

PREFACE

Cancer has always been, and still remains, one of the most feared diseases and a major threat to human life. Although multiple therapeutic targets have been identified to treat tumors, numerous failures of anti-cancer therapies can be observed, even after 50 years of research. Taking into account the importance of the tumor micro-environment has recently made it possible to optimize some treatments. Based on individual tumor characteristics, the best therapeutic strategy to follow can be defined. Numerous experimental or clinical successes have demonstrated the importance of treatment individualization, as each patient can present different responses to the same therapy. Yet, this approach has to be optimized in the future by taking into account the tumor heterogeneity, the dynamic evolution of micro-environmental parameters and the early delineation of treatment-resistant areas in order to redefine the therapeutic strategy as quickly as possible.

Tumor treatment response is usually evaluated by tumor regression that can be shown by morphological imaging techniques like computed tomography and magnetic resonance imaging (MRI). However, reductions in tumor size can be highly variable depending on tumor type and treatment protocol. For instance, post-radiation induced necrosis or fibrosis will impede tumor shrinkage, although they are actually related to successful treatment. Timing of tumor regression is also variable. In some cases it can take several weeks before tumor shrinkage becomes noticeable. Earlier evaluation of treatment efficiency is crucial to increase the chances of a successful therapy. Noticing early that a tumor does not respond, gives the physicians the possibility to immediately pass on to other more aggressive treatments without severe progression of the disease. Therefore, molecular imaging is more suitable for monitoring anti-tumor treatments than morphological assessment, especially in the light of the large therapeutic arsenal available nowadays. Molecular imaging differs from traditional imaging as it uses probes, known as biomarkers, to image particular targets inside organisms in order to give information about fundamental molecular pathways. The non invasive visualization and follow-up of molecular processes will contribute to treatment improvement of diseases such as cancer. Molecular imaging could show if a treatment has induced changes in tumor cell biology, physiology and biochemistry, long before changes in tumor size become visible.

When using MRI, several different types of images can be acquired within the same patient examination. Advantages of MRI are its good penetration depth, its high spatial resolution and its good soft tissue delineation in anatomical imaging. Additionally, MRI allows co-registration of anatomical images with functional information within the same imaging modality, including changes in metabolism or receptor expression associated with tumor response. MRI can be used to image early markers of tumor cell death or inhibition of cell proliferation using targeting ligands that are linked to magnetic labels.

Failure of tumor cells to undergo programmed cell death can cause resistance to treatment. It is becoming increasingly clear that apoptosis is a significant mode of cell death in the response of tumor cells to various anti-cancer therapies. As apoptosis might possibly give an earlier indication of treatment success than tumor shrinkage, in this thesis we tried to assess tumor response to treatment by cell death-targeted MRI. For this purpose we evaluated a new magnetic biomarker based on ultrasmall iron oxide particles, which were targeted at phosphatidylserine, a phospholipid present on the surface of apoptotic cells.

Chapter I: INTRODUCTION

1) Molecular magnetic resonance imaging

The principle of nuclear magnetic resonance (NMR) techniques is based on the resonance phenomenon. This phenomenon occurs when nuclei, previously placed in a homogeneous magnetic field, absorb the energy of an emitted radiowave that has the same frequency as the precession movement of the nuclear spins. When the radiofrequency emission is interrupted, the system liberates the absorbed energy to return to its initial equilibrium state; this is called relaxation. Relaxation is composed of two different phenomena: the longitudinal relaxation and the transversal relaxation. Longitudinal relaxation denotes the return of the nuclei magnetization to its equilibrium in the direction of the magnetic field; it is also called spin-lattice relaxation. This type of relaxation is characterized by the relaxation time T_1 that is the time needed to recover 63% of the initial value of longitudinal magnetization (Fig 1, left). The transversal component depends on the dephasing of the spins due to magnetic field heterogeneities. It is also called spin-spin relaxation and characterized by T_2 , the time needed to drop to 37% of the initial magnitude of transversal magnetization (Fig 1, right). Transversal relaxation time T_2 is much lower than longitudinal relaxation time T_1 .

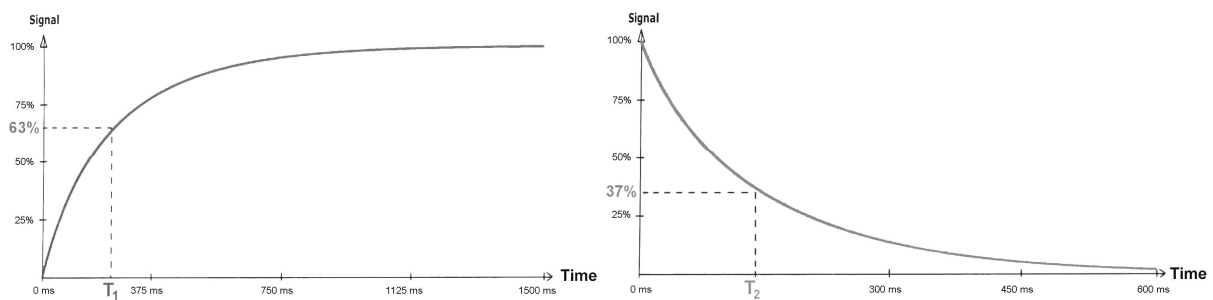


Fig 1: Relaxation curves of the longitudinal (left) and transversal (right) component of the macroscopic magnetization, indicating T_1 and T_2 respectively (Hoa 2007).

Actually, the “real” T_2 is called T_2^* . It is the transversal relaxation time without any compensation for magnetic field inhomogeneities. T_2^* decay is much faster than T_2 decay because it depends on both external field homogeneity and spin-spin interactions, whereas T_2 only depends on spin-spin interactions. T_2^* would only be equal to T_2 if we had a perfect magnetic field without any disturbing inhomogeneities, which is virtually impossible.

In MRI, proton density-weighted images reflect the concentration of mobile hydrogen atoms within tissues. If this is not sufficient, one has to play with the contrast of different tissues by choosing T_1 -, T_2 -, or T_2^* -weighted sequences. In a given magnetic field, the relaxation times are characteristic for specific tissues because they depend on molecular mobility of water protons within tissues. Water for instance has long T_1 and T_2 , whereas fat presents short relaxation times, which is why fat and water appear differently in MR images.

A. Exogenous contrast in magnetic resonance imaging

The term “contrast” denotes the relative difference in intensities between two neighboring regions. The intrinsic contrast in MRI is provided by differences in the local concentration of water protons, but also by differences in the intrinsic relaxation times of tissues. However, the endogenous contrast in MRI is often too weak to enable sensitive and specific diagnosis. For this reason MRI contrast agents (CAs) are increasingly used. Exogenous contrast agents are administered substances, mostly intravenously injected, that allow modification of the contrast of vessels or organs. CAs are considered as drugs and are subject to the same requirements of development and prescription. Unlike the CAs used in other imaging techniques, magnetic probes are indirect CAs because they do not become visible by themselves. Contrast modification comes from a shortening of relaxation times (T_1 and/or T_2), which CAs induce on hydrogen nuclei that are situated nearby (Fig 2). Efficiency of a contrast probe depends on its ability to modify relaxation times, known as their relaxivity “ r ”. The relaxivity (expressed in $s^{-1}.mM^{-1}$) is defined as the increase of the nuclear relaxation rate produced by a CA concentration of 1 mM. The term $1/T$ (inverse of relaxation time) is called the relaxation rate “ R ” with units of s^{-1} (Burtea 2008).

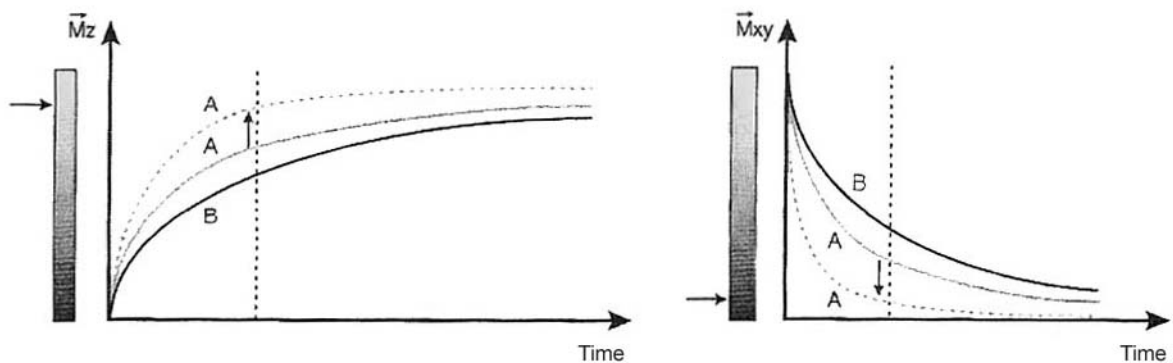


Fig 2: Illustration of CAs with predominant T_1 (left) or predominant T_2 effect (right), producing a brightening or darkening in signal intensity of tissue A respectively (Kastler 2006).

1. Classes of MRI contrast agents

Two major classes of CAs can be distinguished:

- Paramagnetic manganese and gadolinium chelates, generally presenting a major signal enhancement effect on T_1 -weighted images (“positive contrast agents”).
- Superparamagnetic iron oxide particles (SPIOs), mainly used for their signal loss effect on T_2 and T_2^* -weighted images (“negative contrast agents”).

a) Paramagnetic contrast agents

A paramagnetic substance will be magnetized when exposed to a magnetic field. Paramagnetism is the consequence of unpaired electrons of metal ions, such as gadolinium (Gd^{3+}) and manganese (Mn^{2+}). The more unpaired electrons an ion has, the more its magnetic properties are important. The influence of CAs on relaxation rates of tissues is due to an interaction between their electronic spins and the nuclear spin of protons in tissues. For in vivo use though, heavy metals have to be chelated with hydrophilic ligands to reduce their toxicity. Paramagnetic CAs with low molecular weight (MW) have similar effects on R_1 and R_2 , but at the low doses that are usually used, the predominant effect is T_1 shortening. Paramagnetic CAs (most of them complexes of gadolinium) are frequently used in the clinic as extracellular, hepatobiliary or blood pool agents (Hoa 2007).

Manganese chelates

The first metal proposed as paramagnetic probe for proton MRI was the bivalent manganese cation. But even when using the minimum dose necessary to achieve enhancement in tissue relaxation, Mn^{2+} was unacceptably toxic. Therefore, complexation of Mn^{2+} to strong ligands is necessary before considering its in vivo use. After intravenous administration, Mn-DPDP (Teslascan[®]) allows diagnosis of hepatocellular liver tumors (Rofsky 1993). The metabolism of the parent compound Mn-DPDP results in release of free Mn^{2+} ions that are captured by the healthy liver tissue, where they produce a signal enhancement on T_1 -weighted images (Fig 3).

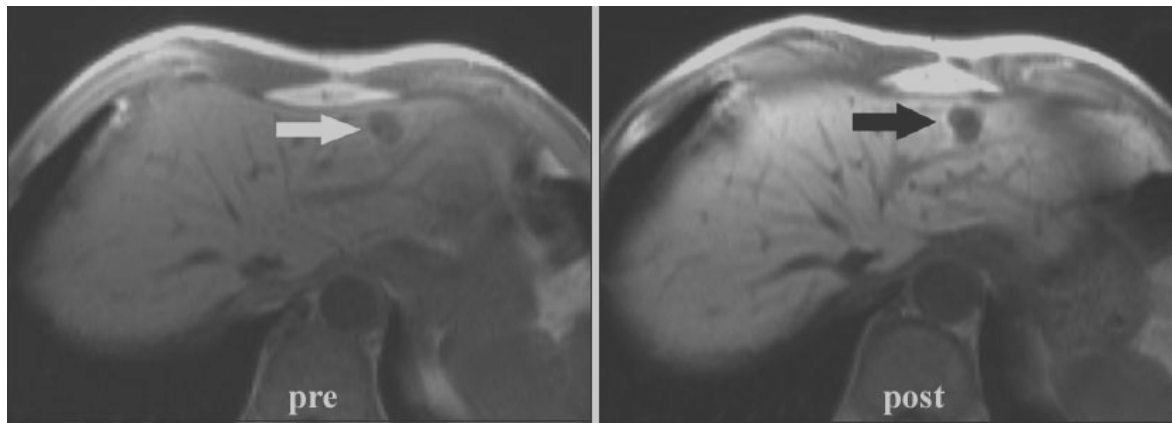


Fig 3: T₁-weighted MR images of a patient with hepatic metastasis taken before and 2 hours after injection of Mn-DPDP (Kim 2006).

Gadolinium chelates

Gadolinium-based CAs are the most frequently used type of contrast media in MRI. Gd³⁺ shortens the relaxation times of surrounding protons, with a predominant T₁ effect that leads to an enhancement on T₁-weighted images. At a low concentration, the T₂ effect of Gd³⁺ chelates is insignificant in comparison to the T₁ effect. The T₂ shortening becomes only visible at high Gd³⁺ concentrations, in the bladder after renal excretion for instance. Gd-DTPA (Magnevist®) and Gd-DOTA (Dotarem®) were the first compounds of this generation of imaging agents (Table 1). They are efficient agents for imaging a blood-brain-barrier leakage because Gd³⁺ chelates are normally not able to pass the intact blood-brain barrier. For this reason, they are typically used for detection of brain tumors (Fig 4).

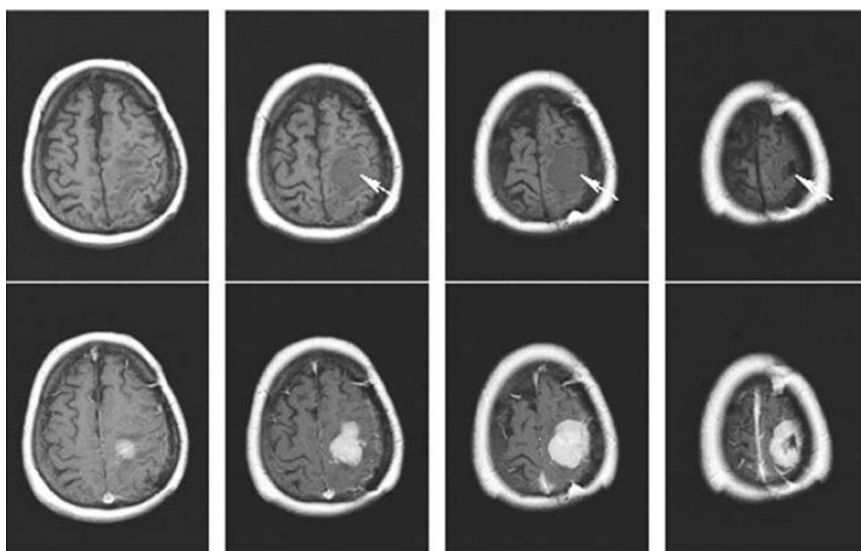


Fig 4: Patient with brain tumor before (top row) and after (bottom row) injection of Gd-DTPA (Burtea 2008).

Major applications of Gd-based contrast media include perfusion and angiographic studies, using extracellularly-distributed agents for dynamic contrast-enhanced MRI (DCE-MRI). They are typically small MW compounds that are distributed non specifically in the blood plasma and extracellular space after administration. New low MW contrast probes with high vascular circulating time are now available (Vasovist[®]). Their residence time in the intra-vascular space is increased due to non covalent binding to serum proteins, which leads to the formation of a macromolecular complex with blood pool distribution. Another approach for DCE-MRI consists in the use of intermediate size blood-pool agents (Vistarem[®]). Their bigger size limits the passage through normal endothelium fenestration; they are therefore also used for characterizing cancerous lesions since they are known to have a higher vascular permeability.

Extracellular agents are rapidly eliminated through the kidneys due to their hydrophilic binding agent, except for Gd-BOPTA and Gd-EOB-DTPA, which are partly eliminated by biliary excretion (Table 1). These lipophilic agents are therefore used as hepato-specific CAs as illustrated in figure 5 (Reimer 2004, van Montfoort 1999).



Fig 5: MR images of a patient with hepatic metastasis acquired at 1.5T before contrast material injection and 20 minutes after administration of Gd-EOB-DTPA (Huppertz 2005).

It is furthermore important to mention the fact that the Gd^{3+} ion is toxic. This is the reason why it is administered in chelated form (linear or macrocyclic, ionic or non ionic), with linear Gd^{3+} chelates being less stable than macrocyclic systems. Besides nausea, headache and taste alterations, free Gd^{3+} can cause nephrogenic systemic fibrosis in patients presenting severe renal failure and in liver transplants, which can lead to death. But in general this group of CAs is considered to be safe and well tolerated in the vast majority of patients.

Table 1: Summary of commercialized Gd-based preparations.

Trade name	Acronym	Structure	Charge	Biodistribution
Magnevist [®]	Gd-DTPA	linear	ionic	extracellular
Omniscan [®]	Gd-DTPA-BMA	linear	non ionic	extracellular
Optimark [®]	Gd-DTPA-BMEA	linear	non ionic	extracellular
Dotarem [®]	Gd-DOTA	macrocyclic	ionic	extracellular
ProHance [®]	Gd-HP-DO3A	macrocyclic	non ionic	extracellular
Gadovist [®]	Gd-BT-DO3A	macrocyclic	non ionic	extracellular
Multihance [®]	Gd-BOPTA	linear	ionic	hepatic/extracellular
Primovist [®] /Eovist [®]	Gd-EOB-DTPA	linear	ionic	hepatic/extracellular
Vasovist [®]	MS-325	linear	ionic	blood pool agent
Vistarem [®]	P792	macrocyclic	ionic	blood pool agent

Abbreviations of Gd-chelating agents: BMA: bismethylamide, BMEA: bismethoxyethylamide, BOPTA: benzyloxy propionic tetraacetic acid, BT: butyrol, DO3A: tetraazacyclododecane, DOTA: tetraazacyclododeca tetraacetic acid, DTPA: diethylene triamine pentaacetic acid, EOB: ethoxybenzyl, HP: hydroxypropyl .

b) Superparamagnetic contrast agents

Characteristics

Paramagnetism is a property of one single atom, whereas superparamagnetism emanates from a group of numerous atoms contained inside particles. The resultant magnetic moment of the entire superparamagnetic iron oxide particle is greater than the sum of the magnetic moments of each single iron atom constituting the particle. Superparamagnetic iron oxide particles possess a high magnetic moment generating microscopic field inhomogeneities (magnetic susceptibility effect), which causes a hyposignal on T_2/T_2^* -weighted images. Although T_2 -weighted contrast generated by these particles has been highly investigated, several examinations demonstrated that there is sufficient T_1 contrast for imaging too. According to their size, these particles are classified into two main groups: superparamagnetic iron oxide particles (SPIOs, >50 nm) and ultrasmall superparamagnetic iron oxide particles (USPIOs, <50 nm). The particles contain a mono- or polycrystalline core of iron oxides and are surrounded by a polymer coating. The core synthesis that is most commonly used, involves an alkaline co-precipitation of ferrous and ferric ions in aqueous solution (Babes 1999). The T_2/T_1 relaxivity will be controlled by the core size (Weissleder 1990). The use of surface coating, typically hydrophilic, is often required to obtain better stability and to improve dispersion of the particles.

Pharmacokinetic profile

After their intravenous administration, SPIOs are covered with plasma opsonins and are rapidly cleared from the circulation by the macrophages of the reticulo-endothelial system (RES). Kinetics of their plasma clearance is dose-dependent and is also much faster in mice and rats than in humans due to differences in RES organ distribution (McLachlan 1994). Whether SPIOs are administered by bolus injection or by slow infusion has no impact on pharmacokinetics; the pharmacokinetic profile is rather determined by the iron oxide particle size and coating. In studies using radiolabeled ^{59}Fe -USPIOs, 25% of the injected dose was found in the liver of rats compared to 89% with SPIO particles, which are mainly taken up by Kupffer cells of the liver within 1-2 hours of administration. The same studies also showed important distribution of USPIOs in spleen (40%) and lymph nodes (10%) compared with 20% in spleen and negligible amounts in lymph nodes for larger SPIOs (Bourrinet 2006, Weissleder 1989b). The half-life of ^{59}Fe -detection in the liver was about 3-4 days, whereas the T_2 effect was only detectable during 1-2 days. This means that after 1-2 days, iron oxides are metabolized into other non magnetic forms of iron. As mentioned earlier, the coatings also play a major role in the in vivo fate of particles. Prevention of particle aggregation and improvement of their hydrophilicity can be obtained by attaching biocompatible polymers to the particle surface by physical or chemical adsorption (Sonvico 2005). Particles with hydrophobic surfaces are covered with opsonins, whereas particles that have hydrophilic surfaces are cleared more slowly from the blood compartment (Gaur 2000). Moreover, the blood half-life is shorter for ionic polymers than for non ionic coatings. The most frequently used polymers for biocompatible SPIOs are derivatives of dextran or polyethylene glycol (PEG) (Di Marco 2007). Feruglose (Clariscan[®]), with a PEGylated starch coating, can be considered as true “stealth nanoparticles” as they are barely recognized by macrophages (Bjornerud 2001). By playing with particle size and nature of coating, it is possible to modify biodistribution and pharmacokinetics, and consequently clinical application.

Medical applications

Liver imaging is the principal application of SPIOs. After injection, the negative contrast agents are recognized by plasma proteins and easily captured by Kupffer cells of the liver. As figure 6 shows, the darkening of signal is only displayed in healthy parts of the liver due to the uptake of SPIOs by functional macrophages, and not in tumor regions, where the signal

intensity remains the same as before (Bach-Gansmo 1993). Liver tumors or metastases even as small as 2–3 mm can be detected with this method (Yoshikawa 2006). The amount of SPIOs administered for imaging of hepatic tumors is 8-15 $\mu\text{molFe/kg}$.

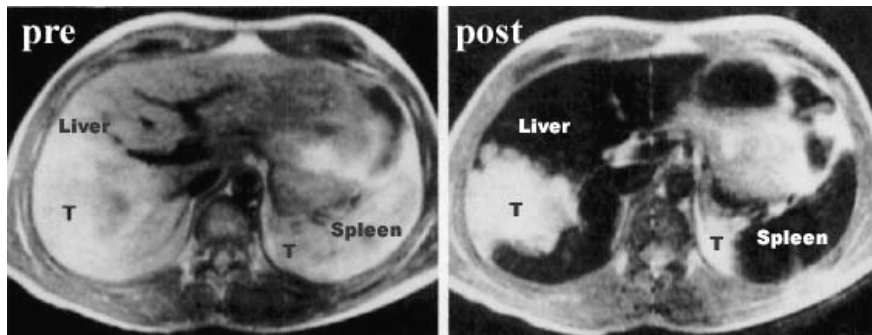


Fig 6: Transversal images of liver and spleen before and after administration of SPIOs. After administration, signal loss of normal spleen and liver occurs and tumors (T) become sharply delineated (Weissleder 1989a).

Furthermore, intracellular macrophagic accumulation of SPIOs via phagocytic internalization allows potential MR imaging of degenerative disorders and inflammation, as well as other diseases associated with high macrophage presence. Regions of up-regulated macrophage activity include sites of atheroma, multiple sclerosis, stroke, graft rejection, glomerulonephritis, and so on (Corot 2004, Dousset 2002, Kooi 2003).

Due to their small size and their hydrophilic coating, USPIOs are generally able to partly avoid their early and massive uptake by the macrophages of the RES and therefore they have a greater vascular persistence. Moreover, the smaller size enables them to reach lymph nodes and bone marrow by crossing the capillary wall (Harisinghani 2006, Seneterre 1991). The intended human dose of ferumoxtran-10 (Sinerem[®]) for lymph node imaging was 45 $\mu\text{molFe/kg}$ (Islam 2009). Even so, these small-sized USPIOs (7–30 nm) can also be used for angiographic applications. Their prolonged half-life presents optimal properties for blood pool imaging. The properties of a new preparation of very small USPIOs (VSOP-C184), in which the particles are stabilized by a citrate coat, are currently being investigated (Table 2) (Kaufels 2008). VSOP-C184 particles produce a signal enhancement on T₁-weighted images because their ratio of T₁- to T₂-shortening is much higher than with other SPIOs.

Another class of SPIOs are orally administered large particles that are used to differentiate between the gastrointestinal tract and surrounding tissues (Table 2) (Jacobsen 1996, Johnson 1996).

Table 2: Currently used iron oxide preparations (already commercialized or still in development).

Generic name	Trade name	Class	Size	Coating
AMI-121/Ferumoxsil	Lumirem [®] /Gastromark [®]	Oral SPIOs	300 nm	silicone
OMP/Ferristene	Abdoscan [®]	Oral SPIOs	> 300 μ m	polystyrene
AMI-25/Ferumoxide	Endorem [®] /Feridex [®]	SPIOs	80-150 nm	dextran
SHU555A/Ferucarbotran	Resovist [®] /Cliavist [®]	SPIOs	62 nm	carboxydextran
AMI-227/Ferumoxtran	Sinerem [®] /Combidx [®]	USPIOs	20-40 nm	dextran
NC100150/Fergulose	Clariscan [®]	USPIOs	20 nm	starch and PEG
CODE 7228/ Ferumoxytol	/	USPIOs	18-20 nm	polyglucose carboxy- methylether
VSOP-C184	/	USPIOs	~8 nm	citrate
SHU-555C	Supravist [®]	USPIOs	20 nm	carboxydextran

Besides all these applications, iron oxide particles can also be used for cell tracking with SPIOs being better suited for cell labeling than USPIOs (Sun 2005). Cells of any particular type can be loaded with nanoparticles ex vivo and then cells can be tracked once implanted in vivo. The ability to follow cells after implantation is useful in therapies including stem cells, dendritic cells or T-lymphocytes (Bos 2004, Daldrup-Link 2005a, Daldrup-Link 2005b, de Vries 2005).

Toxicity

The biocompatibility of SPIOs has been widely investigated (Weissleder 1989b). Cell tracking experiments have shown that SPIOs have little effect on cell viability, proliferation or cell function. However, more recent studies report that SPIOs taken up by cells generally lead to a transient induction of reactive oxygen species (ROS) caused by the Fenton reaction, which can result in DNA damage, impaired cell proliferation, cellular dysfunction, and eventually leads to cell death (Soenen 2009). Interpretation of data is not easy. As results depend on the cell line, particle type and particle concentration used, it is difficult to draw a general conclusion about the biocompatibility of SPIOs. Surface coating of particles can reduce their toxicity by reducing cellular internalization. Given the fact that iron is an essential element for human beings, it is not surprising that after their uptake by the RES, SPIOs can enter the body's normal iron metabolism cycle and are progressively found inside red blood cells (Runge 2000). Iron, from endogenous or exogenous origin, is eliminated very slowly. The safety profile of USPIOs was assessed using pooled data from 37 clinical studies in 1777 adults (Bernd 2009). The reported adverse effect rate was similar to the one reported in the placebo group (23.2% versus 25.3% respectively). Adverse effects reported with this

CA were mainly back pain, headache and hypersensitivity reactions. Symptoms of flushing are probably due to typical infusion-related reactions. Toxicity was only observed after very high concentrations and related to iron overload (Bourrinet 2006). In hemochromatosis, total body iron exceeds 15 g, and cirrhosis and hepatocellular carcinoma can ensue if liver iron concentration exceeds 4000 $\mu\text{g/g}$ wet weight. But the amount of iron administered by a single injection of the CA is negligible and far below the amount needed to induce liver toxicity (Weissleder 1989b). Acute toxicity of SPIOs was not evident at doses up to 3000 $\mu\text{molFe/kg}$ and the LD_{50} would exceed this dose. The safety index (ratio of the toxic dose to the therapeutic dose) is greater than 1:150 (Weissleder 1989b). In experimental conditions SPIOs were also devoid of mutagenic potential, even when injected with doses more than 150 times the human dose. However, they did show maternal toxicity and teratogenic potential in rats and rabbits at dose levels of 15 to 50 mgFe/kg/day (Bourrinet 2006). But in general, USPIOs are well tolerated CAs when administered by slow infusion. They also appeared to have a good safety profile in patients generally considered to be at high risk of adverse events.

2. Targeted contrast agents

Molecular imaging is a fast evolving field that shifts the focus from anatomical imaging to reporting the biological processes. Some successful approaches have involved the development of molecular probes that can address paramagnetic Gd chelates or SPIOs to certain targets in a specific manner.

a) Principles

Passive targeting relies on natural pharmacokinetic mechanisms. For certain applications this may be sufficient, but active targeting allows imaging of biomedically relevant molecules. Active targeting is based on contrast probes coupled to specific ligands that selectively bind to a given target. Information about the localization and expression level of molecular markers could allow the early detection of a disease, accurate pathological staging or monitoring of treatment efficiency, and it can be used as drug development tool as well. High concentrations of CAs at the target site will especially be needed in MRI because of its lower sensitivity compared to other imaging techniques. Targeted magnetic probes should therefore present high relaxivity, high affinity for the target and the target of interest should be overexpressed.

Table 3 summarizes some main criteria that should be taken into account for a successful development of new molecular biomarkers.

Table 3: Considerations for a successful development of molecular biomarkers.

Good signal to noise ratio
Low non specific binding
High target affinity and specificity
Low nano- to micromolar concentrations needed
Wash out sufficiently slow to allow accumulation at the target site
Wash out rapid enough to provide adequate target to background contrast
Favorable pharmacokinetic profile
Labeling should not change the binding properties of the ligand to its target
Binding between ligand and contrast probe should be stable in vivo
Avoid immunogenic reactions (sometimes caused by antibodies)

In this thesis, USPIOs were chosen as magnetic reporters. The following lines will elucidate why these particles are attractive reporters for molecular imaging. First of all, USPIOs are very sensitive to MR detection because of their high relaxivity, thus only low doses are required for efficient detection. Besides, they have a good biocompatibility since they enter the normal metabolic iron pathway of the body. USPIOs are easy to synthesize, inexpensive and have a good capacity for derivatization to targeting particles. The problem arising for in vivo use of USPIOs is their fast capture by macrophages after intravenous injection, even if their size is relatively small. For our studies, the hydrophilic polymer PEG was grafted onto the particles to prevent plasma protein adsorption and to reduce phagocytosis. PEG molecules are neutral and allow relatively strong binding of water molecules around the polymer chain, leading to high steric repulsion. In addition, PEGylated surfaces are biocompatible and not immunogenic (Bhadra 2002, Moghimi 2002). Moreover, surface groups are also useful for derivatization with targeting agents or can be conjugated to other imaging agents.

b) How to assure selectivity

First of all, selectivity of a new biomarker should be tested in vitro. Radiolabels (radioimmunoassay) or target-coated ELISA (enzyme-linked immunosorbent assay) plates can be used to prove selective binding of the biomarker to the molecule of interest instead of to a related molecule, such as phosphatidylserine (PS) and phosphatidylcholine (PtdC) for instance (Laumonier 2006). Competition experiments can also be carried out, observing a displacement of the biomarker from the target molecule when adding increasing

concentrations of its endogenous ligand. A similar experiment can be performed in vivo, when blocking the receptor with the endogenous ligand or even with an excess of non labeled compound that competitively block binding of the targeted contrast probe, and thus demonstrate the specificity of binding (Wang 2009). Biodistribution also plays an important role; the target-to-background ratio should be as high as possible. One should assure oneself that the targeted CA does not accumulate in the corresponding control tissues. Another negative control consists in comparing results to those obtained with non targeted reporters (Kresse 1998). An important validation of new biomarkers is histological correlation, a method used in almost all molecular imaging studies. First, expression of the target molecule in the tissue of interest has to be proved and secondly the co-localization of the biomarker should also be demonstrated, for instance by coupling the probe to a fluorescent or radioactive marker to become detectable by fluorescence microscopy or autoradiography respectively (Jun 2010).

c) Illustrative examples

There are many cancer-specific targets that are clinically relevant. Avidin-Gd-DTPA complexes were shown to allow binding to specific targets, which were pre-labeled with a biotinylated antibody. This technique was used for instance to image tumor cells expressing the HER-2/neu receptor in an animal model of breast cancer (Artemov 2003b). Gd-based CAs were also used for imaging activated vessels in an animal model of angiogenesis by targeting the highly-expressed adhesion molecule $\alpha_v\beta_3$ via its antibody (Anderson 2000). The endothelial integrin $\alpha_v\beta_3$ can also bind with the RGD motif (arginine-glycine-aspartic acid). The highly popular $\alpha_v\beta_3$ was targeted in neoplastic tissues with RGD-Gd conjugates (Ke 2007) and the radioactive tracer ^{18}F -FDG-RGD (fluoro-2-deoxy-D-glucose) has already been used in positron emission tomography (PET) for human trials (Beer 2006). Another study done by Jun et al. has demonstrated the in vivo detection of tumor angiogenesis by using Gd conjugates linked to an anti-VEGF receptor 2 antibody (Jun 2010).

