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(54) Title: ENHANCED SUBSTRATE IMAGING BY REVERSIBLE BINDING TO A PARAMAGNETIC COMPLEX

(57) Abstract: The invention relates to a method for the Magnetic Resonance Imaging and Magnetic Resonance Spectroscopy Imaging of a substrate molecule different from water wherein the NMR signal is enhanced by a reversible binding of the substrate molecule to a paramagnetic chelate complex, as well as to the use of a paramagnetic chelate complex in said method and the pharmaceutical composition for use therein.

**ENHANCED SUBSTRATE IMAGING BY REVERSIBLE BINDING
TO A PARAMAGNETIC COMPLEX**

The present invention relates to the Magnetic Resonance Imaging field. In particular the invention relates to a method for the Magnetic Resonance Imaging and Magnetic Resonance Spectroscopy Imaging of a substrate molecule different from water wherein the NMR signal is enhanced by a reversible binding of the substrate molecule to a paramagnetic chelate complex.

The invention also relates to the use of a paramagnetic chelate complex in said method and the pharmaceutical composition for use therein.

BACKGROUND OF THE INVENTION

The diagnostic imaging based on the Nuclear Magnetic Resonance principles, which includes the Magnetic Resonance Imaging (MRI) and the Magnetic Resonance Spectroscopy Imaging (MRSI), herein after collectively indicated as MRI, is a well established imaging method which represents a powerful tool for everyday clinical investigations.

The large majority of MRI procedures deals with the very intense water signal, the concentration of which in the human body is about 55.6 M. In most cases, ^1H -MR images of the examined organ or body tissue correspond to a topological representations of differences in water density and/or relaxation rates of water protons into the region of clinical interest.

The contrast in these images can be augmented by the use of specific substances, known as contrast agents for MRI, modifying the relaxation times, T_1 and T_2 , of water protons.

Because of their ability to affect relaxation times, many paramagnetic substances have potential as contrast agents. In practice, however, only metal complexes or cluster complexes that are paramagnetic as a result of

containing one or more unpaired electrons are used as MRI paramagnetic contrast agents.

The most important class of contrast agents for MRI is represented by paramagnetic chelates, especially those containing one of the following paramagnetic ions: Gd(III), Mn(II), Fe(III) and Cr(III).

Their effect on the relaxation rate of water protons is usually assessed through the determination of their relaxivity (r_1), i.e. the relaxation enhancement of water protons promoted by the paramagnetic complex at 1 mM concentration, measured at a given observation frequency (often 20 MHz) and temperature (usually 39°C).

The relaxivity r_1 of a given paramagnetic complex may be considered as the sum of different contributions reflecting the different interaction modalities between water protons and the paramagnetic center.

The most important of these contributions, r_1^{is} , directly depends on the ratio between the concentration of water bound to the given paramagnetic chelate complex and the total concentration of water, and inversely depends on the sum of the relaxation time T_{1M} and the residence time τ_M of the protons of the water molecule(s) coordinated to the paramagnetic chelate complex, according to the following equation:

$$r_1^{is} = \frac{[H_2O]_{bound}}{[H_2O]_{total}} \frac{1}{T_{1M} + \tau_M}$$

In this formula $[H_2O]_{bound}$ corresponds to the concentration of the given paramagnetic complex times the number (q) of water molecules directly coordinated to the metal centre. Since in most cases $q = 1$ and $[H_2O]_{total} = 55.6$ M, for 1 mM concentration of the paramagnetic chelate, the ratio $[H_2O]_{bound}/[H_2O]_{total}$ is a fixed value ($1.8 \cdot 10^{-5}$). This means that, in the case of MR images of the water signal, this ratio cannot be increased to a greater extent.

Most paramagnetic metal complexes have been developed for MRI methods based on the resonance of ^1H water protons: examples of said complexes and their use in conventional ^1H water protons magnetic resonance imaging methods are reported, inter alia, in WO 00/38738, US 5 5977353, FR 2725449, FR 2802928 and in J. Alloys and Compounds, 249(1997) 185-190.

A relatively small portion of MRI clinical applications deals with imaging of resonances of ^1H protons other than the ^1H water protons.

In such cases, attention has been focused on species present in 10 relatively large amount like the fatty constituents present in many tissues (E.L. Thomas and J.D Bell in "Methods in Biomedical Magnetic Resonance Imaging and Spectroscopy", Wiley; 2000. Vol. 2, p 837-845 and references therein), or in the use of sophisticated and dedicated techniques and equipments (EP-A-370333 for the in vivo lactate visualization).

15 Also the acquisition of MR images of nuclei different from protons is rather uncommon in biological systems. First of all, it is not easy to find in such systems magnetically active nuclei, i.e. nuclei with nuclear spin $\neq 0$, naturally present at sufficiently high concentration or whose enrichment in the isotope of interest is practically feasible.

20 Secondly, and mainly, the relaxation times of these magnetically active nuclei other than ^1H often exceeds one second, making very time consuming the accumulation of the number of transients to yield a sufficient signal to noise ratio (SNR) for obtaining a good image.

In general, in fact, the generation of a good substrate imaging in a 25 reasonable time requires dealing with signal characterized by short T_1 , in order to accumulate a high number of transients enhancing thereby the signal to noise ratio.

Nonetheless, the possibility of recording images of nuclei different

from water protons appears highly attractive because the absence of signal from background is certainly very useful for improving the diagnostic content of the resulting MR image.

Moreover, there are a number of endogenous substances whose
5 imaging would be extremely interesting and useful from the clinical point of view. A non-limiting list of such substances includes metabolites and substrates like lactate, citrate, carbonate, phosphate, pyruvate, natural amino-acids, oxalate, tartrate, succinate, choline, creatine, acetate, malonate. These molecules are involved in several human metabolic
10 processes and changes in their normal production, as well as in their biodistribution, may be generally related to pathologic conditions.

Lactate, for example, is an endogenous metabolite of particular diagnostic relevance. It is an end product of the anaerobic glycolysis and, therefore, an increase of its concentration in tissues may be a clear
15 indication of hypoxia, as it is found in some solid tumors. High levels of lactate are further present in conditions of reduced blood flow occurring in cerebral strokes or coronary infarcts or in presence of metabolic disorders or type I diabetes.

Another important metabolite, whose *in vivo* mapping would be very
20 useful, is citrate. Its concentration in the case of prostatic tumor differs significantly between benign prostatic hyperplasia (BPH, high concentration) and malignant prostatic carcinoma (low concentration).

Hence, there remains a need for a Magnetic Resonance Imaging method which would allow the imaging of molecules different from water in
25 a time suitable for a conventional medical practice and by conventional mode of operation.

SUMMARY OF THE INVENTION

It has now been found that a marked shortening of the relaxation times (T_1 and T_2) of the magnetic nuclei of a substrate molecule different from water can be obtained by exploiting a non covalent reversible binding
5 between the substrate molecule and a suitable paramagnetic chelate complex.

In particular it has been found that the reversible binding between a substrate molecule and a suitable paramagnetic chelate complex allows the specific and selective high enhancement of the relaxation characteristics of
10 the substrate nuclei of interest for the imaging procedure wherein this results in an easy and direct imaging of the substrate.

Quantitatively, the relaxation enhancement, ε , of the substrate nuclei induced by the paramagnetic complex may be expressed in terms of this
15 ratio:

$$\varepsilon = \frac{R_i^{s*}}{R_i^s}$$

in which R_i^s represents the observed relaxation rate of the substrate nucleus of interest without paramagnetic promotion and R_i^{s*} is the corresponding relaxation rate observed when the paramagnetic chelate is
20 present in solution.

The relaxation rate R_i is equal to $1/T_i$, wherein $i = 1$ or 2 .

A first object of the present invention is therefore a method for the Magnetic Resonance imaging of a substrate molecule different from water in a human or animal body organ or tissue characterised in that a
25 paramagnetic chelate complex is employed which non covalently binds to the substrate molecule and as a result of said binding the relaxation enhancement (ε) of the substrate nuclei of interest for the imaging procedure

is at least 2 at 39°C and pH 7.4.

The invention also refers to the use of a paramagnetic chelate complex or a physiologically tolerable salt thereof, said chelate complex being able to non covalently bind to the substrate molecule so that the relaxation enhancement (ϵ) of the substrate nuclei of interest is at least 2 at 39°C and pH 7.4, for the preparation of an enhancing agent for the magnetic resonance imaging of a substrate molecule different from water in a human or animal body organ or tissue.

Preferably, said relaxation enhancement is of at least 10 and, more preferably, is of at least 30 wherein said relaxation enhancement values are obtained independently from the applied magnetic field strength.

