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Magnetic resonance imaging of tumor cells by targeting the amino acid transport system

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Abstract—An early diagnosis of cancer is crucial in the battle against this disease and the in vivo visualization of tumors at cellular level is still the most challenging goal. In order to target tumor cells, we took into account their increased metabolism and amino acid nutrients or pseudo-nutrients, which are actively transported through the cell membrane, have been chosen as vectors for new MRI contrast agents. For this reason new gadolinium complexes conjugated to agmatine, arginine, and glutamine have been synthesized and studied.

Magnetic resonance imaging (MRI) is a powerful, non-invasive, and widely applied diagnostic technique which allows to obtain images of the inside of the human body.[1,2] Nowadays, more than one-third of the MRI scans are performed by administration of a contrast agent, usually a gadolinium complex.[3,4] Gadolinium (III) ion is able, due to its favorable paramagnetic properties, to increase the relaxation rate of the surrounding water protons, making the region of interest brighter than the background.

Since the approval of the first gadolinium complex for human administration in 1988, several other analogues have reached the market.[5,6] The contrast agents of first generation distribute into the intravascular and interstitial space immediately after injection and in this context are called ''non-specific agent''. The medical need for tissue specific contrast agents has driven researchers to design and synthesize contrast agents of second generation able to visualize selectively the liver or the cardiovascular system, for instance.[7] The ultimate goal is a contrast agent that accumulates specifically in tumor cells, allowing an accurate diagnosis of the disease when it is still treatable.

A well-assessed method of tumor diagnosis is the targeting of over-expressed receptors on the membrane of tumor cells with specific radiopharmaceuticals,[8–11] but in spite of its high sensitivity this technique only gives poor image resolution. In the case of MRI this targeting strategy is hampered by the very low concentration of such receptors[12] and by the poor sensitivity of gadolinium complexes. Among the several possibilities proposed to achieve an efficient cellular uptake of gadolinium complexes[13] we took into account transport systems, which are more efficient in terms of internalization than receptors. Considering that rapidly growing tumors require an increased and continuous supply of both essential and nonessential amino acids, we selected the amino acid transport system. In particular, we focused on glutamine transport system because glutamine is the main source of nitrogen for tumor cells.[14,15] For these reasons DTPA (diethylenetriaminepentaacetic acid) and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) were conjugated to glutamine, arginine, and agmatine to give six new gadolinium complexes that were studied in terms of relaxivity, tumor internalization, and in vitro MRI imaging.

For the synthesis of DTPA derivatives, DTPA-Glu **1**[16] was reacted with *N*-hydroxysuccinimide and *N*-ethyl-

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N′-(3-dimethylaminopropyl)carbodiimide (EDCI) in CH_2Cl_2 to give the *N*-hydroxysuccinimidyl ester **5** which was directly coupled without purification to glutamine *tert*-butyl ester **2**, bis-Boc-agmatine **3**,[17] and arginine(Mtr) *tert*-butyl ester **4**.

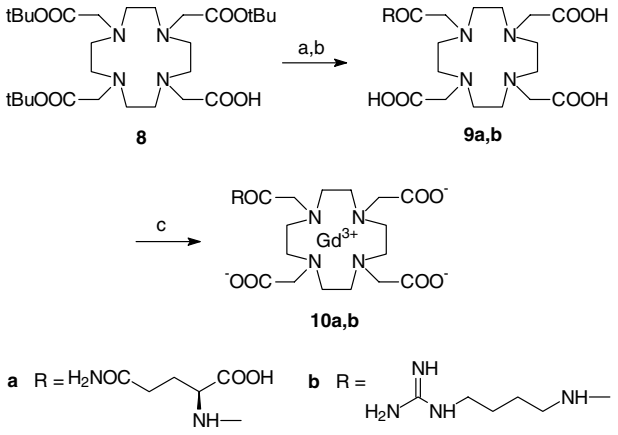
The corresponding amides obtained were deprotected with trifluoroacetic acid (TFA) to give acyclic ligands **6a**–**c**. Complexation was performed by heating the suspension of ligand and Gd_2O_3 in water at 50 °C for 16 h. The excess of Gd_2O_3 was filtered off, 1 M NaOH was added until pH 7, and the solution evaporated to give complexes **7a**–**c** as sodium salts (Scheme 1).