Molecular targeting was also attempted by conjugating specific ligands to iron oxide nanoparticles. Cells with accelerated metabolism, like rapidly dividing cancer cells, have upregulated transferrin receptors. SPIOs conjugated to transferrin accumulated intracellularly by transferrin-receptor-mediated endocytosis (Högemann 2000, Kresse 1998). Folate receptor overexpression is another target for rapidly dividing cancer cells, as folate is needed for the production of new cells (Choi 2004). As already targeted by avidin-Gd-DTPA, the

over-expressed HER-2/neu tyrosine kinase receptor was also targeted by SPIO-herceptin with strong applications in breast cancer imaging (Artemov 2003a). Non receptor ligands have also been imaged. A peptide presenting high affinity for the underglycosylated MUC-1 tumor antigen, a ligand found in numerous tumor types, was bound to SPIOs (Moore 2004). One can also use the inverse strategy: accumulation of contrast media in healthy cells. To highlight pancreatic tumors for instance, SPIOs were labeled with cholecystokinin (CCK), a hormone that specifically binds to CCKA, a receptor that is only expressed on healthy acinar cells (Reimer 1994).

There is a growing arsenal of other ligands that may be interesting for MR imaging. In the course of this thesis, we will especially focus on the detection of tumor cell death after anti-cancer treatment. But molecular imaging of inflammation with targeted USPIOs has also been performed in order to become familiar with the technique of targeted MR contrast probes.

A. Targets of molecular imaging followed in the course of the thesis

1. Inflammation

In the first study of the thesis, we tested a new magnetic reporter targeted at inflammation. However, as early recognition of inflammation does not present our main focus, only a small part of the introduction is dedicated to this matter.

a) Imaging of inflammation

Imaging of inflammation can be useful for pathologies presenting an inflammatory background, such as infection, atherosclerosis, ischemia, multiple sclerosis, graft rejection, rheumatoid arthritis etc. and for monitoring their therapy (Beckmann 2003a, Beckmann 2003b, Dousset 2006, Kleinschnitz 2003, Kooi 2003). Early indication of inflammation, before irreversible anatomical changes occur, is very important (Rennen 2001). Physical examination is quite subjective and serum markers of inflammation do not provide information about the localization of the inflamed zone. Different kinds of approaches have been attempted to visualize inflammation with specific radiopharmaceuticals. Among the strategies used was the labeling of leukocytes moving towards the site of inflammation and the ^{18}F -FDG-PET confirmation of an increased glucose uptake by infiltrated granulocytes and

tissue macrophages (Rennen 2001). As inflammation is associated to a high presence of macrophages, passive macrophage targeting by SPIOs can also enable visualization of inflammatory foci by MRI (Dousset 1999a, Kooi 2003, Ruehm 2001). Cellular imaging of SPIO-labeled macrophages in vivo was already translated to human patients (Kooi 2003, Modo 2005, Saleh 2004), but the sensitivity and specificity of MRI with non specific agents is limited (Rovaris 2000).

b) E-selectin expression during inflammation

During inflammatory processes, cells (leukocytes) and plasma proteins are recruited by vasoactive and chemotactic mediators generated at the inflammation site. Extravasation is facilitated by vasodilation and increased endothelial permeability at the inflamed site. Specific adhesion molecules, such as integrins, selectins, and immunoglobulins mediate the adhesion of leukocytes on the vascular endothelium, as well as their diapedesis through the endothelium as shown in figure 7 (Albelda 1990, Smith 1993). All these adhesion molecules expressed during inflammation present potential targets for molecular imaging of inflammation (Sipkins 2000).

The endothelial (E-)selectin protein is an inflammatory marker expressed on the cell surface of endothelial cells. The members of the selectin family (leukocyte (L-), platelet (P-), and E-selectin) mediate the slow rolling of leukocytes along the endothelium, which constitutes the first step of leukocyte recruitment towards the inflamed site (Ebnet 1999). The natural ligand of these selectins is the sialyl Lewis X (sLe^x), a carbohydrate moiety, located on the surface of leucocytes (Foxall 1992).

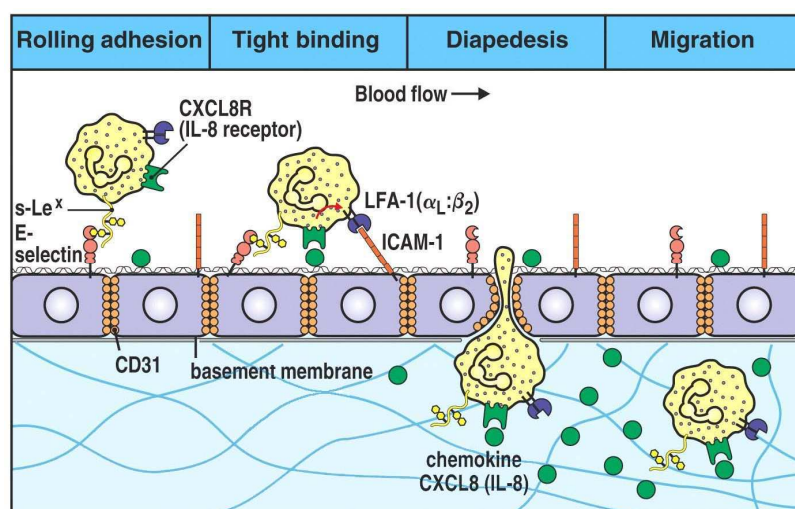


Fig 7: The role of E-selectin in leukocyte recruitment towards sites of inflammation (Janeway 2005).

Both glycoproteins, E-selectin and P-selectin, are expressed on activated endothelium, but not on resting endothelium, and P-selectin is also present on platelets. P-selectin is stored constitutively within endothelial cells and may be recruited to the cell surface within minutes, following stimulation with inflammatory mediators. E-selectin is synthesized de novo by endothelial cells in response to interleukin-1 (IL-1), lipopolysaccharide (LPS), or tumor necrosis factor α (TNF α), and is therefore detectable shortly after P-selectin expression to augment leukocyte recruitment. L-selectin on the other hand is concentrated on most leukocyte surfaces to interact with carbohydrate ligands on activated endothelium at inflammation sites (Kelly 2007). Expression of E-selectin peaks within 6 hours in response to inflammatory stimuli and lasts up to 30 hours (Leeuwenberg 1992). The role of E-selectin in leukocyte extravasation has been affirmed by studies in E-selectin-deficient mice or in mice injected with anti-E-selectin antibodies (Ley 1998). Smaller atherosclerotic lesions were observed with E- and P-selectin knock-out mice, compared with mice lacking either selectin alone (Manka 2001). The specific interaction between E-selectin and the sLe^x molecule can be blocked to efficiently alter the progression of inflammatory disease (Palma-Vargas 1997). Its temporal and spatial expression profile may render E-selectin useful for diagnosis and therapy of inflammatory diseases.

c) Targeting E-selectin

E-selectin expression was imaged in human endothelial cells using cross-linked iron oxide nanoparticles (CLIOs) linked to anti-human E-selectin antibodies (Kang 2002a). The same group also conjugated Gd chelates to a specific antibody fragment to image E-selectin in vitro (Kang 2002b). Reynolds et al. conjugated USPIOs to anti-murine E-selectin monoclonal antibodies in order to measure E-selectin expression by MRI in a mouse model with contact hypersensitivity to oxazolone in the ear (Reynolds 2006). For imaging of inflammation in rheumatoid arthritis, a monoclonal antibody against E-selectin radiolabeled with ¹¹¹In was successfully tested in an animal model (Jamar 1995) and subsequently in humans too (Chapman 1996).

Instead of using anti-E-selectin antibodies, one could also use the endogenous ligand sLe^x, but large-scale synthesis of it is difficult, just like the synthesis of other oligosaccharides. Therefore, mimetic molecules with an active conformation similar to sLe^x have been designed and synthesized (Kogan 1995, Laurent 2004). Synthesis of such new Gd-based CAs has been described to target sites of inflammation in which E-selectin is expressed

(Fu 2002). This synthetic mimetic molecule of sLe^x has shown to target activated endothelial cells in vivo in an inflammatory model of the brain, in a focal stroke model, and in a model of fulminant hepatitis (Barber 2004, Boutry 2005, Sibson 2004). Figure 8 represents an example of MR images obtained after targeting of hepatitis-related E-selectin expression by a magnetically-labeled sLe^x mimetic.

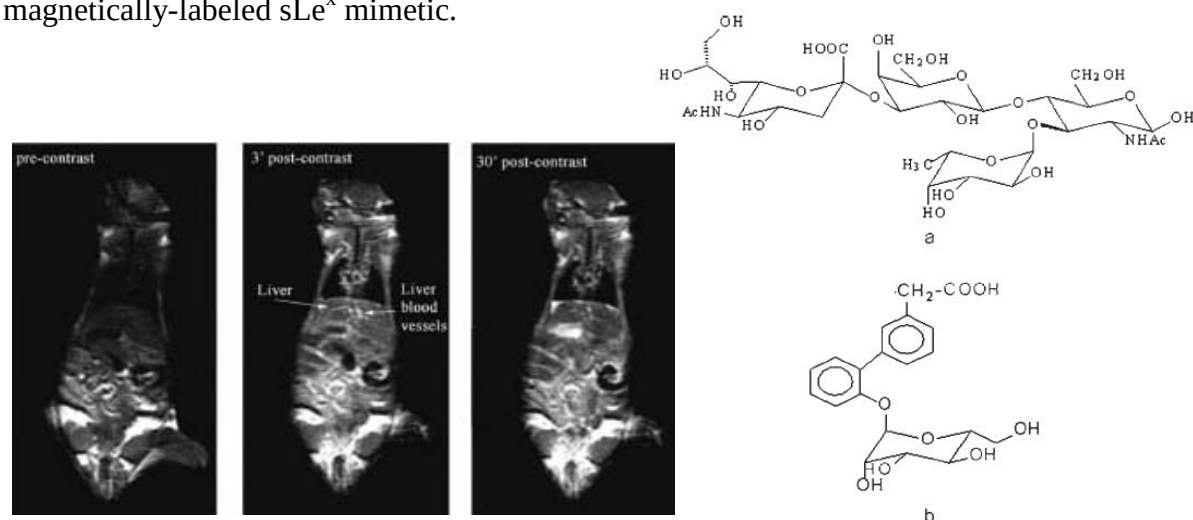


Fig 8: Left: Coronal MR images of a mouse with hepatitis before and at two different points in time after intravenous injection of the Gd-bound sLe^x mimetic (Boutry 2005). Right: Structure of the sLe^x (a) and of the mimetic molecule (b) (Boutry 2006).

The same sLe^x mimetic molecule was also covalently bound to the dextran coating of USPIOs to specifically recognize the E-selectin molecule in a mouse model of hepatitis (Boutry 2006). The same mimetic ligand has been linked to our own PEGylated superparamagnetic particles to provide early detection of inflammation in the rear leg muscle of mice.

2. Tumor cell death

Besides surgery, often used as main cancer treatment, cancer therapy involves chemotherapy, radiotherapy, immunotherapy and monoclonal antibody therapy. Treatment strategy is based on the type of cancer, the location, the grade of the tumor and the stage of the disease, as well as the general condition of the patient (De Saint-Hubert 2009). Unfortunately, treatment is not always effective and can also have severe side-effects. One has to find the most efficient treatment for each individual patient and thereby avoid useless and toxic therapy.

Meyn and his co-workers have suggested that an acute response in the form of apoptosis may occur in some tumors that respond well to irradiation (Meyn 1993). Nevertheless, their study revealed intra- and intertumor heterogeneity in the apoptotic

response. The major form of cell death actually occurring after irradiation was shown to be mitotic catastrophe, which can lead to other forms of cell death at later stages (van der Kogel 2009). Two years later, Meyn et al. have shown that in some tumors apoptosis is also an important response to different cytotoxic agents. The degree of apoptosis occurring after treatment correlated well with tumor growth delay and tumor regression (Meyn 1995). Molecular imaging of cell death could therefore be valuable for the assessment of tumor response to anti-cancer therapy. Not every treatment can be evaluated by cell death of course; some of the more recent treatments are based on cytostatic properties, in which case detection of cell death will be useless.

Apoptosis, the so-called programmed cell death, is a physiological mechanism to control the number of cells. Apoptosis is an organized self-disassembly into small sealed membrane vesicles called apoptotic bodies. Apoptosis is usually characterized by specific changes such as cell shrinking, chromatin condensation, nucleus fragmentation, and morphological changes of the cell membrane. Apoptotic cells are rapidly removed as intact cells or as apoptotic bodies by professional phagocytes or by neighbouring cells. Engulfment prevents inflammation by removing dead cells before they release their intracellular content into the interstitium. Necrosis, on the contrary, is an accidental and passive form of cell death resulting from damage and metabolic failure. Cell swelling, flocculation of chromatin and loss of the cell membrane integrity is characteristic for necrosis. Necrotic cells swell because of water influx and ion redistribution, which ultimately causes the cell to disintegrate. It is assumed that the release of the cell content induces inflammation. However, the two processes of cell death described are not as unconnected to each other as it may seem since ATP (adenosine triphosphate) depletion leads to a switch from apoptosis to necrosis (Eguchi 1997). Another common form of cell death is the active process of autophagy, in which cytosolic components are degraded into essential constituents inside lysosomes in order to protect the damaged cells from altered macromolecules. If the cell is too impaired, it can be removed from tissue by autophagy (Cuervo 2004). Autophagy is frequently referred to as programmed cell death type II. Besides these well-studied types of cell death, many other forms have been discovered and need to be further characterized, such as mitotic catastrophe, which occurs shortly after deregulated mitosis (Bröker 2005). There is not always a clear distinction between these different forms; several types of cell death can share the same features. There were many attempts to discriminate between apoptosis and necrosis, but often these approaches failed because apoptotic pathways are very complex (Grasl-Kraupp 1995).

Therefore, when attempting detection of one specific type of cell death, recognition of other forms of cell death cannot be excluded.

A number of genetic mutations in the cell cycle or in the cell death process contribute to the immortalization and progression of cancers. Imaging of apoptosis or cell death in general could provide an early indication about cancer progression or regression and by this manner it could be used to determine treatment efficacy. Imaging can be used to screen for events that are characteristic of apoptosis, such as caspase-3 activation or phosphatidylserine (PS) exposure. All these emerging methods to image tumor cell death will be elucidated later on in this introduction.

2) Evaluation of anti-cancer treatment outcome

A. Morphological imaging

Most cancer patients will have some kind of surgery, because it offers the greatest chance for cure, especially if the cancer has not yet produced metastases. Surgery can be used alone or together with an adjuvant treatment such as irradiation or chemotherapy. Treatments that are able to reduce tumor size have been considered as beneficial, although tumor shrinkage should not be considered as the only criterion for treatment efficiency. Some treatments may indeed have an impact on tumors, without displaying any obvious reductions in tumor size however.

The World Health Organisation (WHO) wanted to standardize tumor responses in the late 1970s (Miller 1981). According to WHO methodology, one measures the maximum diameter of a tumor and its largest perpendicular diameter. These two diameters are multiplied to obtain a cross product. These two-dimensional criteria were more recently revised and updated into the Response Evaluation Criteria In Solid Tumors (RECIST) that include important changes such as one-dimensional measurements, selection of lesions, details about imaging modalities and new thresholds for determining the progression or regression of the disease (Table 4) (Therasse 2000). One-dimensional measurements (longest diameter) decrease the possibility of errors because measurements are based on adding the lengths of the different lesions and not their surfaces.

Table 4: Comparison of WHO and RECIST response criteria (O'Connor 2008).

	WHO	RECIST
Method		
Lesions	Two dimensional.	One dimensional; target lesions identified.
Measurement	Product of longest diameter and longest perpendicular diameter.	Longest diameter.
Response criteria		
Complete response	Disappearance of all known disease. Confirmed ≥ 4 weeks later.	Disappearance of all known disease. Confirmed ≥ 4 weeks later.
Partial response	$\geq 50\%$ decrease in size.	$\geq 30\%$ decrease in the sum of longest diameters from baseline.
Stable disease	Patient does not meet criteria for partial response or progressive disease.	Patient does not meet criteria for partial response or progressive disease.
Progressive disease	$\geq 25\%$ increase in size of at least one measurable lesion; appearance of new lesions.	$\geq 20\%$ increase in the sum of longest diameters from baseline; appearance of new lesions.

Computed tomography (CT) and MRI are now routinely used to report tumor size, so that clinicians can determine whether patients will continue to benefit from their current therapy (stable or shrinking lesions) or not (new or progressive lesions). But tumors will not uniformly decrease in size as lesions are not perfectly spherical. Due to improvements in imaging methods, new response criteria have to be defined. Volumetric measurements alongside the three cardinal axes have been suggested after advances in CT and MRI technology have been made, but a number of studies have shown conflicting results about the benefit of volumetric tumor measurements added to 1D or 2D measurements. Different formulas for volume estimation are needed when considering non-spherical tumors and drawing tumor margins is time consuming. However, in some clinical studies volume-based measurements were more effective than 1D or 2D measurements. Volumetric changes have predicted overall survival in patients with high-grade glioma and with locally advanced breast cancer, whereas WHO or RECIST criteria did not predict outcome in either study (Dempsey 2005, Partridge 2005).

Recently RECIST criteria were updated. Besides some minor changes, such as the maximum number of target lesions, inclusion of pathological lymph node assessment and the new definition of progressive disease, it was concluded that volumetric measurements of response and functional imaging by PET or by MRI need further investigation and standardization before they can officially be accepted as surrogate endpoints. The only method accepted as complement to CT scan in detection of new lesions is the evaluation of glucose metabolism by ^{18}F -FDG-PET (Eisenhauer 2009).

Timing of tumor shrinkage can be quite variable. Sometimes it can take two or three cycles (6-8 weeks) of therapy before meaningful changes can be expected. Moreover, a small residual tumor mass can be highly aggressive, and on the other hand, a more voluminous mass can be stable because of fibrosis, cysts or necrosis, suggesting potential benefit despite no apparent changes in lesion size. In addition, activity of anti-proliferative, anti-angiogenic, anti-vascular, or immunomodulating agents may not be reflected by changes of size. In summary, the current method accepted as tumor response marker in clinical trials is not without limitations. As a result of these deficiencies, newer imaging methods assessing physiological changes are increasingly investigated. A surrogate endpoint is needed to predict benefit at an earlier time; therefore functional and/or molecular imaging can be used to obtain valuable information about treatment outcome (Brindle 2008).

B. Functional imaging

1. Imaging of glucose metabolism by ^{18}F -FDG

Functional imaging detects molecular signals indicating biochemical activity and changes. The most universally available tracer for molecular positron emission tomography (PET) is ^{18}F -FDG with numerous applications in oncology, cardiology, neurology and imaging of inflammation. PET imaging has a lower spatial resolution than other imaging modalities (autoradiography, CT, MRI), but the sensitivity in the detection of emitted radiations is very high. The development of combined PET and CT scanners has joined the morphological data from CT with the functional images from PET. ^{18}F -FDG is an excellent marker of tumor glycolysis as FDG easily substitutes the glucose in both aerobic and anaerobic conditions. After intravenous bolus injection, cellular FDG uptake is governed by glucose transporter proteins (GLUTs) and, inside the cell, FDG is phosphorylated by a hexokinase to FDG-6-phosphate. Unlike natural glucose, the FDG-6-phosphate will not enter the Krebs cycle. It will rather accumulate inside the cell proportionally to the level of glycolysis of the cell (Fig. 9).

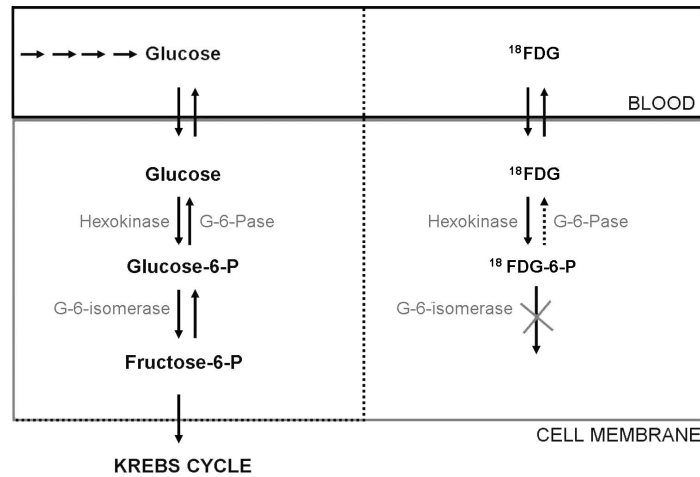


Fig. 9: Mechanisms of ^{18}F -FDG accumulation in tumor cells.

It is well known that tumor cells consume more glucose subsequent to an overexpression of GLUTs and enzymes of the glycolytic pathway. Due to this important accumulation in tumors, ^{18}F -FDG can be detected with high sensitivity by PET-scan. ^{18}F -FDG is not entirely specific for malignant cells and false positives from inflammatory processes can occur. A high uptake can also be seen in highly active healthy tissues such as the heart and the brain. ^{18}F -FDG-PET has shown to be a valuable tool in the differentiation of benign and malignant tissues, in staging, in the detection of recurrent disease, and more recently in the identification of early tumor response (Fig. 10). ^{18}F -FDG-PET is now approved for staging of several tumor types, as well as for monitoring treatment response to breast cancer (Juweid 2006), and standardized response criteria for PET in clinical trials have been published (Young 1999). In Belgium, ^{18}F -FDG is the most utilized PET tracer in clinical routine in the field of oncology.

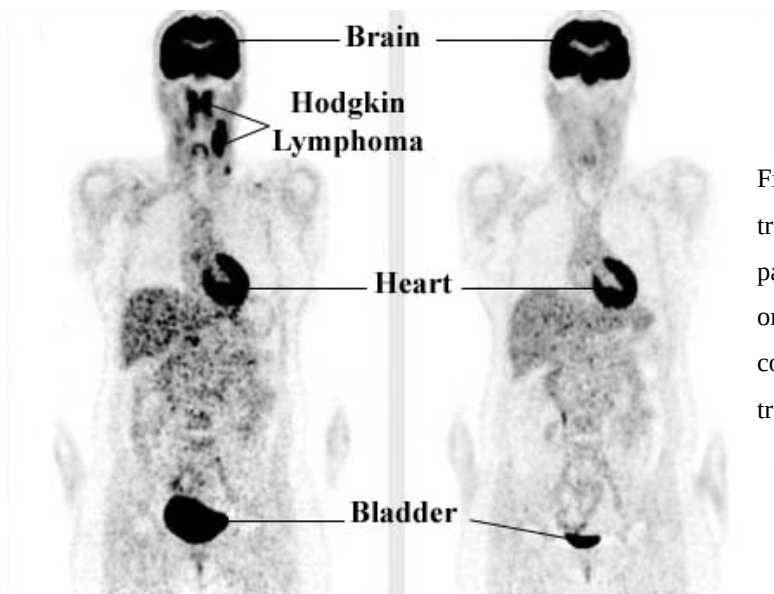


Fig. 10: Example of ^{18}F -FDG-PET used for treatment response evaluation in Hodgkin patients scanned at baseline (left) and after one cycle of chemotherapy (right), with a complete resolution of FDG avidity after treatment (Hutchings 2009).

Functional images of glucose metabolism can already be obtained one hour after ^{18}F -FDG injection. Several studies have demonstrated a negative correlation between ^{18}F -FDG capture by tumor cells and the early response to treatment. ^{18}F -FDG-PET of imatinib-treated gastrointestinal stromal tumors (GISTs) showed little or no ^{18}F -FDG uptake after treatment although lesion size remained large due to necrotic areas (van den Abbeele 2002). Loss in ^{18}F -FDG tumor uptake within hours after gefitinib treatment was also observed in non small cell lung cancer (Su 2006), but this decrease can also represent reduced glucose utilization instead of tumor cell death (Workman 2006). Some targeted therapies have direct effects on glucose metabolism (treatment with tyrosine kinase inhibitors (TKI) such as imatinib and gefitinib) inducing internalization of the glucose-receptor (Prenen 2006). ^{18}F -FDG-PET may also be inaccurate during the first two weeks after chemotherapy because of a temporary metabolic shutdown. For monitoring of chemosensitivity by metabolic imaging it is recommended to wait at least 2-3 weeks (Cremerius 1998). Another fact that should be taken into account is that some slow-growing tumors are not FDG-avid, in which case ^{18}F -FDG is of no use. In patients with high serum glucose levels, tumor-to-background ratio may be reduced because of a possible competition between ^{18}F -FDG and endogenous glucose (Lindholm 1993).

In table 5, the main advantages and disadvantages of using ^{18}F -FDG for evaluation of treatment outcome, and also of the PET technique in general, are listed. Furthermore, possible explanations for false positive and false negative interpretations of ^{18}F -FDG–PET studies are discussed below (table 6).

Table 5: Advantages and disadvantages of the ^{18}F -FDG–PET technique.

Advantages	Limits
High sensitivity	High noise in brain and heart Inflammatory diseases High noise in urinary tract because of urinary elimination Poor sensitivity in tumors with low malignancy or low metabolic activity
Improved by CT-PET coupling	Lack of anatomical details Low spatial resolution, small tumors (4-6 mm) are difficult to detect by PET
Performable in any clinical environment	Exposure to radioactivity
Entire body scan to detect potential metastases	Impossible to distinguish chemical species bound to the radionuclides
Quantification by means of complex kinetic models	

Table 6: Causes of false positives and false negatives in measuring tumor glucose metabolism

False positives	False negatives
Bowel activity Muscle contraction Endometrial and ovarian hotspots Atherosclerosis Infection Inflammation Intervention-related uptake: after surgery, biopsy, radiotherapy or chemotherapy	Low GLUT expression Small lesions (<5 mm) High background (e.g. in the brain) Hyperglycemia (diabetics) Metabolic shut-down after chemotherapy Low grade tumors

2. Cell proliferation markers

Treatment-induced diminutions in cell proliferation may be more specific than changes in glucose consumption (Barthel 2003, Shields 1998). This supports the need for a direct marker to image cell proliferation, which can be measured by tracers involved in DNA synthesis and cell division.

a) ^{18}F - fluorothymidine

Several new PET tracers are now being evaluated to study other metabolic pathways than glucose metabolism. Thymidine and other related molecules have received the most interest as imaging agents of cell proliferation. The pyrimidine analog thymidine is incorporated into DNA, (deoxyribonucleic acid) but not into RNA (ribonucleic acid). The utilization of ^3H - or ^{14}C -labeled thymidine has widely been established for cell proliferation measurements. This led to the development of ^{11}C -labeled thymidine, enabling cellular proliferation measurements in humans by PET-scan (Caroli 2010, Vander Borgh 1991). Although studies using ^{11}C -thymidine have demonstrated the potential of the tracer to image tumor proliferation and response to therapy, the tracer is rapidly metabolized in vivo and the presence of multiple metabolites has disturbed its wider use. This led to the development of 3'-deoxy-3'- ^{18}F -fluorothymidine (^{18}F -FLT), which remains unchanged during in vivo administration, and whose radioisotope has a longer half-life (Buck 2003, Caroli 2010, Francis 2003, Grierson 2000). Cellular uptake of ^{18}F -FLT occurs through facilitated Na^+ -dependent transport or through passive diffusion. Subsequently, FLT is phosphorylated by highly activated thymidine kinase 1 (TK_1) into FLT-monophosphate (FLT-MP), which is trapped inside the cell (Fig. 11).

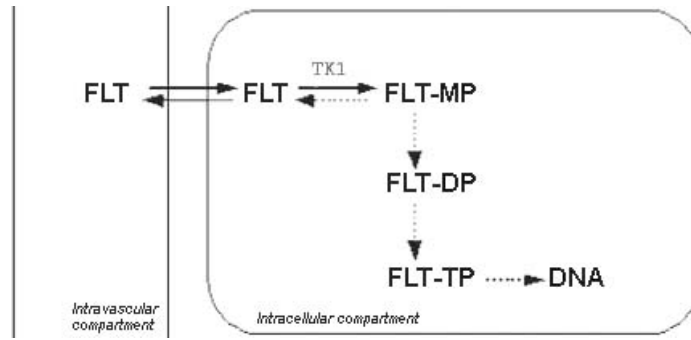


Fig. 11: Mechanisms of FLT uptake into proliferating tumor cells (Been 2004).

TK₁ activity is increased in the late G1 and S phase of non malignant proliferating cells and even higher (3-4 times) throughout the whole cell cycle in malignant cells, whereas its activity is absent in quiescent cells (Boothman 1994, Munch-Petersen 1995). This increase in TK₁ activity in neoplastic tissues is responsible for higher levels of FLT phosphorylation and subsequent intracellular trapping of FLT in tumor cells. FLT is not directly incorporated into the DNA because it acts as a chain terminator due to the absence of the 3'-hydroxyl group. It only visualises the first step of the salvage pathway of DNA synthesis, which makes it an indirect proliferation marker. In one study though, ³H-FLT uptake showed a good correlation with ³H-thymidine uptake, meaning that ³H-FLT uptake is well able to provide a good representation of the salvage pathway (Rasey 2002).

First biodistribution studies using ¹⁸F-FLT in humans have shown physiological uptake in bone marrow (high proliferation rate), in liver (glucuronidation), in the urinary tract (excretion) and in reactive lymph nodes (Shields 1998, Weber 2010). In contrast to ¹⁸F-FDG-PET, no uptake is seen in the brain, skeletal muscles or myocardium. Overall selectivity for tumors is greater for ¹⁸F-FLT than for ¹⁸F-FDG, especially in the brain, but on the other hand, the signal increase induced by ¹⁸F-FDG uptake in tumors was higher than by ¹⁸F-FLT uptake (Fig. 12) (Lee 2009, Seitz 2002, Weber 2010).

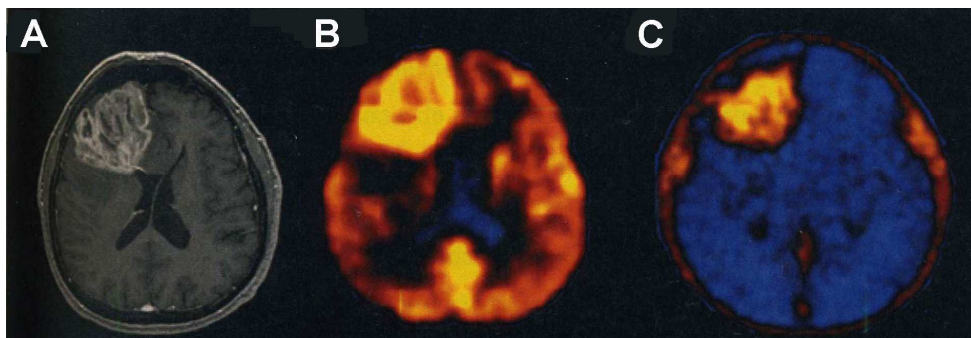


Fig. 12: Imaging of high-grade glioma by A: contrast-enhanced T₁-weighted MRI, B: ¹⁸F-FDG-PET and C: ¹⁸F-FLT-PET (Chen 2005).

PET-images can already be acquired 5 to 10 minutes after ^{18}F -FLT injection. Reduction in ^{18}F -FLT uptake has allowed to identify responders and non-responders in a study of glioma patients treated with bevacizumab and irinotecan (Chen 2007). The same was achieved in another study of non small cell lung cancer patients treated with gefitinib (Sohn 2008). ^{18}F -FLT-PET was also allowed to visualize radiation effects on the bone marrow and to distinguish inflammation from recurrent disease (Yue 2010). Different types of anticancer therapy have been studied and the results of these studies may seem inconsistent. Sometimes TK_1 activity remains high, although tumor cell proliferation is compromised. Drugs such as methotrexate and 5-fluorouracil arrest cells in S phase and impede the de novo pathway of DNA synthesis. The result of this shutdown is a compensatory upregulation of the salvage pathway with increased TK_1 activity (Mirjolet 1998, Dittmann 2002). Different tumors also vary in the respective contributions of the salvage pathway and the de novo pathway to DNA synthesis (Bardot 1994, Cole 2002). Schwartz et al. showed that 30% of the tumor cell types tested showed less TK_1 -dependent proliferation and subsequently no significant correlation was found between TK_1 activity and the S phase fraction of cells (Schwartz 2003). Today, clinical studies about ^{18}F -FLT-PET are still limited, but results are promising in general. Further studies are needed to assess the value of ^{18}F -FLT for evaluating early response to therapy.