This non covalent binding between paramagnetic chelate complex and substrate molecule allows a marked shortening of the relaxation times (T_1 and T_2) of the magnetic nuclei of said substrate molecule allowing, in such a way, the acquisition of a particular high number of transients in a brief time and the registration of clear and diagnostically useful images in a short time, compatible with the clinical practice.

Moreover, by use of the paramagnetic complex according to the method of the invention, it is possible to obtain ^1H images of substrates in spite of the presence of the large signal arising from water. In this respect, in fact, when the substrate of interest interacts with a paramagnetic complex, according to this invention, the time required for the spectra acquisition is considerably shorter. Moreover, the water signal intensity is significantly reduced owing to the very rapid repetition time, and it can be further reduced by the use of a suitable pulse sequence.

Substrates molecules whose Magnetic Resonance Imaging is enhanced by the use of a suitable paramagnetic chelate complex according to the method of this invention include any molecule different from water which

comprises one or more magnetic nuclei, i.e. nuclei with nuclear spin $\neq 0$.

Said magnetic nuclei can also be in natural abundance in the substrate molecule or they can be "ad hoc" introduced or enriched in the substrate molecule of interest for the diagnostic procedure.

5 A non limiting example of substrate nuclei of interest for the imaging procedure includes ^1H , ^{19}F , ^{31}P , ^{13}C , ^{15}N , wherein the substrate molecule is optionally labelled with ^{19}F , ^{31}P , ^{13}C , ^{15}N .

Preferably, said substrate molecule is an endogenous or an endogenously generated substrate, wherein the term "endogenous substrate" 10 as used herein refers to a substrate which results from the normal or pathological behaviour of the biochemical process of cells and tissues and the term "endogenously generated substrate" as used herein refers to a substrate which results, in part or fully, from the transformation of a substance administered for the purpose of the diagnostic assay.

15 More preferably, the endogenous substrates are selected from the group consisting of lactate, citrate, carbonate, phosphate, pyruvate, natural amino-acids, oxalate, tartrate, succinate, choline, creatine, acetate, and malonate and the endogenously generated substrates are selected from the group consisting of lactate, citrate, carbonate, phosphate, pyruvate, natural amino- 20 acids, oxalate, tartrate, succinate, choline, creatine, acetate and malonate generated in the body upon administration of suitably labelled precursors, such as ^{13}C -enriched glucose, to metabolically yield ^{13}C -enriched lactate.

Particularly preferred substrates are human metabolites, wherein lactate, citrate, carbonate or phosphate are the most preferred.

25 Moreover, the substrate molecule of the invention can be a non-endogenous substance, wherein the term "non-endogenous", as used herein, refers to any substance of pharmacological or diagnostic interest, optionally modified in order to allow a suitable binding to the paramagnetic complex

and/or to introduce the magnetically active nuclei of interest for the imaging procedure.

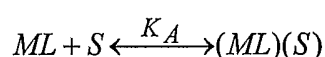
Preferably, the non-endogenous substrate is selected from the group consisting of fluorinated or ^{13}C - or ^{15}N -labelled drugs, fluorinated or ^{13}C - or ^{15}N -labelled metabolites. Trifluoro-lactate, fluorinated citrate, ^{13}C -labelled lactate, ^{13}C -labelled citrate are particularly preferred.

When the substrate molecule to be imaged is non-endogenous, it may be administered to the patient as a sterile aqueous solution or suspension, optionally including a physiologically tolerable carrier.

It can be administered before the imaging, at the same time of the paramagnetic chelate complex. Optionally it can also be administered either before or after the administration of the paramagnetic complex.

To obtain the desired relaxation enhancement for the substrate nuclei, it is necessary that the K_A of the adduct between the paramagnetic complex and the substrate molecule be at least 10 M^{-1} .

The K_A constant, which expresses the thermodynamic stability of the adduct, is given by the usual equation



$$K_A = \frac{[(ML)(S)]}{[ML][S]}$$

Since the relaxivity of the two paramagnetic species involved in the equilibrium, that bound to the substrate molecule and that unbound, are different, the well-established Proton Relaxation Enhancement (PRE) method (Aime S, Fasano M, Terreno E, Botta M in *The Chemistry of Contrast Agents in Medical Magnetic Resonance Imaging*, Merbach AE, Tóth E (eds), Wiley & Sons: Chichester, 2001, 203-209) may be conveniently used for estimating the affinity of the paramagnetic complex

towards the substrate molecule.

The K_A values obtained for the adducts disclosed in the following are measured according to this PRE method.

Moreover, the binding interaction between the substrate molecule and
5 the paramagnetic chelate complex has to be kinetically labile in order to affect all the substrate molecules (S) in the presence of a limited amount of the paramagnetic chelate complex (ML)

Suitable kinetic lability is obtained when the residence lifetime of the substrate molecule bound to the paramagnetic complex, τ_M , is $\leq T_{IM}$ (rapid
10 exchange conditions).

Physiologically acceptable paramagnetic chelate complexes, in which the chelating ligand consists of a system which does not saturate the coordination number of the paramagnetic metal ion, are preferably used.

A further preferred class of physiologically acceptable paramagnetic
15 complex according to the invention is represented by systems able to form a strong association with the substrate of interest, such as LnDOTP⁴⁻ which can form tight ion-pairs with positively charged molecules.

Preferably, the chelating ligand comprises: a linear or cyclic polyaminopolycarboxylic, polyaminopolyphosphonic or
20 polyaminopolyphosphinic acid residue or derivatives thereof including, for example, amide and ester derivatives as well as functional derivatives comprising suitable functional substituents on their structures; macrocyclic Schiff bases; porphyrins, or any ligand whose complex with a given paramagnetic metal ion is able to form a ternary complex with the substrate
25 of interest. The paramagnetic ion is any lanthanide metal ion with an atomic number comprised from 58 to 70 or a transition metal ion with an atomic number from 21 to 29 and from 42 and 44. Preferably, the paramagnetic ion is selected from the group consisting of: gadolinium (III), iron (II), iron

(III), manganese (II), manganese (III), chromium (III), cobalt (III), copper (II), nickel (II), dysprosium (III), erbium (III), terbium (III), holmium (III), thulium (III), ytterbium (III), europium (II) and europium (III). Gadolinium (III), manganese (II) and manganese (III) are particularly preferred.

5 Particularly preferred paramagnetic complexes for use in the method according to the invention are those in which the chelating ligand is selected from EDTA (ethylenediaminetetraacetic acid), PCTA (3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15)11,13-triene-3,6,9-triacetic acid), PCTP (3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15)11,13-triene-3,6,9-
10 tris(methanephosphonic) acid), DOTP (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methanephosphonic) acid) and DO3A (1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid), including DO3A derivatives in which the non carboxylate nitrogen atom is suitably substituted with a pendant or a functional group.

15 A particularly preferred DO3A derivative is 10-[(3-methoxyphenyl)methyl]-1,4,7,10-tetraazacyclododecane-1,4,7-tris-[(aminocarbonyl)methyl]. The gadolinium complex thereof is herein referred to as Gd-1 complex.

Another preferred DO3A derivative is 10-[(3-methoxyphenyl)methyl]-
20 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid. The gadolinium complex thereof is herein referred to as Gd-2 complex.

A third preferred compound is 10-[(4-carboxyphenyl)methyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid, whose gadolinium complex thereof is herein referred to as Gd-3 complex.

25 All these ligands as well as their paramagnetic chelate complexes with gadolinium ion are new and are a further object of this invention.

The physiologically acceptable paramagnetic chelate complex may be employed in the form of salt thereof with suitable organic or inorganic acids

or bases.

Preferred cations of inorganic bases include ions of alkali or alkaline-earth metals such as potassium, sodium, calcium, magnesium, and mixtures thereof.

5 Preferred cations of organic bases include those obtained by protonation of primary, secondary, and tertiary amines such as ethanolamine, diethanolamine, morpholine, glucamine, N-methylglucamine, and N,N-dimethylglucamine.

10 Anions of inorganic acids preferably include the ions of halo acids, i.e. chlorides, bromides, iodides or different ions such as the sulphate ion.

Anions of organic acids preferably include those used for salifying basic substances, such as acetate, succinate, fumarate, citrate and maleate.

Cations and anions of amino acids include, for example, taurine, glycine, lysine, arginine, ornithine, aspartic acid and glutamic acid.

15 The salifying cations and anions will obviously be selected so as not to interfere with the imaging procedure. In particular cations and anions corresponding to the substrate molecule to be imaged should be avoided.

The paramagnetic complex for use in the method according to this invention may be represented by a single molecule or it may optionally be
20 administered under the form of covalent or non-covalent aggregates of different size.

