galactose residues, from serum (9). In addition to asialoglycoproteins, the ASGP-R recognises β-galactoside and N-acetyl-β-galactosaminyl residues appended to a variety of artificial molecular scaffolds (10 -13).

Annually many people die worldwide of liver cancer following hepatitis B. The functional imaging of liver ASGP-R is of both diagnostic and prognostic value during

treatment of liver cancer and other liver conditions (14 -16). Molecular constructs bearing efficient reporter groups and pendant β-galactoside and/or N-Acetyl-β-galactosaminyl residues can lead to hepatocyte-specific imaging agents for liver (11, 14 -16). A conjugate of a neoglycoprotein, galactosylated serum albumin (GSA), with ^{99m}Tc-DTPA (diethylene triamine pentaacetic acid) ([^{99m}Tc]-DTPA-GSA), has been used in SPECT (single photon emission computed tomography) hepatic imaging to assess the ASGP-R function in mice (17). The hepatocyte-specific nature of the ASGP-R and the fact that it is still expressed (although in reduced numbers) on hepatoma cells makes it possible to detect liver cancer metastases to other organs. The same imaging agent, ([^{99m}Tc]-DTPA-GSA), has been demonstrated clinically useful in humans to detect liver cancer metastases to the bones (18) and to assess de dynamics of the ASGP-R internalisation/recycling to the surface of the hepatocytes (19, 20). A Gd-DTPA conjugate of polylysine (PL) derivatized with galactosyl groups (Gd-DTPA-gal-PL) (21), as well as a spin-labeled arabinogalactan (22), have also been developed as macromolecular potential contrast agents for liver MRI by targeting the hepatocyte ASGP-R. A variety of particulate systems targeted to the ASGP-R have also been tested in cells and mice as potential contrast agents for liver MRI: monocrystalline iron oxide nanoparticles (MION) conjugated to the bovine plasma protein asialofetuin (ASF), (MION-ASF) (23), arabinogalactan (AG)-coated ultra-small superparamagnetic iron oxide particles (USPIO), (AG-USPIO) (24 -27). Both the macromolecular and particle-based ASGP-R-targeted imaging agents described above, include carriers bearing multiple reporter groups and a multivalent display of galactosyl targeting groups. However these agents are inherently polydisperse and ill characterised. Chemically well-defined and characterised multivalent agents can be assembled by an alternative molecular design: the conjugation of dendrimeric clustered carbohydrate bifunctional reagents (monodisperse, well defined and characterised) to a reporter group (28). The resulting conjugates are of substantially lower molecular weight, higher stability and greater biological safety.

Previous to our report on the synthesis of dendrimeric multivalent glycoconjugates of metal complexes for molecular imaging (MRI and scintigraphy) (29, 30), only a study describing the use of a low molecular weight, well characterized, monomeric [^{111}In]-radiolabeled galactopyranosyl conjugate of DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) for the targeting of the ASGP-R both in hepatic cell lines and mice had appeared in the literature (31). The design of lectin-targeted MRI imaging agents includes a reporter group, consisting of a chelator binding lanthanide(III) ions with high kinetic and thermodynamic stability, and a targeting group consisting of a clustered carbohydrate of variable valence, containing an increasing number of terminal galactosyl groups, capable of interacting with high affinity and selectivity with endogenous lectins such as the ASGP-R (3, 29, 30).

Here we report the radiolabeling of a series of glycoconjugates based on DOTA-monoamide and bisamide functionalized chelators (Fig. 1) with [^{153}Sm]$^{3+}$ and their evaluation using i*n vitro* studies in the human hepatocyte carcinoma cell line Hep G2. DOTA-like chelators are well known to form lanthanide(III) chelates of high thermodynamic and kinetic stability, which is of crucial importance for *in vivo* applications (32). Dynamic gamma-scintigraphic studies of the pharmacokinetics of the [^{153}Sm]$^{3+}$-labeled glycoconjugates and their biodistribution in Wistar rats are also described, revealing the effect of valence and type of the terminal sugar moiety on their liver targeting efficacy. We also report an *in vivo* MRI study of the contrast effects and pharmacokinetics of the Gd^{3+} complexes of the divalent glycoconjugates $DOTAGal_2$ (**2a**), $DOTAGlc_2$ (**2b**) and $DOTALac_2$ (**2c**) (Fig. 1) in mice, compared with a typical commercial, small molecular weight contrast agent, GdDTPA (Magnevist®).

EXPERIMENTAL

The synthesis of the monovalent DOTAGal, DOTALac and DOTAGlc (**1**), divalent $DOTAGal_2$, $DOTALac_2$ and $DOTAGlc_2$ (**2**) and tetravalent $DOTAGal_4$ (**4**) monoamide

glycoconjugate ligands (29), as well as of the divalent DO2A(cis)Gal_2 bisamide (**3**) ligand (Torres S, Martins JA, André JP, Neves M, Santos AC, Prata MIM, Geraldes CFGC, *Radiochimica Acta*, submitted), have been described previously.

Reagents for cell interaction, gamma imaging and biodistribution studies

[^{153}Sm]Samarium chloride ([^{153}Sm]Cl_3) was produced at the ITN (Instituto Tecnológico e Nuclear), Lisbon, with a specific activity >5 GBq/mg. For this purpose, [^{153}Sm] samarium oxide ([^{153}Sm]$_2O_3$) was prepared from a 98% [^{152}Sm] enriched samarium oxide target, sealed into a quartz vial and welded into an aluminium can, by neutron irradiation using a thermal flux of 2.3 x 10^{13} n/cm^2 s. Following irradiation, the sample was opened, dissolved in 1N HCl and the final [^{153}Sm]samarium chloride ($^{153}SmCl_3$) was brought to a stock concentration of 1.9 x 10^{-3} M.

Radiotracer preparation

In initial experiments, stock solutions of the ligands were prepared in isotonic HEPES (0.1 M 1-piperazineethane sulfonic acid, 4-(2-hydroxyethyl)- monosodium salt) buffer (pH 7), mixed with [^{153}Sm]samarium chloride and the solution was heated at 60° for 3 h. After verifying that that the radiolabeling of the ligands is more efficient at lower pH (to promote the deprotonation of the binding groups), the definitive experiments were carried out using sodium acetate buffer (0.4 M, pH 5). A quality control was performed by thin layer chromatography (TLC), using water: ethanol: dicloromethane: ammonia, 5: 5: 5: 1, as eluent.