Low overall uptake in tumors, the variability in ^{18}F -FLT uptake in tumors after therapy and the indirect measurement of DNA synthesis represent the limitations of ^{18}F -FLT-PET imaging. Newly developed tracers, such as 2'-fluoro-5-methyl-1- β -D-arabinofuranosyluracil (FMAU) and 2'-deoxy-2'-fluoro-5-bromo-1- β -D-arabinofuranosyluracil (FBAU), are incorporated into DNA and therefore directly reflect DNA synthesis, which is a potential advantage over ^{18}F -FLT (Mangner 2003).

b) Radiolabeled amino acids

Additionally to proliferation markers, other targets related to protein synthesis and amino acid transport have been identified (Caroli 2010, Herholz 1998, Narayanan 2002). Proteins are composed of amino acids and several amino acid tracers, such as ^{11}C -methyl-methionine (MET), have been developed. Like ^{18}F -FLT, the primary application of ^{11}C -MET is in brain tumors, since ^{18}F -FDG induces high background signal in normal brains (Kawai 2009). ^{11}C -MET imaging in the abdominal region will be more complicated because natural amino acids, such as methionine, are taken up by pancreas and liver (Kubota 2001).

O- ^{11}C -methyl-L-tyrosine (OMT), a non metabolized amino acid analogue, has also been developed (Ishiwata 2004). When compared with ^{18}F -FDG-PET, ^{11}C -OMT-PET had smaller signal-to-noise ratios but may have potential for improved specificity by reducing the rate of false positives (Fig. 13, left). ^{11}C -OMT showed similar uptake compared to ^{11}C -MET in PET-images of brain tumors (Fig. 13, right). As ^{11}C requires the disposability of a cyclotron nearby, tyrosine has also been labeled with an ^{18}F isotope (Caroli 2010).

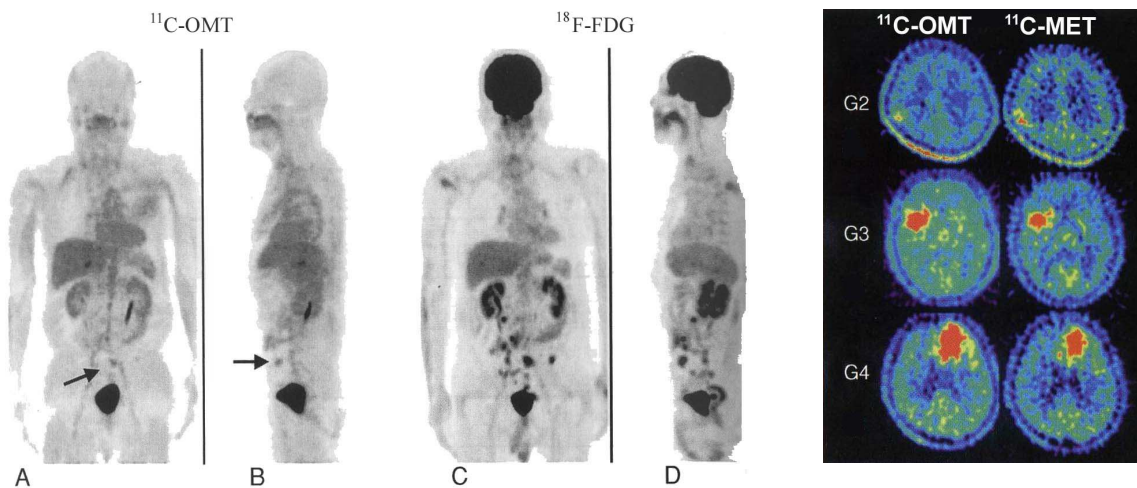


Fig. 13: Left: Coronal (A and C) and sagittal (B and D) slices of whole-body PET-imaging in order to detect tumor metastasis by ^{11}C -OMT or ^{18}F -FDG. Right: Representative PET images of obtained with ^{11}C -OMT and ^{11}C -MET presenting gliomas of different grades (G2, G3, G4) (Hayat 2007).

c) Choline derivatives

Exogenously administered choline actively enters cells via choline transporters, is then metabolized to generate phosphocholine (PC), and is finally transferred to diacylglycerol (DAG) to synthesize phosphatidylcholine (PtdC), which is integrated into the cell membrane (Roivainen 2000). Once ^{11}C -labeled choline has entered tumor cells, it is rapidly phosphorylated by a choline kinase (ChoK) and chemically trapped inside the cells (Hara 1997). An increased choline uptake has been demonstrated in malignant tumor cells, which have higher cell proliferation and an increased synthesis of cell membranes (Podo 1999). Overexpression of ChoK has been found in most tumors, providing the basis for cancer imaging with choline PET (DeGrado 2001). Studies have shown that ^{11}C -choline PET may offer a new approach to follow tumor response, as the decrease of choline uptake represented an early response to treatment (Fei 2010). In prostate cancer for instance, the use of ^{18}F -FDG is limited due to high urinary excretion and frequently occurring inflammatory lesions. ^{11}C -labeled choline has low urinary excretion and its uptake in recurrent prostate cancer after

radiotherapy showed good correlation with PSA (prostate-specific antigen) levels, prostate biopsies and morphological imaging (Breeuwsma 2010). To circumvent the short half-life of ^{11}C -choline, ^{18}F -fluoro(methyl)choline (^{18}F -FCH) was developed. ^{18}F -FCH could be considered for response evaluation with main applications in the pelvic region because of lower urinary elimination compared to ^{18}F -FDG. Until now, ^{18}F -FCH is only used for diagnosis of locally advanced or metastatic prostate cancer and not for response evaluation (Breeuwsma 2010, Cimitan 2006, Kwee 2009).

3. Perfusion imaging by dynamic contrast-enhanced MRI

The main use of DCE-MRI in oncology is to be an early marker of tumor response, notably to anti-angiogenic treatments. The gold standard measurement of angiogenesis is the histological estimation of microvascular density, which is invasive, suffers from sampling bias, and cannot determine parameters such as flow or permeability. DCE-MRI involves the acquisition of sequential images during the passage of an intravenously injected CA through a particular tissue of interest. CAs used for these studies are Gd chelates that do not penetrate inside cells and therefore directly reflect microcirculation. After the passage of the contrast probe into the tumor, parameters were gathered from the kinetic curve. Clinical studies have shown that the parameter K^{trans} , reflecting the “wash in” of the CA into the tumor, could be used for in vivo evaluation of tumor response (Galbraith 2003). K^{trans} can be influenced by blood flow, endothelial permeability and endothelial surface area.

One has to distinguish diffusible probes from blood pool agents, since they behave differently after intravenous injection. Low MW chelates of gadolinium (<1 kDa) are diffusible tracers; blood pool tracers (> 30 kDa) are larger molecules, hence extravasation is reduced. Macromolecular contrast media have less unspecific extravasation in normal vessels (< 1%) compared to small gadolinium chelates (45%), which could enhance the sensitivity of tumor characterization and treatment monitoring by DCE-MRI, at least in experimental models (Brasch 2000).

Similarly, the use of DCE-CT can be a method for assessing tumor perfusion; especially in strongly moving areas such as the lungs where CT images are of better quality than MR images (Marcus 2008). DCE-CT's major advantage over DCE-MRI is a direct linear correlation between signal enhancement and CA concentration. The DCE-ultrasound technique developed more recently uses pure intravascular microbubbles and is therefore suitable for measuring blood flow and perfusion (Broumas 2005).

C. Determination of molecular profiles by MR spectroscopy

Currently, metabolites in tumors are analysed by taking surgical biopsies for western blots or ELISA assays. A major limitation of this approach is the dependence on sampling of a heterogeneous disease, not to mention the risk and discomfort that may be caused to the patient (Workman 2006). The use of plasma samples is another approach providing a source for many serum markers such as circulating tumor cells, proteins, metabolites and so on. However, this technique does not allow in situ detection of tumor cells. MR spectroscopy (MRS) is a non invasive tool, which can be used to repeatedly study metabolic processes in vivo. Initially developed for non-tumoral applications, the technique is now being evaluated for a large number of tumors. Several nuclei (^1H , ^{13}C , ^{14}N , ^{19}F , ^{23}Na , ^{31}P) can be studied by MRS, but ^1H is the most used nucleus for the assessment of tumor response. Metabolites can be identified by their characteristic chemical shift, which corresponds to the modified resonance frequency of nuclei depending on their chemical surrounding. The presence of the electronic surrounding slightly influences the magnetic field experienced by the nuclei. This way, the chemical shift informs the investigator about the molecular group that is carrying the studied nucleus. Results are then displayed on a spectrum, which shows a series of peaks corresponding to the resonance frequencies of the different metabolites. Frequencies are expressed in terms of the dimensionless unit parts per million (ppm), a value independent of the static magnetic field strength (Gadian 1995a). Unlike MRI, that provides imaging of anatomy, MRS does not provide spatial information. Localized spectra are acquired and analysed in order to determine precise chemical shifts and peak integrals. In vivo, results are mostly expressed as metabolite ratios. In vitro acquisitions have improved spectral resolution; furthermore reference compounds can be added to quantify metabolite concentration and to calibrate the chemical shift.

1. ^1H MRS

Although spectroscopy can be performed on different types of nuclei, the major application of this technique is spectroscopy of the hydrogen nucleus. A spectrum is represented by the peak amplitude on the y-axis in function of the chemical shift of metabolites on the x-axis, expressed in ppm (Fig. 14). Previous to spectrum acquisition, the signal of the water peak, situated at 4.7 ppm, has to be suppressed because the signal coming from protons of water molecules is so important that it would mask the spectroscopic signal from the other

metabolites, which is 10,000 times lower (Fig. 14). Excellent field homogeneity is required to ensure that the “wings” of the water signal do not interfere with the metabolite signals, and that there is adequate spectral resolution between the signals of interest. A heterogeneous magnetic field results in a dispersion of resonance frequencies and therefore in peak broadening. The volume of interest is determined by the intersection of three orthogonal sections. The larger a region of interest is, the more difficult it is to homogenize the field inside this region. Spectrum quality is evaluated by two main criteria: signal-to-noise ratio (peak height) and spectral resolution (peak width). The surface area of the peak depends on metabolite concentration and on T_1 of protons carried by the metabolite. The peak width is inversely proportional to the relaxation time T_2^* . Because of the low signal-to-noise ratio, multiple acquisitions have to be done.

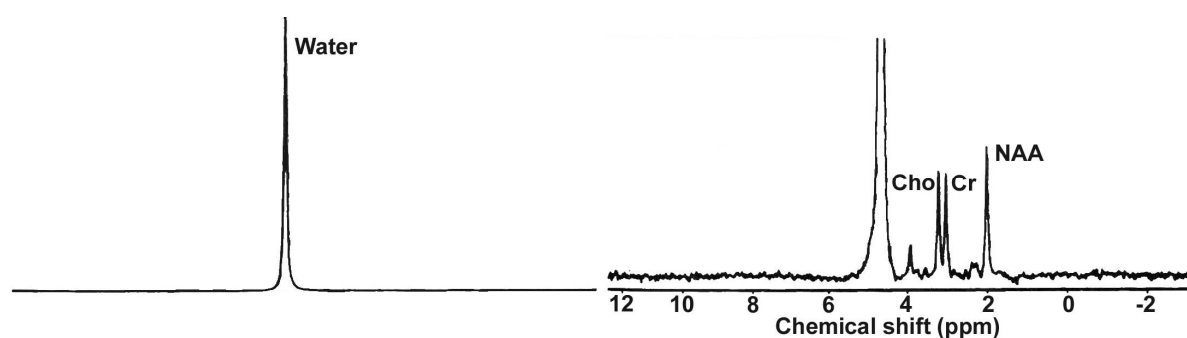


Fig. 14: On ^1H spectra from brain acquired without solvent suppression, only the water signal can be seen (left). With water suppression (right), the spectrum displays signals from N-acetylaspartate (NAA), choline-containing-compounds (Cho) and creatine+phosphocreatinine (Cr) (Gadian 1995b).

Table 7: Most relevant metabolites observed in vivo by ^1H MRS (adapted from Marcus 2008).

Metabolite		Frequency (ppm)	Function	Aberration
Choline	Cho	3,2	Marker of cell membrane metabolism	↑: tumors, demyelination
Creatine, phosphocreatine	Cr, PCr	3,0	Marker of energetic metabolism	Often used as reference peak
N-acetyl-aspartate	NAA	2,0	Marker of healthy neurons	↓: neuronal disease
Myo-inositol	mI	3,6	Glial marker	↑: glioma, MS ↓: herpetic encephalitis
GABA, glutamate, glutamine	Glx	2,1-2,5	Marker of intracellular neurotransmitters	↑: herpetic encephalitis
Citrate	Ci	2,6	Marker of healthy prostate	↓: prostate cancer
Lactate	Lac	1,3	Ischemia, convulsions, tumors	Pathologic
Mobile lipids	Lip	0,9 and 1,3	Apoptosis, necrosis	

The number of metabolites detectable in vivo by ^1H MRS is limited (table 7). Peaks observed by proton MRS in almost all types of tumors are mainly corresponding to choline-containing compounds (Cho), creatine and phosphocreatine (Cr, PCr), lactate (Lac), and CH_2 mobile lipids. The results of metabolic peaks obtained by MRS are often expressed as ratios. Measurement of the real metabolite concentration requires precise determination of the peak area and conversion into metabolite concentration after calibration.

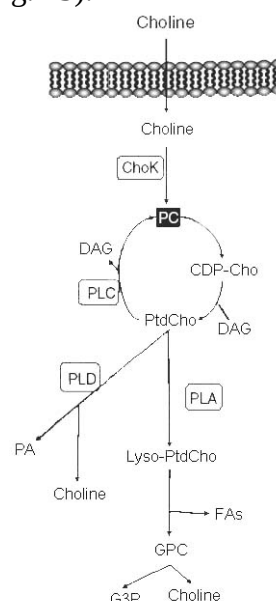
Lipid proton MRS was the first MR technique applied for the detection of apoptosis (Blankenberg 1996, Blankenberg 1997, Engelmann 1996). In these studies, investigators observed an apoptosis-related increase in mobile lipids with an increased methylene (CH_2) peak at 1.3 ppm relative to the methyl (CH_3) peak situated at 0.9 ppm. Both, in vitro and in vivo studies showed accumulation of lipid vesicles during apoptosis (Griffin 2003, Griffin 2006, Schmitz 2005), but also in necrotic tumors (Zoula 2003). The rise in CH_2 resonance occurred with a wide range of anti-cancer treatments and in different cell types. Therefore, an increase of the signal ratio of CH_2 to CH_3 was considered as a measurement of the degree of apoptosis. Although there was a rise in methylene resonance, no detectable changes were found in the total lipid composition or in new lipid synthesis in tumors. The increased methylene signal was shown to come from the formation of cytoplasmic lipid droplets (0.2–2.0 μm) during apoptosis (Hakumäki 1998). Lipids in cell membranes are not detectable by MRS because of their limited mobility. Release of fatty acids from cell membranes and their storage in lipid droplets in the form of triglycerides renders them visible on MR spectra. Overall therapy-induced cell death can thus be detected through an increase in mobile lipids in apoptotic cells (Callies 1993). A major limitation of this method is the relatively small change in lipid signal; therefore clinical studies will be restricted to organs that are detectable to surface coils, or to organs that are free of motion. The lipid methylene signal in tumors may be contaminated by the presence of lactate. The peak of mobile lipids is actually the only peak that is characterized by an increase during apoptotic cell death. During apoptosis, a significant decrease in metabolite concentration can be observed for the other chemical species displayed on ^1H MR spectra. On the other hand, metabolites behave completely different during necrotic cell death: The proton spectrum displays a general increase of metabolite peaks, except for the glutathione peak (Strauss 2008).

Today, many studies are also focusing on measurements of choline-containing metabolites using water suppressed MRS. Several chemical resonances contribute to the total choline peak (tCho), including choline, PC, and glycerophosphocholine (GPC). Separation of the single choline peak into its different contributors can be obtained in ex vivo acquisitions,

where higher spectral resolution can be achieved (Gribbestad 1999, Sitter 2002). The choline level increases with increased tumor cell proliferation and cell density (Jackowski 1996, Rutkowski 2003). High levels of choline are characteristic of increased membrane turnover, possibly representing increased malignant potential of cells (Aboagye 1999, Podo 1999, Ronen 2001). Choline-containing metabolites are essential substrates in the metabolism of lipids. Choline and PC are precursors and breakdown products of PtdCho, the major phospholipid component of the cell membrane. PC can be produced by phosphorylation of choline through ChoK (Beloueche-Babari 2010, Podo 1999) (Fig. 15).

Fig. 15: Metabolic pathways of choline-containing compounds.

ChoK: choline kinase
 PC: phosphocholine
 CDP-Cho: cytidine diphosphate-choline
 DAG: diacylglycerol
 PtdCho: phosphatidylcholine
 PL: phospholipases (A, C et D)
 FAs: fatty acids
 GPC: glycerophosphocholine
 G3P: glyceraldehyde-3-phosphate
 PA: phosphatidic acid
 (adapted from Beloueche-Babari 2010)



In many cancers an increase in choline-containing metabolites was observed by proton spectroscopy (Howe 2003, Leach 1998, Vilanova 2009). Al-Saffar et al. applied the approach of choline spectroscopy to mice treated by the ChoK inhibitor MN58b and found a positive correlation between choline decrease and tumor growth inhibition (Al-Saffar 2006). Furthermore, it has been reported that hypoxia can be a regulating factor of ChoK expression (Glunde 2008). This observation could explain the decrease detected in choline-containing metabolites in mice treated by PX-478, an inhibitor of HIF-1 α (hypoxia-inducible factor) (Jordan 2005a). After several preclinical studies, decreases in choline signals were also observed in patients treated by chemo- or radiotherapy (Table 8).

High glucose consumption, forming the basis for metabolic imaging by ^{18}F -FDG-PET, and lactate production are other characteristics of cancer cells. Glucose metabolism was also monitored by ^1H -MRS in animal cancer models (Aboagye 1998, Rivenzon-Segal 2002). PX-478 treatment, inhibiting HIF-1 α , led to a trend of decreased glucose uptake and lactate production in tumor xenografts (Jordan 2005a). However, the clinical measurement of lactate production by MRS in humans requires further development.

Table 8: Listing of human studies wherein response to anti-cancer treatment was evaluated by changes in the total choline peak as measured by proton MRS.

Author	Tumor type	Treatment	Follow-up	Result
Kurhanewicz 1996	Prostate tumor	Cryosurgery	3-6 months	↓ [Cho+Cr]/Ci = necrosis
Parivar 1996	Prostate tumor	Cryosurgery	10-79 weeks	↓ [Cho+Cr]/Ci > 0.86 = recurrence
Yoshino 1996	Brain tumor	RT	5-19 months	↓ Cho
Nelson 1999	Brain tumor	RT	2-12 months	↓ Cho in responders ↑ Cho when progression/recurrence
Preul 2000	Glioma	Tamoxifen	Pretherapy and 2-4 weeks after	↓ Cho in responders
Coakley 2001	Prostate tumor	RT	14-48 months	↑ Cho when recurrence
Graves 2001	Brain tumor	CT + radiosurgery	2-14 months	↑ Cho when recurrence
Jagannathan. 2001	Breast tumor	CT	After 3 cycles	↓ Cho in responders
Mueller-Lisse 2001	Prostate tumor	HT	1-16 weeks	Loss of Cho, Cr, Ci = atrophy
Schwarz 2002	Lymphoma	CT	during first cycle	↓ Cho in responders
Kimura 2004	Brain tumor	Radiosurgery	1-3 months	Early ↓ Cho
Meisamy 2004	Breast tumor	CT	24h of cycle 1-end of cycle 4	↓ Cho in responders
Murphy 2004	Brain tumor	CT	3-12 months	↓ Cho at 12 month
Pucar 2005	Prostate	RT	1-6 months	↑ [Cho+Cr]/Ci when recurrence
Chen 2006	HCC	CE	2-3 days	↓ Cho
Wu 2006	HCC	CE	1 week	↓ Cho and ↑ lipids
Zeng 2007	Glioma tumor	RT	6 weeks 4 months	↑ Cho when recurrence
Haddadin 2009	Breast tumor	CT	At 24h of cycle 1	↓ Cho in responders

CE: chemoembolization, Cho: choline, Ci: citrate, Cr: creatine, CT: chemotherapy, HCC: hepatocellular carcinoma, HT: hormonotherapy, RT: radiotherapy.

2. ^{31}P MRS

^{31}P MRS was first used to study cellular energetics in tissues like the heart and the brain (Gadian 1981). The sensitivity of ^{31}P MRS is only about 7% of the sensitivity reached in ^1H MRS but its chemical shift range is larger (30 ppm) and does not present the problem of water suppression. The most important peaks displayed by ^{31}P MRS originate from PCr, nucleotide triphosphates (NTP) or inorganic phosphate (Pi). Other main contributors of the ^{31}P spectrum are metabolites of membrane phospholipids including phosphomonoesters (PMEs), which comprise PC and PE, and phosphodiester (PDEs), comprising glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE) (Cox 1992, Daly 1989, Steen 1989).

Characteristic for cancer are elevated concentrations of PMEs and PDEs as observed by in vivo ^{31}P MRS, since PC and PE contribute to membrane synthesis (Leach 1998, Negendank 1992). These changes are often reversed on successful treatment with chemo- or radiotherapy (Fig. 16) and are now explored as biomarkers for tumor diagnosis, staging and

clinical response monitoring (Payne 2006, Shah 2006). A review of the literature has revealed that a decrease in PME occurred in 38 of 47 cancers that responded to treatment (Negendank 1992), but as this technique is relatively insensitive, it requires long acquisition times and has a poor spatial resolution. With this technique, it will be difficult to detect tumor apoptosis in vivo since the percentage of apoptosis in treated tumors is relatively low.

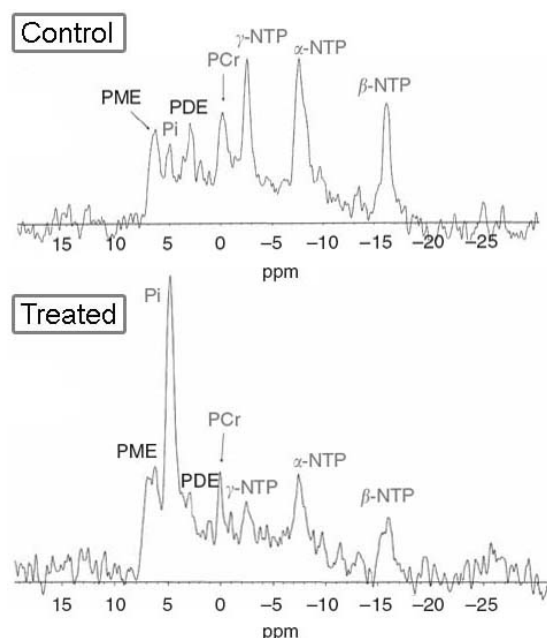


Fig. 16: In vivo ^{31}P MRS of tumor xenografts before and after anti-cancer treatment (Chung 2008). Metabolites: PME: polymonoester, Pi: inorganic phosphate, PDE: phosphodiester, PCr: phosphocreatine, NTP: nucleotide triphosphate.

D. Molecular imaging of cell death

As stated previously, many anti-cancer therapies exert their therapeutic effect by inducing apoptosis. For this reason, imaging of apoptosis or of cell death in general allows an early evaluation of treatment outcome in cancer patients. A number of steps in the cell death process could potentially be imaged. DW-MRI is sensitive to a loss in tumor cellularity and proton MRS can measure lipid droplets formed during programmed cell death. Besides, changes in cell membrane or activation of apoptosis-related enzymes can also be imaged by targeted CAs. Major modifications of apoptotic cells include: (1) activation of cysteine protease enzymes (caspases) to cleave specific intracellular proteins; (2) degradation of DNA molecules into 50- to 300-kb-sized pieces by activated endonucleases; (3) PS expression on the external leaflet of the cell membrane, (4) decrease in cell size due to loss of intracellular water. Pieces of cells undergoing apoptosis are packaged in small vesicles that are derived from the cell membrane. These pieces of cells are called “apoptosomes.” Unlike in necrosis, apoptotic cells are removed without release of cytosolic content and without induction of inflammation. Some of the following techniques try to selectively image apoptosis. As the

hallmarks of apoptosis are often not really specific for apoptosis, except for DNA laddering, detection of cell death in general is more probable. Since every type of cell death is beneficial to anti-cancer treatment response, it does not matter very much by which kind of cell death tumor cells actually die.

1. Diffusion-weighted MRI

Randomly moving water molecules (Brownian motion) encounter different obstacles within an organism (cell membranes, proteins, macromolecules, fibres...) that vary depending on the type of tissue or pathological modifications of tissues. It can be considered, approximately, that mainly extracellular water is explored in diffusion imaging, thus diffusion gives indirect information about the structure surrounding these water molecules. Tumor treatments that damage or kill cells increase the fractional volume of interstitial space.

In DW-MRI, two symmetrical gradients are added to the MR sequence. The first gradient induces a phase shift of the water protons and the second rephases them. The water protons will only be completely rephased if they have stayed at the same initial location, in which case signal intensity will be maximal. Any molecular movement during the period of time elapsing between the application of the two gradients will lead to signal attenuation. Water mobility will thus be displayed as signal loss. The degree of diffusion-weighting is expressed by the b-value (in s/mm²), a parameter that depends on strength, duration and separation of the two additional gradients. A b-value of 0s/mm² represents no diffusion-weighting and a b-factor of 1000s/mm² indicates high diffusion-weighting. As DWI is based on T₂-weighted sequences, the contrast on DW images has a diffusion component but also a T₂ component, which has to be considered for interpretation of the results. This means that a hypersignal on DW images can signify restricted diffusion but also a lesion that appears as hypersignal on T₂-weighted images (T₂-shine-through). With two diffusion images acquired at different b-values, the apparent diffusion coefficient (ADC, expressed in mm²/s) can be calculated to quantitatively evaluate mobility of water molecules. The ADC does not depend on T₂ any more and it can be represented as an ADC map. The relation between b-value and signal intensity on DWI is: $S(b) / S(0) = e^{-b \cdot ADC}$, where S(b) is the signal intensity in DW images of a given b-value and S(0) the initial signal intensity on images without diffusion-weighting (b-value of 0s/mm²). ADC can be calculated based on this equation. A hyposignal on DW images is displayed as hypersignal on ADC maps, corresponding to an increase in diffusion (Fig. 17).

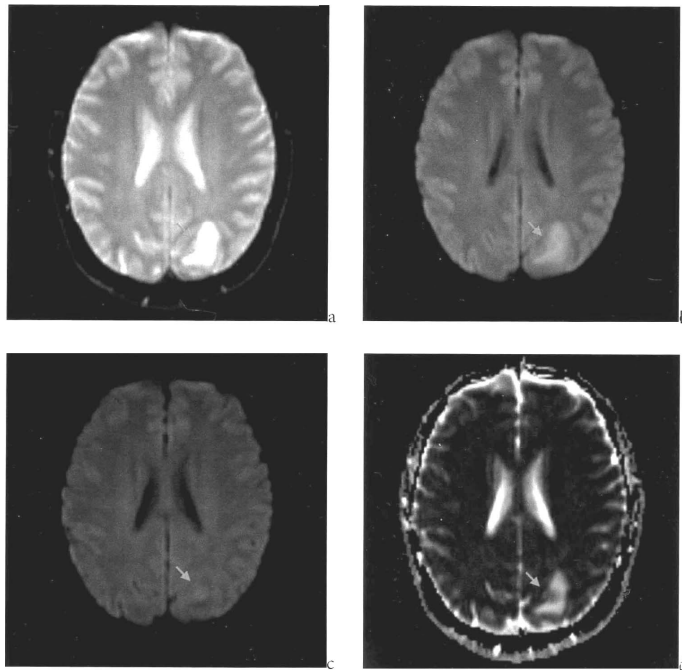
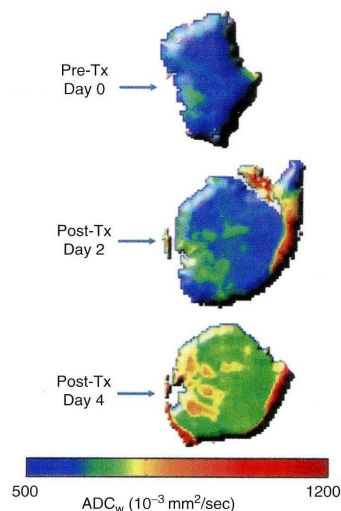


Fig. 17: Diffusion imaging of cerebral edema presenting increased diffusion (arrow). a: diffusion-weighted image with $b=0\text{s/mm}^2$, b: $b=500\text{s/mm}^2$, c: $b=1000\text{s/mm}^2$, d: ADCmap (Hoa 2007).

For the moment, clinical DWI is essentially used for cerebral applications (stroke, multiple sclerosis, traumas...). However, new applications other than those in neuropathology emerge in the field of oncology. Loss of membrane integrity and increased cell death caused by cytotoxic treatments increase the diffusion of water molecules and therefore also increase the ADC value.

Apoptosis and necrosis have the same effect on ADC, as they both have a decreased intracellular volume either through shrinkage in the case of apoptosis or through membrane disruption in necrosis. ADC is an attractive marker of response because it is independent of the magnetic field, scanner and operator and does not require injection of external contrast probes, allowing longitudinal follow-up studies (Fig. 18). The difficulty is to detect microscopic water movements while physiological macroscopic motions, such as respiration, occur (Bammer 2003).

Fig. 18: ADC maps of tumor before and after docetaxel treatment at day 0, 2, and 4 displaying increased ADC values after therapy (Jennings 2002).



The first preclinical detection of tumor response by DW-MRI was shown in chemotherapy-treated brain tumors (Ross 1994). The work of Zhao and co-workers also showed an increased ADC prior to tumor shrinkage in a mouse RIF-1 tumor model after cyclophosphamide treatment (Zhao 1996). Chenevert et al. estimated that an ADC increase of 60% corresponds to 10% of remaining tumor cells after treatment (Chenevert 1997). Because of strong preclinical findings, DW-MRI monitoring of therapy has been investigated in clinical settings (Table 9).

Table 9: Summary of some human studies wherein treatment outcome was assessed by changes in ADC.

Author	Tumor type	Treatment	N	Follow-up	Result
Dzik-Jurasz 2002	Rectal cancer	CT-RT	14	Before and after CT/RT	↑ in ADC in responders
Hein 2003	Rectal cancer	CR	9	During chemoradiation	↓ in ADC due to edema and fibrosis
Kamel 2003	HCC	CE	8	27-42 days	↑ in ADC correlated with necrosis
Kremser 2003	Rectal cancer	CR	8	1 week	↑ in ADC in responders
Mardor 2003	Brain cancer	RT	8	1 week	↑ in ADC in responders
DeVries 2004	Rectal cancer	CR	34	Pretherapy	ADC predictive for outcome
Mardor 2004	Brain cancer	RT	12	Pretherapy	ADC predictive for outcome
Theilmann 2004	Breast (hepatic metastases)	CT	13	4 days	↑ in ADC correlated to ↓ in tumor size
Hamstra. 2005	Glioma	RT or CT	34	3-10 weeks	↑ in ADC predictive of overall survival
Moffat. 2005	Primary brain tumors	CT or RT	20	3 weeks	↑ in ADC predictive of response
Chen 2006	HCC	CE	20	2-3 days	↑ in ADC after CE
Pickles 2006	Beast cancer	CT	10	After cycle 1 and 2	↑ in ADC before ↓ in tumor size
Tomura 2006	Brain cancer	RT	19	2-4 weeks	↑ in ADC correlated to ↓ in tumor size
Uhl 2006	Osteosarcoma	CT	8	After CT	↑ in ADC correlated to ↓ in tumor volume
Lee 2007	Prostate (bone metastases)	HT	1	2 and 8 weeks	↑ in ADC correlated to ↓ in tumor size
Yankeelov 2007	Breast tumor	CT	11	3-6 weeks after the last cycle	↑ in ADC after CT
Lambrecht 2010	Rectal cancer	CR	22	Pretherapy	ADC predictive for outcome

ADC: apparent diffusion coefficient, CE: chemoembolization, CR: chemoradiation, CT: chemotherapy, HCC: hepatocellular carcinoma, HT: hormonotherapy, RT: radiotherapy.

Chinnaiyan et al. observed higher water diffusion in breast cancer treated with a combination of radiotherapy and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) (Chinnaiyan 2000). The taxol response in brain tumor patients was also monitored by DW-MRI, and increases in ADC value were correlated with treatment-induced necrosis (Mardor 2001). The same group also monitored the effects of irradiation on early malignant brain lesions and all responding patients showed increases in ADC (Mardor 2003). However, another study monitoring response of primary rectal carcinoma to chemoradiation showed a decrease of ADC (Hein 2003). The investigators concluded that treatment-related fibrosis should be responsible for this decrease. These results were consistent with other studies monitoring chemoradiation response of rectal cancer (Dzik-Jurasz 2002, Kremser 2003). In treated glioblastoma multiforme, reduced interstitial edema has also been postulated as an explanation for reduced ADC (Batchelor 2007). In general, interpretation of changes in water ADC has shown to be hampered by a reduction in tumor size (Hakumäki 2002). Although decreases and increases in ADC can have multiple interpretations, DWI might be a useful technique in targeted therapeutics.