For instance, it may be bound to a molecule endowed with specific pharmacological or diagnostic properties or it may be covalently or non-covalently bound to a protein, to a polyaminoacid, to a macromolecular
25 substrate. Analogously, upon introducing suitable functionalisations on the surface of the chelate, it may form supramolecular adducts with other molecules, proteins, polyaminoacids or macromolecular systems.

Moreover, the paramagnetic complex or a suitable aggregate thereof

can optionally be conjugated to a suitable moiety responsible for its targeting to the desired body region, tissue or organ. A non limiting example of suitable moieties includes peptides and fragments thereof and targeting receptors over-expressed in the presence of pathological
5 conditions.

Further, the paramagnetic complex can optionally be inglobated into liposomes, vesicles or other suitable carriers.

The ultimate choice of the paramagnetic metal complex suitable for the claimed use and method will depend on several properties including the
10 electric charge and the solution structure of both the metal complex and the substrate, the concentration of the interacting partners in the tissue or organ to be imaged, the instrumental conditions of the intended analysis. The skilled practitioner will be anyhow able to select a suitable complex by merely applying routine methods, as detailed in the Examples. In particular,
15 a first selection will be made by taking into account the structural features of the intended substrate, particularly its electric charge, the donor ability of its chelating moieties (e.g. carboxy-, hydroxy-, amino-, phosphonate, thio-, sulfonate groups etc.) and its overall steric hindrance. A paramagnetic metal complex matching said structural features of the substrate can usually be
20 selected out from the known classes of paramagnetic metal complexes, for example from complexes of linear or cyclic polyaminopolycarboxylic, polyaminopolyphosphonic or polyaminopolyphosphinic ligands such as EDTA, PCTA, PCTP, DOTP and DO3A. If necessary, the ligand core may be modified by inserting suitable functional groups expected to improve the
25 non-covalent binding and/or increase the exchange rate between the free- and the metal complex bound- form of the substrate (e.g. by means of hydrogen bonds or by exploiting other ionic and/or hydrophobic interactions). The determination of the relaxation enhancement ϵ and of the

affinity constant K_A is then carried out, to confirm the theoretical expectations made in the selection phase.

The paramagnetic complex used in the method according to the invention can suitably be formulated as sterile aqueous solutions or suspensions, whose pH can range from 6.0 to 8.5, either alone or optionally with other physiologically acceptable ingredients. The pharmaceutical preparations can be suitably injected intravasally (for instance intravenously, intraarterially, intraventricularly, and so on) or used by way of intrathecal, intraperitoneal, intralymphatic, intracavitary, oral or enteral administration.

These aqueous solutions or suspensions are stable and can be formulated as such or they can be formulated as a lyophilisate and reconstituted just before use. They can be administered in a concentration ranging between 0.0001 and 1.0 M.

The paramagnetic chelate complex is administered early enough before the MRI monitoring to allow for the distribution of the agent to the body organ or tissue to be imaged.

The method of the invention includes the steps of generating a magnetic field around the human or animal body, body organ or tissue of interest by use of a large magnet which forces the magnetic nuclei possessing a magnetic moment $\neq 0$ of said human or animal body, body organ or tissue to align themselves with the field direction, applying radio pulses of suitably selected frequency which move said magnetic nuclei out of alignment, and collecting the radiofrequency emitted when the same come back into equilibrium conditions containing spatial and intensity information and processing them by a suitable computer to produce the body organ or tissue desired image.

The magnitude of the applied magnetic field is at least 60.000 times

more intense than the Earth's one but it is not substantially greater than 3 Tesla for *in vivo* imaging, while it could be even higher for the *ex vivo* samples. The frequency of the applied radio pulses is suitably selected according to the precession frequency of the magnetic nuclei of interest for the imaging procedure. The selection of a proper pulse frequency allows, for example, the direct ^{13}C observation and the direct imaging based on these observations.

When ^1H -image are desired, the fully exploitation of the imaging method of the invention is preferably based, even if not limited to, on the possibility to record an inverse-detected image. Through this experimental modality, it is possible, by the use of a proper pulse sequence, to obtain an ^1H -image collecting the signal only from the protons bound to ^{13}C nuclei. This procedure still results in an image without background signals, as in the case of a direct ^{13}C observation, but with a great sensitivity enhancement due to the observation of a considerably more sensitive nucleus.

^1H -images can also be obtained exploiting different experimental modality such as, for example, the MRSI imaging technique which can be advantageously used even if the concentration of the substrate molecule is not abundant and which does not request the concomitant presence of a suitably labelled substrate.

The Magnetic Resonance Imaging is an imaging procedure available only since 1980. This technology is however well known to those skilled in the art and will not be detailed here. Quite important, anyway, is to point out that the imager can operate in various modes depending on, for example, the exciting radio frequency selected pulses, which may be varied according to the information sought from the organ or tissue under examination, as well as the MR imaging parameters adopted. All these can be selected, within a given limit, by the operator, optionally on the basis of available

protocols, resulting in flexibility.

A further object of the invention is a pharmaceutical composition comprising a paramagnetic chelate complex or a physiologically acceptable salt thereof, together with a physiologically tolerable carrier, wherein the
5 paramagnetic chelate complex non covalently binds to the substrate molecule, thereby enhancing the relaxation of the substrate nuclei at least 2 at 39°C and pH 7.4.

The pharmaceutical compositions can also optionally comprise a non-endogenous substrate molecule, such as a substrate molecule artificially
10 enriched in or labelled with a particular substrate nucleus or isotope, or a suitable pro-drug of the same, which transforms into the suitable diagnostically active form upon time, by action of agents such as enzymes, oxidants, reductants, acids, bases or ions.

The invention moreover concerns a pharmaceutical composition
15 comprising a non-endogenous substrate molecule such as a molecule artificially enriched in or labelled with a particular substrate nucleus or isotope, or a suitable pro-drug of the same, together with a physiologically tolerable carrier, which can advantageously be employed, for example, when the suitable paramagnetic chelate complex and the non-endogenous
20 substrate molecule need to be administered at different times.

The pharmaceutical compositions can be administered either intravascularly, i.e. intravenously, intraarterially, intracoronarily, or used by way of intrathecal, intraperitoneal, intracavitary, intralymphatic, enteral or oral administration.

25 When the substrate molecule to be imaged is non-endogenous, it may be administered to the patient before the imaging, at the same time of the paramagnetic chelate complex or, optionally, either before or after its administration.

A further object of the invention is a therefore kit for use in a method of the invention which comprises a first pharmaceutical composition including a non-endogenous substrate molecule, or a pro-drug thereof, together with a physiologically tolerable carrier, and a second
5 pharmaceutical composition which includes a physiologically acceptable paramagnetic chelate complex or a salt thereof, together with a physiologically tolerable carrier, wherein said non-endogenous substrate molecule and said paramagnetic chelate complex are suitably selected so that the paramagnetic chelate complex non covalently binds to the non-
10 endogenous substrate and as a result of said binding the relaxation enhancement (ϵ) of the substrate nuclei of interest for the imaging procedure is at least 2 at 39°C and pH 7.4.

Preferred non-endogenous substrates for the kits of the invention are: trifluoro-lactate, fluorinated citrate, ^{13}C -labelled lactate, ^{13}C -labelled citrate.

15 These kits allow the selection of the most suitable non-endogenous substrate, providing the best visualization of the body organ or tissue of interest, while allowing the best choice of the timing of injection of the paramagnetic complex versus the substrate molecule.

BRIEF DESCRIPTION OF THE FIGURES

20 Figure 1: ^{13}C -NMR Spin-Echo spectra of a solution of lactate (10 mM, 50% ^{13}C -enriched at the methyl position) with (trace B) and without (trace A) GdDO3A 1 mM. Acquisition conditions: 7.1 T, 312K, solution pH 7.

Figure 2: MRI images recorded at 11.75 T on a 5 mm micro-imaging probe. Left: ^1H -MRI of water obtained by means of a conventional GE pulse
25 sequence. All the capillaries contain 10 mM of lactate 100% ^{13}C -enriched at the methyl position and different concentrations of GdDO3A (pH of the solutions = 7). A = no GdDO3A, B = 0.25 mM, C = 0.5 mM, D = 1 mM, E = 2 mM. Right: ^1H -MRI "inverse-detected" of lactate of the same phantom

obtained by means of a pulse sequence consisting of a HMQC-like pulse sequence followed by a GE sequence.

Figure 3: ^{19}F -NMR Spin-Echo spectra of a 10 mM solution of trifluoro-lactate with (trace B, 0.9 mM) and without (trace A) GdDO3A (acquisition
5 conditions: 7.1 T, 312K, pH 7).