Serum stability

To 3 mL of fresh human serum previously equilibrated in a 5% CO_2 (95% air) environment at 37°C, was added 5 μCi of the [^{153}Sm]DOTAGal standard solution. The mixture was stored in a 37°C environment. At appropriate periods of time (0, 30 min, 1 h and 4 h) 100 μL aliquots (in triplicate) were removed and treated with 200 μL of ethanol. Samples were then cooled (4°C) and centrifuged for 15 min, at 4000 rpm and 4°C, to

precipitate serum proteins. 100 μL of supernatant was removed for activity counting in a γ well-counter. The sediment was washed twice with 1 mL of ethanol and counted. The activities in the supernatant and in the pellet were compared as (activity in the pellet/ activity in supernatant) ∗100.

***In vitro* cell interaction studies**

Hep G2 human hepatoma cell line (ECACC, United Kingdom) was grown in 75 mL bottles with MEM (minimum essential medium) from Sigma, which contains 18 mM $CaCl_2$, supplemented with 10% FBS (fetal bovine serum) from Gibco Invitrogen, and non-essential aminoacids, glutamine and penicillin/streptomycin, all from Sigma. The cells were counted and transferred to a 6-hole multiwell plate (0.2 million cells/hole), 2 mL of medium were added to each hole and then the cells were incubated at 37 °C, under 5% CO_2 for the period necessary to obtain cell confluence. This is needed because the human hepatocyte carcinoma line Hep G2 expresses maximum receptor activity only in confluent cultures (33). The medium was changed every day. In the day of the experiments the medium was removed and the cells were washed twice with 1.5 mL of medium before adding 1.2 mL of medium and they were allowed to adjust for 1 h at 37 °C. Approximately 1 μCi of radiolabeled ligand was added in each well to the medium and the cells were incubated for 30 min, 1 h and 2 h at 37 °C. To three of the holes in each plate an excess of asialofetuin (Sigma) (200 μL of a 0.2 mg/mL solution) (31) was added simultaneously with the radiolabeled ligand in order to evaluate the contribution of non-specific contributions to the cell uptake of the glycoconjugates. At appropriate time periods the experiment was stopped by removal of the medium and washing the cells twice with 750 μL ice-cold phosphate buffered saline. The medium plus PBS was collected to tubes to measure the radioactivity. Finally, the cells were treated with 1 N NaOH (1 mL) and incubated at 37 °C for 10 min, to detach them from the

plates, and the radioactivity was measured in a γ-counter. Each experiment was carried out simultaneously in triplicate.

***In vivo* gamma imaging**

A gamma camera-computer system (GE 400 GenieAcq, from General Electric, Milwaukee, USA) was used for acquisition and pre-processing. Data processing and display were performed on a personal computer using homemade software developed for the IDL 5.2 computer tool. A well counter (DPC-Gamma C_{12}, LA, USA) with a Compaq DeskPro compatible computer was used for activity counting in the biodistribution studies.

Gamma images and biological distribution for the $[^{153}Sm]^{3+}$complexes were determined using (200-250 g) Wistar rats. All animal studies were carried out in compliance with procedures approved by the appropriate institutional review committees. Conscious rats were allowed free access of food and water *ad libitum*. Groups of four animals (one group for each complex) were anaesthetized with Ketamine (50 mg/ml) /chloropromazine (2.5%) (10:3) and injected in the femoral vein with ca. 400 μCi of the respective $[^{153}Sm]^{3+}$chelate. The animals were then positioned in dorsal *decubitus* over the detector. Image acquisition was initiated immediately after radiotracer injection. Sequences of 180 images (of ten seconds each), were acquired to 64×64 matrices. In addition, static data were acquired 24 h after the radiotracer injection.

Images were subsequently processed using an IDL based program (Interactive Data Language, Research Systems, Boulder, CO, USA). In order to analyze the transport of radiotracer over time, three regions of interest (ROI) were drawn on the image files, corresponding to the thorax, liver and left kidney. From these regions, time-activity curves were obtained.

Biodistribution studies

Groups of four animals were injected in the tail vein with ca. 100 μCi of the respective $[^{153}Sm]^{3+}$ tracer and sacrificed 1 h later. The major organs were excised, weighted and tissue

radioactivity measured in a γ well-counter. Similar biodistribution studies were also performed with the animals referred in the previous section sacrificed at 24 h. For determination of non-specific uptake on receptor-positive organs, a group of four animals was co-injected in the femoral vein with a solution of asialofetuin in NaCl (2 mg/mL) (injected volume, 200 μL, 0.2 mg asialofetuin/100 g body weight) and $[^{153}Sm]^{3+}$-DOTAGal$_2$ (200 μL).

Magnetic resonance imaging

The MRI experiments were performed on a Bruker Pharmascan system (Bruker Medical Gmbh, Ettlingen, Germany) using a 7.0-T horizontal-bore superconducting magnet, equipped with a ^{1}H selective birdcage resonator of 38 mm and a Bruker gradient insert with 90 mm of diameter (maximum intensity 300 mT/m). All the data were acquired using a Hewlett-Packard console running Paravision software (Bruker Medical Gmbh, Ettlingen, Germany) operating on a Linux platform. All MRI studies were performed in accordance with the Spanish law for animal protection. *In vivo* experiments were performed on albine Swiss male mice, weighing 19-26 g. One animal was measured by MRI for each compound tested. Anesthesia was initiated by inhalation in an induction box with O_2 (1 l/min) containing 3 % of isofluorane, and maintained during the experiment employing a mask and 1-2 % of isofluorane in O_2. The mice were taped down gently into a holder, to minimize breathing-related motion, and were then placed in a heated probe, which maintained the core body temperature at approx. 37 ºC, monitored by a rectal probe. The physiological state of the animal was monitored by a Biotrig physiological monitor (Bruker Medical Gmbh, Ettlingen, Germany) using the respiratory rate and body temperature. The contrast agents were injected as a bolus of 50 μl via a tail vein catheter at a dose of 0.3 mmol Gd Kg^{-1} BW, except for GdDTPA (Magnevist®, Schering AG, Berlin, Germany) where a dose of 0.2 mmol Gd kg^{-1} BW was used.