Determining diffusion coefficients of intracellular metabolites can also be attractive because their extracellular concentration is insignificant. Choline-containing species, for instance, are strictly cytoplasmic (Kent 1990). When cells are dying, one could expect that choline is trickling out to the interstitium, which leads to an increased diffusion of choline-containing metabolites. Contrary to expectations, choline diffusivity has shown to be restricted within apoptotic cells because of reduced intracellular volume and an intact cell membrane (Hakumäki 1998).

2. Targeting phosphatidylserine (PS)

a) PS exposure

PS is one of the four major phospholipids that make up the cell membrane; the other three are PtdE, PtdC, and sphingomyelin (SM). PS usually constitutes less than 10% of the total phospholipid content in the membrane. PS plays a role in blood coagulation, phagocytosis of red blood cells and activation of protein kinase C (Zachowski 1993). The removal of apoptotic cells by macrophages involves the formation of apoptotic bodies and the exposure of specific molecules on the surface of the apoptotic cell. Among these molecules, the exposure of the anionic phospholipid PS is the best characterized (Fadok 1992). The lipid

composition of plasma membranes is asymmetric in healthy mammalian cells. An ATP-dependent aminophospholipid translocase, called flippase, ensures that PS and PtdE remain on the inner leaflet of the plasma membrane (Seigneuret 1984). Floppase, also an ATP-dependent enzyme, pumps the cationic phospholipids (PtdC and SM) to the cell surface to maintain the asymmetry between inner and outer leaflets (Tait 1994). Cells undergoing apoptosis lose this polarization, resulting in the redistribution of PS from the inner to the outer leaflet of the cell membrane. However, inhibition of flippase and floppase alone cannot cause phospholipid scrambling (Bratton 1997). The simultaneous activation of scramblase, a hypothetical protein, is responsible for the facilitated transport and the symmetrical repartition of phospholipids between the two leaflets of the cell membrane (Beyers 1999, Zhou 1997) (Fig. 19). The enzymatic activity of scramblase is believed to depend on an increase in intracellular Ca^{2+} concentration (Beyers 1999, Gleiss 2002, Martin 1995). However, some studies suggest that increased cytosolic calcium is well needed, but not enough to stimulate scrambling (Wurth and Zweifach 2002).

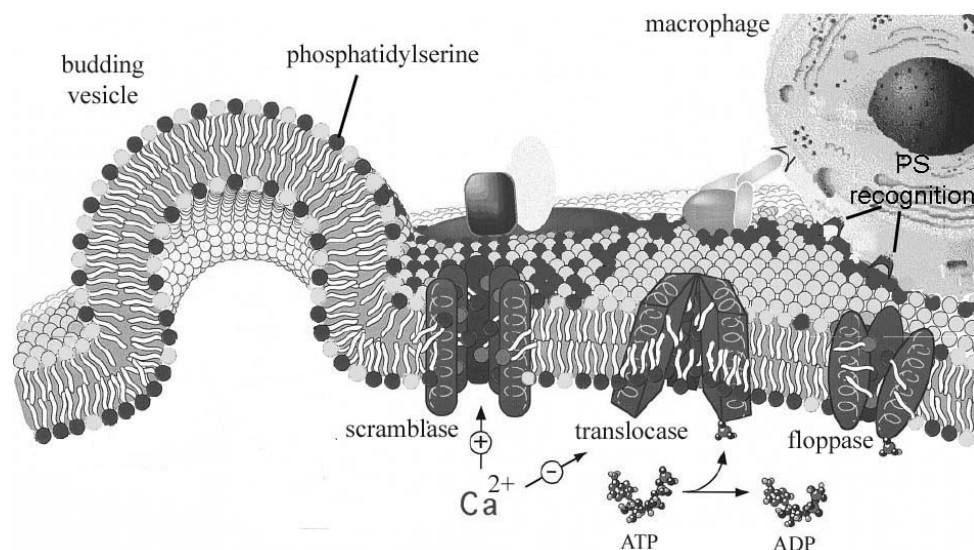


Fig. 19: Schematic illustration of enzymes involved in the phospholipid scrambling process (Zwaal 1997).

Although PS has widely been known as “eat-me” signal of apoptotic cells that have to be cleared by phagocytes (Fadok 1992), Böse et al. demonstrated that PS-receptor-deficient macrophages were well able to engulf apoptotic cells (Böse 2004). Nevertheless, the exposure of PS remains one of the hallmarks of apoptosis. During apoptosis, PS scrambling occurs immediately after activation of the caspase cascade, but before DNA fragmentation takes place. Even if PS exposure is not the earliest event in the apoptotic process, it is an easily accessible target, since PS is situated on the outer membrane surface and the probe does not need to enter the cell. The PS molecule can be detected in necrosis too because of cell

membrane rupture. It has even been documented in necrotic cells before membrane disintegration (Cocco 2001). This same feature was also demonstrated in other forms of cell death, including mitotic catastrophe, autophagy, and cell senescence (Corsten 2006, Verheij 2008). The ability to recognize all forms of cell death is an advantage in treatment monitoring. Transient PS exposure can also be observed in non apoptotic cells, including platelets, lymphocytes, granulocytes, mast cells, during myotube formation or after B cell receptor activation without any ensuing cell clearance (Dillon 2001, van den Eijnde 2001). Vascular smooth muscle cells (VSMC) have been observed to express PS in tumor vessels while alive (Ran 2002). This is further evidence that PS exposure alone is not sufficient to promote engulfment. PS can reversibly be expressed under conditions of cell stress, which does not necessarily mean that cells will die, but PS rather defines tissues at risk for cell death (Hammill 1999, Lejeune 1998, Martin 2000, Yang 2002).

b) The natural ligand annexin V

Annexin V (AnxV), a 36-kDa endogenous human protein, is known to bind PS exposed on apoptotic cell surfaces with a very high affinity ($K_d = 6 \times 10^{-9} \text{M}$). For this reason, this molecule has been widely used for imaging of apoptosis. Owing to its PS-binding, AnxV plays a role in the inhibition of blood coagulation, of protein kinase C and of phospholipase A2 (Boersma 2005). Exogenously administered AnxV, normally found inside cells, is suggested to bind to PS and enter the cell by an energy-dependent internalization (Kenis 2004). The extent to which AnxV binds to a membrane depends on the free AnxV concentration, membrane PS content, and Ca^{2+} concentration (Tait 1992).

The first apoptosis detection assay was designed in vitro by fluorescence-labeled AnxV, allowing identification of apoptotic cells with fluorescence microscopy or flow cytometry (Koopman 1994). The first imaging studies of therapy-induced tumor apoptosis were carried out with human recombinant AnxV radiolabeled with $^{99\text{m}}\text{Tc}$ (Blankenberg 1998). Another group investigated the kinetics of $^{99\text{m}}\text{Tc}$ -AnxV uptake and observed two peaks of AnxV uptake in tumors after doxorubicin treatment: an early increase in AnxV between 1 and 5 hours and a second peak lasting from 9 to 24 hours (Mandl 2004). After 48 hours AnxV uptake became insignificant. This emphasizes the importance of early AnxV imaging before clearance of PS-expressing cells by macrophages occurs. Preparation of the radiolabeled material is quite complex, therefore hydrazino nicotinamide (HYNIC) was selected as alternative vectorizing approach (Fig. 20) (Kemerink 2003).

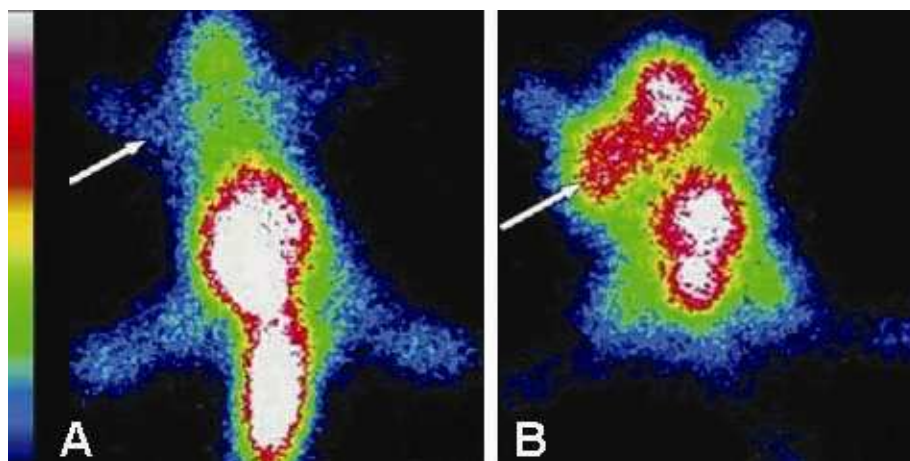


Fig. 20: ^{99m}Tc -HYNIC-AnxV scintigraphic images of lymphoma-bearing mice before (A) and after irradiation (B). The white arrows indicate the implanted tumor (Guo 2009).

Multiple clinical trials have confirmed the clinical utility of ^{99m}Tc -HYNIC-AnxV in determining the efficacy of chemotherapy in oncology patients. The first clinical ^{99m}Tc -AnxV study was carried out before and within 3 days after chemotherapy (Belhocine 2002). After therapy, increased uptake of radiolabeled AnxV was detected and results were correlated with tumor response evaluation by CT and ^{18}F -FDG-PET 3 months later, suggesting that ^{99m}Tc -AnxV may be an early marker for tumor response. Another observation was that the presence of ^{99m}Tc -AnxV uptake in the lesions shortly before chemotherapy also correlated with tumor response. These data suggested that the lesions with high spontaneous levels of apoptosis have a higher probability of responding to therapy. Another explanation could be the targeting of PS, which is expressed by VSMC of the tumor vasculature, since highly perfused tumors show a better response to chemotherapy (van de Wiele 2003). Other clinical studies in patients with head and neck cancer (van de Wiele 2003), lymphoma (Haas 2004) and breast cancer (Rottey 2006) also demonstrated the feasibility of evaluating tumor treatment response by ^{99m}Tc -labeled AnxV (Table 10). However, tumor to background ratio and biodistribution profile were not optimal with ^{99m}Tc -AnxV, therefore investigation into better suited radiolabels continues. Tait et al. investigated the structural requirements of AnxV and developed mutant forms of AnxV with endogenous chelating sites for the ^{99m}Tc -labeling (Tait 2005). PET imaging is more sensitive and has a better spatial resolution than SPECT, therefore researchers designed ^{18}F -labeled AnxV probes (Grierson 2004, Lorberboym 2006, Murakami 2004). AnxV clearance from blood is slow, requiring imaging studies at 4 to 6 hours after administration of the radiotracer, which is not compatible with the short half-life of radionuclides used in PET. Nevertheless, ^{18}F -AnxV was able to successfully highlight apoptotic regions by PET in cycloheximidine-induced hepatic apoptosis and myocardial

ischemia (Murakami 2004, Yagle 2005). Further optimization and standardization of the labeling procedure of ^{18}F -AnxV are necessary however. Other investigators labeled AnxV with another PET-isotope, ^{124}I , and measured its accumulation in the liver of anti-Fas treated mice, but this radionuclide was rapidly abandoned because of high radiation doses, a cumbersome labeling method and enzymatic deiodination (Keen 2005). ^{64}Cu -labeled streptavidin PET imaging after pretargeting of PS with biotinylated AnxV has also been attempted (Cauchon 2007). In a similar approach, near-infrared probes and magnetic CAs were conjugated to AnxV to target apoptotic cells in vitro and in vivo (Hiller 2006, Petrovsky 2003, Schellenberger 2002, van Tilborg 2009). In several studies bimodal probes such as AnxV-CLIO-Cy5.5 or AnxV-conjugated paramagnetic quantum dots were used for combined MR and fluorescence imaging of apoptosis (Schellenberger 2004, Sosnovik 2005, Sosnovik 2009, van Tilborg 2006). So far, AnxV is the most extensively used means of measuring apoptosis in vitro and in vivo, and it has been coupled to several contrast agents (magnetic, radiolabeled or fluorescent probes) to image apoptosis in vivo.

Table 10: Summary of AnxV studies performed in patients in order to evaluate the therapeutic impact on tumors.

Author	Tumor type	Treatment	N	Follow-up
Belhocine 2002	SCLC and NSCLC, metastatic breast cancer, lymphoma, hodgkins	CT	15	After first cycle of CT
Belhocine 2003	Lung cancer, breast cancer and lymphoma	CT	15	1-2 days after CT
Haas 2004	Follicular lymphoma	RT	11	24-48h after RT
Kartachova 2004	Malignant lymphoma, leukaemia, NSCLC, H&NSCC	RT , CT, CR	33	Before and early after the start of treatment
Rottey 2006	Breast cancer, melanoma, H&NSCC, bladder cancer, liver metastases, esophageal cancer, SCLC, colon cancer...	CT	19	5-7h and 40-44h after treatment
Kartachova 2007	NSCLC	CT	16	Before and within 48 hours after CT
Kartachova 2008	Lymphoma, NSCLC, H&NSCC	CT	38	2 days after treatment
Hoebbers 2008	H&NSCC	CR	13	48h after CR

CE: chemoembolization., CR: chemoradiation, CT: chemotherapy, H&NSCC : head and neck squamous cell carcinoma, NSCLC: non small cell lung cancer, RT: radiotherapy, SCLC: small cell lung cancer.

c) Recognition of PS by the C₂A domain of synaptotagmin I

Proteins such as protein kinase C, a regulator of signaling pathways, and synaptotagmin I, implicated in membrane fusion and proposed as regulator in neurotransmitter release, specifically interact with the PS molecule in the presence of calcium (Davletov1993). The C₂A domain in protein kinase C and in synaptotagmin I was revealed as the sequence responsible for PS-binding. Like AnxV, the 14 kDa C₂A domain binds in a Ca²⁺-dependent manner and with nanomolar affinity to PS, but displays a 10-fold weaker PS binding capacity than AnxV (Zhao 2006). This C₂A domain was at first conjugated with SPIOs to specifically bind to apoptotic tumor cells in vitro and in vivo, and to permit detection of treatment efficiency by reducing signal intensities on T₂-weighted MR images (Zhao 2001). The same group also used avidin-labeled Gd chelates complexed to biotinylated C₂A domains to detect cell death by MRI. The advantage of this latter marker is the small size of the imaging complex (110 kDa, ca. 5 nm) and positive contrast enhancement in T₁-weighted images (Jung 2004, Neves 2007). The same C₂A domain was also labeled with ^{99m}Tc to image apoptosis by SPECT in acute myocardial infarction and in paclitaxel-treated NSCLC xenografts (Fang 2007, Wang 2008, Zhao 2006).

d) Cationic contrast agents targeting negatively charged cell membranes

The anionic nature of the PS molecules is largely attributed to its negatively-charged CO₂ and PO₄ groups (Kimura 2006). Therefore Bose et al. used non selective cationic liposomes composed of a cationic surfactant and a fluorescent dye, thereby suggesting an electrostatic interaction between the positively charged head group of cationic liposomes and the translocated anionic phospholipid PS (Bose 2004). The liposome diameter varied from 400 nm to 2.5 μm. The major limitation of this method is the fact that liposomes are widely known to passively accumulate in tumors, which are characterized by excessively leaky microvasculature. Over-expression of VEGF in tumors results in both increased vascular permeability and chaotic tumor vessel architecture. In combination, these effects cause enhanced permeation and retention (EPR) (Yaghini 2009), resulting in high local liposome concentrations, regardless of the fact if tumor cells are undergoing apoptosis or not (Fig. 21). Based on these findings, the development of cationic liposomes has contributed to novel formulations for encapsulation of cytotoxic drugs such as paclitaxel, etoposide and cisplatin to obtain superior therapeutic efficacy (Thurston 1998).

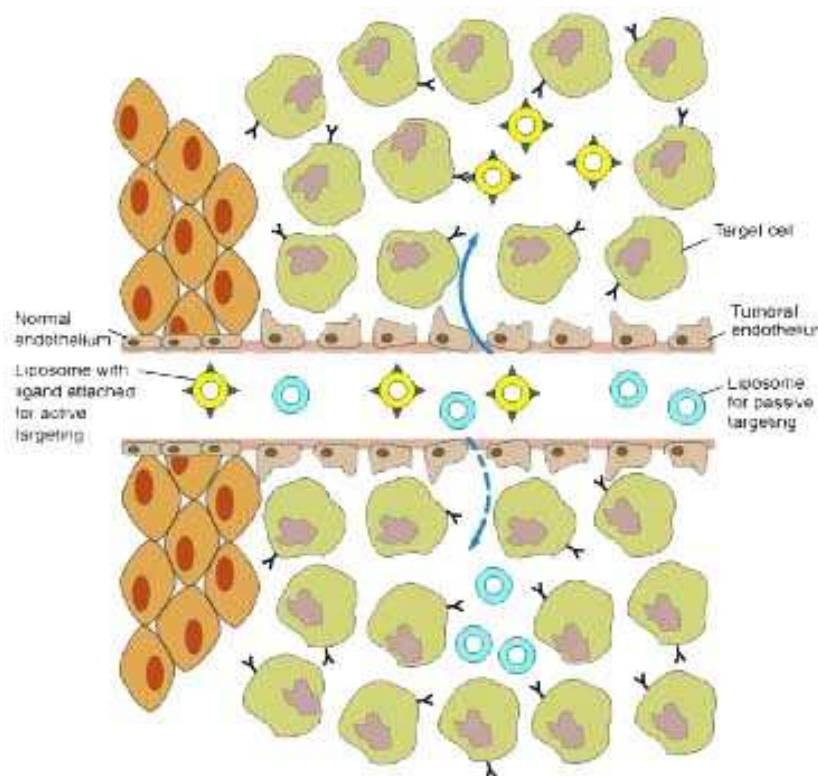


Fig. 21: Illustration of the enhanced permeation and retention effect in tumor tissues that are characterized by leaky endothelium (Malam 2009).

More recently, Zn(II) di-2-picolyamine (DPA) coordination complexes have shown to target anionic PS-enriched membrane surfaces, which could be useful for the detection of apoptosis (Hanshaw 2005, Quinti 2006). Multiple zinc complexes are able to bind to one single PS molecule, thereby increasing the generated signal. Hanshaw et al. monitored apoptosis in treated murine lung carcinoma by using Zn^{2+} -DPA coordination compounds with fluorescein or biotin groups as reporter elements, or conjugated directly to quantum dots.

e) PS-binding peptides discovered by phage display screening

Peptides discovered by the phage display technique represent an alternative solution for imaging of apoptosis. Phage display is an extremely promising technology because it enables to select peptides interacting with any given target. (Laumonier 2006). This technique is based on a phage library, where different foreign DNA inserts are incorporated into the DNA of different phages and therefore each phage displays different peptides on its surface. The expressed peptide is fused to coat proteins of phages, resulting in several copies of the displayed peptide per phage (Fig. 21).

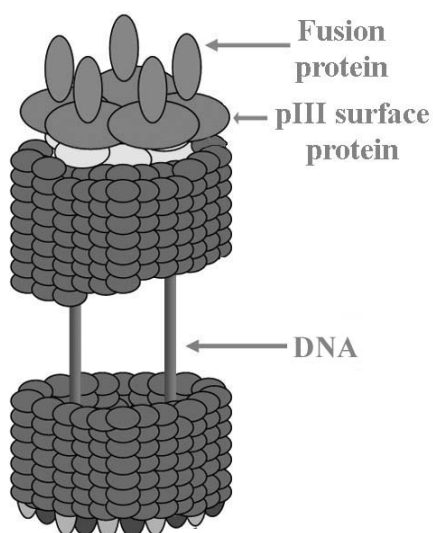


Fig. 21: Illustration of an M13 bacteriophage expressing the fusion protein after insertion of the foreign DNA into the gene coding for the coat protein pIII (Yoo 2006).

Selection of the specific peptide is carried out by “panning” the phage library against purified receptors, intact cells, or organs. The advantages of small peptides over bigger antibodies are better stability, lower immunogenicity, lower cost and facilitated tissue penetration (Ladner 2004). Thus, the discovery of a peptide with PS avidity could be useful, and may result in the development of a diagnostic means for non-invasive imaging of apoptosis.

Laumonier and colleagues found a PS-binding phage through ex vivo biopanning of a hexapeptide phage library in perfused apoptotic mouse livers. The phage clone that exhibited the best PS-binding capacity carried the TLVSSL peptide, called E3. In our thesis, we will use this PS-targeted hexapeptide (K_d for PS: $4 \times 10^{-7} \text{M}$) as targeting moiety of PEGylated USPIOs. Another study also identified a PS-binding peptide by panning a 15-mer phage library (Nakai 2005). In most reported sequences, the basic amino acids arginine and lysine appeared to be critical for interaction with PS, and ionic interaction was suggested. PS3-10 also possesses two basic amino acids (lysine and arginine), suggesting that its binding to negatively charged PS may be mediated through ionic interaction (Shao 2007). However, since the scrambled peptide, containing two Arg and two Lys units, presented significantly lower binding to PS than the PS3-10 peptide, it is likely that ionic interaction is not the only factor determining binding activity. Results of other studies using neutral peptides support the notion that the presence of charge is not an absolute requirement for selective binding of peptides to PS (Laumonier 2006, Thapa 2008). Thapa and co-workers were the first group evaluating treatment efficiency in tumor-bearing mice with a fluorescein-labeled peptide, which had previously been discovered by phage screening on PS-coated plates. The same group also assessed a ^{131}I -labeled 9-mer peptide in a murine stroke model by autoradiography (Hong 2008).

3. Other methods used for assessment of cell death

a) Targeting phosphatidylethanolamine

Concurrently to PS exposure in apoptotic cells, PtdE also flips from the inner to the outer leaflet of the membrane by virtue of the same mechanism. PtdE is thought to play a regulatory role in membrane structure remodeling during apoptotic blebbing. PtdE exposed on dividing Chinese hamster ovary cells during telophase was specifically recognized by cinnamycin (Ro09-0198), an antibiotic acting against Gram-positive bacteria (Emoto 1996). Cinnamycin also enabled binding to apoptotic cells via formation of a tight equimolar complex with PtdE and recognition could be specifically inhibited by PtdE-containing liposomes (Emoto 1997). Closely related to cinnamycin is duramycin, another 19-amino-acid antibiotic, that was also able to target PtdE-containing liposomes (Iwamoto 2007). Both antibiotics require the hydrophilic headgroup and the hydrophobic tail of PtdE for specific recognition and their binding seems to be dependent on membrane curvature (Iwamoto 2007). ^{99m}Tc-labeled duramycin was recently shown to selectively bind to PtdE in vivo, as demonstrated in a rat model of acute myocardial infarction (Zhao 2008). Like PS-binding compounds, duramycin recognizes an overall degree of cellular injury, rather than an exact mode of cell death.

b) Visualization of caspase-3 activation

Other new tracers of apoptosis focus on the development of molecules binding to caspase-3, as the initiation of the caspase cascade occurs prior to the exposure of PS. The caspases, responsible for the regulation and execution of apoptosis, exist as inactive pro-caspases in the cytosol and become activated when cells receive an apoptotic signal. Activated caspases are responsible for the proteolytic cleavage of proteins necessary for cellular function. Caspases can serve as potential targets inside the apoptotic cell. The hurdle of this method is the entrance of the caspase activity reporter into the cell. Currently, a couple of caspase-substrates based on luminescent, fluorescent or radioactive probes are investigated (Bauer 2005, Bullok 2005, Hickson 2010, Laxman 2002, Lee 2007). These agents most commonly include a small membrane permeate, a Tat-based permeation peptide, fused to the caspase-cleavable peptide sequence Asp-Glu-Val-Asp (DEVD) (Bullok 2005, Coppola 2008). Perhaps the biggest challenge for these probes is to develop peptide sequences that are specific for caspases,

because the DEVD sequence is also efficiently recognized by several other cysteine proteases, including the cathepsins and legumain (Kato 2005).

Apoptosis has also been detected *in vivo* by optical imaging using luminescent reporter systems. In one approach, luciferase activity was abolished by its fusion to two estrogen-regulated domains, which were linked to the enzyme by a DEVD cleavage site (Laxman 2002, Lee 2007). Other preclinical methods for *in vivo* monitoring of apoptosis used a split luciferase model and its utility was shown in a glioma model responding to various chemotherapy and radiotherapy regimens (Coppola 2008). In this system, two domains of luciferase are fused to strongly interacting peptides that are linked by a DEVD sequence. Cleavage of the linker will permit the interacting peptides to associate, allowing reconstitution of luciferase activity. Hickson et al. showed the effect of docetaxel on two oncology models by using a bioluminescence marker, which was this time a modified firefly luciferase substrate (Z-DEVD-aminoluciferin) instead of a split luciferase protein (Hickson 2010). After cleavage by caspase-3, the aminoluciferin can be consumed by luciferase. Similarly, a multimodal imaging reporter was also recently described to be composed of fluorescent, bioluminescent, and PET reporters, all separated by DEVD linkers (Ray 2008). Bioluminescent contrast agents can potentially serve as preclinical tool for drug development, but they cannot be used in clinical studies because this method requires reporter transfection.

Besides substrates, several small inhibitors have been used to label caspase activity. These include agents such as membrane-permeant fluorochrome-labeled inhibitors of caspases (FLICA), the ^{125}I -labeled irreversible caspase inhibitor M808, and WC-II-89, a non peptidic inhibitor based on isatin sulfonamide analogs (Bedner 2000, Lee 2008, Methot 2004, Smolewski 2001, Zhou 2006). Apoptosis induced in the thymi of mice, as well as in mouse tumors, was detected by FLICA probes that covalently bind to the active center of caspases, but these irreversible caspase inhibitors also recognize cathepsins and legumain (Edgington 2009). All these fluorescent strategies need yet to be entirely validated for longitudinal *in vivo* imaging of apoptosis, because tissue absorption of the emitted fluorescence will lead to detection problems. Though, small inhibitors of caspase-3 were also labeled with PET tracers, such as ^{11}C or ^{18}F , for potential imaging of apoptosis *in vivo* (Chen 2009). WC-II-89, an isatin analogue, has been labeled with ^{18}F for PET imaging of cycloheximidine-induced apoptosis in livers (Zhou 2006). Although this molecule has to pass the cell membrane to recognize activated caspases, a huge advantage is its non peptidic nature and its favorable biodistribution (Haberkorn 2001). Further pre-clinical research is necessary to show the potential of ^{18}F -WC-II-89 as a PET radiotracer for imaging apoptosis in patients.

c) The ApoSense family

A family of small amphipathic molecules discriminating between vital and apoptotic cells has been developed (Reshef 2010). These non peptidic fluorescent molecules selectively target and accumulate within apoptotic and/or necrotic cells and are suitable for attachment of different reporters allowing imaging of cell death. It should be further investigated to which target ApoSense molecules bind and how they cross the cell membrane in early apoptosis. Hydrophobic moieties seem to provide a membrane anchor, while charged moieties prevents the crossing of the hydrophobic cell membrane in viable cells. Authors suggested that membrane crossing in apoptotic cells is associated with the scrambling process of the membrane phospholipids. Intracellular accumulation of ApoSense molecules may produce a stronger signal compared to AnxV, which only binds peripherally.

First results showed that ApoSense is able to recognize apoptosis in different animal models, independent of cell type or trigger. One member of the ApoSense family, NST-732, was developed with a fluorophore for fluorescence detection and a fluorine atom, which can potentially be replaced by an ^{18}F -isotope for PET imaging. NST-732 recognition of dying cells was evaluated in several rodent models of apoptosis: irradiated lymphoma, renal ischemia/reperfusion and cerebral stroke (Aloya 2006), as well as in diagnosis of neurodegenerative disorders such as Alzheimer's disease or amyotrophic lateral sclerosis (Shirvan 2009). Another non peptide compound with inherent fluorescent properties, didansyl cystine (DDC), allowed evaluation of cell death in various preclinical models of disease, such as acute renal failure (Damianovich 2006), cerebral stroke (Reshef 2007), brain injury (Reshef 2008a), chemotherapy-induced enteropathy (Levin 2009) and also in a murine melanoma model treated with chemotherapy (Cohen 2007). The same group also designed the even smaller ML-9 (butyl-2-methyl-malonic acid), based on the alkyl-malonate motif of the amino acid γ -carboxyglutamic acid (Gla) that has previously be shown to mediate binding of clotting factors to anionic phospholipids (Sahu 2007). Ex vivo assessment of ^3H -ML-9 distribution and uptake following chemotherapy was tumor-specific. The radiolabeled analog of γ -carboxyglutamic acid (^{18}F -ML-10) was specifically designed for clinical imaging of apoptosis by PET. After observation of selective uptake in the region of cerebral ischemia in mice (Reshef 2008b), ^{18}F -ML-10 has now entered phase II in clinical trials to detect brain metastasis response to treatment. However, its applicability as regards detection of cell death in other diseases is difficult to estimate, as information about its mode of action is still limited.

d) Necrosis-avid contrast agents

In most cases, in vivo imaging of necrosis has taken advantage of the loss of cell membrane integrity, which allows access to intracellular macromolecules. For years, porphyrin derivatives have been used for photodynamic therapy, based on tumor accumulation and photosensitizing properties of the agents (Gomer 1989, Kessel 1984, Neslon 1990). In consequence of their tumor-localizing properties, metalloporphyrins were developed for tumor detection by MRI (Nelson 1991, Organ 1987, Saini 1995, Van Zijl 1990). Ni and co-workers found out that the reported specific contrast enhancement could only be attributed to non viable (typically necrotic) instead of viable tumor cells, an observation just opposite to the assumption raised earlier (Ni 1995). As the dark porphyrin-based pigments are responsible for the adverse reactions caused by these necrosis-avid contrast agents (NACAs), less-colored derivatives were developed, as the porphyrin structure does not seem to be required for tumor targeting. A few promising compounds such as the light yellowish ECIII-60 (bis-Gd-DTPA-pamoic acid) and the colorless ECIV-7 (bis-Gd-DTPA-bisindole) have been synthesized by the same group (Ni 2002). In most studies NACAs were used for identifying acute myocardial infarction by discriminating between viable and non viable myocardium. The targeting mechanisms of NACAs still remain to be elucidated. Most probably, NACAs interact with tissue debris after disintegration of the cell membrane (Ni 2005).

Among the numerous other molecules that firmly bind to nonviable tissues, or more specifically to necrotic tissues, are the synthetic dye Evans Blue used for intravital staining (Hamer 2002), the botanical extract hypericin, and glucarate, the natural endogenous end-metabolite catabolized from uridine-diphosphate-glucose. It was shown that ^{99m}Tc -glucarate selectively recognizes necrotic cells in the infarcted myocardium, because it binds to positively charged histones after disintegration of the nucleus (Okada 2004, Orlandi 1991). Hypericin is a polyaromatic quinone that can be isolated from St. John's wort (*Hypericum perforatum*). Hypericin was labeled with ^{123}I to detect necrotic cells in reperfused hepatic infarctions in rats (Estorch 1990) and also in acute myocardial infarction in rabbits (Fonge 2008). Markers of necrosis are mainly used in models of acute myocardial infarction because infarction induces necrosis rather than apoptosis due to intensive energy exhaustion. However, all these necrosis-detecting contrast probes could also potentially be used as imaging agents of tumor necrosis.

Chapter II: OBJECTIVE OF THE THESIS

The aim of this thesis is to validate new non invasive biomarkers of therapeutic response. In recent advances, various targeted magnetic probes were shown to be useful for imaging of tumor response to treatment. However, as specificity and sensitivity of these biomarkers are often insufficient, new targeted contrast agents are still greatly needed.

This work is actually a collaboration between our group and the NMR and Molecular Imaging Laboratory at the University of Mons (Professor R. N. Muller), where the new targeted contrast agents were synthesized and characterized. Our specific role was to assess the value of these agents in vitro and in animal models.

In a first study, we evaluated a new molecular biomarker of inflammation in order to become familiar with the techniques needed for the assessment of targeted magnetic nanoparticles. More specifically, USPIO particles, used as negative contrast agents in MRI, were linked to a sLe^x mimetic in order to selectively recognize E-selectin, an adhesion molecule implicated in the early inflammatory process.

The main emphasis though, was the early detection of tumor response, and thus the prediction of the likely outcome of anti-cancer treatments, with particular focus on cell death. It is now well established that cytotoxic anti-cancer therapies induce apoptosis, or cell death in general, and that disruption of apoptotic programs can reduce treatment sensitivity. In our studies, we attempted to investigate whether or not we were able to detect cell death in treated tumors, which accounts for the tumor response. A hallmark of apoptosis is the exposure of phosphatidylserine on the outer leaflet of the plasma membrane bilayer. This event can be detected by MRI when using appropriately targeted magnetic agents that bind to this externalized phospholipid. In the course of this thesis, we followed the accumulation of phosphatidylserine-targeted and control USPIO particles in treated and untreated tumors by using a combination of electron paramagnetic resonance (EPR) and MRI and results were correlated with apoptotic fractions in tumors. We were also interested in the value of the biomarker in tumor models that have a totally different response to various anti-cancer treatments. First we evaluated the value of this biomarker in different tumor models presenting different sensitivities to radiotherapy, and then in tumors treated with different chemotherapeutic agents. Results were correlated with survival curves or with the fraction of dead cells after treatment. We also compared this new targeted system with other markers of therapeutic response, such as diffusion MRI and choline spectroscopy, in terms of specificity, selectivity and local heterogeneities of response.