Figure 4: MRI images recorded at 7.1 T on a 5 mm micro-imaging probe. Left: ^1H -MRI of water obtained by means of a conventional GE pulse sequence. All the capillaries contain 10 mM of trifluoro-lactate (pH 7) and different concentrations of GdDO3A. A = no GdDO3A, B = 0.25 mM, C =
10 0.5 mM, D = 1 mM, E = 2 mM. The solution outside the five capillaries is represented by water doped with CuSO_4 . Right: ^{19}F -MRI of trifluoro-lactate of the same phantom obtained by means of a conventional GE sequence.

Figure 5: ^{31}P -MRI SE image recorded at 11.75 T on a 5 mm micro-imaging probe.

15 All the five capillaries contain a 20 mM solution of inorganic phosphate (pH 7.4) and different concentrations of GdDO3A. A = no GdDO3A, B = 0.25 mM, C = 0.5

Imaging of Lactate

20 As a non limiting example of the wide range of applications of the present invention, the enhancement of the relaxation rates of lactate, and therefore of its signal in a MR image, by the use of a paramagnetic complex is reported hereinafter.

25 As previously pointed out, lactate is an endogenous metabolite, which is produced at increased rate whenever glycolysis accelerates. Its presence and accumulation in some body organs or tissue changes during pathological activation and generally indicates the presence of a pathologic state.

Under normal conditions, for example, lactate is not detected in the

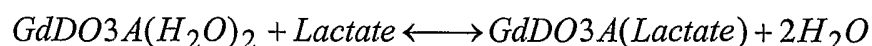
brain. Its presence indicates that the normal oxidative respiration at the cellular level has been altered and that carbohydrates are being catabolised by an anaerobic pathway. This situation occurs in several cellular and metabolic lesions that are thought to outgrow their blood supply. The presence of lactate may therefore indicate a high degree of malignancy. As such this metabolite is found in multiform glioblastoma.

An increase of the lactate level is also caused by acute brain infarction and stroke.

The imaging of this substrate, enhanced by the use of a paramagnetic complex according to the invention, surely provides a powerful aid in the clinical investigation of the related pathologies.

Being lactate a bidentate ligand, good binding is reasonably obtainable when two coordination vacancies at the metal centre of the paramagnetic complex are available. Lactate coordinates through two oxygen donor atoms and therefore Gd(III) ion, which is an "hard" ion, is well suitable. In order to prevent problems associated with the release of toxic Gd(III) ions, the metal ion has to be administered under suitable forms. Among several possibilities the neutral complex formed by Gd(III) and the ligand DO3A (1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid) represents a good choice to illustrate the purpose of this invention. In fact, DO3A is an heptadentate ligand based on a tetraazacyclododecane ring in which only three nitrogens bear a coordinating acetic arm. The coordination number of Gd(III) ion with this ligand is nine, so the two vacancies in GdDO3A are occupied by two water molecules. The formation constant of GdDO3A is ca 10^{21} , i.e. a very safe value for "in vivo" applications.

By adding lactate to an aqueous solution of GdDO3A the following equilibrium takes place:



Since the relaxivity of the two Gd(III) species involved in the equilibrium are different (higher for GdDO3A(H₂O)₂), the well-consolidated Proton Relaxation Enhancement (PRE) method (Aime S, Fasano M, Terreno E, Botta M in *The Chemistry of Contrast Agents in Medical Magnetic Resonance Imaging*, Merbach AE, Tóth E (eds), Wiley & Sons: Chichester, 2001, 203-209) may be conveniently used for estimating the affinity of the GdDO3A complex towards Lactate.

On this basis, a K_A value of 150 M⁻¹ at pH 7 and 25°C has been determined. Then, in a solution containing 10 mM of Lactate and 1 mM of GdDO3A, the ratio $[Lac]_{bound}/[Lac]_{total}$ is $5.8 \cdot 10^{-2}$, i.e. more than three order of magnitude higher than the value of the $[H_2O]_{bound}/[H_2O]_{total}$ ratio in the experiment dealing with water protons and carried out with the same concentration of GdDO3A.

The condition of fast exchange between free and bound Lactate has been verified.

The reversible interaction of lactate at the paramagnetic Gd(III) centre promotes a dramatic reduction of T_1 and T_2 of its resonances. In fact, in the solution containing GdDO3A 1mM and Lactate 10 mM, the T_1 (at 7.05 T and 39°C) of the ¹H-methyl resonance passes from 2.9 s to 8.0 ms, whereas in an analogous experiment T_1 of the ¹³C-methyl resonance passes from 3.5 s to 30.0 ms wherein the relaxation enhancement for ¹H-methyl, ${}^1H\varepsilon$ is 362.5 while ${}^{13}C\varepsilon$ is 116.7.

Figure 1 shows the ¹³C NMR spectrum (¹H-decoupled) of 10 mM of Lactate, 50% ¹³C-enriched at the methyl group, in the absence (trace A) and in the presence (trace B) of GdDO3A 1 mM.

At 7.05 T and 25°C, we carried out the acquisition of 1k transients in 1'17'' through a conventional T_1 -weighted spin-echo (SE) sequence. The long T_1 of the ¹³C-methyl resonance of Lactate does not allow the clear

detection of any signal in the absence of paramagnetic complex. On the contrary, when the same experiment is repeated in the presence of GdDO3A a relatively intense signal comes out.

Besides the direct ^{13}C observation, an alternative approach for fully exploiting the marked relaxation enhancement of the ^1H and ^{13}C -lactate resonances in the presence of a paramagnetic relaxation agent is based on the possibility to record an inverse-detected image. Through this experimental modality, it is possible, by the use of a proper pulse sequence, to obtain an ^1H -image collecting the signal only from the protons bound to ^{13}C nuclei. This procedure still results in an image without background signals, as in the case of a direct ^{13}C observation, but with a great sensitivity enhancement due to the observation of a considerably more sensitive nucleus. Moreover, a further shortening of the acquisition time for obtaining the image results from the fact that the acquired signal is dependent on the ^1H relaxation times, which are significantly shorter than those ones of the coupled ^{13}C nuclear spin.

A T_1 -weighted image, based on a HMQC pulse sequence inserted before the conventional gradient echo (GE) pulse sequence, has been recorded on a high resolution spectrometer endowed with a 5 mm micro-imaging probe, operating at 11.75 T.

The HMQC (Heteronuclear Multiple Quantum Coherence) pulse sequence provides correlation between protons and their attached heteronuclei (^{13}C in this case) through the heteronuclear scalar coupling. This sequence is very sensitive as it is based on proton detection instead of the detection of the least sensitive low gamma heteronuclei.

The result is reported in Figure 2 (right). On the left, a conventional ^1H -image is shown reporting the signal from water protons in order to localise the different samples. The phantom was formed by 5 capillaries

(diameter about 1 mm) inside a 5 mm NMR tube containing water. The samples consisted of aqueous solutions of Lactate 10 mM (100% ^{13}C enriched at the methyl group) with different concentrations of GdDO3A (A = no GdDO3A, B = 0.25 mM, C = 0.5 mM, D = 1 mM, and E = 2 mM).

5 The inverse-detected image was obtained in 5'20" by collecting 512 transients with a repetition time (TR) of 20 ms and an echo-time (TE) of 1.8 ms. Whereas no signal is detected for the sample A, there is enough signal intensity in the other samples. Interestingly, sample E, which contains the highest amount of GdDO3A shows less signal than C and D. This is a
10 consequence of T_2 -effects due to the very short T_2 relaxation time of the ^1H -methyl lactate signal in the presence of relatively high concentration of the paramagnetic relaxation agent. In other words, higher GdDO3A concentrations, by keeping fixed the total amount of lactate, lead to an increase of the $[\text{Lac}]_{\text{bound}}/[\text{Lac}]_{\text{total}}$ ratio, thus, reducing both T_1 and T_2 of the
15 ^1H -methyl resonance. On this basis, the relatively weak signal emerging from sample E is the result of the broadening of the resonance. The signal detected for sample B, which contains the lowest GdDO3A concentration, is quite similar to sample E, but now this is due to the relatively long T_1 of the ^1H -methyl resonance of lactate in the presence of a small amount of the
20 relaxation agent. This is an interesting result because it means that the maximum contrast to noise ratio (CNR) is achieved at relatively low concentration of GdDO3A.

As mentioned above, an object of this invention is the use of a paramagnetic complex to obtain images of non-endogenous substrates.

25 As a non limiting example for illustrating this further aspect of this invention we used Trifluoro-lactate as imaged substrate.