For the synthesis of DOTA derivatives, DOTA tris *tert*-butyl ester **8**[18] was coupled to amines **2** and **3** using EDCI, 1-hydroxybenzotriazole (HOBt), and diethylisopropylamine (DIEA) in CH_2Cl_2. The corresponding amides obtained were deprotected with TFA and the cyclic ligands **9a**,**b** so obtained were reacted with Gd_2O_3 to give gadolinium complexes **10a**,**b** (Scheme 2).

Complex **10c** was obtained with a different synthetic strategy. Arginine(Mtr) *tert*-butyl ester **4** was acylated with chloroacetyl chloride to give chloroacetamide **11**. DO3A tris *tert*-butyl ester **12**[18] was thus alkylated with **11** and the product obtained deprotected with TFA to give cyclic ligand **9c**. Final complexation with Gd_2O_3 gave product **10c** (Scheme 3).

Deprotection of DTPA-Glu **1** with TFA and complexation with Gd_2O_3 gave the reference compound **13** (Scheme 1).

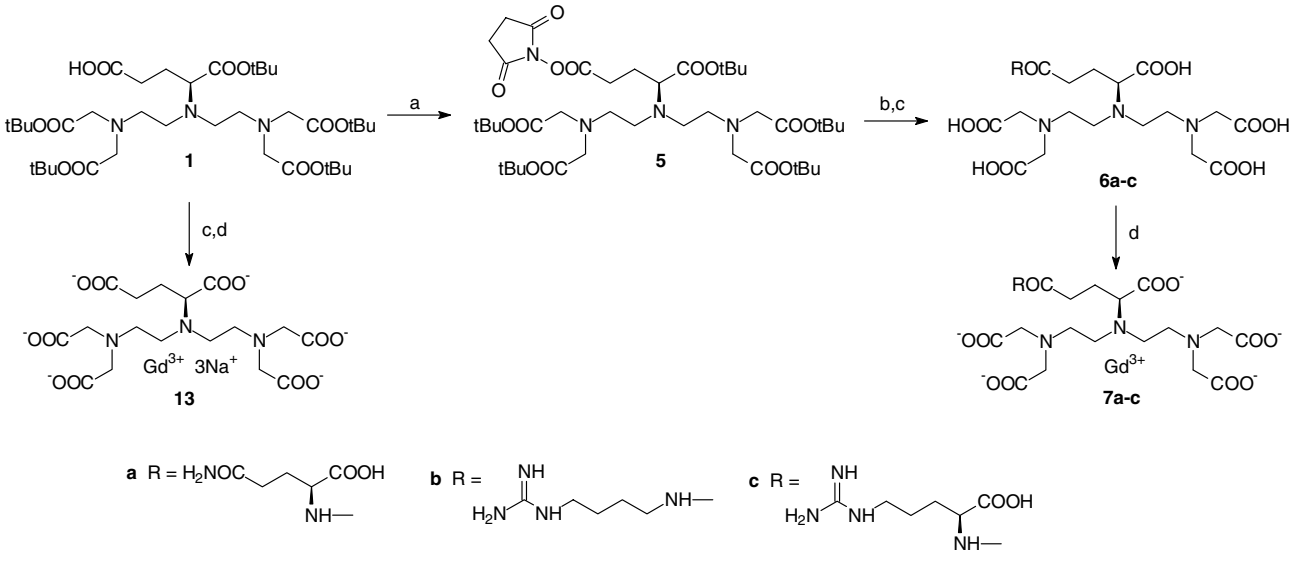
Relaxivity (r_1) of all gadolinium complexes (Table 1) was determined by titration of an aqueous solution of each corresponding ligand with a standard solution of $GdCl_3$. All complexes show relaxivities significantly higher than the ones of the parent Gd-DTPA