For anatomical reference, a multi-slice rapid acquisition and relaxation enhancement (RARE) sequence (34) was performed with the following parameters: RARE factor = 8, TR = 4000 ms, effective TE = 65 ms, averages = 2, 6 coronal slices, slice thickness 1.2 mm, FOV = 3.8 cm, matrix = 256 x 256. Then, regional contrast agent uptake was assessed. A series of T_1-weighted spin echo images were sequentially acquired over one hour following the injection of the contrast agent. The acquisition parameters were: TR = 155 ms, TE = 11.76 ms, averages = 2, 6 coronal slices, slice thickness 1.2 mm, FOV = 3.8 cm, matrix = 256 x 256, 60 repetitions with temporal resolution 59.31 s.

MRI Data processing

Data were analyzed with software written in house in IDL. With the aim of comparing the pharmacokinetics obtained from different animals, the data were normalized by calculating the relative, rather than the absolute, enhancement (RE):

$$RE = \frac{(I - I_0)}{I_0} \times 100$$

where I is the signal intensity at any given time after CA injection and I_0 is the intensity before injection.

Pharmacokinetics were analyzed by calculating the average enhancements within two different ROIs placed on the liver, kidney medulla, kidney cortex and muscle.

Statistical Analysis

All the data obtained in the biodistribution and cell experiments are expressed as means ± standard deviation (SD). For the cell studies results a two tailed t-test was employed to evaluate the signficance of differences of individual pairs of values, and $p < 0.05$ was considere significant.

RESULTS AND DISCUSSION

Studies of $[^{153}Sm]^{3+}$-radiolabeled glycoconjugates

Quality control analysis: All the radioactive complexes where analysed by TLC for radiochemical purity determination as described in the Experimental section. The radiochemical purity was > 98 % for all the [$^{153}Sm^{3+}$] complexes with a specific activity *ca* 2mCi/μmol.

Stability in Serum. The percentage of radioactivity in the non-proteic fraction of human serum incubated with 5 μCi of the [$^{153}Sm]^{3+}$-labeled chelates slowly decays as a function of time (data not shown). For example, in the case of [$^{153}Sm]^{3+}$-DOTAGal *ca* 90 % of the activity remained in the non-proteic fraction after 4 hours.

Cell uptake. The Hep G2 human hepatocyte carcinoma cell line is known to express ASGP-R both *in vivo* and *in vitro* (32, 34). Fig. 2 shows the time dependence of the radioligand [$^{153}Sm]^{3+}$-$DOTAGal_2$ uptake in Hep G2 at 37°C with and without ASGP-R blocking, studied in a MEM medium containing 16 mM $CaCl_2$. In these conditions, unblocked cell uptake increases with time, reaching 12.6 % of the added activity after 2 h, but without reaching a plateau within the 2 h of incubation. The *in vitro* cell uptake of the radioligand activity was reduced in the presence of an excess of the ASGP-R blocker asialofetuin, but much less than expected from ASGP-R mediated specific uptake of the radioligand in these ASGP-R rich cells, especially at short incubation times (31). (Fig. 2). The non-blocked and blocked internalization values are at the limit of significant difference at the short times 30 min and 60 min (ratios of 1.4 and 1.2, respectively), as indicated by a value of $p > 0.04$ using the two tailed t–test, and are significantly different at 2 h ($p = 0.002$), when the non-blocked/blocked ratio reaches a maximum value of 2.3. Hep G2 cell uptake studies of an [$^{111}In]^{3+}$- labeled DOTA-Gal monoamide, using a RPMI-1640 medium containing 50 μM $CaCl_2$, gave a 3.1 ratio of non-blocked/blocked uptake at 30 min (31). The discrepancy between the two studies at low incubation times could be attributed to the larger concentration of $CaCl_2$ in the cell medium used in the present study,

although the litterature study, which used much lower $CaCl_2$ concentration also reported that ion baseline uptake was drastically reduced in the absence of Ca^{2+} (31).

***In vivo* gamma imaging and biodistribution.** Fig. 3 shows scintigraphic images of Wistar rats obtained 30 minutes after tracer injection for $[^{153}Sm]^{3+}$-labeled $DOTAGal_2$ (**2a**) and $DOTAGlc_2$ (**2b**) conjugates, selected from dynamic acquisitions consisting of frames obteined at every 10 seconds. At 30 minutes, there is a noticeably high uptake of $[^{153}Sm]^{3+}$-$DOTAGal_2$ by liver (left image), correlating with the fact that the surface of liver parenchimal cells contain a very high density of ASGP-R, about 500,000 per cell (36, 37), which can selectively take up the radiotracer by receptor mediated endocytosis (31). In the left image the bladder is also seen, which reflects the renal excretion pathway of these hydrophilic compounds. In addition, a very rapid clearance occurs from all other organs, including the kidneys, in accordance with the biodistribution data obtained in the same animals (see below). A quite different behaviour is found in rats injected with $[^{153}Sm]^{3+}$-$DOTAGlc_2$ (right image), as the 30 minutes activity is localized almost exclusively in the bladder. Virtually no liver uptake is observed, which is consistent with the fact that glucosyl glycoconjugates are not recognized by the ASGP-Rs (38). The time/activity curves for the $[^{153}Sm]^{3+}$-labeled glycoconjugates were obtained from the dynamic acquisition experiments (Fig. 4). The curves were smoothed and normalized in relation to the maximum activity obtained for each radiolabeled glycoconjugate. The kidneys are well visible in the images at early times, allowing the definition of their ROIs. The time/activity kidney curves of Fig. 4 correspond to the left kidney but the profile is similar for both kidneys. While the activity of $[^{153}Sm]^{3+}$-$DOTAGal_2$ is higher in the liver than in the kidney, and steadily increases up to the 30 minutes time interval in both organs, the activity of $[^{153}Sm]^{3+}$-$DOTAGlc_2$ quickly rises in kidney to a much higher value than in liver, reaching a maximum at 5 minutes, and later decays slowly. The initial behaviour of $[^{153}Sm]^{3+}$-$DOTALac_2$, with a quick activity increase in kidney, is close to that of $[^{153}Sm]^{3+}$-

DOTAGlc$_2$, but after attaining a maximum at 4 minutes, the kidney activity quickly decreases, while the liver activity steadily rises, like in [^{153}Sm]$^{3+}$-DOTAGal$_2$. This result might be explained by the higher hydrophilicity of the lactosyl conjugate relative to the galactosyl conjugate, which determines its initial concentration in the kidney, after which the terminal galactosyl groups of the lactosyl derivative binds to the liver ASGP-Rs. A number of lactosyl conjugates have been described for liver targeting (40, 41).