^{19}F has a nuclear spin of $\frac{1}{2}$ and a natural abundance of 100 %.

At 7.05 T and 312°K, the relaxation time T_1 of ^{19}F resonance of a 10

mM solution of $\text{CF}_3\text{-CH(OH)-COO}^-$ is 4.5 s.

Upon the addition of GdDO3A 0.9 mM the T_1 value becomes 23.0 ms wherein the relaxation enhancement $^{19}\text{F}\epsilon$ is 195.6.

Figure 3 shows the ^{19}F -NMR spectra (T_1 -weighted SE sequence) of these two aqueous solutions (trace A = no GdDO3A).

The observed enhancement promoted by the interaction with the paramagnetic substrate is even higher than the one previously shown in Figure 1 for the ^{13}C -methyl resonance, either because of the higher γ -value for ^{19}F nuclear spin or for the presence of three equivalent ^{19}F nuclei in each trifluoro methyl moiety.

Figure 4 (right) shows a ^{19}F image of trifluoro-lactate obtained at 7.1 T on a 5 mm micro-imaging probe. On the left in the same figure it is also reported a conventional GE image for water protons. All the five capillaries (about 1 mm diameter) contain a 10 mM solution of the non endogenous substrate trifluoro-lactate and different concentrations of the relaxation agent GdDO3A (A = no GdDO3A, B = 0.25 mM, C = 0.5 mM, D = 1 mM, and E = 2 mM). The capillaries have been introduced into a 5 mm NMR tube containing water doped with copper(II) sulfate. The left image allows the identification of the different samples. The right image (^{19}F) was obtained with a conventional GE sequence (TR = 20 ms, TE = 1.1 ms) in 5'15" through the acquisition of 512 transients. In this case the best CNR value corresponds to the sample E, which contains the highest concentration of GdDO3A, but a relatively good contrast is still observable for sample B, whereas no signal is detected in the sample without the paramagnetic complex.

A marked increase of the relaxation enhancement of ^{19}F nuclei in Trifluoro-lactate may be obtained by using a Gd(III) complex able to stronger interact with the non-endogenous substrate. Gd-2 complex has a K_A

value with Trifluoro-lactate of 60 at 298 K, i.e. about 6 times larger than GdDO3A.

For this reason a T_1 value of 11.8 ms and a relaxation enhancement of 381.3 has been measured at 312 K for the ^{19}F nuclei of the substrate (10 mM) in a 0.9 mM solution of Gd-2.

The citrate issue

In addition to the enhanced imaging reporting the lactate distribution, the present invention further allows an advantageous visualization of citrate in human body tissues, mainly addressed to the prostate gland.

As discussed before, the citrate concentration in the normal prostate gland differs significantly from corresponding concentration in benign prostatic hyperplasia (BPH) and in malignant prostatic carcinoma. Prostate cancer is now the most commonly occurring malignancy in man, exceeding even lung cancer, and it represents the second biggest cause of cancer-related death.

The enhanced imaging of the citrate molecule obtained according to this invention will allow the mapping of the distribution and concentration of this metabolite in the prostatic tissue and, consequently, will lead to the differentiation between the prostatic carcinoma and the benign pathology, without need of surgical biopsies which are invasive and traumatic for the patient.

Chemically, citrate is an hydroxy-tricarboxylic acid and, in principle, it may bind the paramagnetic complex with different modalities. Nevertheless, citrate binds GdDO3A weaker than lactate (K_A of 40 M^{-1} at pH 7 and 25°C). It is likely that citrate may interact with GdDO3A mainly through the carboxylate and the hydroxy group in position 3, similarly to lactate, but here the two acetate groups of citrate could represent a steric hindrance for the binding.

An enhanced affinity towards citrate is shown by a derivative of GdDO3A, the gadolinium complex of the 10-[(3-methoxyphenyl)methyl]-1,4,7,10-tetraazacyclododecane-1,4,7-tris-[(aminocarbonyl)methyl] chelating ligand, in which the three carboxylic groups have been transformed into carboxoamide groups, and the fourth nitrogen atom of the macrocyclic ring (N-H in GdDO3A) has been substituted with a 3-(methoxyphenyl)methyl substituent. The resulting three-positively charged paramagnetic chelate Gd-1 binds citrate very strongly, with a K_A value $\geq 20000 \text{ M}^{-1}$ at pH 7 and 25°C.

The large difference in the affinity towards citrate between GdDO3A and Gd-1 is ascribed to the different electric charge and, likely to a smaller extent, to the presence of the non coordinating substituent on the nitrogen of the macrocyclic ring. In fact, we observed that the introduction of a pendant substituent in such position may enhance the binding between the substrate and the paramagnetic GdDO3A derivative.

The ternary complex [Gd-1(citrate)] deriving from the interaction of a citrate with Gd-1 complex is new and is a further object of this invention

Other substrates

The use of a paramagnetic complex according to the present invention further allows the enhanced imaging of a number of substrates. A non-limiting list includes alanine, a non-essential amino acid which has been found to be present at high concentration in some meningiomas, choline, one of the most important metabolite the presence and concentration of which increases in primary and secondary brain tumors; creatine and phosphocreatine, which are involved in the production of adenosine-triphosphate and are present at decreased concentration in several brain tumors; N-acetyl aspartate, an intracellular metabolite whose detection may provide information on the neuronal loss; inorganic phosphate and phosphorylated metabolites, which are involved in the energetic metabolic

processes.

For the latter substrates, GdDO3A represents again a good candidate as relaxation agent. The binding strength between GdDO3A and inorganic phosphate is 50 M^{-1} at pH 7.4 and 25°C , and the substrate acts as
5 monodentate towards the paramagnetic complex. This means that one residual water molecule is still present in the inner coordination sphere of the Gd(III) ion in the ternary complex.

^{31}P has a nuclear spin of $\frac{1}{2}$, a natural abundance of 100 %, but a γ -value smaller than ^1H and ^{19}F .

10 The T_1 of a 20 mM solution of inorganic phosphate at 7.1 T, pH 7.4, and 39°C is very long (about 10 s), but in the presence of 0.2 mM of GdDO3A it dramatically shortens to 36 ms ($^{31}\text{P}\epsilon = 277.8$). The very short T_1 of ^{31}P -phosphate, with respect ^1H -, ^{13}C -, and ^{19}F -lactate, is due to the closer proximity of the ^{31}P nucleus to the metal centre in the ternary complex.

15 Figure 5 ^{31}P shows images of inorganic phosphate 20 mM in the presence of different concentrations of GdDO3A. The images were obtained on a 5 mm micro-imaging probe at 11.75T by using a conventional SE sequence (1 hour of acquisition time, TR = 13 ms, TE = 1.5 ms, 10000 transients acquired).

20 The phantom consists of five 1 mm capillaries introduced in a 5 mm NMR tube containing water. The GdDO3A concentration inside the capillaries is: 0 (sample A), 0.25 mM (sample B), 0.5 mM (sample C), 1 mM (sample D) and 2 mM (sample E). In analogy with the image shown in Figure 2, the best CNR is not observed in the sample with the highest
25 GdDO3A concentration, owing to T_2 effects.

Other examples dealing with the recognition ability of Gd(III) complexes toward substrates of biological relevance include Gd-2 and tartrate (K_A of 3000 at 298 K and pH 7), Gd-3 and oxalate (very high

affinity, $K_A > 50000$ at 298K and pH 7) and Gd-3 and aspartate (K_A of 120 at 298 K and pH 7).

In addition to the enhanced imaging of metabolites of particular clinical interest exemplified so far, the method of the invention further
5 allows the visualisation of substances of different nature and biological function, such as antigens and receptors.

In a further aspect, the present invention also relates to the targeting and the enhanced visualization of a human or animal organ or tissue component and, particularly, of an antigen or a receptor, by use, in the
10 method of the invention, of a suitable paramagnetic chelate complex which non covalently binds to a substrate molecule according to said method and, further, is conjugated to or functionalized with a suitable targeting vector well recognised by the organ or tissue component of interest in the imaging procedure.

In particular, the use in the method of the invention of a
15 pharmaceutical composition including this targeted paramagnetic complex allows the enhanced visualisation of membrane receptors, such as PSMA, a cellular membrane receptor of the prostate cells; EGF, which is the endothelial grown factor responsible for the development of the
20 microvascularized system in malignant tumors; or MUC1, a membrane receptor whose glycosylation degree changes in the presence of some tumors and malignancies.

The imaging of such membrane receptors can be practically obtained by administration to the patient of a pharmaceutical composition for use in
25 the method of the invention including, for instance, GdDO3A complex, and preferably a polymer containing a number of GdDO3A or GdDO3A derivative units, conjugated to or functionalized with a suitable targeting vector selected among a polypeptide, an antibody or a suitable Fab, which

are well recognised by the receptor of interest, and which binds to it.