Chapter III: RESULTS

1) Early in vivo detection of inflammation

***In vivo* Detection of Inflammation Using Pegylated
Iron Oxide Particles Targeted at E-selectin.
A Multimodal Approach Using MR Imaging and EPR Spectroscopy**

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Invest Radiol. 2009 Jul;44(7):398-404.

In Vivo Detection of Inflammation Using Pegylated Iron Oxide Particles Targeted at E-Selectin

A Multimodal Approach Using MR Imaging and EPR Spectroscopy

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Objectives: Ultrasmall particles of iron oxide (USPIO) possess superparamagnetic properties and are used as negative contrast agent in magnetic resonance imaging (MRI) because of their strong T_2 and T_2^* effects. Besides this method, electron paramagnetic resonance (EPR) offers the unique capability to quantify these particles. The objective of this study was to evaluate a molecular marker for non invasive diagnosis and monitoring of inflammation. During inflammation cell adhesion molecules such as E-selectin are expressed on the endothelial cell surface. An E-selectin ligand was coupled to pegylated USPIO particles.

Materials and Methods: Inflammation was induced by intramuscular injection of Freund's Complete Adjuvant in male NMRI mice. After intravenous injection of grafted or ungrafted USPIO particles, iron concentration in inflamed muscles was quantified ex vivo by X-band EPR. Particle accumulation was also assessed in vivo by L-Band EPR, as well as by T_2 -weighted MRI.

Results: We determined the mean iron oxide concentration in inflamed muscles after injection of grafted or ungrafted USPIO particles, which was 0.8% and 0.4% of the initially injected dose, respectively. By L-band EPR, we observed that the concentration of the grafted USPIO particles in inflamed muscles was twice higher than for the ungrafted particles. Using MRI experiments, a higher signal loss was clearly observed in the inflamed muscle when grafted particles were injected in comparison with the ungrafted particles.

Conclusion: Even taking into account a non specific accumulation of iron oxides, the targeting of USPIO particles with E-selectin ligands significantly improved the sensitivity of detection of inflamed tissues.

Key Words: EPR, MRI, USPIO, molecular imaging, E-selectin

(*Invest Radiol* 2009;44: 398–404)

Molecular markers of inflammation are needed to be able to diagnose and monitor inflammation in a noninvasive manner before the occurrence of irreversible anatomic changes.¹ Physical examination is often subjective and serum markers of inflammation do not provide information about the localization of the inflamed zone.² Molecular markers for imaging could, therefore, be helpful in diseases where the inflammatory action of misdirected leukocytes can destroy healthy tissues, such as in atherosclerotic plaques,³ graft

rejection,⁴ ischemia-reperfusion injury,⁵ multiple sclerosis,⁶ and rheumatoid arthritis.⁷ Ultrasmall particles of iron oxide (USPIOs) have been proposed as molecular markers as these particles are phagocytosed by macrophages recruited during the inflammatory process.^{3,6,8–10} This approach is, however, limited in sensitivity and specificity due to the lack of a specific target. An alternative and promising approach is to develop MR contrast agents that are selectively targeted at receptors involved in the inflammatory process.^{11–13} Several cell adhesion molecules are overexpressed on leukocytes and endothelial cells during the inflammatory process.¹⁴ Among these molecules, endothelial selectin (E-selectin) mediates the “slow rolling” of leukocytes in blood by encouraging adhesion to the vascular endothelium. This adhesion process provides the first step of granulocyte, monocyte, and T lymphocyte recruitment toward an inflamed site.^{15,16} De novo E-selectin expression is stimulated by proinflammatory cytokines, like tumor necrosis factor (TNF)- α or interleukin-1 β .^{11,16} The up-regulation of E-selectin lasts from 4 to 30 hours after induction of inflammation and can be used as an early marker of inflammation.¹⁵ The natural ligand of E-selectin is the tetrasaccharide, sialyl Lewis X (sLeX).¹⁷ This carbohydrate structure is linked to certain leukocyte surface proteins by specific glycosylation enzymes.¹⁸

In the present study, multimodal molecular approach, with MRI and electron paramagnetic resonance (EPR) using a mimetic of the sLeX molecule coupled to a USPIO, was used to detect inflammation. This mimetic has previously been coupled with other magnetic reporters, such as gadolinium complexes, and successfully used to detect inflammation.^{5,19,20} USPIOs directed against E-selectin have been proposed recently, using a coating functionalized with antibodies against E-selectin²¹ or with the sLeX mimetic molecule.²² In this study, we coupled the sLeX mimetic on USPIOs that were also coated with the hydrophilic polyethylene glycol (PEG) polymer to prolong the plasma circulation time and minimize the nonspecific accumulation of iron oxide in tissues.^{23–25} The functionalized particles were characterized for their relaxometry properties and their hydrodynamic size. The ability of these functionalized particles to detect inflammation was evaluated in an in vivo model of local inflammation in muscle induced by injection of Freund's complete adjuvant (local stimulation of TNF α production). The quantitative detection of iron oxide particles in the tissues was carried out ex vivo using EPR spectroscopy. Finally, the efficacy of the targeted MR contrast agent was evaluated using in vivo EPR spectroscopy and T_2 -weighted imaging.

MATERIALS AND METHODS

Synthesis of the Contrast Agent, USPIO-PEG-sLeX

USPIO-PEG750-sLeX was prepared from nanoparticles with carboxylated groups on their surface (previously described in Reference 26). The USPIOs were modified in 2 successive steps (Fig. 1): First, the USPIOs were grafted with the mimetic of the sLeX

Received December 17, 2008; and accepted for publication (after revision) March 8, 2009.

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Supported by the Belgian National Fund for Scientific Research (FNRS), Televie grants (7.4597.06), the Fonds Joseph Maisin, the Saint-Luc Foundation, the “Actions de Recherches Concertées-Communauté Française de Belgique-ARC 04/09-317 and 05/10-335,” and the Pôle d'attraction Interuniversitaire PAI VI (P6/38 and P6/29).

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ISSN: 0020-9996/09/4407-0398

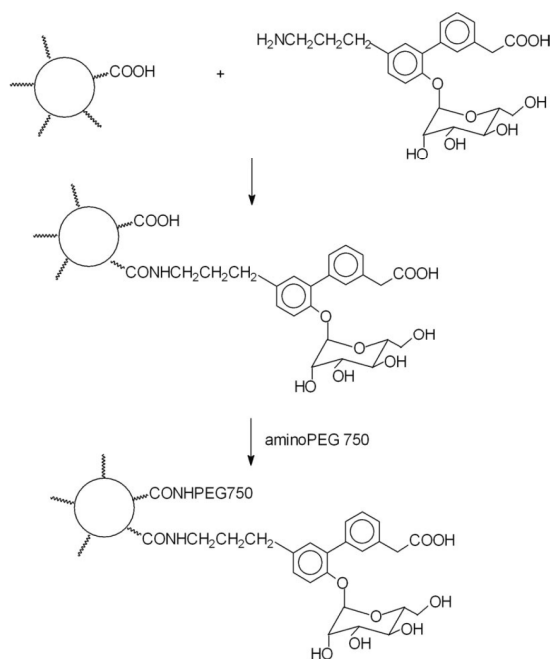


FIGURE 1. Synthetic preparation of USPIO-PEG-sLeX.

molecule;²⁷ second, they were coupled to aminoPEG 750 (Fluka, Bornem, Belgium).

Briefly, 10 mg of the sLeX mimetic and 6 mg of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride were added to 15 mL of nanoparticles ($[\text{Fe}] = 0.175 \text{ M}$), and the mixture was stirred overnight. The nanoparticle suspension was ultrafiltered on a 30-kD membrane. AminoPEG750 (0.503 g) and ethylcarbodiimide hydrochloride (0.327 g) were added to the nanoparticles and the reaction was stirred for 17 hours. The mixture was ultrafiltered on a 30 kD membrane to remove low-molecular weight material.

Characterization of the USPIO-PEG-sLeX

Hydrodynamic size was measured by photon correlation spectroscopy on a Brookhaven system BI-160 (New York) equipped with a He-Ne laser ($\lambda = 633 \text{ nm}$, 35 mW), a goniometer, and a correlator BI-9000AT-BC. The Nuclear Magnetic Relaxation Dispersion (NMRD) profile was recorded at 37°C on a Fast Field Cycling Relaxometer (Stelar, Mede, Italy). Longitudinal relaxivities were measured over a magnetic field range from 0.24 mT to 0.24 T. The data obtained at 0.5 and 1.5 T were measured with Minispec PC-20 and Mq 60 Series systems (Bruker, Karlsruhe, Germany). The magnetization curve was recorded on a VSM-NUVO magnetometer (MOLSPIN, Newcastle Upon Tyne, UK).

Inflammatory Model

Freund's complete adjuvant was obtained from Sigma-Aldrich (Schnellendorf, Germany). FCAs are water-in-mineral oil emulsions (W/O emulsions) containing heat-killed mycobacteria (*Mycobacterium tuberculosis*, H34Ra, ATCC25177) at 1 mg/mL.

Animals and Protocol

Male NMRI mice (20–25 g) were provided by Janvier (France). For all experiments 5 to 6 mice were used per group. FCA (50 μL) was injected intramuscularly into the right rear leg (gastrocnemius muscle) of mice to stimulate E-selectin expression.²⁸ The left, noninflamed muscle served as control muscle. Twenty-four hours after induction of inflammation, the USPIO-PEG-sLeX or the ungrafted USPIO-PEG was injected intravenously into a tail vein at a dose of 7.7 mg Fe/kg body weight. The animal protocols were

approved by the ethical committee of the Université catholique de Louvain. All experiments were conducted according to national animal care regulations.

Some commercial iron oxide particles were also studied to assess the influence of their size and coating on their kinetics. We used 3 types of preparation: ferumoxide (Guerbet; mean particle size: 80–150 nm, coating: dextran), ferucarbotran (Schering; mean particle size: 60 nm, coating: carboxydextran), and ferumoxtran-10 (Guerbet; mean particle size: 20–40 nm, coating: dextran). Each preparation was injected intravenously (7.7 mg Fe/kg) in mice. Mice were killed at different time points (5 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 5 hours, 18 hours, and 24 hours) and the iron concentrations in blood, liver, and spleen were measured by X-band EPR as described for the USPIO-PEG-sLeX.

Histology

Muscles were sampled 1, 3, and 5 hours after FCA administration, and on days 1, 3, and 7. Two mice were used for each time interval. The mice were killed by cervical dislocation, and the muscle was carefully removed. For comparison, the left gastrocnemius muscle of each mouse served as control. Muscle specimens were fixed in 10% neutral buffered formalin for 24 hours, and then embedded in paraffin. Tissues were sectioned at 5 μm and the slides were stained with hematoxylin-eosin. Analyses were performed on an optical microscope (Nikon Alphashot-2 Y52).

Ex Vivo EPR

Twenty-four hours after induction of inflammation, iron oxides were injected into the tail vein of the mice. Blood was collected by retro-orbital puncture and afterward the animals were immediately killed by cervical dislocation (time delays: 1, 3, and 6 hours). The liver, spleen, and both muscles (inflamed and control muscle) were excised. The liver and spleen samples were homogenized in NaCl 0.9% (5 times dilution). The tissue homogenates were aspirated into a Teflon capillary (5 cm long and 0.625 mm diameter), and inserted into a quartz tube that passed through the EPR cavity. As muscles cannot be homogenized easily, the muscle samples were freeze-dried, and the lyophilisate was crushed to obtain a fine powder. A Bruker EMX X-band spectrometer was used for all measurements. The instrument settings were: Fixed excitation frequency, 9.4 GHz; microwave power, 5 mW; modulation field, 100 kHz; center field, 3150 G; field width, 5000 G; modulation amplitude, 30.81 G; conversion time, 20.48 milliseconds; time constant, 20.48 milliseconds; 4096 data points and a total acquisition time of 84 seconds. Measurements were performed at room temperature. Calibration curves (using aqueous suspensions or solid material) were built with known amounts of iron oxide particles. The calibration curve for an aqueous suspension of USPIO-PEG-sLeX is shown in Figure 2.

In Vivo EPR

In vivo EPR spectra were recorded using an EPR spectrometer (Magnetech, Berlin, Germany) equipped with a low frequency microwave bridge operating at 1.2 GHz and an extended loop resonator. Twenty-four hours after FCA injection, anesthesia was induced using 3% isoflurane (in air), and then maintained using 1.8% isoflurane. USPIO-PEG-sLeX or nongrafted USPIO-PEG were injected into the tail vein. The muscles were inserted into the loop resonator, keeping the same position for all mice. In vivo EPR spectra were recorded at 1, 2, 3, and 4 hours under the following conditions: Fixed excitation frequency, 1.2 GHz; microwave power, 24 mW; modulation field, 100 kHz; center field, 53 mT; field width, 20 mT; modulation amplitude, 0.42 mT; 1024 data points and a total acquisition time of 60 seconds.

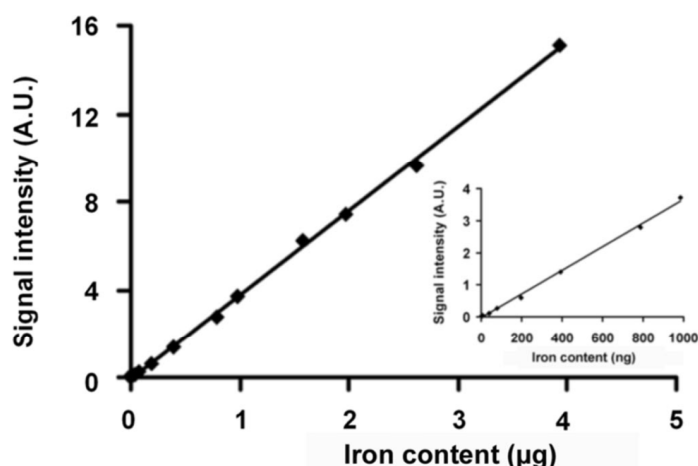


FIGURE 2. Quantitative analysis of iron oxide by EPR spectrometry. Calibration curve of the signal intensity as a function of iron content.

In Vivo MRI

Twenty-four hours after FCA injection, the animals were anesthetized with isoflurane (3% induction, 1.8% maintenance). MR images were obtained on a Bruker BIOSPEC system equipped with a horizontal 4.7 T magnet. The images were acquired using a T_2 -weighted RARE sequence. The parameters were repetition time, TR = 3.2 seconds; echo time, TE, 66.9 milliseconds; bandwidth, 50 kHz; FOV, 5 cm; matrix size, 128 × 128; 2-mm slice thickness; acquisition time, 6 minutes 49 seconds. After a precontrast sequence, USPIO-PEG-sLeX or nongrafted USPIO-PEG were injected into the tail vein of the mice. Postcontrast images of the muscles were acquired at different time delays (every 30 minutes until 3 hours) after injection of the iron oxides. As a control, we placed a phantom tube containing CuSO_4 (100 mg/L) alongside the animals and measured the phantom signal before and after the contrast media injection. For the analysis, Paravision software was used to measure the signal intensities (SI) in the different regions of interest chosen in the control muscle, the inflamed muscle, and the phantom. Muscle SI was normalized to the SI of the phantom, yielding a relative intensity (RI) for each muscle. Negative contrast enhancement values were calculated for each time point using the following equation:

$$\% \text{ enhancement values} = ([\text{RI post} - \text{RI pre}]/\text{RI pre}) \times 100.$$

For MRI data analysis the T_2 relaxation time has been calculated from the multispin-echo datasets using in-house software

based on the interactive data language (Research Systems, Boulder, CO). Regions of interest were drawn within the inflamed tissue and the mean T_2 value was calculated before and after contrast-enhancement of the inflamed muscles to evaluate the T_2 shortening produced by the grafted or ungrafted USPIO particles.

Statistical Analysis

All results are given as mean values $\pm$ SEM from 5 to 6 animals per group. Comparisons between groups were made with Student 2-tailed t test and a $P < 0.05$ was considered as significant. The following symbols are used in the figures: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RESULTS

Characterization of the USPIO-PEG-sLeX

The hydrodynamic size of the synthesized USPIO-PEG-sLeX was 23 nm (polydispersity index = 0.128). The R_1 -NMRD profile of USPIO-PEG-sLeX is shown in Figure 3 (left). The relaxivity values at 37°C were: $r_1 = 36.8 \text{ mM}^{-1}\text{s}^{-1}$ and $r_2 = 87.2 \text{ mM}^{-1}\text{s}^{-1}$ (at 20 MHz), and $r_1 = 15.4 \text{ mM}^{-1}\text{s}^{-1}$ and $r_2 = 87.8 \text{ mM}^{-1}\text{s}^{-1}$ (at 60 MHz). The relaxivity is defined as relaxation rate of water protons in 1 mmol/L solution of contrast agents and its value is expressed in mmol/L in iron. Interpretation of the relaxation profiles (ie, evolution of the relaxivity as a function of the magnetic field) with appropriate theoretical models^{29,30} provides information about the main properties of the superparamagnetic crystals, such as their specific magnetization ($51.7 \text{ Am}^2/\text{kg}$) and their size ($d = 11.6 \text{ nm}$). The corresponding values obtained by magnetometry (Fig. 3, right) were $M_s = 68.3 \text{ Am}^2/\text{kg}$ for the magnetization and $d = 9.8 \text{ nm}$ for the crystal diameter. As previously observed, the mean diameter obtained by magnetometry was less than that obtained by relaxometry. This difference can be explained by a distribution of crystal sizes, which influences the mean size obtained by the various methods.³¹ This size dispersion is also responsible for the lower specific magnetization obtained by relaxometry compared with that obtained by magnetometry.

Inflammatory Model

Histologic analysis confirmed the presence of a large area of inflammation in the muscle injected with FCA. Inflammatory cells were recruited near the site of injection which was identified by remaining droplets of mineral oil. Inflammatory cell recruitment was already visible 1 hour after administration of FCA, increased progressively up to 1 day after injection, and then remained unchanged on days 3 and 7. A typical histologic image (obtained 1 day after injection of FCA) is shown in Figure 4. As it was established that E-selectin is highly up-regulated in this inflammatory model,²⁸ and as the up-regulation lasts from 4 to 30 hours after induction of the

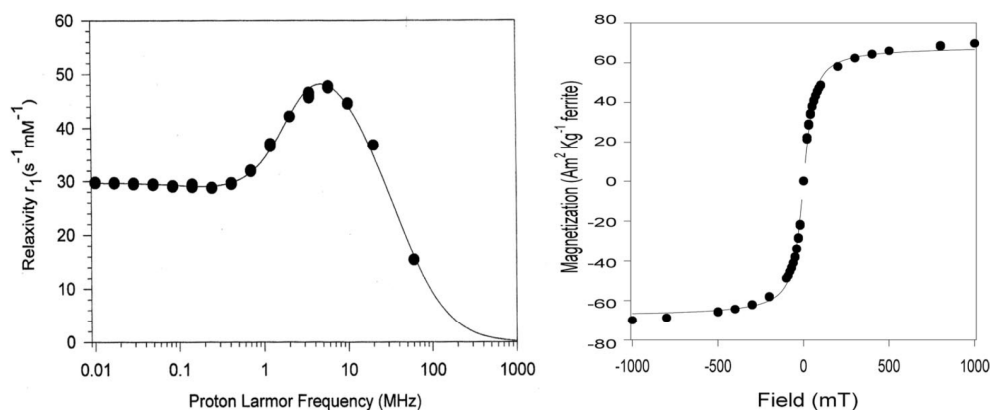


FIGURE 3. Magnetic characterization of USPIO-PEG-sLeX. Left: NMRD profile. Right: magnetometry curve.

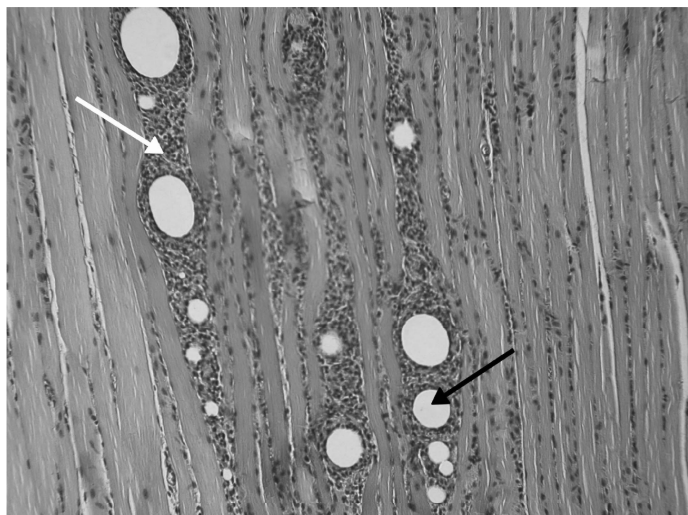


FIGURE 4. Histologic image of an inflamed mouse muscle 24 hours after administration of 50 uL of Freund's Complete Adjuvant. The white arrow indicates the inflammatory cells recruited on the inflamed site and the black arrow shows small lipid bubbles of remaining mineral oil.

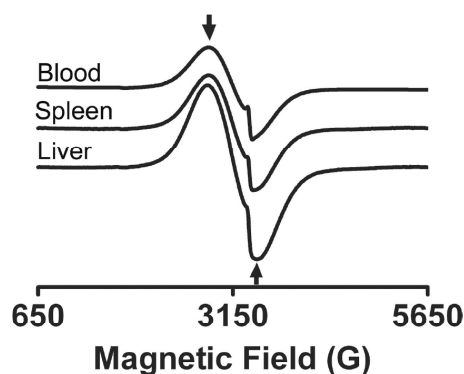


FIGURE 5. Typical EPR X-band spectra of the different tissues recorded ex vivo at 3 hours after IV injection of USPIO-PEG-sLeX.

inflammation, we further used 24 hours after injection of FCA to evaluate the distribution of the USPIOs.

Ex Vivo EPR

USPIOs exhibit an EPR spectrum, which directly reflects the number of iron oxide particles in a sample (Fig. 2). EPR spectroscopy has already been proposed as a method of quantifying the accumulation of iron oxide inside tissues.³² Calibration curves were built from saline and liver homogenates containing known concentrations of iron oxide particles. The calibration curves did not change when the particles were dispersed in saline or in liver homogenates (data not shown). The EPR spectra were characterized by an average g value of 2.13. Typical EPR spectra recorded in different tissues (blood, spleen, and liver) are shown in Figure 5. The evolution of the concentration of USPIO-PEG-sLeX in different tissues is shown in Figure 6. In blood (Fig. 6, left), we observed a decrease in circulating USPIO-PEG-sLeX over time. The iron oxide remaining in the circulation 1 hour after IV injection, expressed as a percentage of the initial blood concentration just after injection, was $17.7\% \pm 0.7\%$ for USPIO-PEG-sLeX and $17.5\% \pm 0.8\%$ for USPIO-PEG (mean $\pm$ SEM, $n = 5$). Under the same conditions, the remaining circulating

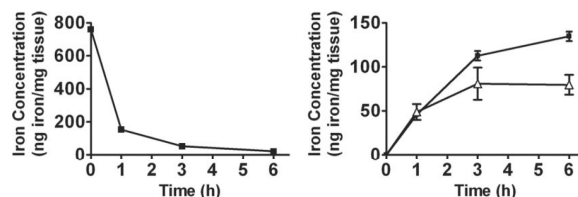


FIGURE 6. Time course of USPIO-PEG-sLeX concentration in different tissues as measured by ex vivo X-band EPR spectroscopy. Left: measurements in blood (■). Right: measurements in the liver (▲) and in the spleen (Δ). The results are expressed as mean $\pm$ SEM, $n = 5$.

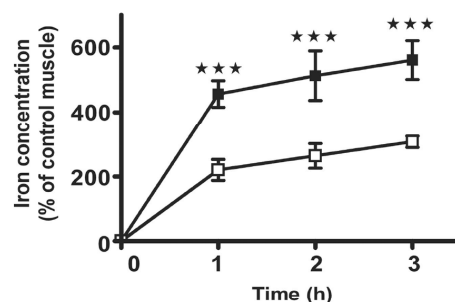


FIGURE 7. Time course of USPIO-PEG (□) and USPIO-PEG-sLeX (■) concentration in inflamed muscle as measured by X-band EPR ex vivo. The results are expressed as % of the concentration found in control muscle (mean $\pm$ SEM, $n = 5$).

iron oxides were $4.7\% \pm 0.4\%$, $2.7\% \pm 0.2\%$, and $0.9\% \pm 0.1\%$ of the initial blood concentration for ferumoxtran-10, ferucarbotran, and ferumoxide, respectively. The concentration of USPIO-PEG-sLeX was less than 3% after 6 hours, and it was completely cleared from the blood 24 hours after administration (data not shown). In the spleen, the concentration of USPIO-PEG-sLeX reached a plateau by 3 hours after injection and then remained stable for the rest of the observation period (6 hours). In the liver, the concentration of USPIO-PEG-sLeX increased over time, but the rate of accumulation appeared to slow slightly 3 hours after administration. After this preliminary USPIO-PEG-sLeX biodistribution study, we evaluated the inflamed muscles. The results are expressed as a percentage of the iron oxide concentration detected in the noninflamed control muscle (Fig. 7). The accumulation of iron oxide particles in the inflamed muscle was significantly higher after administration of USPIO-PEG-sLeX compared with ungrafted USPIO-PEG. Three hours after injection of the contrast media, the mean muscular iron content was $561\% \pm 60\%$ (mean $\pm$ SEM, $n = 5$) for mice injected with USPIO-PEG-sLeX, and $308\% \pm 18\%$ for mice injected with ungrafted USPIO-PEG. In other words, the iron content was about 6 times higher in the inflamed muscle than in the control muscle, using USPIO-PEG-sLeX, and when using USPIO-PEG control particles, iron concentration is still 3 times higher in inflamed muscles than in control muscles. The amount of iron accumulated in the inflamed muscle was about 0.8% of the injected dose when using USPIO-PEG-sLeX compared with 0.4% obtained with USPIO-PEG particles.

In Vivo EPR

Using EPR spectrometers operating at low frequency, it is possible to record EPR spectra directly in vivo. We looked at the feasibility of detecting USPIO directly in vivo using these systems. Interestingly, the EPR signal was easily visible in inflamed muscles,

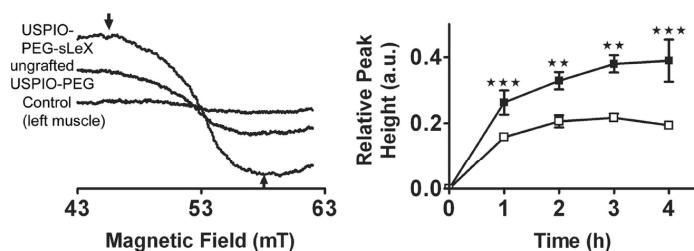


FIGURE 8. In vivo EPR study after administration of the USPIOs. Left: typical EPR L-band spectra recorded at 3 hours after USPIO injection. Right: Mice with inflamed muscle were injected with either USPIO-PEG-sLeX (■) or with USPIO-PEG as control particles (□) and time course of iron concentration in inflamed muscles was measured by L-band EPR. Results are expressed as mean $\pm$ SEM, $n = 6$ mice/group.

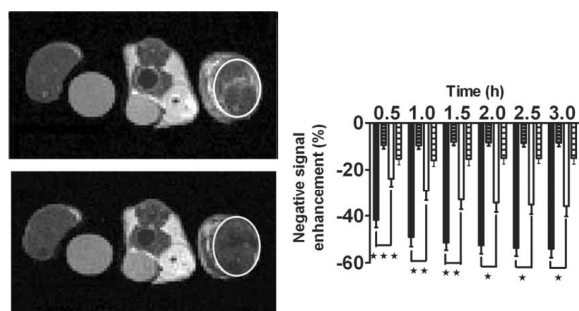


FIGURE 9. Effect of the USPIOs on the MR contrast in inflamed muscle. Left: typical T_2 -weighted images before (top) and 3 hours after (bottom) the administration of USPIO-PEG-sLeX. The control muscle is located on the left, the inflamed muscle on the right. The white circles indicate the regions of interest in the inflamed muscles with an obvious signal decrease after administration of USPIO-PEG-sLeX. Right: bar graph showing the time course of the negative contrast enhancement produced by USPIO-PEG-sLeX (black) and ungrafted USPIO-PEG (white) in T_2 -weighted images. The effect on the control muscle is indicated by the striped bar graph. Results are mean $\pm$ SEM, $n = 5$ mice/group.

compared with control muscles (Fig. 8, left). The SI was measured as a function of time (Fig. 8, right). Between 3 and 4 hours after the injection, the SI recorded in the inflamed muscle was twice as high with USPIO-PEG-sLeX as with ungrafted USPIO-PEG.

In Vivo MRI

Iron oxide particles are characterized by a large magnetic moment and a large size that leads to a shortening of the transverse relaxation time (T_2) and to considerable signal loss in T_2 -weighted MRI sequences. MR images were obtained before and at different times following the intravenous administration of iron oxide particles (Fig. 9, left). The SI decreased rapidly in the inflamed muscle after administration of USPIO-PEG-sLeX. The signal loss was $-49\% \pm 4\%$ 60 minutes after the administration of the contrast agent, and remained stable until the end of the 3 hours imaging period ($-54\% \pm 4\%$). The decrease in SI was less pronounced when using the ungrafted USPIO-PEG ($-36\% \pm 4\%$). There was also a small, but significant, decrease in SI in the control muscle (around 10%) after injection of both contrast media.

Calculated T_2 values were obtained from mice before and 3 hours after administration of either USPIO-PEG-sLeX or USPIO-PEG. The shortening of the transversal relaxation time T_2 in in-

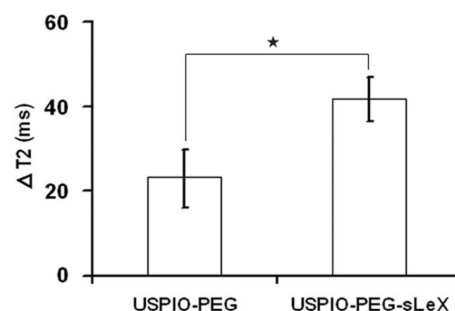


FIGURE 10. The graph shows mean ΔT_2 measurements of either targeted or untargeted USPIO particles, with $\Delta T_2 = T_2$ precontrast— T_2 3 hours postcontrast.

flamed mice muscle is about 41.8 ± 5.2 milliseconds when targeted particles were injected and only 21.1 ± 5.1 milliseconds for the untargeted ones (Fig. 10). Compared with the T_2 value measured on precontrast images the particles produce a T_2 shortening of 62.9% and 37.3%, respectively, which kind of reconfirms the percentages obtained for the negative signal enhancement.

DISCUSSION

The major insights of the present study are (a) EPR and MRI can be used complementarily to assess the amount and localization of targeted iron oxide particles; (b) USPIO-PEG-sLeX accumulate preferentially in inflamed areas, and may be used as inflammation-selective MR contrast agents.

Multimodality approach involves the incorporation of at least 2 measuring modalities using labeled “reporter” agents. Useful complementary information coming from a multimodal approach may be related to localization (macroscopic imaging or histology), absolute quantification, metabolism, or gene activation. NMR spectroscopy and imaging are able to provide important anatomic, functional, and metabolic imaging, with very high spatial resolution for several nuclei. To measure real concentrations of iron oxide in tissues, other authors have used the incorporation of radioactive ^{59}Fe in the USPIO core³³ or inductively coupled plasma-atomic emission spectrometry.³⁴ Here, we used EPR, a magnetic resonance method that detects the presence of species carrying unpaired electrons with high sensitivity. EPR spectrometry is interesting because it is more sensitive than NMR (due to the difference in gyromagnetic ratio) and because the EPR signal intensity quantitatively reflects the amount of iron oxide particles, without any confounding effect. Calibration curves were built with standard amounts of iron oxide contrast agent to check the linearity of the response (Fig. 2). EPR is generally used in vitro or ex vivo on freeze-dried samples or thin tissue homogenates to avoid the dielectric loss observed in large aqueous samples. Early work carried out on magnetite particles in the nineties indicated that X-Band EPR was useful to monitor the clearance of iron oxide particles from the blood³² and their accumulation in the liver and in the spleen by ex vivo sampling.³⁵ To correlate the dynamics observed in MRI with EPR, we also used a low frequency EPR spectrometer (1GHz) that allows a wave penetration of 1 cm inside tissue, which was typically the size of the muscles analyzed in the present study. To our knowledge, only one in vivo study has reported the detection of superparamagnetic iron oxide particles in the liver using an L-band spectrometer.³⁶ So far, EPR has never been used in the evaluation of targeted iron oxide particles developed for molecular imaging.