Biodistribution data for the [^{153}Sm]$^{3+}$-radiolabeled glycoconjugates obtained in Wistar rats are presented in Figs. 5 and 6 and Table 1 as percentage of injected dose per gram of tissue (% ID/g). The most striking feature shown by the biodistribution studies at one hour post-injection is the high liver targeting ability of the galactosyl conjugates (Fig. 5a). In sharp contrast, the glucosyl conjugates show no liver uptake, as they locate mainly in the kidneys due to their hydrophilicity. The biodistribution data for the lactosyl conjugates confirm the intermediate behaviour between the galactosyl and glucosyl conjugates seen in the dynamic acquisition curves described above.

Another noteworthy finding is the effect of the valence of the glycoconjugates on the biodistribution pattern. Whereas the monovalent conjugate [^{153}Sm]$^{3+}$-DOTAGal shows some spleen and lung uptake, the divalent compound, apart from the kidney, does not appear in a significant extent in any other organ. The behaviour of the tetravalent compound is very similar to that of the divalent one (e.g. liver uptake corresponds to 0.91 % of the ID/g for [^{153}Sm]-DOTAGal$_2$ and to 0.82 % of the ID/g for [^{153}Sm]$^{3+}$-DOTAGal$_4$), indicating that the effect of the increase in valence from two to four does not seem to improve liver uptake after one hour. This might be explained by the high number of ASGP-Rs expressed by each hepatocyte cell (36, 37) and/or some degree of steric crowding of the galactose residues. However, the effect of the valence of synthetic and natural glycoconjugates on the affinity to lectins has been fully demonstrated (7, 8) and thus the expected order of increasing affinity of the glycoconjugate ligands for the ASGP-

R is tetra>tri>di>mono (7, 8, 41, 42). This is confirmed by the observed contrast in the biodistribution behaviour between the $[^{153}Sm]^{3+}$-DOTAGal$_2$ and $[^{153}Sm]^{3+}$-DOTAGal$_4$ derivatives after 24 hours (Fig. 5b, Table 1). The activity remaining on liver at 24 hours for the tetravalent radiolabeled glycoconjugate $[^{153}Sm]^{3+}$-DOTAGal$_4$ (0.2 % of the ID/g) is much higher than for the divalent $^{153}Sm^{3+}$-DOTAGal$_2$ (0.002 % of the ID/g), suggesting a greater metabolic stability of the former compound.

The data in Fig. 5a also shows that the three ligands bearing only one sugar moiety (DOTAGal, DOTAGlc and DOTALac) stay longer in the blood stream and have a more dispersed biodistribution pattern than the higher valence analogs. Binding to some plasma component might be responsible for this finding. Thus, in addition to the multivalence effect, subtle structural effects arising from the dendrimeric framework of these ligands, especially the tertiary amine nitrogen, might be responsible for the observed differences of biodistribution between the monovalent and multivalent compounds.

Fig. 5c shows the bioditribution results of an *in vivo* competition experiment using large excess of asialofetuin, a specific blocker of the ASGP-R, co-injected with the radioligand $[^{153}Sm]^{3+}$-DOTAGal$_2$. In animals injected with the blocker, activity uptake in liver was reduced by 90 % ($p < 0.001$) compared with the blocked animals (31). The injection of the blocking dose also increased the uptake in the kidneys by a factor of three, suggesting that more activity is cleared instead of binding to the ASGP-R. These results strongly suggest that the *in vivo* uptake of the radiolabeled glycoconjugate is specific to the ASGP-R endocytosis. These *in vivo* results give a much higher 1 hour non-blocked/blocked uptake ratio of 9 that obtained for the Hep G2 cell line *in vitro*, even at 2 hours. This could in part result from the high Ca^{2+} concentrations in the cell medium used in the later studies and also from the fact that the total number of ASGP-R receptors in the human hepatocyte carcinoma cell line HepG2 is 140,000 +/- 65,000 sites per cell (43), significantly lower than the value obtained for isolated rat hepatocytes (36, 37).

A DOTA bisamide glycoconjugate bearing two galactosyl moieties in a cis position, DO2A(cis)Gal_2 (Fig. 1) was also evaluated. Fig. 6 compares the biodistribution of this radiolabeled ligand in Wistar rats at one hour post-injection with that of DO3AGal_2. It shows that the two different topologies of the targeting groups do not change their very marked preference for preferential liver uptake, but the radiolabeled DO2A(cis)Gal_2 compound shows only half liver enhancement of the DOTA monoamide conjugate, indicating reduced liver targeting ability.

Magnetic resonance imaging

Effect of the Gd chelates on vital functions. The CAs are well tolerated by the mice. No gross side effects were observed during injection, immediately or after the experiment. The animal temperature remained stable throughout the entire experiment, whereas the respiration rate slightly increased during the first 2-3 minutes post-injection.

MRI *in vivo*. Series of T_1-weighted spin echo images of the dynamic contrast enhanced (DCE) MRI experiments with the three divalent DOTA-glycoconjugate chelates of Gd^{3+}, GdDOTALac_2, GdDOTAGal_2 and GdDOTAGlc_2 (dose 0.3 mmol kg^{-1} BW), and GdDTPA (dose 0.2 mmol kg^{-1} BW), are shown in Fig. 7. Before injection, kidney, liver and muscle did not show any contrast difference, due to the short TR (155 ms) used for the acquisition of the T_1-weigthed images. After injection, a strong signal enhancement was observed in the kidneys and vena cava, for the various CAs, as a result of the T_1 shortening. A slighter signal enhancement was observed in liver and muscle for all the CAs.