Alternatively, the accumulation of GdDO3A may be pursued through the set-up of non covalent supramolecular adducts. Moreover, one may pursue the visualisation of the cells bearing the receptor of interest through
5 the internalisation of a high number of paramagnetic agents.

About 24 hours after the administration of this pharmaceutical composition, ^{13}C -enriched Lactate or trifluoro-lactate is administered to the patient under examination. The labelled lactate uniformly distributes in the body organs and tissues and exchanges with the endogenous lactate. When
10 the organ or tissue of interest is imaged, only the ^1H -, ^{13}C -coupled, methyl lactate resonance or the ^{19}F resonance of trifluoro-lactate, bound to the GdDO3A complex targeted to the membrane cell may be detected in a short time, allowing the target visualisation.

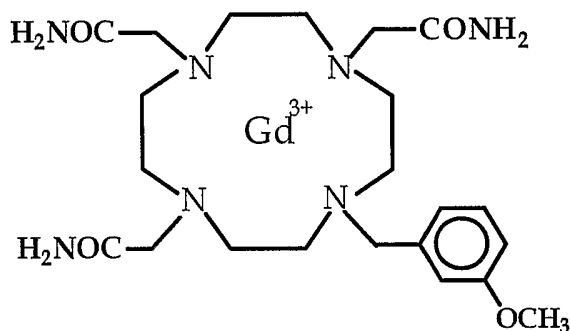
In addition, a GdDO3A-like complex, suitably functionalised with the
15 polypeptide or the antibody able to target the complex to the membrane receptor of interest, is administered to a patient. Because of the presence of the targeting moiety, the GdDO3A complex derivative is internalised into the targeted cells.

^{13}C -methyl enriched lactate or trifluorolactate is then administered to
20 the patient and it distributes in the body organs and tissues. When the inverse detected $^1\text{H}/^{13}\text{C}$ image (or direct ^{19}F image) of the body region of interest is acquired, the recovered signal only derives from the labelled lactate molecules internalised into the targeted cells. The short T_1 of the nucleus examined, caused by the lactate-paramagnetic complex interaction,
25 allows the collection of a huge number of transients in a short time, thus enhancing the SNR.

EXPERIMENTAL SECTION

a) Preparation of 10-[(3-methoxyphenyl)methyl]-1,4,7,10-tetraazacyclododecane-1,4,7-tris-[(aminocarbonyl)methyl] gadolinium complex (Gd-1 complex) of formula:

5

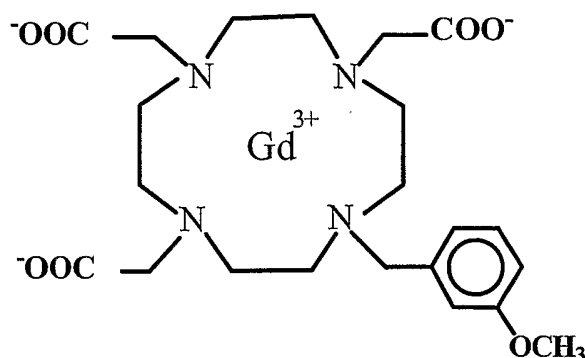
Gd-1

The chelating ligand was synthesised according to the following steps:

- 10 a. 1,4,7,10-tetraazacyclododecane (TAZA) was N-alkylated by reaction with 3-(methoxyphenyl)methyl bromide in a 5:1 ratio (solvent acetonitrile/water 1:1, room temperature);
- b. acetonitrile was removed and the remaining aqueous solution was extracted with ethyl acetate. The organic fractions were evaporated giving a product (pale yellow oil) containing both the mono- and the bis-alkylated TAZA derivatives.
- 15 c. bromo-acetamide was added to the intermediate of step b) in dry acetonitrile in the presence of K_2CO_3 as base and the resulting solution was refluxed for 30'. The mono-alkylated ligand was obtained from the reaction mixture as white solid by filtration.

20 b) Preparation of 10-[(3-methoxyphenyl)methyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid gadolinium complex (Gd-2

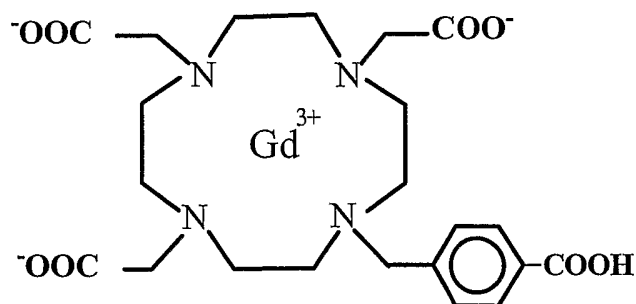
complex) of formula:



Gd-2

The synthesis of this chelating ligand was carried out by starting from
5 the intermediate of step b) (synthesis of ligand 1). Bromoacetic acid was
added to this product (molar ratio 4:1) and the pH of the solution was
brought up to 10 by adding NaOH 10 N. After heating (50-60°C) for 6 hours
the solution was filtered and acidified with HCl 10% up to pH 2.5. The final
product was purified by liquid chromatography. A first elution was carried
10 out by using Amberlite® XAD 1600 (eluent: gradient of methanol up to 40
% in water) in order to separate the product from the bis-alkylated TAZA
derivatives. Then, a second elution was performed by using Duolite® C20
MB (eluent: NH_4OH 2.5 M) in order to separate the ligand 2 from salts and
from the excess of Bromoacetic acid.

15 c) Preparation of 10-[(3-carboxyphenyl)methyl]-1,4,7,10-
tetraazacyclododecane-1,4,7-triacetic acid gadolinium complex (Gd-3
complex) of formula:



Gd-3

The chelating ligand was synthesised according to the following steps:

- 5 a) 1,4,7,10-tetraazacyclododecane (TAZA) was N-alkylated by reaction with 3-(carboxyphenyl)methyl bromide in a 5:1 ratio (solvent water, room temperature, overnight);
- b) The pH was brought to 7 by adding HCl and the solution was concentrated. The alkylated products are extracted with absolute ethanol and the resulting solution is evaporated to dryness. The solid
10 was dissolved in water and the alkylated products are separated by cationic-exchange chromatography (eluent: NH₄OH 2N).
- c) Bromoacetic acid was added to the alkylated products product (molar ratio 4:1) and the pH of the solution was brought up to 10 by adding NaOH 10 N. After heating (50-60°C) for 6 hours the solution
15 was filtered and acidified with HCl 10% up to pH 2.5. The final product was purified by liquid chromatography. A first elution was carried out by using Amberlite[®] XAD 1600 (eluent: gradient of methanol up to 40 % in water) in order to separate the product from the bis-alkylated TAZA derivatives. Then, a second elution was
20 performed by using Duolite[®] C20 MB (eluent: NH₄OH 2.5 M) in order to separate the ligand 2 from salts and from the excess of Bromoacetic

acid.

All the Gd(III) complexes were prepared in water (room temperature, pH 7-8) by addition of Gd(III) chloride to an aqueous solution of the given ligand in stoichiometric ratio 1:1. After reaction, the solution was filtered
5 (Millipore®), concentrated and desalted through a Dowex® column to give the title complex compound.

CLAIMS

1. The use of paramagnetic chelate complexes having the following characteristics:
 - 5 - non-covalent binding ability to a substrate of diagnostic or pharmacological interest other than water, with a strength of at least 10M^{-1} , measured by the Proton Relaxation Enhancement method ;
 - relaxation enhancement ($\varepsilon = \frac{R_i^{s*}}{R_i^s}$ wherein R_i^s represents the observed relaxation rate of the substrate nucleus of interest without
10 paramagnetic promotion and R_i^{s*} is the correspondent relaxation rate observed when the paramagnetic chelate is present in solution) of the nuclei of said physiological substrate, of at least 2 at 39°C and pH 7.4, as a result of said non-covalent binding;
for the preparation of a diagnostic composition for the magnetic resonance
15 imaging of said substrate molecule different from water in a human or animal body organ or tissue.
2. The use according to claim 1 wherein said substrate is selected from lactate, citrate, carbonate, phosphate, pyruvate, natural amino-acids, oxalate, tartrate, succinate, choline, creatine, acetate, and malonate either
20 endogenous or endogeneously generated from labelled precursors thereof.
3. The use according to claim 2 wherein said substrate is lactate.
4. The use according to claim 3 for the imaging of brain tumors.
5. The use according to claim 2 wherein the substrate is citrate.
6. The use according to claim 5 for the imaging of prostate tumors.
- 25 7. The use according to claim 2 wherein the substrate is phosphate.
8. The use of claim 1 wherein the substrate molecule is selected from the group consisting of fluorinated or ^{13}C - or ^{15}N -labelled drugs and fluorinated

or ^{13}C - or ^{15}N -labelled metabolites.