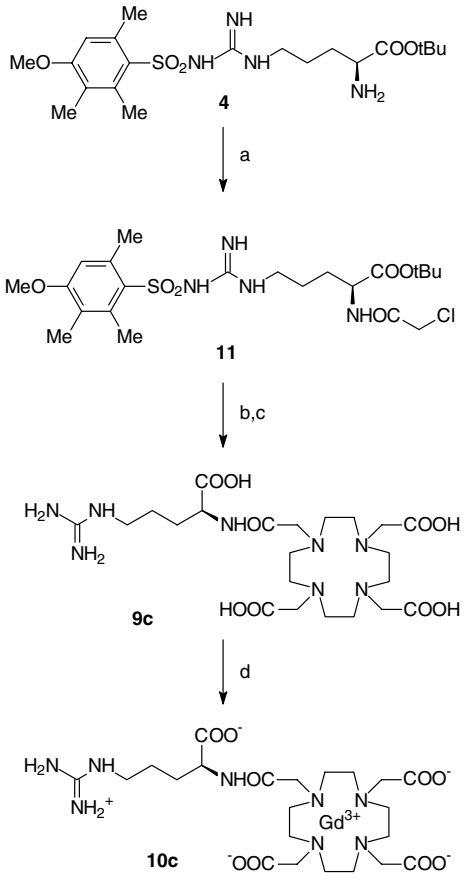
Scheme 2. Reagents and conditions: (a) amine **2** or **3**, HOBt, EDCI, DIEA; (b) TFA, (c) Gd_2O_3, H_2O, 50 °C.

(4.3 s^{-1} mM^{-1})[19] and Gd-DOTA (4.2 s^{-1} mM^{-1})[19] complexes. Since the increase in relaxivity is higher than that expected from the effects associated to the elongation of the molecular reorientational time, a possible explanation may be ascribed to a contribution arising from water molecules/exchangeable protons in the second coordination sphere due to the presence of the aminoacid residue.

Cellular uptake (Fig. 1) was performed with rat hepatocarcinoma cell line (HTC).[20] The cells were seeded in 75 cm^2 flasks at a density of about 25,000 cells/cm^2. After 24 h, cells were washed with 5 mL phosphate-buffered saline (PBS) and then incubated at 37 °C for 6 h with 5 mL of Earle's balanced salt solution (EBSS) in the presence of 1.6 mM concentration of each Gd(III) complex. At the end of the uptake/binding experiment the medium was removed; the cells (ca. 5×10^6) were washed three times with 5 mL ice-cold PBS and collected in 200 μL PBS. Cells were then treated with 200 μL of



Scheme 1. Reagents and conditions: (a) *N*-hydroxysuccinimide, EDCI, CH_2Cl_2; (b) coupling with amines **2**–**4**; (c) TFA; (d) Gd_2O_3, 1 M NaOH, pH 7, 50 °C.



Scheme 3. Reagents and conditions: (a) chloroacetylchloride, DIEA, CH_2Cl_2; (b) DO3A tris *tert*-butyl ester **12**, K_2CO_3, MeCN; (c) TFA; (d) Gd_2O_3, H_2O, 50 °C.

Table 1. Relaxivity of the gadolinium complexes studied

Compound	R_1 (s^{-1} mM^{-1})[a]
Gd-DTPAGlu-Gln **7a**	6.5
Gd-DTPAGlu-Agm **7b**	6.8
Gd-DTPAGlu-Arg **7c**	6.8
Gd-DOTA-Gln **10a**	5.0
Gd-DOTA-Agm **10b**	5.1
Gd-DOTA-Arg **10c**	5.6
Gd-DTPAGlu **13**	6.2

[a] Values obtained at 20 MHz and 25 °C.