The time course of the averaged signal intensity of several ROIs of the T_1-weighted spin echo MR post- contrast images obtained for the different CAs, normalized to the respective intensities of the pre-contrast images, are displayed in Fig. 8. The scattering in the curves was caused by animal respiratory motion. The time course of the averaged enhancement after the injection of 0.2 mmol/kg of GdDTPA (Fig. 8a) is in good agreement with the literature (44). The signal enhancement increased immediately after injection from

0 up to about 300 % in the kidney medulla and about 150 % in the kidney cortex, liver and muscle. While the enhancement in the later organs remained constant for about 40 min, the kidney medulla value slowly decreased in that time interval to about 200 %. The kidney medulla/liver enhancement ratio decreased from 2.2 at 4 minutes to 1.5 at 35 minutes. After injection of $GdDOTALac_2$ (Fig. 8b), the relative enhancement increased up to approx. 350 % in the kidney medulla, 260 % in the kidney cortex, 300 % in the liver and 180 % in the muscle. These maximum enhancements were reached at 20, 10, 3-4 and 4-5 minutes post-injection, respectively, and then decreased slowly. The kidney medulla/liver enhancement ratio increased from 0.6 at 4 minutes to 4.1 at 35 minutes. In the case of $GdDOTAGal_2$ (Fig. 8c), more moderate signal enhancements were observed in all the organs. The relative enhancement increase was approx. 220 % in the kidney medulla, 150 % in the kidney cortex, 100 % in the liver and 80 % in the muscle. In all ROIs a plateau was reached within the first 5 minutes post-injection and remained constant until the end of the pharmacokinetics experiment, with a constant kidney medulla/liver enhancement ratio of 1.7. Finally, immediately after the injection of $GdDOTAGlc_2$ (Fig. 8d) a very strong signal rise was observed in the kidney cortex, medulla and liver, followed after 3 minutes by signal decay, especially in the kidney cortex and medulla, due to an effect of T_2 shortening induced by the extremely high concentration of the CA. This circumstance did not allow the maximum enhancement to be measured. Nevertheless, from the measurable points the kidney medulla/liver enhancement ratio increased from 1.6 at 3 minutes to 5.3 at 35 minutes.

This qualitative comparison of the pharmacokinetics data of the four compounds shows that the glycoconjugate chelates $GdDOTAGlc_2$ and $GdDOTAGal_2$ behave quite similarly to GdDTPA throughout the whole time course of the experiment, with predominant kidney enhancement. In the case of $GdDOTALac_2$ the initial enhancement of

liver is higher than of kidney, but after 4 minutes the kidney cortex and medulla effect becomes predominant.

Although the r_1 relaxivities at 300 MHz (7.0 T) and 37 ºC of the CAs used in this work have not been published, the estimated values from their published NMRD curves and the respective fitting parameters are r_1 = 7.0 $mM^{-1}.s^{-1}$ for $GdDOTAGal_2$ and $GdDOTAGlc_2$ and r_1 = 8.0 $mM^{-1}.s^{-1}$ for $GdDOTALac_2$ (29), and r_1 = 4.0 $mM^{-1}.s^{-1}$ for GdDTPA (45). The r_2/r_1 ratio should be 7/6, giving in each case r_2 values 16 % higher than r_1. The gadolinium concentration time courses for each ROI were not calculated from the T_1-weighted image intensities, as there is no garantee that the *in vitro* and tissue relaxivities of the compounds are the same. However, the ratio of the product (r_1 x dose) is 2.6 for $GdDOTAGal_2$ and $GdDOTAGlc_2$ versus GdDTPA and 3.0 for $GdDOTALac_2$ versus GdDTPA. These values are much larger than the ratios of the maximum image intensity enhancements attained by those CAs relative to GdDTPA, which are 0.7 – 0.9 for the kidney medulla and 1.3 – 1.6 for the cortex (Fig. 8) ($GdDOTAGlc_2$ is excluded because its maximum T_1 enhancement effect could not be measured, as discussed above). This shows that the maximum concentration/dose ratio attained in the kidneys by the Gd^{3+}- based CAs is considerably lower than for GdDTPA, indicating that part of the injected CAs become T_1 contrast-silent, presumably when taken up into the liver. Such a considerable uptake of the galacosyl containing chelates was demonstrated by the gamma scintigraphic and biodistribution studies of the $[^{153}Sm]^{3+}$-labeled ligands.

CONCLUSIONS

Hepatobiliary CAs, with selective hepatocyte uptake by organic anion carriers and biliary excretion, provide a means of obtaining specific liver MRI enhancement and improved detection and characterization of small hepatic lesions (46). Several potential CAs for liver MRI have also been developed by targeting the hepatocyte ASGP-R and uptake via its endocytic pathway, such as targeted particulate T_2 relaxation agents (23, 27)

and macromolecular T_1 relaxation agents (21, 22). This approach is also useful in the detection and characterization of hepatic lesions, as they strongly affect the hepatocyte receptor levels (21). Appending a small glycodendrimer ending with galactosyl unit(s) to a GdDOTA scaffold via an amide bond generates a new class of low molecular weight, stable, hydrophilic glycoconjugates, of greater biological safety than the polymeric Gd-DTPA-gal-PL (21), and with targeting ability for the liver via recognition of the ASGP-R, as suggested *in vitro* by Hep G2 cell uptake, and specially *in vivo* gamma-imaging and biodistribution studies of the $[^{153}Sm]^{3+}$-labeled compounds in Wistar rats. The glycodendrimer architecture allows variation of the sugar type and valence in an interactive way from a reduced number of building blocks. The effect of the increase of valence on the targeting efficiency is clear when going from monoderivatives to divalent glycoconjugates. The performance of the next generation, tetravalent glycoconjugates, is similar to that of the divalent one, at one hour post-injection. However this compound is retained much longer at its target organ, as demonstrated by the activity remaining in the liver after 24 hours. Moreover, there are important biodistribution differences between mono- and multivalent glycoconjugates (31). A critical feature in the glycoconjugate-ASGP-R interaction is the topology of both the glycoconjugate and lectin. In this respect, the relative orientation and spacing of the carbohydrate residues in the glycoconjugate in relation to the distribution of the Carbohydrate Recognition Domains (CRDs) on the ASGP-R is of fundamental importance in the optimization of their interaction (47, 48).