9. The use according to claim 8 wherein the substrate is a trifluoro-lactate or a fluorinated citrate or a ^{13}C -labelled lactate or a ^{13}C -labelled citrate.

10. The use according to any one of claims 1 to 9 wherein the chelating
5 ligand is a system which does not saturate the coordination number of the chelated paramagnetic metal ion.

11. The use according to any one of claims 1 to 10 wherein the chelating ligand is selected from linear or cyclic polyaminopolycarboxylic, polyaminopolyphosphonic or polyaminopolyphosphinic acid residues or
10 derivatives thereof, macrocyclic Schiff bases, porphyrins.

12. The use according to any one of claims 1 to 11, wherein the paramagnetic ion is either a lanthanide metal ion with an atomic number ranging from 58 to 70 or a transition metal ion with an atomic number from 21 to 29 and from 42 and 44.

15 13. The use according to claim 12, wherein the paramagnetic ion is selected from the group consisting of: gadolinium (III), iron (II), iron (III), manganese (II), manganese (III), chromium (III), cobalt (III), copper (II), nickel (II), dysprosium (III), erbium (III), terbium (III), holmium (III), thulium (III), ytterbium (III), europium (II) and europium (III).

20 14. The use according to claim 13, wherein the paramagnetic ion is Gadolinium (III), manganese (II) or manganese (III).

15. The use according to any one of claims 1-14 wherein the chelating ligand is selected from of 10-[(3-methoxyphenyl)methyl]-1,4,7,10-tetraazacyclododecane-1,4,7-tris-[(aminocarbonyl)methyl], 3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15)11,13-triene-3,6,9-triacetic acid (PCTA), 3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15)11,13-triene-3,6,9-tris(methanephosphonic) acid (PCTP), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methanephosphonic) acid (DOTP), ethylenediaminetetraacetic

acid (EDTA), and 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A).

16. A method for the Magnetic Resonance Imaging of an endogenous or endogenously generated substrate molecule different from water in a human or animal body organ or tissue, characterised in that a paramagnetic chelate complex which non covalently binds with a strength of at least 10 M^{-1} to the substrate molecule and inducing, as a result of said binding, a relaxation enhancement (ϵ) of the substrate nuclei of at least 2 at 39°C and pH 7.4 is used as contrast agent in the Magnetic Resonance Imaging procedure.
17. A method for the Magnetic Resonance imaging according to claim 16 wherein the chelating ligand of the paramagnetic metal complex consists of either a linear or a cyclic polyaminopolycarboxylic or polyaminopolyphosphonic or polyaminopolyphosphinic acid residue or a derivative thereof.
18. A pharmaceutical composition which comprises a paramagnetic chelate complex or a physiologically acceptable salt thereof, together with a physiologically tolerable carrier, wherein the paramagnetic chelate complex non covalently binds to the substrate molecule and as a result of said binding the relaxation enhancement of the substrate nuclei of interest for the imaging procedure is at least 2 at 39°C and pH 7.4, further optionally comprising a non-endogenous substrate molecule formulated in a suitable form allowing its direct interaction with the paramagnetic complex or, optionally, in a suitable pro-drug form.
19. A pharmaceutical composition of claim 18 wherein the non-endogenous substrate molecule is artificially enriched in or labelled with the particular substrate nucleus or isotope of interest for the imaging procedure.
20. A kit for use in a method of claim 16 which comprises a first pharmaceutical composition including a non-endogenous substrate

molecule, or a pro-drug thereof, together with a physiologically tolerable carrier, and a second pharmaceutical composition which includes a physiologically acceptable paramagnetic chelate complex or a salt thereof, together with a physiologically tolerable carrier, wherein said non-
5 endogenous substrate molecule and said paramagnetic chelate complex are suitably selected so that the paramagnetic chelate complex non covalently binds to the non-endogenous substrate and as a result of said binding the relaxation enhancement (ϵ) of the substrate nuclei of interest for the imaging procedure is at least 2 at 39°C and pH 7.4.

- 10 21. A compound selected from: 10-[(3-methoxyphenyl)methyl]-1,4,7,10-tetraazacyclododecane-1,4,7-tris-[(aminocarbonyl)methyl] and the gadolinium complex thereof optionally in form of the ternary complex with citrate, 10-[(3-methoxyphenyl)methyl]-1,4,7,10-tetraazacyclododecane-
1,4,7-triacetic acid and the gadolinium complex thereof, 10-[(4-
15 carboxyphenyl)methyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid and the gadolinium complex thereof.