37% HCl and sonicated at 120 °C. After 16 h, all Gd^{3+} is dissolved as free aqua-ion.

By measuring the proton relaxation rate (R_{1obs}) of these solutions it is possible to determine the Gd^{3+} concentration. In fact, R_{1obs} is proportional to the concentration of the paramagnetic Gd^{3+} ion according to the formula:

$$R_{1obs} = R_{1d} + [Gd^{3+}] \cdot r_{1p}^{Gd3+}$$

where R_{1d} is the relaxation rate obtained with the same number of untreated cells, and r_{1p}^{Gd3+} the millimolar relaxivity of the Gd ion (13.5 s^{-1} mM^{-1} in a 6 M HCl solution). The method has been checked out by using atomic standard solutions of Gd^{3+} ion and double-checked with parallel ICP-MS measurements.

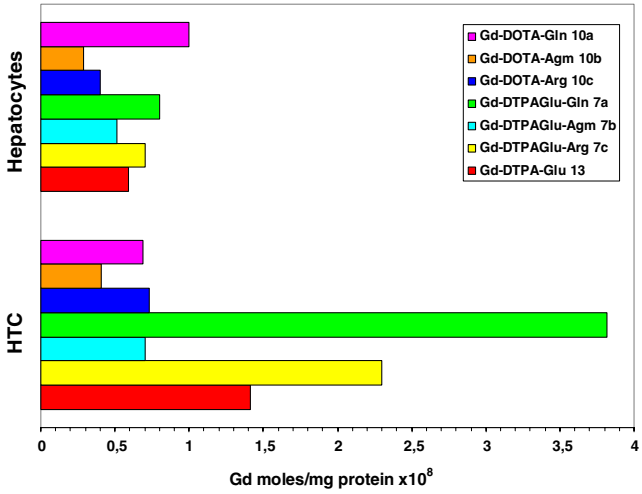


Figure 1. Comparison of cellular uptakes of the gadolinium complexes synthesized in this work on hepatocytes (as model of healthy cells) and HTC (as model of tumor cells). Each value is the average of three experiments.

For MRI of cell pellets (Fig. 2), cells were incubated with the contrast agent, extensively washed, detached, and again washed as described above. The cells were pelleted within a glass capillary (1 mm diameter) that was then inserted in 2% agarose within a 5 ml tube. The imaging protocol consisted of an Inversion-Recovery T_1-weighted spin-echo.[21] The T_1 of the hepatocytes blank is shorter than HTC blank because of their different iron content. The membrane binding (4 °C) of compound **7a** is higher in HTC cells because of the over-expression of the amino acid transport systems on these tumor cells.[22]

Upon incubation, all complexes enter into HTC cells in amounts that appear definitively higher than the ones that could be expected by simple pinocytosis (Fig. 1).

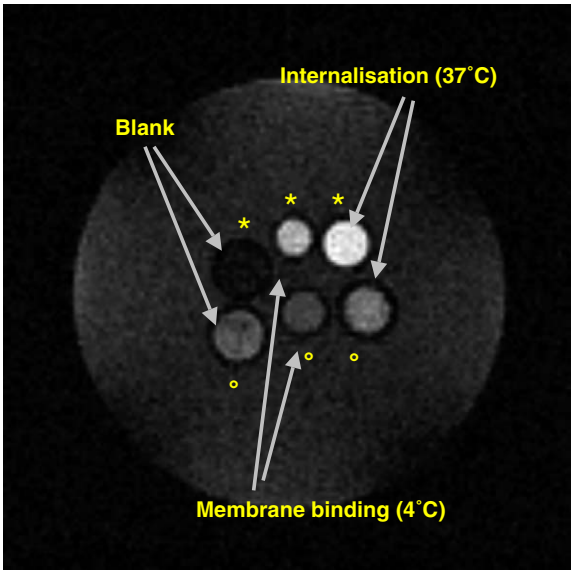


Figure 2. MRI images of cellular pellets in agar (7 T). The HTC (∗) and hepatocytes (°) have been incubated in EBSS medium added with Gd-DTPAGlu-Gln **7a**. T1 of HTC pellets: blank = 1.99 (s), binding = 1.15 (s), uptake = 0.96 (s). T1 of hepatocyte pellets: blank = 1.64 (s), binding = 1.65 (s), uptake = 1.45 (s).