The superior performance of the multivalent compounds validates our approach to lectin-mediated targeting for molecular imaging. The specific uptake of the galactosyl-bearing compounds via the ASGP-R opened an opportunity for the use of their Gd^{3+} chelates as CAs for ASGP-R mediated liver MRI. However, despite the promising scintigraphic performance of the galactosyl-bearing multivalent compounds, the animal MRI assessment of the corresponding Gd^{3+} chelates shows only limited liver-to-kidney

contrast effects, similar to the GdDTPA effects. We found evidence that the maximum concentration/dose ratio attained in the kidneys by the Gd^{3+}- based CAs is considerably lower than for GdDTPA, indicating that part of the injected CAs become T_1 contrast-silent, presumably when taken up into the liver. It is possible that the T_1 shortening effect of the Gd^{3+}-labeled Gal-containing compounds is strongly diminished upon hepatocyte internalization into an endossomic vesicle via receptor-mediated endocytosis of the ASGP-R. Several studies have shown that the molar relaxivity r_1 of a CA is strongly reduced upon uptake into an intracellular compartment (49-51), which restricts the efficiency of extracellular water exchange with the paramagnetic center in the vesicle compartments through the cytoplasm due to the effects of the membranes (51). As opposed to cell internalization into the cytoplasm by electroporation, where the observed relaxation rates are proportional to the amount of internalized paramagnetic complex, internalization into intracellular vesicles quenches the relaxation enhancement to a plateau value (about 3 s^{-1}) when the entrapped amount of Gd^{3+}-chelate is higher than 1 x 10^{10} Gd/cell (51).

The fast kidney uptake of these highly hydrophilic complexes will also limit the extent to which they can be concentrated within the hepatocytes up to a concentration high enough to yield a detectable image contrast (52), limiting their use as CAs for ASGP-R - mediated molecular imaging**.** The design of more efficient hepatocyte-target MRI agents of this kind may be pursued by tuning the hydrophilicity/hydrophobicity balance of the dendrimeric compounds, through either structural modification of the dendrimeric backbone or the sugar-linker moiety, while improving the targeting ability of the multivalent architecture, and enhancing their relaxivity by centrally positioning the Gd^{3+} to provide a more effective motional coupling of the chelate with the Gd^{3+}-proton vector (53).

Acknowledgments

This work was financially supported by the Foundation of Science and Technology (FCT), Portugal (project POCTI/QUI/47005/2002) and FEDER. MLGM acknowledges financial support from the Institute of Health Carlos III, Spain and TBR a FCT grant (SFRH/BD/5407/2001). The work was carried out within the EC COST Action D18 "Lanthanide chemistry for diagnosis and therapy", the European-funded EMIL programme (LSCH-2004-503569) and the Acção Integrada Luso-Espanhola E-10/04.

REFERENCES

1. Lis H, Sharon N. Lectins: carbohydrate-specific proteins that mediate cellular recognition, *Chem. Rev*., 1998, 98: 637-674.

2. Gabius H-J, André S, Kaltner H, Siebert, H-C, The sugar code: functional lectinomics, *Biochim. Biophys. Acta*, 2002, 1572: 165-177.

3. Dam TK, Brewer CF. Thermodynamic studies of lectin-carbohydrate interactions by isothermal titration calorimetry. *Chem. Rev*., 2002, 102: 387-429.

4. Loris R, Principles of structures of animal and plant lectins, *Biochim. Biophys. Acta*, 2002, 1572: 198-208.

5. Sharon N, Lis H**,** Lectins, 2nd Ed., Kluwer Academic Publishers, Dordrecht, 2003.

6. Yamazaki N, Kojima S, Bovin NV, André S, Gabius S, Gabius H-J, Endogenous lectins as targets for drug delivery. *Adv. Drug Deliv. Rev.*, 2000, 43: 225-244.

7. Mammen M, Choi S-K, Whitesides G M, Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors, *Angew. Chem. Int. Ed.*, 1998, 37: 2754-2794;

8. Lundquist JJ, Toone EJ, The cluster glycoside effect, *Chem. Rev*., 2002, 102: 555-578.

9. Weigel PH, Yik JHN. Glycans as endocytosis signals: the cases of the asialoglycoprotein and hyaluronan/chondroitin sulfate receptors. *Biochim. Biophys. Acta (General Subjects)*, 2002, 1572: 341-363.

10. Gregoriadis G, Targeting of drugs: implications in medicine. *Lancet*, 1981, *2*, 241-246.

11. Deal KA, Criste ME, Welsh MJ, Cellular distribution of ^{111}In-DTPA galactose BSA in normal and asialoglycoprotein receptor-deficient mouse liver, *Nucl. Med. Biol*., 1998, 25, 379 –385.

12. Ishibashi S, Hammer RE, Herz J. Asialoglycoprotein receptor deficiency in mice lacking the minor receptor subunit. *J. Biol. Chem*., 1994, 269: 27803-27806.

13. Koyama Y, Ishikawa M, Yeda A, Sudo R, Kojima S, Sugunaka A, Body distribution of galactose-containing synthetic-polymer and galactosylated albumin, *Polymer J*., 1993, 25: 355-361.

14. Vera DR, Stadalnik R, Krohn K, Technetium-99m galactosyl-neoglycoalbumin: preparation and preclinical studies *J. Nucl. Med*., 1985, 10: 1157-1167.

15. Nakagima K, Kinuya K, Mizutani Y, Hwang E H, Michigishi T, Tonami N, Kobayashi K, Simple scintigraphic parameters with Tc-99m galactosyl human serum albumin for clinical staging of chronic hepatocellular disfunction, *Ann. Nucl. Med*., 1999, 13: 5-11.