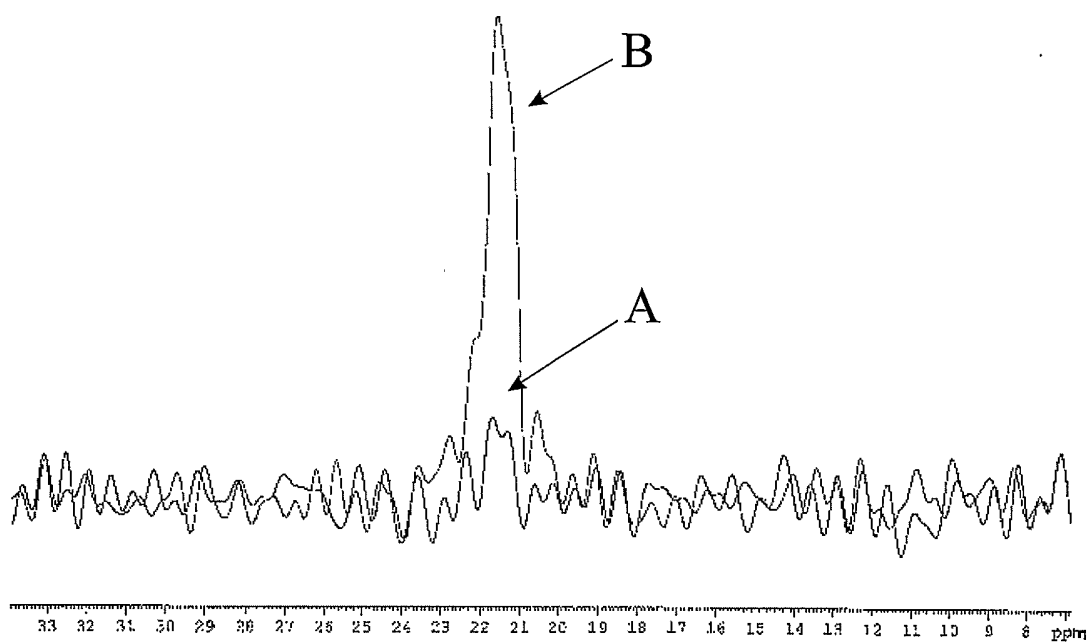


Figure 1

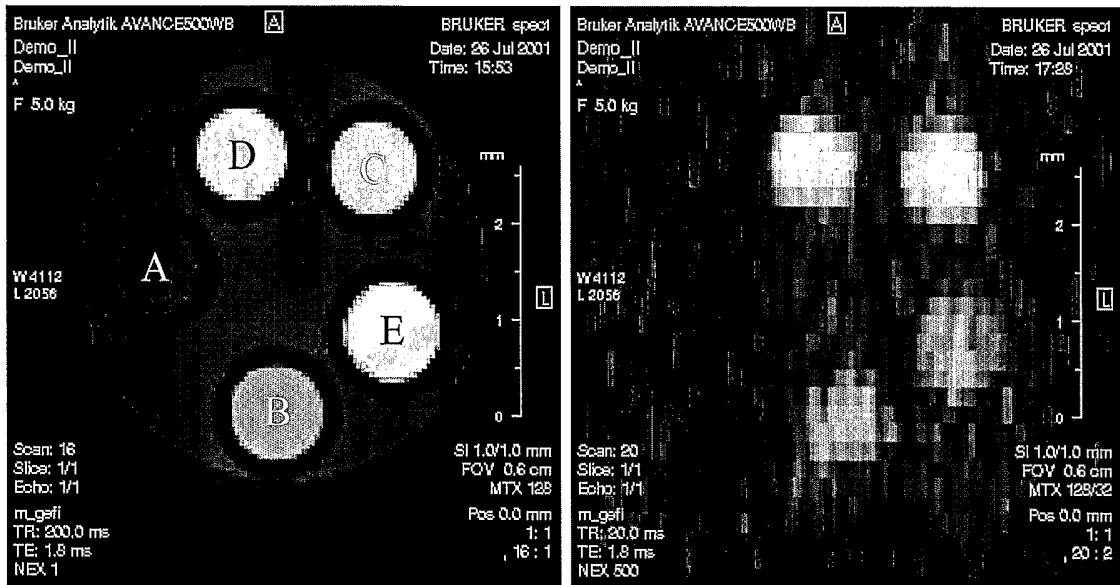


Figure 2

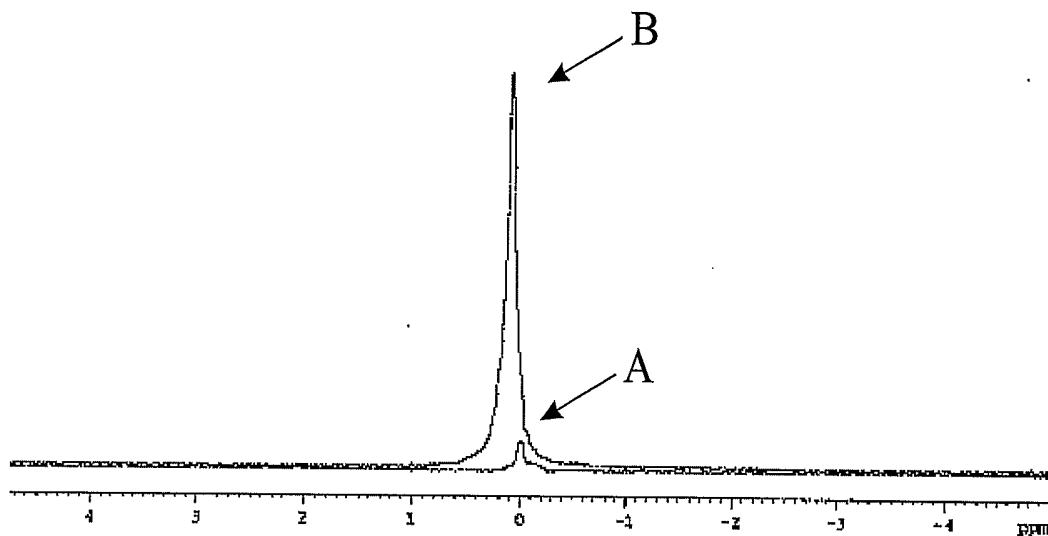


Figure 3

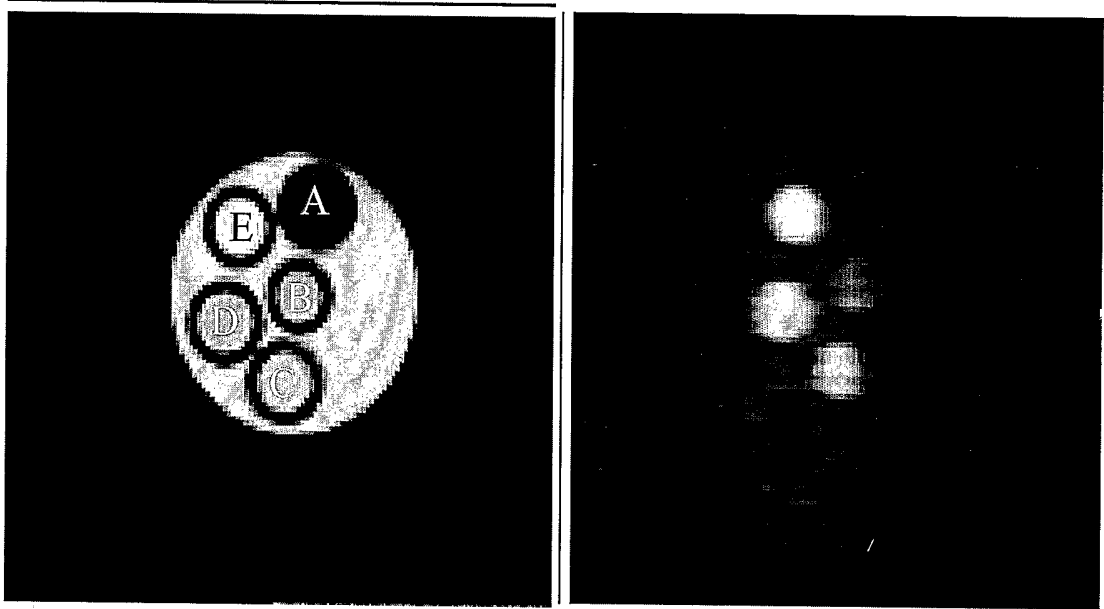


Figure 4

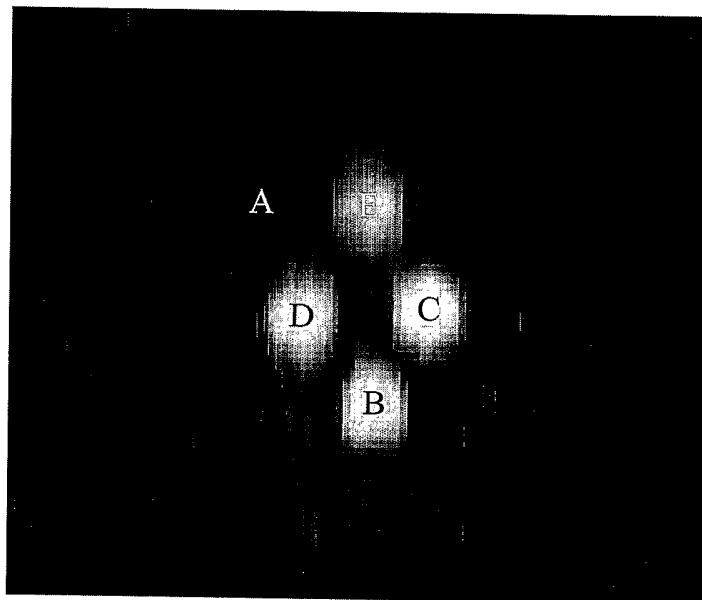


Figure 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/11271A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D257/02 A61K49/08 A61K49/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, MEDLINE, BIOSIS, PAJ, SCISEARCH, BEILSTEIN Data, EMBASE, PASCAL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARGERUM L D ET AL: "Gadolinium(III) D03A macrocycles and polyethylene glycol coupled to dendrimers. Effect of molecular weight on physical and biological properties of macromolecular magnetic resonance imaging contrast agents" JOURNAL OF ALLOYS AND COMPOUNDS, ELSEVIER SEQUOIA, LAUSANNE, CH, vol. 249, no. 1-2, 15 March 1997 (1997-03-15), pages 185-190, XP004083563 ISSN: 0925-8388 * abstract; Scheme 1; 3.4 Biological Studies *	1-20
Y	the whole document --- -/--	21

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

° Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

6 February 2003

Date of mailing of the international search report

26/02/2003

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/11271

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 977 353 A (ARGESE MARIA ET AL) 2 November 1999 (1999-11-02) * col.2, 1.53-col.3, 1.5; claims 1-4 *	1-20
Y	the whole document	21
X	FR 2 725 449 A (AIR LIQUIDE) 12 April 1996 (1996-04-12) claims 1-8	1-20
Y	the whole document	21
X	FR 2 802 928 A (AIR LIQUIDE) 29 June 2001 (2001-06-29) claims 1-21	1-20
Y	the whole document	21
X	WO 00 38738 A (ANELLI PIER LUCIO ;BROCCHETTA MARINO (IT); HAEN CHRISTOPH DE (IT);) 6 July 2000 (2000-07-06) claims 1-28	1-20
Y	the whole document	21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/11271

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 16 and 17 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: 1-14(part.) and 16-20(part.)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-14(part.) and 16-20(part.)

Present claims 1-14 and 16-20 relate to compositions containing a compound or uses of said compound which compound is defined by reference to a desirable characteristic or property, namely,

"....the paramagnetic chelate complex non-covalently binds the substrate molecule and as a result of said binding the relaxation enhancement of the substrate nuclei of interest for the imaging procedure is at least 2 to 39 C and pH 7.4....."

The claims cover all compositions containing said compounds, uses of said compositions, compounds or methods using said compositions/compounds having this characteristic or property, whereas the application provides support within the meaning of Article 84 EPC and/or disclosure within the meaning of Article 83 EPC for only a very limited number of such compositions containing said compounds, uses of said compositions, compounds or methods using said compositions. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 84 EPC). An attempt is made to define the compositions containing said compounds, uses of said compositions, compounds or methods using said compositions by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds identified in claims 15 or 21, compositions containing them and their use in diagnostic.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No
PCT/EP 02/11271

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