In fact the latter process, that leads to a nonspecific internalization of portions of the incubation medium, may yield to significant entrapments only at concentration of imaging agents much higher than that those used in the present work. Thus, on the basis of the amounts of internalized Gd, one may conclude that the internalization path involves the transport system of choice. By comparing the MR images of HTC pellets incubated at 4 °C with those incubated at 37 °C (Fig. 2) one may assess the relative effects on the attainable contrast arising from the simple binding on the outer cellular membrane and the overall uptake process. At low temperature (4 °C), the energy-dependent pathways involved in the cellular uptake are not activated and therefore the observed signal enhancement is limited to the small amount of paramagnetic agent bound on the outer cell membrane. Support to the view that glutamine transporters are involved in the uptake process has been gained by measuring a decrease in the amounts of internalized gadolinium complexes **7a** when free glutamine was added to the incubation medium (Fig. 3). Clearly the free glutamine binds better to its transporter and causes a significant decrease of the cellular uptake of **7a**. On the basis of the available results it is not possible to say anything concerning the eventual changes in the internalization pathway of the amino acid-functionalized agents in respect to the free amino acids. Complex **7a** gave the best performance being internalized to an extent of about 5×10^9 Gd atoms per HTC cell. The observation that complex **10a** does not show an analogous uptake outlines how the structure of the chelate (DTPA vs DOTA), the residual charge, and the nature of the spacer may be important in determining the efficiency of the uptake. Moreover, one or more of these parameters, together to the increased pinocytic activity of tumor cells, could be responsible for the different HTC uptake of compounds **7b**, **7c**, and **13**.

On the basis of the results of the in vitro uptake experiments, the complex **7a** has been chosen as a lead compound for further in vivo experiments on tumor bearing animal models.

In summary, the results reported in this work show that the conjugation of amino acids to DTPA and DOTA Gd(III) complexes leads to products that display improved relaxometric properties in respect to the parent non-conjugated complexes. Moreover, the complex **7a** is able to recognize the *trans*-membrane glutamine transport system and the in vitro experiments show that the amount of internalized Gd is sufficient for the MRI visualization of tumor cells in respect to healthy ones. Further studies are ongoing with compound **7a** on other tumor cell lines.

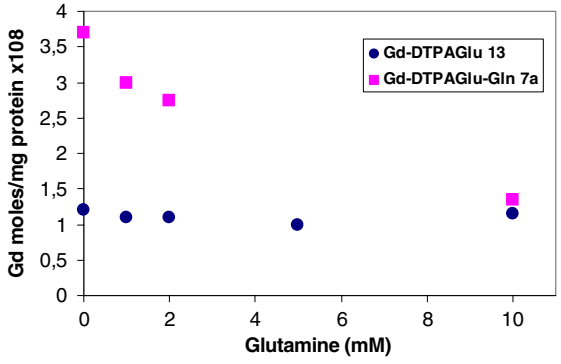


Figure 3. Competition assay between complexes **7a** and **13** and glutamine for the uptake into HTC cells (6 h, 37 °C, complex concentration 1.3 mM).

Acknowledgment

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21. Parameters used: repetition time (TR) = 10,000 ms; echo-time (TE) = 3.3 ms; inversion-time (TI) = 1000 ms; number of excitation (NEX) = 1; FOV = 1 × 1 cm; data matrix = 128 × 128; slice thickness = 1 mm.
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