To conduct a fair comparison between USPIO-PEG-sLeX and USPIO-PEG, we first checked the EPR and NMR characteristics of both iron oxide contrast agents. The r_2 relaxivities obtained for

USPIO-PEG-sLeX are comparable to values obtained previously with ungrafted USPIO-PEG or USPIO grafted with sLeX without pegylation of the particles.²² This finding confirms that the branching did not induce a major change in the relaxometric properties of these compounds. We also observed that the EPR signal intensity and shape were not dependent on the coating of the surface of the particle, nor on the tissue in which the USPIO was dispersed. This observation allowed us to quantify and compare directly the content of the contrast agent in different tissues.

The change in blood clearance as a result of the branching of PEG polymers at the surface of the particles is obvious when the amount of iron oxide particles remaining in the circulating blood is compared for different preparations. Comparison with ferumoxtran-10 is the most relevant as this compound has a size (20–40 nm) comparable to that of USPIO-PEG-sLeX (23 nm). One hour after administration, the blood concentration of USPIO-PEG-sLeX (and of the ungrafted USPIO-PEG) was about 4 times higher than the blood concentration of ferumoxtran-10. The PEG chains, which are known to reduce plasma protein binding (opsonization), do indeed delay clearance by macrophages. It should be noted that the pegylation did not preclude the final accumulation of USPIOs in the reticulo-endothelial system as we observed a large uptake by the liver and the spleen (Fig. 6). Nevertheless, the increased half-life in the blood is beneficial to the possible molecular recognition of targeted sites.

To estimate the ability of USPIO-PEG-sLeX to specifically detect local inflammation, we used ex vivo EPR, in vivo EPR and MRI. Ex vivo EPR allowed the absolute quantification of iron oxide particles in tissues by comparison with standard calibration curves (Fig. 2). Interestingly, we obtained in vivo EPR signals from USPIOs with a high signal-to-noise ratio using rapid scans (1 minute) in a noninvasive manner. It should be emphasized that the absolute quantification by EPR is theoretically more difficult to obtain in vivo than ex vivo, as the signal strongly depends on the geometry of detection. However, we obtained a reasonable quantification by carefully placing the leg muscle in exactly the same position from one study to another. The results obtained by both techniques (Figs. 7, 8) are remarkably concordant. Finally, MRI studies were carried out to evaluate the ability of this new contrast agent to improve the contrast in inflamed tissues. The 3 methods led to the same conclusions: (1) the ungrafted USPIO-PEG was more concentrated in inflamed muscles than in control muscles; (2) there was a higher accumulation (by a factor 2) of USPIO-PEG-sLeX compared with ungrafted USPIO-PEG in inflamed areas. The large concentration of ungrafted USPIO-PEG in inflamed muscles as compared with control muscles can be explained by the fact that inflamed muscle will recruit a large number of macrophages. These macrophages will take up USPIOs in a nonspecific manner. Furthermore, inflammation causes vasodilation and increased vascular permeability. The resulting improvement in blood flux and transendothelial passage enables a greater accumulation of iron oxide particles in the inflamed tissue. Even taking into account the nonspecific accumulation of USPIOs in local macrophages, our results clearly indicate that the use of USPIO-PEG-sLeX improved the ability to detect inflammation by a factor of 2 compared with ungrafted USPIO-PEG.

In conclusion, USPIO-PEG-sLeX shows promise as an in vivo tool for the noninvasive analysis of the expression of E-selectin in different disorders. The long intravascular persistence time, selective accumulation in inflamed tissues, and the high and prolonged contrast provided in MRI studies support further studies using this new type of contrast agent.

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2) Magnetic resonance imaging of cell death: a proof of principle

**Iron Oxide Particles Covered with Hexapeptides Targeted at
Phosphatidylserine as MR Biomarkers of Tumor Cell Death**

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Contrast Media Mol Imaging. 2010; in press.

Iron oxide particles covered with hexapeptides targeted at phosphatidylserine as MR biomarkers of tumor cell death

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The aim of the study was to evaluate the ability of a new MR contrast agent to detect cell death as a biomarker of the efficacy of anti-cancer treatment. The phosphatidylserine-targeted hexapeptide (E3) was coupled to pegylated ultrasmall iron oxide nanoparticles (USPIO) that can be detected by magnetic resonance imaging (MRI) and by electron paramagnetic resonance (EPR). USPIO binding to staurosporine-treated TLT (transplantable liver tumor) cells, evaluated by X-Band EPR, indicated twice as much binding of USPIO grafted with the E3 peptide, compared with USPIO grafted with a scrambled peptide or ungrafted USPIO. *In vivo* experiments were carried out using TLT cells implanted intramuscularly into NMRI mice, and tumor cell death was induced by irradiation. After intravenous injection of the different types of USPIO, the accumulation of contrast agent was evaluated *ex vivo* by X-band EPR, *in vivo* by L-band EPR and by T_2 -weighted MRI. In irradiated tumors there was greater accumulation of the targeted USPIO particles compared with control particles or compared with the targeted particles in untreated tissues. In conclusion, phosphatidylserine-targeting of USPIO particles can detect dying tissues. This molecular targeted system should be evaluated further as a potential biomarker of tumor response to treatment. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: MRI; EPR; molecular imaging; cell death; ultrasmall iron oxide particles

1. Introduction

The prediction of tumor response to a treatment is usually based on reductions in tumor size evidenced by morphological imaging (1). However, it can take several weeks before tumor shrinkage becomes evident. There has been considerable interest in obtaining an earlier indication of therapeutic efficacy and evaluation of tumor progression. One method to track individual tumor response for many types of cancer involves detecting a decrease in [^{18}F]-fluorodeoxyglucose (FDG) uptake in treatment-sensitive tumors by positron-emission tomography (PET) (2). An alternative method is to image tumor cell death as a prognostic factor of treatment outcome since radiotherapy and different forms of chemotherapy have a common ability to induce apoptosis and necrosis (3–5).

There are two major types of cell death: organized and programmed cell death, called apoptosis, and chaotic injury-induced cell death, called necrosis. Early in the apoptotic process, the membrane-bound phospholipid, phosphatidylserine (PS), flips from the inner layer to the outer layer of the plasma membrane, regardless of how apoptosis is induced (6–9). Although during necrosis PS remains restricted to the inner leaflet of the plasma membrane, membrane rupture allows access to PS and PS will inevitably also be detected in necrotic cells (10). However, the ability to recognize all forms of cell death would be advantageous when monitoring the efficacy of an anti-cancer treatment.

Annexin A5 (anxA5), a PS-binding protein, has been developed into a molecular imaging probe to measure apoptosis *in vitro* and *in*

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Contract/grant sponsor: Belgian National Fund for Scientific Research.

Contract/grant sponsor: Televie grants; contract/grant number: 7.4597.06.

Contract/grant sponsor: Fonds Joseph Maisin.

Contract/grant sponsor: The Saint-Luc Foundation.

Contract/grant sponsor: The Fondation contre le Cancer, the 'Actions de Recherches Concertées-Communauté Française de Belgique'; contract/grant number: ARC 04/09-317 and 05/10-335.

Contract/grant sponsor: 'Pôle d'attraction Interuniversitaire PAI VI'; contract/grant number: P6/38 and P6/29.

The aim of the present study was to couple the PS-targeted E3 hexapeptide (peptide sequence: TLVSSL), previously selected by phage display (24), to pegylated USPIO particles (USPIO-PEG750). Pegylation of particles was carried out to decrease the rapid uptake of USPIO by the reticulo-endothelial system, to prolong blood circulation time, and hence to increase the probability of tumor access and specific tumor targeting. Validation of this new MR contrast agent was achieved by inducing cell death *in vitro* and *in vivo* in the transplantable liver tumor model (TLT). The quantification of iron oxides bound to dying tumor cells was achieved using electron paramagnetic resonance (EPR) spectroscopy and T_2 -weighted MR imaging.

Figure 1 displays the experimental scheme to provide an overview of all experiments carried out on mice within the scope of this study.

USPIO particles were first covalent-bonded to the peptide and then particles were pegylated to prolong their blood circulation time. Four or five peptides were grafted per USPIO nanoparticle.

The physicochemical and magnetic properties of USPIO-PEG750-E3 and USPIO-PEG750-E3scramble are shown in Table 1. The difference in hydrodynamic size between native and conjugated nanoparticles is not significant. The magnetometry and nuclear magnetic resonance dispersion (NMRD) profiles (i.e. evolution of the longitudinal relaxivity, r_1 , as a function of the magnetic field) are shown in Figs 2 and 3, respectively. The parameters obtained from the fitting of the NMRD curves were the crystal radius and the specific magnetization. The corresponding values obtained by magnetometry were lower for the mean radius and higher for the specific magnetization. These differences can be explained by the size dispersion, which influences the mean size obtained by the two methods (28).

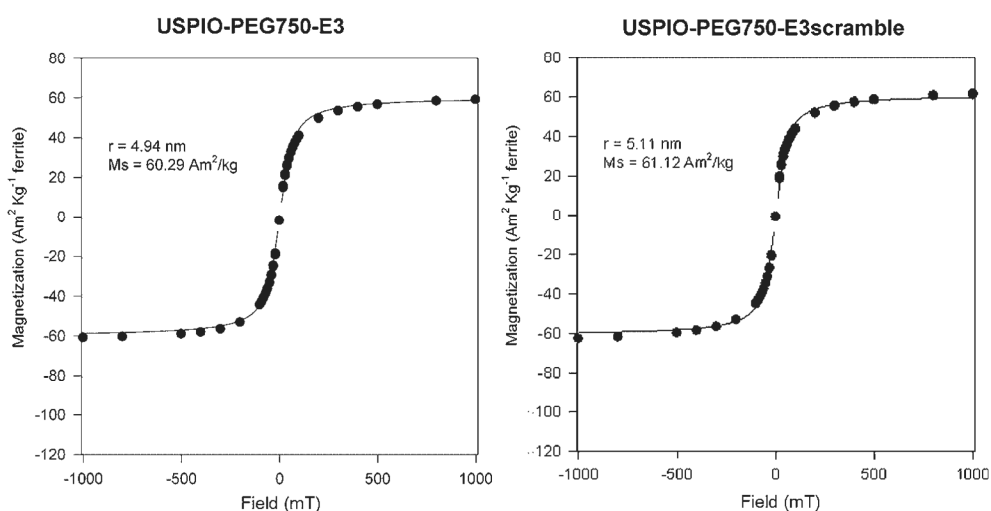
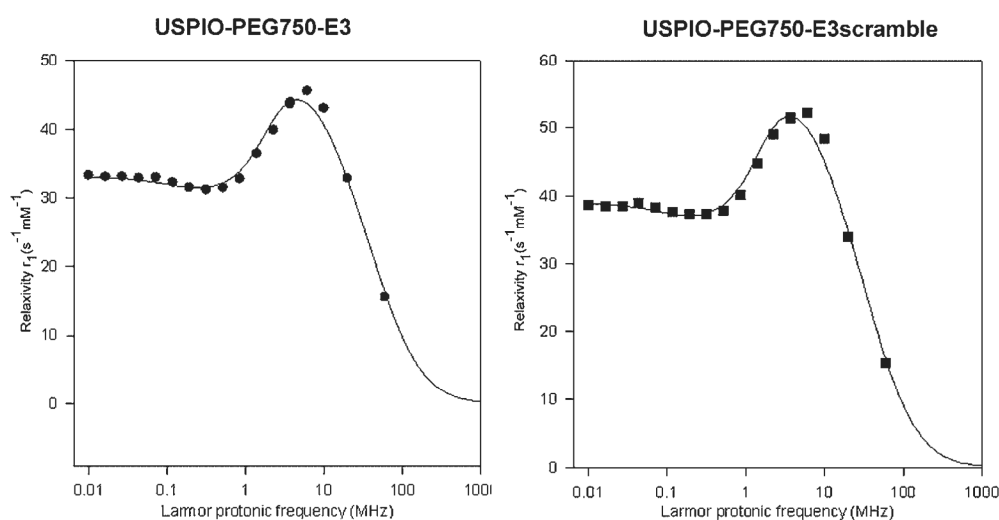
We incubated fluorescein isothiocyanate (FITC)-anxA5 and propidium iodide (PI) with untreated (Fig. 4A), serum-deprived (Fig. 4B) and staurosporine-treated cells (Fig. 4C), and submitted them to flow cytometry analysis. In Fig. 4(A), the majority of cells were anxA5⁻ and PI⁻, indicating an intact plasma membrane and a lack of PS exposure on the outer membrane. These cells appear in the lower left quadrant of the plot, where the cells are considered viable. Treatment with staurosporine and/or serum deprivation was accompanied by an increase in the number of cells in the lower right quadrant which are stained anxA5⁺ and PI⁻ considered as apoptotic cells because they have lost their membrane phospholipid asymmetry. For cell cultures grown in serum-deprived medium and treated with staurosporine for 24 h, more than 90% of the cells exhibited properties of apoptotic cells (Fig. 4C).

EPR spectroscopy has already been proposed as a method of quantifying the accumulation of iron oxide inside tissues (29). Calibration curves were built from saline containing known concentrations of iron oxide particles (data not shown). Iron oxide uptake in cells was calculated in $\mu\text{g Fe}/10^6$ cells (Fig. 5). Staurosporine-treated cells that were incubated with E3-functionalized USPIO appeared to have the highest iron oxide binding (4.10 ± 0.14 , $n = 5$) compared with untreated control cells and also compared with apoptotic cells incubated with control particles. For cells in serum-free medium incubated with USPIO-PEG750-E3 (2.86 ± 0.27 , $n = 5$), there was also a significantly



Table 1. Characteristic data of USPIO-PEG750-E3 and USPIO-PEG750-E3 scramble

	Method	USPIO-PEG750-E3	USPIO-PREG750-E3scramble
Hydronamic size	Photon correlation spectroscopy Malvern system (Zetasizer Nanoseries ZEN 3600)	24 nm	29 nm
r_1 at 20 MHz	Minispec MQ 20 spin analyzers	32.9/mm/s	33.9/mm/s
r_2 at 20 MHz	(Bruker, Karlsruhe, Germany)	72.1/mm/s	70.3/mm/s
r_1 at 60 MHz	Minispec MQ 60 spin analyzer	15.5/mm/s	15.4/mm/s
r_2 at 60 MHz	(Bruker, Karlsruhe, Germany)	70.4/mm/s	71.4/mm/s
Crystal radius r	NMRD profile Field Cycling	5.41 nm	5.71 nm
Specific magnetization, M_s	Relaxometer (Stelar, Mede, Italy)	52.7 Am ² /kg	51.3 Am ² /kg
Crystal radius, r	Magnetometry Vibrating sample	4.94 nm	5.11 nm
Specific magnetization, M_s	magnetometer (VSM-NUOVO MOLSPIN/Newcastle Upon Tyne, UK)	60.29 Am ² /kg	61.12 Am ² /kg

**Figure 2.** Magnetometry of USPIO-PEG750-peptides.**Figure 3.** NMRD profiles of USPIO-PEG750-peptides.

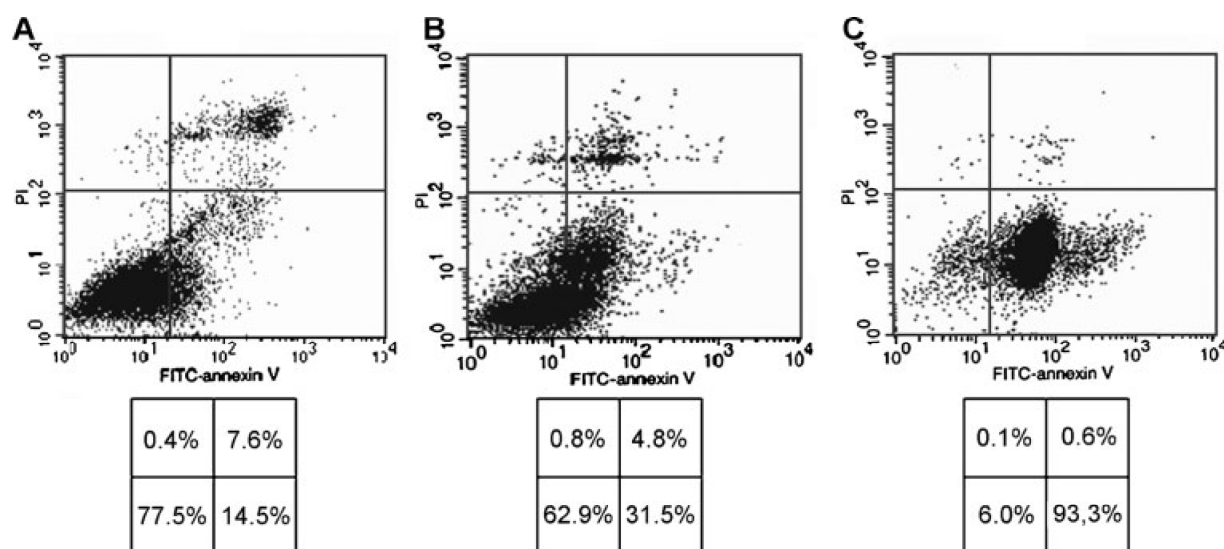


Figure 4. Induction of apoptosis as measured by flow cytometric analysis: TLT cells were grown in normal medium (A), in serum-free medium (B) or in serum-free medium treated with 5 μM of staurosporine (C). Scatterplots show log Annexin A5 fluorescence emission (x-axis) vs log propidium iodide fluorescence emission (y-axis). Quadrants contain living (lower left), apoptotic (lower right), necrotic (upper left) and late apoptotic cells (upper right). The percentage of events is indicated in the schematic boxes at the bottom of the figure.

higher particle binding than with USPIO-PEG750-E3scramble (1.61 ± 0.09 , $n = 5$) and ungrafted USPIO-PEG750 (1.54 ± 0.09 , $n = 5$) because serum deprivation induces cell death, but to a lesser extent than staurosporine treatment. We also observed a small nonspecific binding of USPIO-PEG750 in apoptotic cells compared with control cells (Fig. 5). The amount of USPIO-PEG750 bound to apoptotic cells was, however, very low compared with USPIO-PEG750-E3.

2.4. Histology

Frozen tumor sections obtained from irradiated and untreated mice were stained by the TUNEL method and then examined by fluorescence microscopy (Fig. 6). Histological analysis confirmed the presence of larger areas of apoptosis in tumors 24 h after irradiation (Fig. 6B) when compared with nonirradiated tumors (Fig. 6A). The proportion of apoptotic regions compared with the

total area of tumor tissue were $1 \pm 1\%$ for control tumors and $10 \pm 3\%$ after irradiation, this difference was significant.

A hematoxylin–eosin staining was also carried out on paraffin-embedded tumor slices to determine the necrotic fraction before and 24 h after X-ray irradiation. Here, contrarily to the quantification of apoptosis, no difference could be observed between irradiated (Fig. 6D) and control tumors (Fig. 6C). Percentages of necrotic fractions were $23 \pm 8\%$ and $23 \pm 9\%$ respectively.

2.5. Ex vivo monitoring of tumor cell death after irradiation

To determine when irradiation-induced tumor cell death reached a maximum, the iron concentration in excised tumors was measured by X-band EPR at different time points after irradiation. The accumulation of iron oxide particles in the irradiated tumors was significantly higher after administration of USPIO-PEG750-E3 compared with nonfunctionalized USPIO-PEG750 or USPIO-PEG750-E3scramble for all time delays after tumor irradiation (Fig. 7). The maximal concentration of USPIO-PEG750-E3 in the irradiated tumor was observed 7 h after irradiation (3 h after contrast media injection). The iron content per mg of tissue was 87 ± 7 ng of iron ($n = 8$) for mice injected with USPIO-PEG750-E3, 18 ± 1 and 18 ± 3 ng for mice injected with ungrafted USPIO-PEG750 ($n = 8$) or USPIO-PEG750-E3scramble ($n = 7$), respectively. In other words, the iron content was about five times higher in the irradiated tumor using USPIO-PEG750-E3 than when using nontargeted control particles. In nonirradiated tumors, the iron content per mg of tissue was 33 ± 6 ng, ($n = 6$), 17 ± 2 ng ($n = 6$) and 15 ± 2 ng ($n = 10$) for USPIO-PEG750-E3, USPIO-PEG750 and USPIO-PEG750-E3scramble, respectively. This is consistent with ‘physiological’ apoptosis occurring during the tumor development.

2.6. In vivo time course of iron accumulation in tumors

L-band EPR enabled us to detect USPIO particles directly *in vivo*. Consistent with our previous *ex vivo* experiment, the contrast

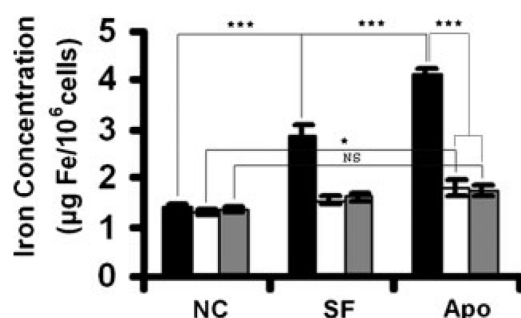


Figure 5. Iron uptake (μg) in 10^6 TLT cells as measured by X-band EPR ($n = 5$). Negative control cells (NC), cells in serum-free medium (SF) and apoptotic cells (Apo) treated with 5 μM of staurosporine in serum-deprived medium were incubated with USPIO-PEG750-E3 (black bars), nonfunctionalized USPIO-PEG750 (white bars) or with USPIO-PEG750-E3scramble (gray bars).

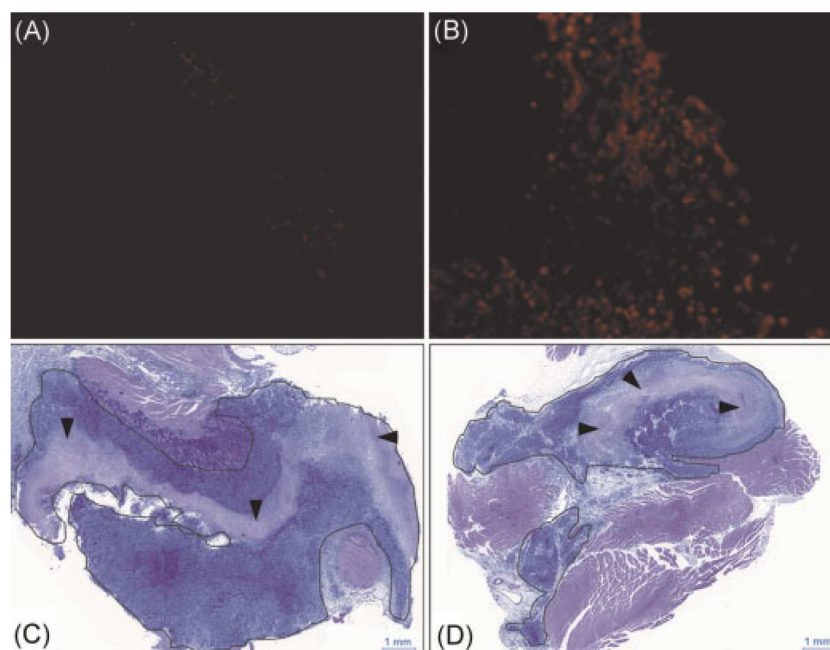


Figure 6. Fluorescent TUNEL-stained section from untreated mouse (A) and from treated mouse, 24 h after X-ray irradiation (B). TUNEL-positive nuclei are stained in red (magnification: 200 ×). The figure also shows hematoxylin/eosin-stained slices of irradiated (D) and control tumors (C). The encircled area delimits the tumor region and the arrowheads the necrotic fraction.

agent was injected 4 h after irradiation. The signal intensity (SI) of iron oxide content in tumors was measured as a function of time up to 24 h after contrast media injection in order to establish a kinetics curve (Fig. 8). The only group significantly different from the others was the irradiated animals injected with the PS-targeted USPIO particles ($n = 6$). This difference became significant 2 h after contrast agent injection and signal intensity dropped to the same level as the control groups at 24 h.

2.7. In vivo imaging

To examine whether USPIO-PEG750-E3 might serve as an MRI contrast agent, mice were imaged 4 h after tumor irradiation. MR images were obtained from irradiated or untreated (image not shown) animals before intravenous administration of USPIO-PEG750-E3 (Fig. 9 A1), USPIO-PEG750 (image not shown) or USPIO-PEG750-E3scramble (Fig. 9 B1). A signal decrease was

observed in tumors 7 h after irradiation and 3 h after injection of the USPIO-PEG750-E3 contrast agent (Fig. 9 A2) compared with the mice injected with control particles (Fig. 9 B2). The use of T_2 -weighted subtraction images highlighted even better the difference in USPIO-PEG750-E3 accumulation in irradiated tumors (Fig. 9 A3) compared with USPIO-PEG-E3Scramble (Fig. 9 B3). Negative signal enhancement data were normalized to the reference tube and calculated as a percentage of the pre-injection SI values (Fig. 10, left). The SI decreased rapidly after administration of USPIO-PEG750-E3. For irradiated animals, the signal loss was $-45 \pm 3\%$ ($n = 6$) 180 min after the administration of the PS-targeted contrast agent and $-20 \pm 5\%$ ($n = 6$) for the nonirradiated control tumors. The decrease in SI was much less pronounced in the control particle groups.

Calculated T_2 values were obtained from tumors before and 3 h after administration of USPIO-PEG750-E3, USPIO-PEG750 or USPIO-PEG750-E3scramble. The shortening of the transversal relaxation time, ΔT_2 , in irradiated tumor was about 33 ± 2 ms when targeted particles were injected and only 9 ± 2 ms for the

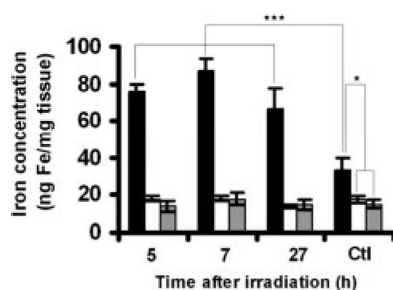


Figure 7. EPR X-band spectra of the different tumors were recorded at 5, 7 or 27 h after X-ray irradiation. Nonirradiated tumors served as negative control (Ctl). Black bars represent animals injected with USPIO-PEG750-E3, white bars unlabeled USPIO-PEG750 particles and gray bars USPIO-PEG750-E3scramble. Iron concentration is expressed as ng of iron in 1 mg of tumor tissue ($n = 6-10$).

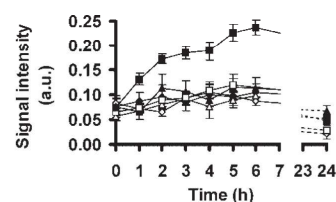


Figure 8. Kinetics curve of iron accumulation in tumors as measured by L-band EPR *in vivo*. The results are expressed as signal intensity as a function of time ($n = 5-6$). Mice with irradiated (closed symbols) or untreated (open symbols) tumors have been injected with USPIO-PEG750-E3 (■, □), USPIO-PEG750-E3scramble (▲, △) or USPIO-PEG750 (●, ○).

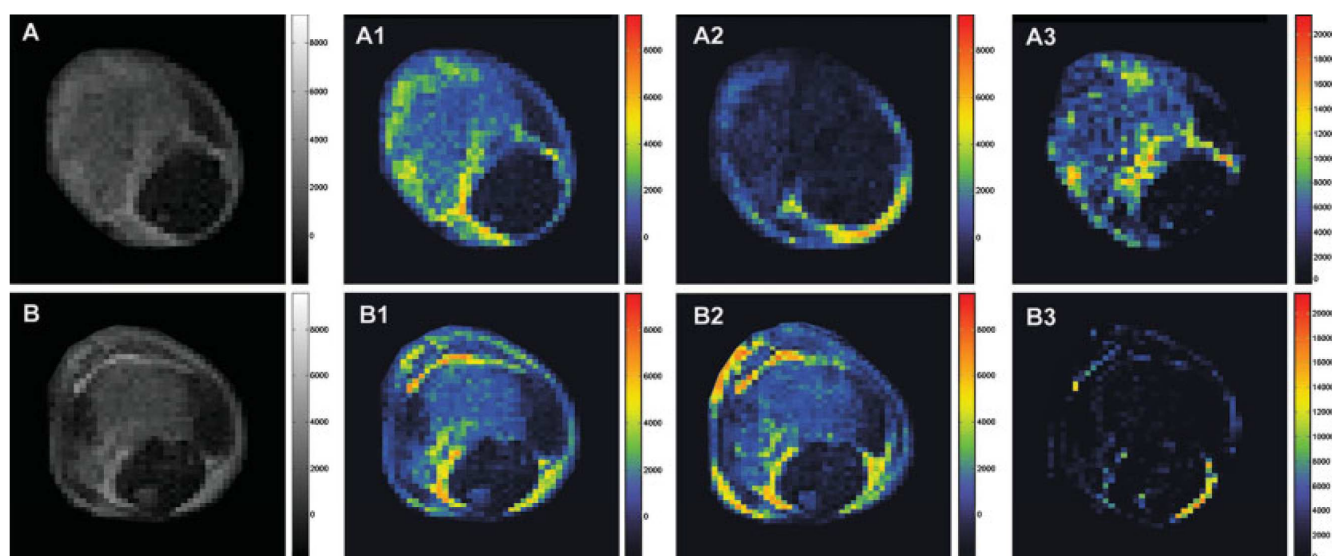


Figure 9. Axial slices of irradiated murine tumors obtained by T_2 -weighted MRI. The images of the first row come from a mouse that was injected with USPIO-PEG750-E3 (A) and those in the second row from a mouse where USPIO-PEG750-E3scramble was injected (B). The black and white images show anatomical images of irradiated tumors (A, B). At the side are the pre-contrast (A1, B1) and the 3 h post-contrast images (A2, B2). Subtraction images (A3, B3) were obtained by subtracting the corresponding post-contrast images from the pre-contrast images.

nontargeted control particles (Fig. 10, right). Compared with the T_2 value measured on pre-contrast images the particles produced a T_2 shortening of 50 and 15%, respectively.

3. Discussion

We used a multimodal approach to validate this new contrast agent. The ultimate goal was to benefit from the superparamagnetic properties of the USPIO and to use them as a negative contrast agent in MRI because of their strong T_2 and T_2^* effects. In addition to these effects on NMR images, we also used the presence of unpaired electrons in these systems to detect and quantify the particles (29). EPR spectrometry is interesting because it is more sensitive than NMR (due to the difference in gyromagnetic ratio) and because the EPR signal intensity is directly proportional to the amount of iron oxide particles, without any confounding effect. However, EPR is generally used

in vitro or *ex vivo* on freeze-dried samples or on thin tissue homogenates to avoid the dielectric loss observed in large aqueous samples. To correlate the dynamics observed in MRI with EPR, we used a low frequency EPR spectrometer (1 GHz) that allows a wave penetration of 1 cm inside tissue, which was typically the size of the tumors analyzed in the present study. To our knowledge, only one *in vivo* study has reported the detection of superparamagnetic iron oxide particles in the liver using an L-band spectrometer (30). In a previous study, EPR was used in the evaluation of targeted iron oxide particles developed for molecular imaging (recognition of E-selectin) (31). In the same study, we showed that branching of PEG polymers at the surface of USPIO produced a dramatic change in blood clearance. This increased half-life in the blood is beneficial to the potential molecular recognition of targeted sites. In another study, using phage display technology, our group at the University of Mons identified the E3 peptide as a peptidic vector for molecular imaging of apoptosis. Competition studies between annexin A5 and the E3 peptide confirmed the specificity of interaction (24). Here, we functionalized the surface of USPIO with PEG750 and the E3 peptide.