16. Miki K, Kubota K., Inoue Y., Vera DR., Makuuchi M, Receptor measurements via Tc-GSA kinetic modeling are proportional to functional hepatocellular mass *J. Nucl. Med*., 2001, 42: 733-737.

17. Colquhoum SD, Conelly CA, Vera DR, Portal–systemic shunts reduce asialoglycoprotein receptor density in rats. *J. Nucl. Med*., 2001, 42: 110-116.

18. Sueyoshi K, Narabayashi I, Aratani T, Doi K, Komori T, Ogura Y, Utsunomiya K, Shimidzu T, Utility of Tc-99m GSA whole-body scintigraphy in detecting bone metastases from hepatocellular carcinoma, *Clinical Nucl. Med*., 2001, 26: 221-224.

19. Miki K, Kubota K, Kokudo N, Inoue Y, Bandai Y, Makuuchi M, Asialoglycoprotein receptor and hepatic blood flow using technetium-99m-DTPA-galactosyl human serum albumin, *J. Nucl. Med*., 1977, 38: 1798-1807.

20. Shuke N, Okizaki A, Kino S, Sato J, Ishikawa Y, Zhao CL, Kineya S, Watabane N, Yokoama K, Aburano T, Functional mapping of regional liver asialoglycoprotein receptor amount from single blood sample and SPECT *J. Nucl. Med*., 2003, 44: 475-482.

21.Vera DR, Buonocore MH, Wisner ER, Katzberg RW, Stadalnik RC. A molecular receptor-binding contrast agent for magnetic resonance imaging of the liver. *Acad. Radiol.*, 1995, 2: 497-506.

22. Gallez B, Lacour V, Demeure R, Debuyst R, Dejehet F, Dekeyser JL, Dumont P. Spin labeled arabinogalactan as MRI contrast agent. *Magn. Reson. Imag*., 1994, 12: 61-69.

23. Schaffer BK, Linker C, Papisov M, Tsai E, Nossiff N, Shibata T, Bogdanov Jr. A., Brady TJ, Weissleder R, Mion-ASF: biokinetics of an MR receptor agent. *Magn. Reson. Imag*., 1993, 11: 411-417.

24. Weissleder R, Reimer P, Lee AS, Wittenberg J, Brady TJ. MR receptor imaging: ultra small iron oxide particles targeted to asialoglycoprotein receptors. *Am. J. Roentgenol*., 1990, 155: 1161-1167.

25. Reimer P, Weissleder R, Lee AS, Buettner S, Wittenberg J, Brady TJ. Asialoglycoprotein receptor function in benign liver disease: evaluation with MR imaging. *Radiology*, 1991, 178: 769-774.

26. Reimer P, Weissleder R, Wittenberg J, Brady TJ. Receptor-directed contrast agents for MR imaging: preclinical evaluation with afinity assays. *Radiology*, 1992, 182: 565-569.

27. Reimer P, Kwong KK, Weisskoff R, Cohen MS, Brady TJ, Weissleder R, Dynamic signal intensity changes in liver with superparamagnetic MR contrast agents, *J. Magn. Reson. Imag.,* 1992, 2: 177-181.

28. Connoly DT, Townsend RR, Kawagushi K, bell Wr, Lee Yc. Binding and endocytosis of cluster glycosides by rabbit hepatocytes. *J. Biol. Chem*., 1982, 257: 939-945.

29. André JP, Geraldes CFGC, Martins JA, Merbach AE, Prata MIM, Santos AC, de Lima JJP, Tóth E. Lanthanide(III) complexes of DOTA–glycoconjugates: a potential new class of lectin-mediated medical imaging agents. *Chem. Eur. J*. 2004, 10: 5804–5816.

30. Baía P, André JP, Geraldes CFGC, Martins JA, Merbach AE, Tóth É, Lanthanide(III) complexes of DTPA-bisamide glycoconjugates: potential imaging agents targeted for the asyaloglycoprotein receptor, *Eur. J. lnor. Chem*, 2005, 2110-2119.

31. Alauddin MM, Louie AY, Shahinian A, Meade TJ, Conti PS. Receptor mediated uptake of a radiolabeled contrast agent sensitive to □-galactosidase activity. *Nucl. Med. Biol*., 2003, 30: 261 –265.

32. Brücher E, Sherry AD, Stability and toxicity of contrast agents, in *The Chemistry of Contrast Agents in Medical Magnetic Resonance Imaging*, Ed. Merbach AE, Tóth É, 2001, John Wiley and Sons, Ltd, Chapter 6, 243-279.

33. Theilman L, Teicher L, Schildkraut CS, Stockert RJ, Growth-dependent expression of a cell surface glycoprotein. *Biochim. Biophys. Acta*, 1983, 762: 475-477.

34. Hennig J, Nauerth A, Friedburg H. RARE imaging: a fast imaging method for clinical MR. *Magn. Reson. Med*., 1986, 3: 823-833.

35. Collins JC, Paietta E, Green R, Morell AG, Stockert RJ. Biotin –dependent expression of the asialoglycoprotein receptor in ethanol-fed mice. *J. Biol. Chem.* 1988, 263: 11280-11283.

36. Schwartz AL, Rup D, Lodish HF. Difficulties in the quantification of asialoglycoprotein receptors on the rat hepatocyte, *J. Biol. Chem.* 1980, 255: 9033-9036.

37. Weigel PH, Oka JA, The large intracellular pool of asialoglycoprotein receptors functions during the endocytosis of asialoglycoproteins by isolated rat hepatocytes. *J. Biol. Chem.* 1983, 258: 5095-5102.

38. Vaino AR, Depew WT, Szarek WA, Synthesis of a D-lactosyl cluster–nucleoside conjugate. *Chem. Commun* 1997, 1871-1872.

39. Krebs A, Depew WT, Szarek WA, Hay GW, Hronowski LJJ, Binding of D-galactose-terminated ligands to rabbit asialoglycoprotein receptor. *Carbohydr. Res.,* 1994, 254: 257-268.

40. Lee YC, Townsend RR, Hardy MR, Lönngren J, Arnarp J, Haraldsson M., Lönn H., Binding of synthetic oligosaccharides to the hepatic Gal/GalNAc lectin. Dependence on fine structural features. *J. Biol. Chem.,*1983, 258: 199-202.