The ability of pegylated USPIO-E3 to specifically detect cell death was demonstrated *in vitro*, *ex vivo* and *in vivo*. A set of control experiments was performed to confirm the selectivity of recognition: control groups using cells or tumors without treatments, and nontargeted particles using USPIO-PEG750 and USPIO-PEG750-E3scramble. *In vitro*, the USPIO-PEG750-E3 binding was the highest in the staurosporine-treated cell group where the number of apoptotic cells present in the media was very important (90%), as observed by flow cytometry. Even so, TLT cells grown in different conditions do not show an USPIO-PEG750-E3 accumulation that is exactly proportional to the amount of apoptotic cells obtained by flow cytometry analysis. This could be explained by the fact that we used different samples of cells for these two experiments. Also some nonspecific nanoparticle adsorption to control cells could be observed during the *in vitro* EPR experiment. In the *ex vivo* study, the highest

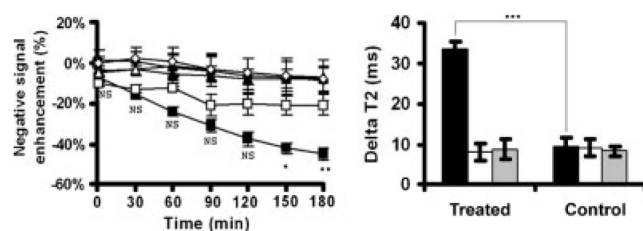


Figure 10. Left: mean negative contrast enhancement values were obtained from regions of interest in MRI images that encompassed the entire tumor region ($n = 5-6$). Mice with irradiated (closed symbols) or untreated (open symbols) tumors were injected with USPIO-PEG750-E3 (■, □), USPIO-PEG750-E3scramble (▲, △), or USPIO-PEG750 (●, ○). Right: diminution of T_2 relaxation times in irradiated and control tumors after contrast agent injection. The graph shows mean ΔT_2 measurements of USPIO-PEG750-E3 (black bars), nonfunctionalized USPIO-PEG750 (white bars) or with USPIO-PEG750-E3scramble (gray bars), with $\Delta T_2 = T_2$ pre-contrast – T_2 3 h postcontrast.

concentration of targeted particles in tumors was measured 7 h after irradiation. This information was useful for the subsequent experiments on the *in vivo* time course. The results obtained *ex vivo* and *in vivo* (using both EPR and MRI) are remarkably concordant for demonstrating the selectivity of USPIO-PEG750-E3 for radiation-induced cell death compared with USPIO-PEG750 and USPIO-PEG750-E3scramble. Subtle differences observed between the different techniques can be ascribed to changes in the relative sensitivity of the techniques. For instance, *ex vivo* X-band EPR is about 10 times more sensitive than *in vivo* L-band EPR; therefore it is not astonishing that for the X-band experience the difference between apoptotic tumors injected with targeted particles and the control groups is more significant than the difference obtained by L-band. The effect on the MR images can be visualized directly in T_2 -weighted images or by measuring the changes in T_2 in the tumor regions. The subtraction images are also useful to visualize the selective accumulation of targeted USPIO in irradiated tumors.

The ability of nanoparticles to pass across the leaky tumor endothelium but not across the vessels in normal tissues may prevent the interaction of targeted particles with phosphatidylserine-expressing cells in normal tissues (EPR effect). It should be emphasized that irradiation may produce changes in tumor blood flow, as demonstrated previously in the same tumor model (32,33). However, we can exclude a flow effect as the cause of the accumulation of USPIO-PEG750-E3 in irradiated tumors as we did not observe the same effect when using nontargeted USPIO, such as USPIO-PEG750 and USPIO-PEG750-E3scramble. It is well known that irradiation may also cause elevated occurrence of macrophage that can take up iron oxide particles in a nonspecific manner. However, in our case we could not observe a difference in binding of control particles for irradiated and untreated tumors, which led us to assume that USPIO-PEG750-E3 accumulation in irradiated tumors is effectively due to PS exposure. It is also known that iron oxide particles can be endocytosed by tumor cells in a nonspecific manner (34). Concerning the unspecific portion of USPIO-PEG750-E3 accumulation in treated tumors, we cannot exclude endocytosis of the contrast probe by tumor cells. However, in our study we could demonstrate that the accumulation of targeted particles in treated tumors was 2–3 times higher with targeted particles than when control particles were used. This let us assume that at least this part of particle accumulation is due to our PS-binding peptide, otherwise the control particle would have been accumulated to the same extent.

The difference in apoptosis induced by the irradiation was confirmed by the immunohistochemistry analysis of tumor samples. We cannot exclude that USPIO-PEG750-E3 particles may accumulate in necrotic areas as the membrane rupture could allow access to PS (10). However, this cannot be considered as a disadvantage as the clinical end-point, when using a cytotoxic treatment, is the ability to recognize all forms of cell death, of whatever origin.

4. Conclusion

We demonstrated that the targeting of USPIO to cell death regions can be achieved by grafting the hexapeptide, E3, to the surface of pegylated particles. EPR quantification and MR imaging studies demonstrate that E3-labeled USPIO is a promising molecular probe for imaging phosphatidylserine expression in

living mice. Future works will assess the value of this new contrast agent to monitor cell death in different tumor models presenting different types of response to cytotoxic treatments. In the future, it would also be interesting to compare this biomarker to other metabolic markers such as [^{18}F]-FDG uptake using micro-PET studies.

5. EXPERIMENTAL

All chemicals used in this study were purchased from Sigma-Aldrich (Bornem, Belgium) unless otherwise indicated.

5.1. Characterization of the contrast material

USPIO-PEG750-E3 and USPIO-PEG750-E3scramble were prepared from nanoparticles with carboxylated groups on the surface as previously described (35). USPIO particles were functionalized in two successive steps with the E3 peptide (TLVSSL) or the E3scramble peptide (SVSLLT, Neomps, Strasbourg, France), and then with an aminoPEG 750 (Fluka, Bornem, Belgium).

Hydrodynamic size measurement was carried out by photon correlation spectroscopy (PCS). The NMRD profiles were recorded at 37°C over a magnetic field range from 0.24 mT to 0.24 T. Additional longitudinal (R_1) and transverse (R_2) relaxation rate measurements were measured at 0.47 and 1.41 T. Fitting of the NMRD profiles using a theoretical relaxation model (36) allows determination of the crystal radius (r), the specific magnetization (M_s), and the Neel relaxation time. The magnetization measurements were performed on a known amount of ferrofluid using a vibrating sample magnetometer. Fitting of the profiles according to the *ad hoc* theory provides several parameters, including the crystal radius (r) and the specific magnetization (M_s) (37).

5.2. Cell culture

Semi-adherent TLT cells were grown in Dulbecco's modified Eagle medium (4.5 g/l glucose without pyruvate, Invitrogen) supplemented with 10% fetal bovine serum (PAA) and 1% of penicillin/streptomycin (Invitrogen). For our experiments, cells were grown to confluence and different groups of cells were prepared: a control group, a serum-free group with cells deprived of serum for 24 h, and an apoptotic group where cells remained for 24 h in a serum-free medium containing 5 μM of staurosporine.

5.3. Flow cytometry

Flow cytometry was performed to determine whether the cell population was successfully stimulated to undergo apoptosis. A total of 5×10^5 cells were resuspended in binding buffer and incubated for 10 min with 1 μl of 50 $\mu\text{g/ml}$ FITC-conjugated anxA5 and with 2.5 μl of 100 $\mu\text{g/ml}$ PI to detect apoptosis or necrosis (Annexin A5-FITC Apoptosis Detection Kit). Cells incubated without any fluorescent label served as a negative control. The resulting mixture was then subjected to flow cytometry. For each sample, 10 000 events were counted using a FACScan apparatus (BD Biosciences) and were analyzed by the CellQuest software (BD Biosciences).

5.4. *In vitro* studies

EPR spectrometry can quantify iron oxide content with high sensitivity. Measurements were performed on an X-band EPR

spectrometer (Bruker, EMX[®], 9.4 GHz). A calibration curve was first established by measuring the signal intensity of different USPIO concentrations in saline. A total of 10^6 cells were incubated in calcium-containing buffer in the presence of 4 mM of USPIO-PEG750, USPIO-PEG750-E3 or USPIO-PEG750-E3scramble. The mixture was incubated for 2 h at room temperature with gentle orbital mixing. The separation between free USPIO and USPIO bound to cells was achieved by using three successive centrifugations (10 min, 3000g) and resuspensions. We checked that there was no sedimentation of USPIO using this centrifugation speed. Finally, cells were resuspended in a saline solution containing 20% dextran to avoid cell sedimentation. Ten microliters of the cell suspension were aspirated through a Teflon tube (length 3 cm; diameter 0.625 mm) and the tube was inserted into the cavity of the X-band EPR spectrometer. The parameters used were: frequency, 9.4 GHz; microwave power, 5.05 mW; center field, 3150 G; field width, 5000 G; modulation amplitude, 30.81 G; time constant, 20.48 ms; conversion time, 20.48 ms; modulation field, 100 kHz; total acquisition time, 83 s. Measurements were performed at room temperature.

5.5. Animals

TLT cells were intramuscularly injected into the right gastrocnemius muscle of 5–6 week old male NMRI mice (Janvier, France). At a size of 8 ± 0.5 mm, tumors received an X-ray-dose of 10 Gy using an RT-250 device (Philips Medical Systems, Hamburg, Germany) to induce apoptosis. Mice were anesthetized using isoflurane (induction 2%; maintenance 1.4%) and the tumor was centered in a circular irradiation field (diameter 3 cm). At different time points after irradiation the animals received the contrast agent (USPIO-PEG750, USPIO-PEG750-E3 or USPIO-PEG750-E3scramble) by tail vein injection at a dose of 7.7 mg Fe/kg. The procedure was approved by a local ethics review committee according to national animal care regulations.

5.6. Histology

The induction of apoptosis was verified by staining with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Irradiated and untreated tumors were excised, embedded in OCT compound, frozen in liquid nitrogen-cooled isopentane and cut into 5 μ m sections. Frozen slices were probed for apoptosis using a commercially available *in situ* cell death detection kit (Roche Diagnostics, Vilvorde, Belgium) according to the manufacturer's protocol; nuclei were also counterstained with 4,6-diamidino-2-phenylindole (DAPI) (data not shown). Slides were photographed using a Zeiss Axioskop microscope equipped for fluorescence. Twenty-four hours after irradiation, irradiated tumors and control tumors were excised, fixed in 10% neutral buffered formalin for 24 h and then embedded in paraffin. Tissues were sectioned at 5 μ m and stained with hematoxylin and eosin, and slides were photographed using a Zeiss Mirax microscope. For both, TUNEL and hematoxylin/eosin staining, slides from three different tumors per group were used to calculate mean apoptotic or necrotic levels in tumors respectively using Image J.

5.7. Ex vivo monitoring of tumor cell death after irradiation

Iron oxide particles were injected into the mice at different time points after tumor irradiation (2, 4 or 24 h). The animals were killed 3 h after contrast agent injection and tumors were excised.

For the control group, animals were not irradiated. The excised tumors were freeze-dried and then crushed into a fine powder. The powder was weighted and then placed into the X-band EPR cavity. We calculated the iron oxide content in each tumor by comparison with the earlier calibration curve. The instrument settings were the same as used for *in vitro* measurements.

5.8. In vivo time course of iron accumulation in tumors

L-band EPR (Magnettech, Germany) was used to track the changes in tumor iron oxide content in real time. Four hours after X-ray irradiation of the tumors, the different USPIO particles were injected intravenously. Mice were anesthetized using isoflurane and their right rear leg was passed through the loop surface coil of the EPR spectrometer. Because the antenna has a loop shape, the leg was held by the antenna and the leg was always placed in the same way to insure similar tumor position during the 24 h of measurements. The signal was recorded every hour up to 6 h and once again at 24 h. Experimental parameters were: frequency, 1.2 GHz; microwave power, 24 mW; center field, 50 mT; sweep, 26 mT; modulation field, 100 kHz; modulation amplitude, 0.42 mT; acquisition time, 60 s.

5.9. In vivo imaging

All MR imaging experiments were performed at 4.7 T on a Bruker Biospec (Ettlingen, Germany) with a whole-body radiofrequency coil for excitation and signal reception. A T_2 -weighted rapid acquisition relaxation-enhanced sequence (relaxation time, TR , 4090.7 ms; echo time, TE , 50.5 ms) was used to provide anatomical images of the mice. Then a multi-spin-echo-sequence was applied for accurate assessment of signal intensities (SI) and T_2 relaxation times before and after contrast agent injection: TR , 3197.4 ms; TE , 6.69 ms; number of echoes, 30; field of view, 4 cm; matrix, 128×128 ; slice thickness, 2 mm; bandwidth, 50 kHz; average, 1; total acquisition time, 6 min, 49 s. Mice were imaged by MRI 4 h after the irradiation. Mice were anesthetized using isoflurane and a pre-contrast image was taken. At time 0, the USPIO were injected through a catheter and an acquisition was launched every 30 min for up to 3 h. Contrary to the L-band EPR experiment, 3 h was the endpoint of this MR imaging study because it was not possible to keep the mouse under anesthesia any longer. It was important to keep the mouse in exactly the same position in order to allow ensuing image subtraction between pre- and post-contrast images. As a control, we placed a phantom tube containing CuSO_4 (100 mg/l) alongside the animals. The mean SI of the tumors was measured by hand-drawn regions of interest (Paravision, Bruker) on images arising from the tenth echo ($TE = 67$ ms) of the multi-echo sequence. Subtraction images were obtained by subtracting post-contrast images from corresponding pre-contrast images. T_2 relaxation times for tumors were calculated from the multi-spin-echo datasets using MATLAB. T_2 values were obtained by an exponential fit of the signal amplitudes versus echo time.

5.10. Statistical analysis

All data are shown as means $\pm$ standard errors of the mean. Comparisons between groups were made with a two-way ANOVA test and a p -value less than 0.05 was considered as significant. The following symbols are used in the figures: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Acknowledgements

This work is supported by grants from the Belgian National Fund for Scientific Research (FNRS), Televie grants (7.4597.06), the Fonds Joseph Maisin, the Saint-Luc Foundation, the Fondation contre le Cancer, the 'Actions de Recherches Concertées-Communauté Française de Belgique-ARC 04/09-317 and 05/10-335', and the 'Pôle d'attraction Interuniversitaire PAI VI' (P6/38 and P6/29).

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- 3) Early assessment of tumor response to irradiation: comparison of tumor models presenting different sensitivities to radiotherapy

Early assessment of the degree of tumor response to irradiation by using MRI and phosphatidylserine-targeted USPIO particles.

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Submitted to MRI.

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Running title:

Early assessment of tumor response to irradiation.

Grant support:

This work is supported by grants from the Belgian National Fund for Scientific Research (FNRS), Televie grants (7.4597.06), the Fonds Joseph Maisin, the Saint-Luc Foundation, the Fondation contre le Cancer, the "Actions de Recherches Concertées-Communauté Française de Belgique-ARC 09/14-020 and 05/10-335", and the « Pôle d'attraction Interuniversitaire PAI VI (P6/38 and P6/29) »

ABSTRACT

Purpose: In a previous study, a new molecular marker of apoptosis has been evaluated in vivo by MRI. This new contrast probe is based on ultrasmall particles of iron oxide particles (USPIO) that were coupled to the phosphatidylserine-targeted peptide E3. In the present study, the impact of irradiation on different tumor models, presenting different responses to radiotherapy, was assessed by monitoring tumor cell death.

Materials and methods: Three murine tumor models presenting different degrees of radiosensitivity were used: a radioresistant fibrosarcoma (FSaII), a moderately radiosensitive hepatocarcinoma (TLT), and a highly radiosensitive lymphoma (EL4). Five animals per group were used and tumor cell death was induced by X-ray irradiation (10 Gy). Mice were bearing two tumors, one in each leg. One tumor was irradiated while the other tumor was not treated and was used as an internal control. The accumulation of the contrast agent in tumors was determined on T₂-weighted MR images, by histology and by Electron Paramagnetic Resonance (EPR) spectroscopy 24 hours after irradiation.

Results: Radioresistant FSaII tumors accumulated USPIO-E3 to the same extent when irradiated or untreated, whereas TLT and EL4 tumors showed a marked increase in USPIO-E3 uptake after irradiation. These MRI results were confirmed by histology and EPR.

Conclusions: USPIO-E3 particles were able to distinguish different levels of radiotherapy-induced response in experimental tumor models. This molecular targeted system could constitute a potential biomarker for the early assessment of tumor response to treatment, and should be further characterized with a larger panel of cytotoxic treatments.

Key words: MRI, EPR, cell death, molecular imaging, tumor response.

INTRODUCTION

The standard procedure in the evaluation of tumor response to treatment is based on measurements of tumor size (1). Functional imaging, assessing physiological or molecular processes, could assess therapeutic response long before the occurrence of morphological changes. To assess treatment efficacy, in addition to morphological imaging, several techniques are currently available: dynamic imaging of tumor vascularisation, diffusion-weighted magnetic resonance imaging (DW-MRI) of tumor cellularity, magnetic resonance spectroscopy (MRS) of molecular components in tumors, positron emission tomography with radiolabeled [^{18}F]-fluoro-deoxy-glucose (FDG-PET) to provide information about the glucose metabolism of tissues or apoptosis detecting imaging using radiolabeled annexin A5 (anxA5) (2-6), caspase-3 seeking tracers (7-9), ApoSense probes (10,11) or phosphatidylserine (PS) recognizing peptides and proteins. Among the latter, novel approaches include the C2A domain of synaptotagmin I that binds to anionic phospholipids in cell membranes (12,13), zinc coordination complexes (14) and cationic liposomes (15).

In a previous study, peptide-expressing bacteriophages were used to detect phosphatidylserine in some treatment-receptive tumor cells. After phage display, the affinity and specificity of the hexapeptide E3 (TLVSSL) for the PS molecule was tested and competition assays with anxA5 were carried out on PS-coated ELISA plates (16). In our previous study, the PS-targeted peptide E3 was conjugated to the surface of PEGylated ultrasmall iron oxide particles (USPIO), a magnetic reporter detectable by MRI and also by electron paramagnetic resonance (EPR). EPR spectroscopy has already been proposed as a method of quantifying the accumulation of iron oxide inside tissues (17). PEGylation of USPIO particles improved blood circulation time and therefore potential nanoprobe accumulation in the tumor (18, 19). The ability of PEGylated USPIO-E3 to specifically detect cell death by EPR and MRI has already been demonstrated *in vitro*, *ex vivo* and *in vivo*. In that former study, a set of control experiments was performed to confirm the selectivity of recognition; including control groups and non-targeted USPIO particles (20). We also previously established that the accumulation of USPIO-E3 in irradiated tumors correlated well with an increase in apoptotic cells (20).

The aim of the present study was to assess the value of USPIO-E3 to detect different degrees of responsiveness to radiotherapy. For that purpose, three experimental tumor models presenting different degrees of sensitivity to radiotherapy were used to evaluate whether this contrast agent was able to distinguish between different levels of cell death. Tumor models

include a highly radiosensitive EL4 lymphoma (21), a moderately radiosensitive TLT tumor (22), and a radioresistant fibrosarcoma FSaII (23). Two tumors were injected per mouse, one in each gastrocnemius muscle to provide a non irradiated control tumor for each mouse. Conjugation of FITC, a fluorescent dye, to the USPIO-E3 nanoparticles enabled to achieve histological correlation to magnetic resonance imaging.

MATERIALS AND METHODS

Targeted iron oxide nanoparticles

PEGylated nanoparticles grafted with the E3 peptide (with or without FITC) were synthesized as previously described (20). The physicochemical properties of the USPIO-E3 included a hydrodynamic size of 24 nm, an r_1 of 32.9 mM⁻¹s⁻¹ and r_2 of 72.1 mM⁻¹s⁻¹ at 20 MHz (37°C), and an r_1 of 15.5 mM⁻¹s⁻¹ and r_2 of 70.4 mM⁻¹s⁻¹ at 60 MHz (37°C).

Animal tumor models

Three different murine tumor models were implanted i.m. into both rear leg muscle of 4-weeks-old male mice (Janvier, France): hepatocarcinoma cells “TLT” (ascites; 24) were injected in NMRI mice, fibrosarcoma cells “FSaII” ($2 \cdot 10^7$ cells/ml; 25) were injected into C3H mice and lymphoma cells “EL4” ($2 \cdot 10^7$ cells/ml; ECACC, United Kingdom) in C57/Bl6 mice. Minimum five mice were used for each group. The procedure was approved by a local ethics review committee according to national animal care regulations.

In vivo magnetic resonance imaging

All tumor models were used for in vivo MRI experiments (n=5-7). When tumor diameters reached the size of 8 ± 0.5 mm, tumors were treated by X-ray irradiation (RT-250 device, Philips Medical Systems, Hamburg, Germany). Animals were anesthetized by isoflurane inhalation (2.5% in air for induction and 2% for maintenance). The tumor of the right rear paw was placed in a 3x3 cm lead-shielded irradiation field and tumors were irradiated with 10Gy (1.2Gy/min). The tumor of the left paw muscle was used as an internal control. The day after radiotherapy, mice were again anesthetized with isoflurane and placed in a whole-body antenna of a MRI system (Bruker Biospec 4.7T, Ettlingen, Germany). A CuSO₄ (100mg/liter) phantom, used as reference tube, was placed between the paws of the mouse. The position of the mouse was checked by coronal and axial anatomical slices using a T₂-weighted fast spin-echo sequence (repetition time (TR): 4090.7 ms; echo time (TE): 50.5 ms; matrix:128x128;

field of view (FOV): 4x4cm; slice thickness: 2mm; total acquisition time (TA): 1 min 2 sec). Pre-contrast images were taken by a multi-echo T₂-weighted fast spin echo sequence (TR: 3197.4 ms; TE: 6.69 ms; number of echoes 30; matrix: 128x128; FOV: 4x4cm; slice thickness: 2mm; bandwidth 50 kHz; average 1; TA: 6 min 49 sec). The multi-echo sequence collects several data points at different echo times and T₂-weighting increases with the echo time (30 TE points from 6.69-200.7ms with a 6.69ms interval). After the pre-contrast image the PS-targeted contrast agent USPIO- E3 or USPIO-E3-FITC for ensuing histological correlation was injected at a dose of 7.7mgFe/kg through the tail vein of mice via a catheter to maintain the animal in the same position. Several post-contrast images were acquired by the same multi-echo sequence at 0, 0.5, 1, 2 and 3 hours post contrast. Mean signal intensity was measured within hand-drawn regions of interest (ROI), delimitating the tumor region and expressed as a percentage of the signal intensity measured in the corresponding pre-contrast images (Paravision, Bruker). Relative intensities were calculated using the intensity of the phantom tube as reference. Subtraction of images, evaluation of the transversal relaxation time T₂ and T₂ mapping were obtained using a home-developed routine in MATLAB.

Iron oxide determination by X-band EPR

After MRI experiments, the same tumors were also used for iron oxide measurement by EPR spectroscopy. For that purpose, mice were sacrificed immediately after MRI experiments, tumors were excised, weighted and frozen at -180°C. After freeze-drying, the tumors were crushed into a fine powder and inserted in the cavity of an X-band EPR spectrometer (Bruker, EMX®, 9.4 GHz) for sensitive quantification of the iron concentration (n=3-5). Except for thin tissue homogenates, freeze-drying is an essential step for in vitro or ex vivo X-band EPR because of the dielectric loss observed in large aqueous samples. Iron content per mg of tumor was calculated using an established calibration curve, as previously described (24). The EPR parameters used were: frequency, 9.4 GHz; microwave power, 5.05 mW; center field, 3150 G; field width, 5000 G; modulation amplitude, 30.81 G; time constant, 20.48 ms; conversion time, 20.48 ms; modulation field, 100 kHz; total acquisition time, 83 s. Measurements were performed at room temperature. Iron content ranged from 20 to 50 ng of iron per mg of tissue.

Histology

After MRI experiment, two mice per tumor model were assigned to histology. Tumors were rapidly excised at the end of the in vivo imaging experience, embedded in OCT freeze medium (Tissue Tek®, Sakura Finetek, Zoeterwoude, The Netherlands) and frozen at -180°C.

Five-micrometer tissue slides were cut, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and slides were observed under a fluorescence microscope (Zeiss Axioskop) to confirm presence of USPIO-E3-FITC in the tumor sections.

Statistical Analysis

All results were expressed as mean $\pm$ SE. EPR and T_2 results were compared by a two-tailed student test and MRI signal loss curves by a two-way ANOVA with $p < 0.05$: ★, $p < 0.01$: ★★; and $p < 0.005$: ★★★.

RESULTS

In vivo magnetic resonance imaging

Pre- and post-contrast images were compared to evaluate the contrast enhancement in untreated tumor and irradiated tumor grown in the same animal. The signal reduction in T_2 -weighted imaging and shortening of the T_2 value were used to follow and estimate the accumulation of USPIO-E3 nanoparticles in the tumor area. After image subtraction (pre-post), the strongest signal was obtained for the irradiated EL4 tumor which is the most radiosensitive tumor (Fig 1A). The analysis of the regions of interest showed that, 3 hours after injection of the contrast agent, signal loss in treated versus untreated mice was 54% versus 39% for EL4, and 44% versus 31% for TLT (Fig 1B). In FSaII-bearing mice, the signal loss was identical in irradiated and untreated tumors. Multi-echo T_2 images of axial slices were used for calculation of T_2 maps (Fig 2A). Quantitative analysis of the changes in T_2 relaxation times ($\Delta T_2 = T_2$ before injection – T_2 after injection of USPIO-E3) in tumors revealed significantly higher shortening of T_2 in irradiated TLT and EL4 tumors than in control tumors, indicating a higher uptake of targeted nanoparticles (Fig 2B). Again, there was no difference in ΔT_2 between irradiated and untreated radioresistant FSaII tumors. We also observed that the effect of USPIO-E3 on T_2 -shortening in FSaII tumors was comparable to the effect observed in the treated radiosensitive tumors EL4 and TLT (Fig 2B).

Iron oxide determination by X-band EPR

EPR provides a simple and highly sensitive approach to determine the iron oxide concentration inside the tumor (Fig 3). Differences in iron oxide accumulation (untreated versus irradiated) were significant only for the two radiosensitive tumor models EL4 and TLT, where the iron concentration doubled after irradiation. The differences between

irradiated tumors and untreated tumors were not significant for the radioresistant FSaII model. Moreover, it could be observed that the % increase in iron oxide content in tumors in irradiated tumors compared to untreated tumors was in accordance with their radiosensitivity (EL4>TLT>FSaII) (Fig 3).

Histology

Histology on tumor specimens harvested after MRI studies confirmed the presence of USPIO-E3-FITC in irradiated tumors (Fig 4). In control tumors, only a few nanoparticles were found for EL4 and TLT. There was a large increase in USPIO-E3-FITC after irradiation in these two tumor models. In the radioresistant FSaII model, the fluorescent staining was almost the same for treated and untreated tumors. Moreover, the fluorescent staining was rather large in untreated FSaII tumors.

DISCUSSION

Individualization of treatment in patients will largely benefit from the availability of biomarkers that can early assess the response of tumors to cytotoxic treatments. In our previous work, we already evaluated the value of iron oxide particles targeted at phosphatidylserine, USPIO-E3, to detect treatment-induced apoptosis in vitro and in vivo: Increased accumulation of PS-targeted particles in treated TLT cells correlated well with FITC-labeled AnxA5 binding in vitro, as shown by flow cytometry, and with apoptotic cell fractions quantified on TUNEL-stained histological slices, whereas no increase was found in irradiated tumors when using ungrafted control particles or USPIO particles linked to a scrambled peptide (20).

To mimic different clinical situations in this new study, we used 3 different tumor models which present different degrees of response after irradiation. To get an unambiguous assessment, we used a multi-modal approach: the accumulation in treated and untreated tumors was achieved by the measurement of iron oxide by EPR spectroscopy; fluorescent particles were visualized by histology; and the effect of the contrast agents was determined by T₂-weighted MRI sequences. Every mouse was injected with two tumors, whereof one was irradiated and the other one served as untreated control tumor within the same animal. In all experiments, it was observed that accumulation of USPIO-E3 was increased in irradiated tumors, compared to untreated tumors, in the two radiosensitive tumor models TLT and EL4. This was in sharp contrast with the results obtained using the radioresistant fibrosarcoma

FSaII: the irradiation did not lead to any increase in the accumulation of USPIO-E3 in irradiated tumors compared to untreated tumors. Another observation is the rather large accumulation of USPIO-E3 in untreated FSaII tumor model. Histology and analysis of MRI measurements (signal loss, T_2 evaluation and T_2 mapping) showed clearly that accumulation of particles targeted to cell death is as high in treated and control FSaII tumors as in irradiated TLT and EL4 tumors. We first assumed that the initial level of PS-exposure in this type of tumor is relatively high even before radiotherapeutic treatment. This result would be concordant with some publication, wherein authors found out that treatment-resistant solid tumors can paradoxically present high levels of apoptosis (26). On the other hand, other groups correlated high apoptotic levels at baseline with positive treatment outcome, as demonstrated by tumor regrowth delay (27,28). After browsing the literature, we found two publications that reported apoptotic fractions of 0.3% for FSaII and 6% for EL4 in untreated control tumors (29,30), meaning that higher spontaneous levels of apoptosis in FSaII can not be the correct explanation for the observed results. Possibly, the increased accumulation is simply due to the fact that different mouse strains were used in this study, which can lead to an inter-strain variability in kinetic profiles, perfusion or permeability. Nevertheless, the USPIO-E3 biomarker enabled to point out treatment resistance of the FSaII model, as the accumulation of targeted particles did not increase after irradiation, contrary to the radiosensitive models TLT and EL4.

Some minor discrepancies were observed using the different techniques. The relative difference between treated and untreated EL4 and TLT tumors was more important with X-band EPR than with MRI. With EPR, we could observe that the USPIO-E3 accumulation was twice as high in irradiated tumors compared to control tumors, whereas for MRI the signal loss effect after irradiation was only about 40% higher than for untreated tumors. This is probably due to the fact that EPR measurements have no background signal in the conditions used (room temperature, EPR signal specific of iron oxide particles).

A likely limitation in the use of USPIO-E3 is the partly non specific uptake of this compound by the tumors. Previously, we tried to improve the pharmacokinetic properties by grafting PEG chains to the particle surface, and we reported that the accumulation in liver and spleen was less pronounced and that plasma half-time was longer for PEGylated USPIOs compared to unPEGylated USPIOs (19). Yet, the accumulation in untreated tumors could be due to several factors: accumulation can stem from apoptotic and necrotic cells already present in the tumor before any treatment was launched; it could be due to an unspecific nanoparticle-uptake by the macrophages present at the tumor site; another possible role is

probably played by the so called enhanced perfusion and retention (EPR) effect (31). The nanoparticles will be guided in a non specific manner to the tumor site because they can extravasate through the leaky endothelium of tumor vessels but not penetrate through vessels in normal tissues. This could cause non specific retention of the contrast agent inside the tumor region because of slow venous return and low lymphatic drainage.

Another question is to know if the accumulation of USPIO-E3 in irradiated tumors correctly reflects the cytotoxic effect of the irradiation. Besides cell toxicity and induced apoptosis, X-ray irradiation may produce changes in tumor blood flow (32,33) and increase macrophage presence at the tumor site (34,35). This fact could misleadingly increase the accumulation of the targeted contrast agent in irradiated tumors. However, in our previous study on TLT tumors (20), we found out that there was a large increase in uptake USPIO-E3 after irradiation, an effect that was not observed using non-targeted USPIO particles or particles grafted with a scrambled peptide. Now, transferring this approach into a clinical tool will still face the problem of correlating the accumulation of USPIO-E3 to cell death. In our study, we used two tumors in the same animal which gave us a direct comparison of the distribution of the contrast agent in control and treated tumors. In practice, there will be the difficult task to link a given parameter (i.e. change in T_2 after administration of USPIO-E3) and the response of the tumor to the treatment. In patients, it could be interesting to have longitudinal follow-up of a tumor during the treatment to monitor its response. After injection of magnetic reporters such as USPIO-E3, longitudinal follow-up will require several days between MRI protocols to allow the clearance of the contrast agent. This specific point will require further research to characterize the pharmacokinetic properties of USPIO-E3 in slowly growing tumors to determine the wash-out period. Another interesting perspective will be to compare the value of USPIO-E3 with other markers of cell death such as DW-MRI. The combination of several imaging technologies could also be helpful to evaluate anticancer treatment outcome.

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FIGURE LEGENDS

Figure 1:

A: Axial slices of T_2 -weighted MR imaging in tumor-bearing mice before (pre) and 3 hours after injection of the PS-targeted contrast agent (3h post). The column “pre-post” shows the resulting subtraction images, on which the highest accumulation of USPIO-E3 is represented by red-colored areas. The red encircled area indicates the irradiated tumor (right paw), the white line delimits the zone of the untreated tumor which served as control (left paw) and a reference tube (R) was placed between both rear paws.

B: MR signal intensity loss was followed in function of time in murine tumors after i.v. injection of the phosphatidylserine-targeted contrast agent USPIO-E3. Black squares represent the irradiated tumors and white squares the untreated tumors. Three tumor models with different radiosensitivities were used: EL4 (a), TLT (b) and FSaII (c).

Figure 2:

A: Anatomical MR black and white images of mice overlaid by colored T_2 maps of the tumors. Images show the treated (right paw, red circle) and untreated (left paw, white circle) tumors of the three different tumor models (EL4, TLT, FSaII).