41. Lee RT, Lee YC, Rabbit and rat hepatic lectins have two sugar-combining sites per monomeric unit. *Biochem. Biophys. Res. Commun.,* 1988, 155: 1444-1451.

42. Biessen EAL, Broxterman H, VanBoom JH, VanBerkel TJ, The cholesterol derivative of a triantennary galactoside with high affinity for the hepatic asialoglycoprotein receptor: a potent cholesterol lowering agent. *J. Med. Chem.,* 1995**,** 38: 1846-1852.

43. Eisenberg C, Seta N, Appel M, Feldmann G, Durand G, Feger J Asialoglycoprotein receptor in human isolated hepatocytes from normal liver and its apparent increase in liver with histological alterations. *J Hepatol.,* 1991,13:305-309.

44. Raghunand N, Howison C, Sherry AD, Zhang S, Gillies RJ. Renal and systemic pH imaging by contrast-enhanced MRI, *Magn. Reson. Med*., 2003, 49: 249-257.

45. Koenig SH, Baglin C, Brown III RD, Magnetic Field dependence of solvent proton relaxation induced by Gd^{3+} and Mn^{2+} complexes, *Magn. Reson. Med*., 1984, 1: 496 -501.

46. Van Beers BE, Gallez B, Prigot J. Contrast-enhanced MR imaging of the liver. *Radiology*, 1997, 203: 297-306.

47. Vrasidas I, André S, Valentini P, Bock C, Lensch M, Kaltner H, Liskamp RMJ, Gabius H-J, Pieters RJ, Rigidified multivalent lactose molecules and their interactions with mammalian galectins: a route to selective inhibitors. *Org. Biomol. Chem.,* 2003, 1: 803-810.

48. Meier M, Bider MD, Malashkevich VN, Spiess M, Burkhard P. Crystal structure of the carbohydrate recognition domain of the H1 subunit of the asialoglycoprotein receptor. *J. Mol. Biol.*, 2000, 300: 857-865.

49. Geninatti Crich S, Biancone L, Cantaluppi V, Duò D, Esposito G, Russo S, Camussi G, Aime S, Improved route for the visualization of stem cells labeled with a Gd-/Eu-chelate as dual (MRI and fluorescence) agent, *Magn. Reson. Med*., 2004, 51: 938-994.

50. Geninatti Crich S, Barge A, Battistini E, Cabella C, Coluccia S, Longo D, Mainero V, Tarone G, Aime S, Magnetic Resonance Imaging visualizatio of targeted cells by the

internalization of supramolecular adducts formed between avidin and biotinylted Gd^{3+} complexes., J. Biol. Inorg. Chem., 2005, 10: 78-86.

51. Terreno E, Geninatti Crich S, Belfiore S, Biancone L, Cabella C, Esposito G, Manazza AD, Aime S, Effect of the intracellular localization of a Gd-based imaging probe on the relaxation enhancement of water protons, *Magn. Reson. Med*., 2006, 55: 491-497.

52. Aime S, Barge A, Cabella C, Geninatti Crich S, Gianolio E, Targeting cells with MR imaging probes based on paramagnetic Gd(III) chelates, *Curr. Pharm. Biotechnol*. 2004, 5: 509-518.

53. Fulton DA, Elemento EM, Aime S, Chaabane L, Botta M, Parker D, Glycoconjugates of gadolinium complexes for MRI applications, *Chem. Commun*., 2006, 1064-1066.

Table 1 – Biodistribution of $[^{153}Sm]^{3+}$-DOTA glycoconjugates at 1 h and 24 h post-injection in Wistar rats. Results are expressed as percentage of injected dose per gram of tissue and are the mean of groups of four animals.

Tissue	Glc_2(**2b**)	Lac_2(**2c**)	Gal (**1a**)	Gal_2 (**2a**)		Gal_4(**3**)	
	1h	1h	1h	1h	24h	1h	24h
Blood	0.002± $5X10^{-4}$	0.015±0.005	0.158±0.01	0.057±$9X10^{-4}$	$5X10^{-5}$±$2X10^{-5}$	0.028±$8X10^{-4}$	$1X10^{-4}$±$4X10^{-5}$
Kidney	1.48±0.001	1.13±0.240	0.0540±0.01	0.075±0.009	0.007±$5X10^{-5}$	0.091±0.015	0.022±0.007
Liver	0.011±0.002	0.106±0.011	0.66±0.09	0.9062±0.073	0.023±$3X10^{-4}$	0.428±0.035	0.201±0.008
Spleen	0.012±0.003	0.046±0.002	0.49±0.12	0.012± 0.006	0.003±$2X10^{-6}$	0.006±0.0005	0.020±0.001
Heart	0.006±0.0006	0.047±$2X10^{-4}$	0.026±0.0006	0.0138±0.006	$2X10^{-4}$±$6X10^{-4}$	0.010±0.0022	0.002±$4X10^{-4}$
Lung	0.022±0.0002	0.051±0.003	0.1590±0.003	0.008±0.002	$5X10^{-4}$±$3X10^{-6}$	0.027±0.008	0.002±0.0002
S.Intest	0.009±$5X10^{-4}$	0.025±0.016	0.0110±0.005	0.0167±0.0084	$3X10^{-4}$±$2X10^{-5}$	0.021±0.0021	0.002±$4X10^{-4}$
L.Intest	0.014±0.0027	0.029±0.017	0.011±0.004	0.0029±0.001	$6X10^{-4}$±$3X10^{-6}$	0.013±0.0018	0.003±$6X10^{-4}$
Stomach	0.0190±0.004	0.004±0.002	0.012±0.0001	0.0121±0.005	$4X10^{-4}$±$2X10^{-5}$	0.014±0.0018	0.002±$7X10^{-4}$
Brain	$3X10^{-4}$±$2X10^{-5}$	$2X10^{-4}$±$9X10^{-5}$	$3X10^{-4}$±0.0005	0.0007± 0.0002	$6X10^{-5}$±$6X10^{-6}$	$4X10^{-4}$±$1X10^{-4}$	$2X10^{-5}$±$4X10^{-6}$