B: Diminution in transversal relaxation time T_2 caused by the presence of the negative contrast agent USPIO-E3 3 hours after injection. Black bars represent the irradiated tumors and white bars the untreated tumors and $\Delta T_2 = T_{2\text{pre}} - T_{2\text{post}}$.

Figure 3:

Measurement of USPIO-E3 accumulation in tumors ex vivo by X-band EPR. Mice bearing three different types of tumors (EL4, TLT, FSaII) were intravenously injected with the contrast agent targeting cell death. The bars represent the iron oxide accumulation in irradiated tumors expressed as percentage relative to the accumulation in untreated control tumors.

Figure 4:

Histologic evaluation of USPIO-E3-FITC accumulation in irradiated (D, E and F) and untreated control tumors (A, B and C). Fluorescent staining corresponding to FITC-labeled USPIO-E3 accumulation was observed in FSaII (A, D), TLT (B, E) and EL4 (C, F) tumor models 3 hours after i.v. injection of the contrast probe. PS-positive nuclei are stained in green (magnification: 400x).

FIGURES

Figure 1

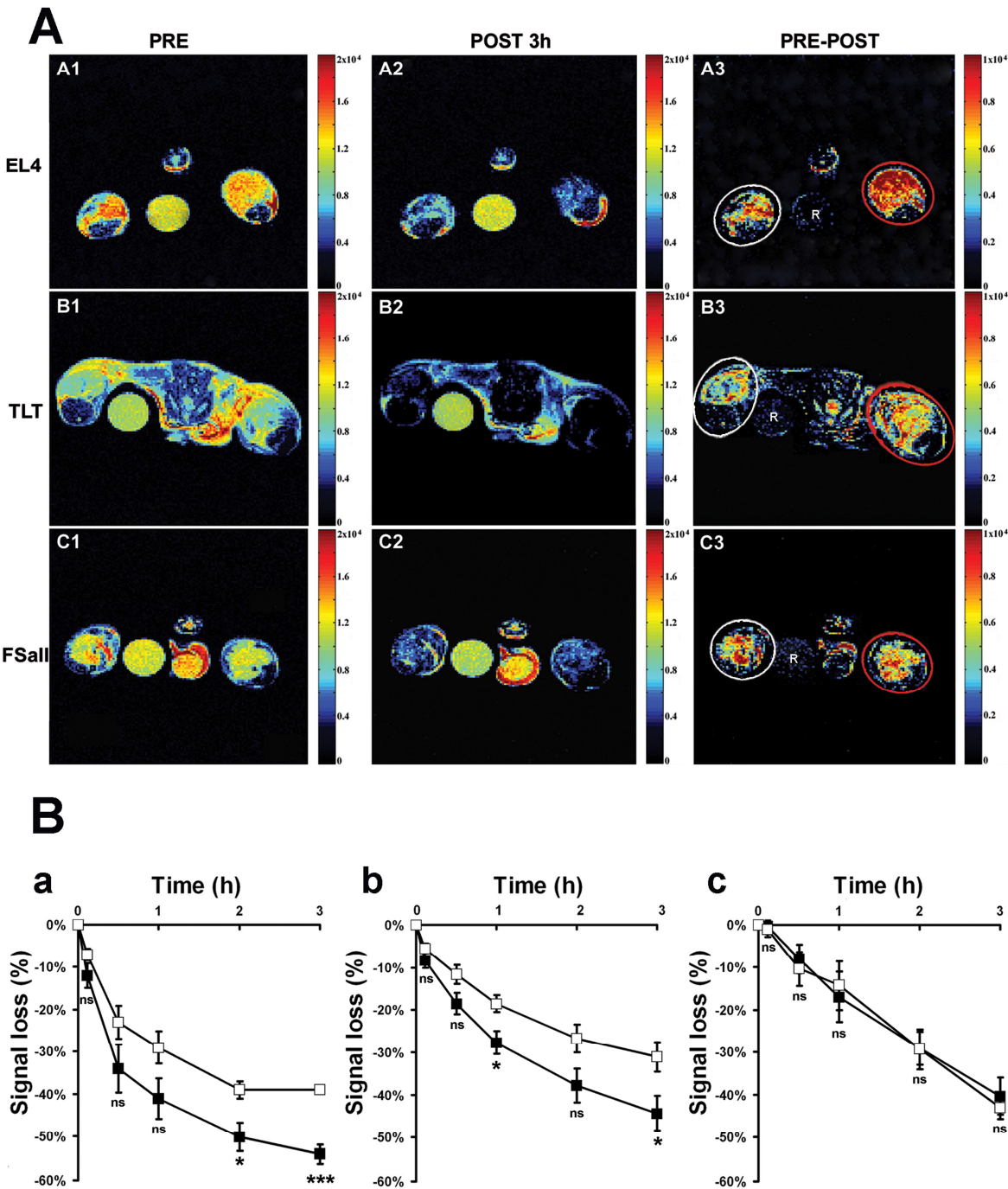


Figure 2

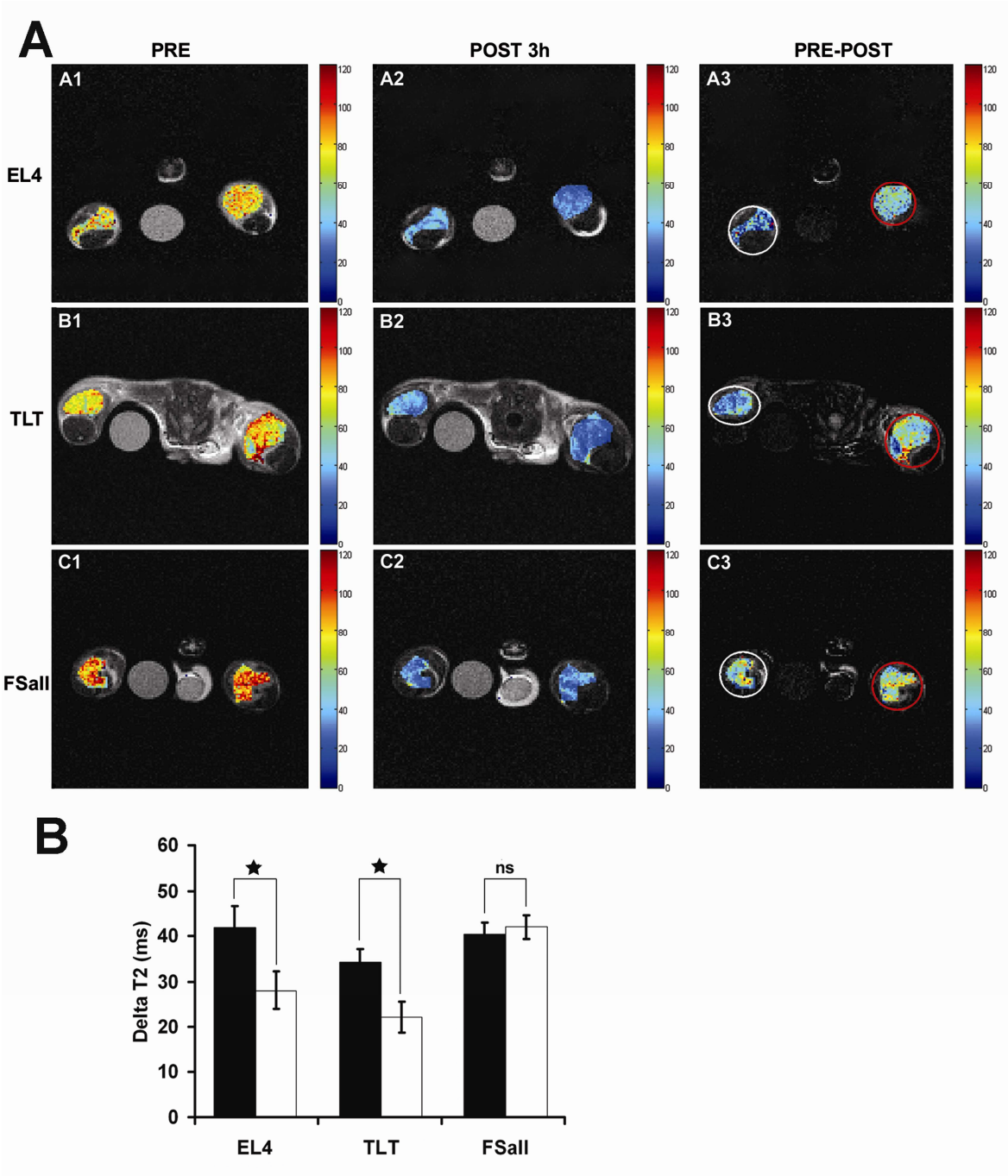


Figure 3

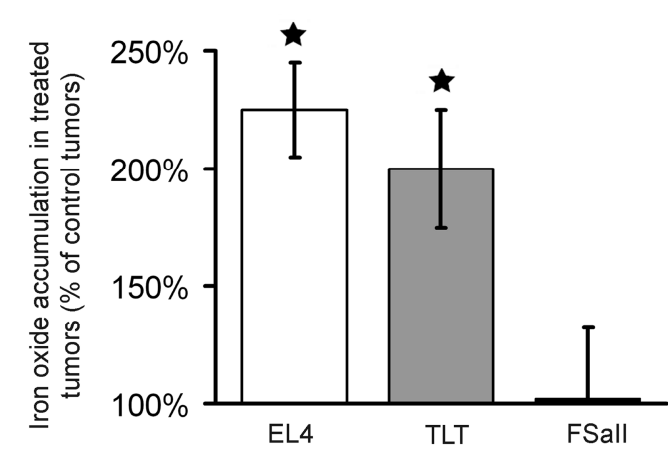
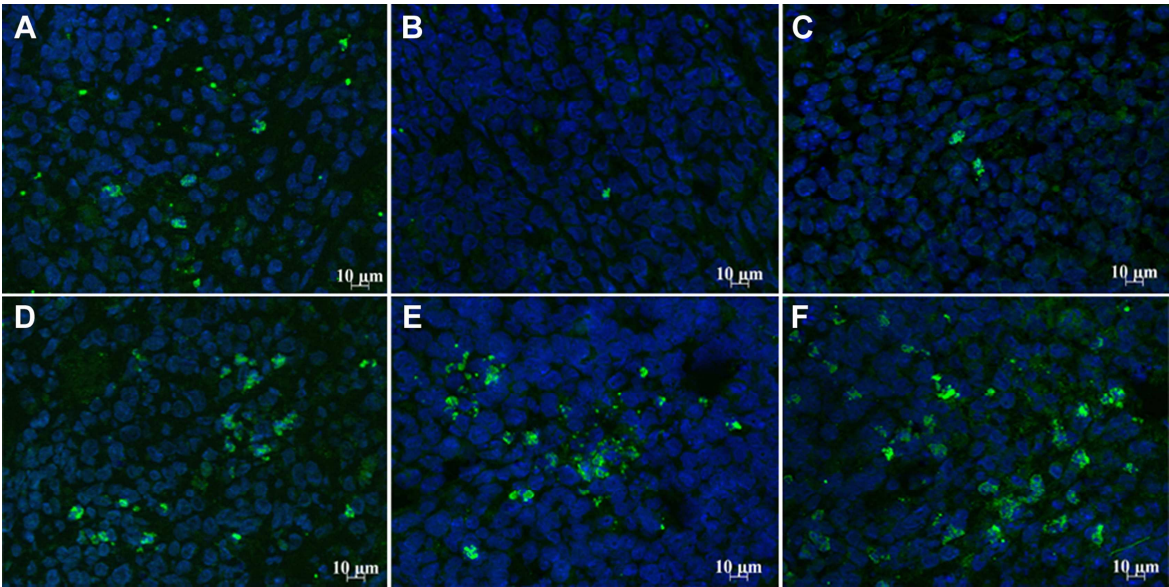


Figure 4



- 4) Comparing the new biomarker with other MR modalities used for evaluation of anti-cancer treatment outcome, such as proton spectroscopy of choline and diffusion-weighted MRI

**Multi-modal Assessment of Early Tumor Response to Chemotherapy:
Comparison between Diffusion-Weighted MRI, ¹H-MR Spectroscopy of Choline and
USPIO Particles Targeted at Cell Death.**

K.A. Radermacher¹, J. Magat¹, C. Bouzin³, S. Laurent², T. Dresselaers⁴,
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Short title: Early assessment of tumor response to chemotherapy.

Keywords: Tumor response, EPR, MRI, diffusion-weighted imaging, choline MR spectroscopy.

Acknowledgements: This work is supported by grants from the Belgian National Fund for Scientific Research (FNRS), Televie grants (7.4547.08), the Fonds Joseph Maisin, the Saint-Luc Foundation, the Fondation contre le Cancer, the "Actions de Recherches Concertées-Communauté Française de Belgique-ARC 09/14-020 and 05/10-335", and the « Pôle d'attraction Interuniversitaire PAI VI (P6/38 and P6/29) ».

ABSTRACT

The aim of the present study was to determine the value of different Magnetic Resonance protocols to assess the early tumor response to chemotherapy. For that purpose, we used a murine tumor model (TLT) presenting different degrees of response to three different cytotoxic agents. As demonstrated by survival curves, cyclophosphamide (CP) was the most efficient drug, followed by 5-fluorouracil (5-FU), whereas the etoposide treatment had almost no impact on TLT tumors. Different MR protocols were used at 9.4 Tesla 24 hours post-treatment: Diffusion-Weighted (DW)-MRI, choline measurement by ^1H MRS and contrast-enhanced MRI using ultrasmall iron oxide nanoparticles (USPIO) targeted at phosphatidylserine. The accumulation of the contrast agent in apoptotic tumors was monitored by T_2 -weighted images and quantified by EPR spectroscopy. Necrosis and apoptosis were also assessed by histology. Large variations were observed in the measurements of choline peak area and could not be directly correlated to tumor response in our study. Although the targeted USPIO particles were able to significantly differentiate between the efficiency of each cytotoxic agent and correlated the best with the survival endpoint, they present the main disadvantage of non specific tumor accumulation which could be a source of problems when transferring the method into the clinic. DW-MRI presents a better compromise by combining the advantage of allowing longitudinal studies along with a high dynamic range, but DW-MRI was not able to show any significant effect for 5-FU. This work illustrates the need for multi-modal imaging in assessing tumor response to treatment to compensate for individual limitations.

Abbreviation list: 5-FU, 5-fluorouracil; ADC_w, apparent diffusion coefficient of water; CP, cyclophosphamide; CTL, control; DW, diffusion-weighted; EPR, electron paramagnetic resonance; ET: etoposide; FLASH, fast low angle shot; H&E, haematoxylin and eosin; PEG, polyethylene glycol; PRESS, point-resolved spectroscopy; PS, phosphatidylserine; RARE, rapid acquisition with relaxation enhancement; ROI, region of interest; SI, signal intensity; TLT, transplantable liver tumor; TUNEL, terminal deoxyribosyl transferase dUDP nick end labeling; USPIO, ultrasmall particles of iron oxide.

INTRODUCTION

The strategy of anticancer treatments is based on the type of cancer, the location and the grade of the tumor, stage of disease, as well as the general condition of the patient (1). For this reason the best treatment has to be found for each individual patient. The prediction of tumor response to therapy is usually determined from measurements of tumor size evidenced by morphological imaging (2). However, tumor shrinkage can take several weeks to manifest. Therefore earlier indication of tumor treatment response has become a major area of research. One method widely used in clinic relies on the decrease in [^{18}F]-fluorodeoxyglucose uptake in treatment-sensitive tumors by positron-emission tomography (3,4).

Imaging of apoptosis also allows an early evaluation of treatment response in cancer patients. During early apoptosis, the phosphatidylserine (PS) exposure on the outer leaflet of the cell membrane serves as a recognition signal for phagocytes (5). To assess apoptosis *in vivo*, the use of radiolabeled annexin A5 has been the most popular. Clinical trials using radiolabeled annexin A5 have been carried out in cancer patients (6-10). Other compounds targeting the early loss of cell membrane asymmetry and exposure of phosphatidylserine on the cell surface are peptides isolated by phage display (11-13) and the C2A domain of synaptotagmin I (14). Other potential tracers are caspase-3 specific binding molecules (18,19), or small amphiphatic molecules of the so-called ApoSense group that accumulate inside the cell from where they emit a fluorescent signal (20,21). The PS-targeting hexapeptide, E3, has been isolated by phage display on apoptotic murine livers and tested for its selectivity and binding capacity to PS (11). This peptide has been coupled to pegylated ultrasmall particles of iron oxides (USPIO), since it has previously been demonstrated that USPIO pegylation was able to prolong blood circulation time and therefore to increase the possibility of achieving its target by a decreased hepatic capture (22). This new MR contrast agent (USPIO-E3) was already tested *in vitro*, *ex vivo* and *in vivo* on irradiated transplantable liver tumors (23). Results from this previous study showed that due to the targeted superparamagnetic particles, distinction between irradiated and control tumors could be made by magnetic resonance imaging (MRI) and electron paramagnetic resonance (EPR).

Other MR methods, such as diffusion-weighted MRI (DW-MRI) and ^1H MR spectroscopy, have been proposed to assess tumor response to treatments (24). Biophysical phenomena such as molecular diffusion can be useful in assessment of cell viability and integrity (25). Physical boundaries such as cell membranes and subcellular structures restrict diffusion. DW-MRI can evaluate tumor response to radiation and chemotherapy (26-29).

Biochemical processes such as metabolism of membrane choline can be monitored by MR spectroscopy in tumors in vivo to provide metabolic information on tumor cell proliferation and membrane degeneration (30-32). High levels of choline-containing compounds have been observed in tumors by in vivo ^{31}P - and ^1H MRS (33-35) which can be reversed after successful treatment. Choline is being explored as biomarker for tumor diagnosis, staging and clinical response monitoring (36,37).

Because there is a critical need for comparing the respective value of imaging biomarkers that may early assess the response of tumors to cytotoxic treatments, our study was designed to compare various MR methods applied to one tumor model that presents different degrees of response to different chemotherapeutic agents (cyclophosphamide, 5-fluorouracil and etoposide). In this study, we compared DW-MRI, ^1H MRS and the recently developed contrast agent USPIO-E3 targeted at phosphatidylserine.

EXPERIMENTAL

Animals

Transplantable liver tumors (TLT) were induced in the gastrocnemius muscle of 5-week-old male NMRI mice (Elevage Janvier, France). Mice were divided in 4 groups, one untreated control group and three different treatment groups. When the hind leg diameter reaches a size of $8\pm 0.5\text{mm}$, mice were either treated with cyclophosphamide (CP: 160mg/kg i.p.), with 5-fluorouracil (5-FU: 100mg/kg i.p.) or with etoposide (ET: 20mg/kg i.p.). All cytotoxic agents used in this study were obtained from St. Luc Hospital (Brussels, Belgium). USPIO-E3 particles were prepared from nanoparticles with carboxylated groups on the surface as previously described (38). USPIO particles were functionalized in two successive steps with the E3 peptide (TLVSSL, Neomps, Strasbourg, France), and then with an aminoPEG 750 (Fluka, Bornem, Belgium). Characterization of the contrast probe has previously been described (23). For EPR and T_2 -weighted MRI experiments, the contrast agent was intravenously injected (7.7mg Fe/kg) 21 hours after injection of the respective treatment. The procedure was approved by a local ethics review committee according to national animal care regulations.

Survival Curve

At day 0, tumor-bearing mice (n=8-9/group) were intraperitoneally injected with one of the chemotherapeutic drugs or remained untreated. The diameter of the hind leg was measured every day in order to visualize a potential tumor growth delay occurring after treatment. When the rear leg diameter reached a size of 15mm, mice were sacrificed and this day was considered as endpoint of the survival curve.

USPIO-E3 quantification by EPR

Iron oxide particles were injected into the tail vein of mice, 21 hours after injection of the chemotherapeutic agent. The animals (n=10-11/group) were sacrificed three hours after contrast agent injection and tumors were excised and weighted. The excised tumors were freeze-dried and crushed into a fine powder. The powder was weighted and then placed into the X-band EPR cavity. We calculated the iron oxide content in each tumor by comparison with a calibration curve. The instrument settings were: Frequency: 9.4GHz; microwave power: 5.05mW; center field: 3150G; field width: 5000G; modulation amplitude: 30.81G; time constant: 20.48ms; conversion time: 20.48ms; modulation field: 100kHz; total acquisition time: 83s. Measurements were performed at room temperature.

MR

All MR experiments were performed using the same 9.4T small animal Biospec MR system (Bruker, Ettlingen, Germany) equipped with a 20cm horizontal bore, a whole body transmitter and a surface coil receiver for signal reception. The animals (n=4-5/group) were anesthetized by isoflurane inhalation (4% in O₂ for initiation and 1-2% in O₂ for maintenance; isoflurane was provided by St Luc Hospital, Brussels, Belgium) and their hind legs were fixed by clay. Animals were lying on a warm water bed to maintain their body temperature at 37°C. A temperature probe was inserted in the rectum of mice and a pressure cushion was placed near to the chest of animals to monitor respiration. Positioning of the mice was checked using a FLASH sequence in the three directions of the space (TR/TE: 3000/200ms). A T₂-weighted axial turbo RARE sequence (TR/TE: 3500/9.065ms; effective TE: 27.20ms; RARE factor: 7) was used to provide anatomical images of the mice.

T₂-weighted MRI and T₂ measurements

A multi-spin-echo-sequence was applied for accurate assessment of signal intensities (SI) and T₂ relaxation times before and three hours after contrast agent injection (TR/TE: 2500/10ms; number of echoes: 16; echo spacing: 10ms; slices: 7; slice thickness: 1mm; interslice distance: 1.5mm; in-plane resolution: 0.117mm²/pixel; acquisition time: 8min). Mean SI of the tumors was measured within manually-drawn regions of interest (ROI) on images arising from the 7th echo (TE: 70ms) of the multi-echo sequence and normalized to the noise using a MatLab program (version 7.9). Mean T₂ relaxation times for tumors were also calculated from the multi-spin-echo datasets using MatLab. T₂ values were obtained by an exponential fit of the signal amplitudes versus echo time. T₂ maps were established on a pixel-by-pixel basis in the ROI.

Diffusion-weighted imaging

For DW-MRI, a transverse echo planar imaging sequence was utilized with the following acquisition parameters: TR/TE: 3000/27.96ms; duration of diffusion gradients (δ): 7ms; separation of diffusion gradients (Δ): 14ms; slice number: 7; slice thickness: 1mm; interslice distance: 1.5mm; in-plane resolution: 0.234mm²/pixel; acquisition time: 4min12s. DW images were acquired using b values of 0, 200, 400, 600, 800 and 1000 s/mm². The b value is equal to $\gamma^2 G_d^2 \delta^2 (\Delta - (\delta/3))$, where G_d is the strength of the diffusion weighting gradient and γ is the gyromagnetic ratio for protons. Mean apparent diffusion coefficient (ADC_w) and ADC_w maps were calculated from the DW images. By using a MatLab program, the exponential decay of signal as a function of the b-value was measured according to the Stejskal-Tanner equation,

$S = S_0 e^{-b \text{ADC}_w}$, where S_0 is the signal intensity achieved without gradient and S is the signal intensity with diffusion weighting.

Single voxel ¹H MRS

For single voxel spectroscopic data acquisition, voxel localization was performed on the corresponding anatomical images obtained by the FLASH and turbo RARE sequences. Proton MR spectra were acquired using a PRESS sequence (TR/TE: 2500/15ms). Voxel size of 2x2x2mm³ and 256 averages were used for MRS studies in all cases, the water-fat-shift was

0.52mm and a spectral resolution of 0.98Hz/point was achieved. Spatially localized saturation bands were used to suppress signal from surrounding areas (gap to voxel: 0.5mm). Field homogeneity was optimized over the selected volume of interest by shimming on the water signal for each tumor to a line width of <20 Hz. Automatic shimming and water suppression (VAPOR) were used. Acquisition time for spectroscopic studies was about 11 minutes.

MR data analysis

Review of T₂-weighted and DW images was performed on an independent workstation using the MatLab software to define ROIs. The same program was also used to establish signal intensity, T₂ and ADC_w maps and to calculate signal loss, transversal relaxation time T₂ and mean ADC_w values. Signal intensities, T₂ and ADC_w values were determined and averaged for every slice on which the tumor was seen. MR spectra were also analysed on an independent workstation using the Topspin software (version 2.0, Bruker Biospin). For data processing, Fourier transformation, 5Hz-linebroadening and phase and baseline correction were applied. Peak areas were measured for the choline peak (at 3.21 ppm). Metabolic ratios were calculated based on measurements of the unsuppressed water peak area. All results were finally expressed as percentage compared to the values obtained for the untreated control mice in order to allow an easier comparison between the different MR methods used in this study.

Histology

Tumor-bearing animals were treated with CP, 5-FU, ET or remained untreated and tumors were excised 24 hours post-treatment. Tumors (n=3/group) were either fixed in 4% paraformaldehyde for standard paraffin sections or embedded in O.C.T. compound for cryosectioning. In both cases the samples were cut into 5 µm sections. The paraffin sections were stained with H&E and photographed on a Zeiss MIRAX microscope to have a global overview of the necrotic regions in the tumors. The frozen slices were probed for apoptosis by TUNEL assay using a commercially available in situ cell death detection kit (Roche Diagnostics, Vilvoorde, Belgium). Nuclei were also counterstained with 4,6-diamidino-2-phenylindole (DAPI). Slides were photographed using a Zeiss Axioskop microscope equipped for fluorescence. Quantification of necrotic and apoptotic regions, expressed as percentage of the whole tumor area, was obtained using Image J.

Statistical analysis

All values were expressed as mean $\pm$ standard error. A logrank test was used to compare the survival curves. For other experiments the statistical differences between groups were evaluated by a two-way ANOVA. A P-value less than 5% was considered as significant, the following symbols are used in the figures: ★ $P \leq 0.05$, ★★ $P \leq 0.01$, ★★★ $P \leq 0.001$.

RESULTS

Survival curve

The chemosensitivity of TLT tumors to the different cytotoxic drugs (cyclophosphamide CP, 5-fluorouracil 5-FU, etoposide ET) was compared by evaluating survival after treatment. As shown in Fig.1, the response was higher when using CP compared to 5-FU, the response to 5-FU being higher than using ET. The ET curve was almost superimposed to the one obtained for the control tumors (CTL), thus the impact of ET treatment on these tumors at the dose of 20mg/kg is minimal. Compared to the CTL group, the survival was significantly higher for the CP-treated group ($p \leq 0.001$), significant for 5-FU-treated group ($p \leq 0.05$), and non-significant for the ET-treated group.

USPIO-E3 quantification by EPR

X-band EPR was used to quantify the accumulation of the targeted iron oxide particles. The amount of USPIO-E3 particles in excised TLT tumors was measured three hours after injection of the contrast agent and 24 hours after treatment administration (Fig. 2). The signal intensity of the iron oxide spectrum acquired in excised tumors was correlated to a calibration curve established before (data not shown). The accumulation of the cell death targeted USPIOs was significantly higher in CP- and 5-FU-treated tumors (74 ± 7 ng/mg and 64 ± 9 ng/mg, respectively) than in ET-treated and CTL tumors (40 ± 5 ng/mg and 33 ± 6 ng/mg, respectively).

T₂-weighted MRI and T₂ measurements

To examine further whether USPIO-E3 might serve as a selective MR contrast agent for cell death, mice were imaged 24 hours after injection of the chemotherapeutic drugs. MR images were obtained before and three hours after i.v. injection of the USPIO-E3 particles. On all post-contrast images, a signal decrease was observed in treated and control tumors (Fig 3A-B). However, the signal loss was more pronounced after CP- and 5-FU treatment than after ET treatment or in control tumors (CTL). Signal loss, normalized to the noise and expressed as percentage compared to the signal loss of untreated control tumors, are shown in Fig. 3C. Three hours after administration of the PS-targeted contrast probe the signal loss was $-11\pm1\%$ for CP-treated animals, $-3\pm2\%$ for 5-FU-treated mice and $-1\pm2\%$ for the ET-treated ones, when compared to the untreated tumors. To confirm these results, T₂ maps were established for each tumor slice and mean T₂ values were obtained from tumors before and 3 hours after administration of USPIO-E3. When compared to the T₂ shortening of control tumors (Fig. 3D), the T₂ shortening in CP-treated tumors was about $-11\pm1\%$, $-5\pm2\%$ in 5-FU-treated mice and $1\pm2\%$ for the ET-treated mice. The results are consistent with the ones obtained for tumor signal loss.

Diffusion-weighted imaging

After the use of an efficient anticancer treatment, tumor cellularity should be reduced and should result in an increase of the apparent diffusion coefficient of water (ADC_w). As a matter of fact, ADC_w maps show a higher intensity 24 hours after CP- and 5-FU treatment when compared to the inefficient ET treatment or to control tumors (Fig. 4A). When average ADC_w values of tumors were compared pre- and 24h post-treatment (Fig. 4B), a diminution of ADC_w was observed for inefficient etoposide treatment and for untreated control tumors, whereas the ADC_w value remained the same after 5-FU-treatment and even increased after CP treatment, representing a loss in tumor cellularity. All ADC_w values were also compared to the ADC of control tumors. As shown in Fig. 4C, mean ADC_w values were the highest in CP-treated tumors ($37\pm4\%$), then followed by the 5-FU-treated mice ($18\pm8\%$) and the lowest value was obtained for ET-treated mice ($10\pm6\%$).

Single Voxel ^1H MRS

Figure 5A displays typical ^1H MR spectra obtained from tumor-bearing mice before and 24 hours after CP treatment. It can be observed that the total choline peak is slightly decreased for the post-treatment spectrum compared to the pre-treatment spectrum. The choline peak areas were normalized to the corresponding unsuppressed water signal and then again compared to the control group (Fig. 5B). CP- and 5-FU-treated animals showed a higher decrease of the choline peak area after treatment ($-19\pm 22\%$ and $-18\pm 5\%$ respectively) than ET-treated and control mice ($-10\pm 14\%$ and $0\pm 4\%$ respectively). But because of the high standard error of the data obtained from the CP group, only the 5-FU-treated group was significantly different compared to the control group.

Histology

Figure 6 displays representative tumor slices after TUNEL (left column) and H&E (right column) staining for quantification of apoptosis and necrosis respectively. TUNEL staining of untreated and treated tumors clearly shows more positively stained cells after CP- and 5-FU treatment ($26\pm 8\%$ and $21\pm 4\%$ respectively) than after ET treatment ($10\pm 5\%$) or than in control tumors ($0.5\pm 0.4\%$). For quantification of necrosis, areas occupied by necrotic regions were measured on H&E stained slices. None of the three chemotherapeutic drugs was able to increase the initial level of necrosis compared to the control tumor where $20\pm 4\%$ of the tumor area displayed necrotic features (CP: $20\pm 4\%$, 5-FU: $21\pm 1\%$ and ET: $23\pm 3\%$).

DISCUSSION

The activity of different clinically relevant chemotherapeutic drugs (cyclophosphamide, 5-fluorouracil and etoposide) was evaluated on TLT tumor models using a cell-death targeted MR contrast agent, DW-MRI and single voxel ^1H MR spectroscopy of choline. Survival curves were first established to evaluate the impact of the treatment. Etoposide treatment had almost no effect on these tumors, and mice died at the same rate than untreated tumor-bearing mice. 5-fluorouracil treatment induced a short delay in tumor growth and cyclophosphamide was the most efficient treatment (two mice out of nine were even cured). Overall, this offered a panel of tumor responses to evaluate the efficacy of MR methods to early assess the response to a treatment.

USPIO-E3 particles induce considerable signal loss on T₂-weighted MR images due to strong magnetic susceptibility effects. We also used EPR spectrometry as a highly sensitive method to quantify the iron oxide content in the tumor without any interference from background (39,40). The iron content in tumors was correlated with signal loss and T₂ shortening measured on T₂-weighted MR images pre and post contrast. For both techniques, we observed statistically significant differences between the most successfully treated groups (CP and 5-FU) and the control group or the group treated inefficiently by etoposide. However, there was also a rather large iron oxide accumulation in untreated control tumors resulting in a large decrease in signal intensity. Overall, although significant, the difference in loss of signal intensity between responsive and unresponsive tumors was relatively small. In our previous study (23), we found out that USPIO-E3 particles presented a much higher affinity for dying cells (treated in vitro by staurosporine or in vivo by irradiation) than USPIOs grafted with a scrambled peptide or ungrafted USPIOs. Yet, the accumulation of USPIO-E3 in untreated or in unresponsive tumors is likely due to a non selective uptake by macrophages involved in tumor-related inflammation and to a rather large basal necrosis present in tumors before starting any treatment. The level of necrosis was indeed about 20% in untreated TLT tumors as shown by H&E histological staining. Since cell membrane integrity is lost during necrosis, the PS molecule becomes accessible and USPIO-E3 will potentially target necrotic cells. In practice, the basal uptake by untreated tumors may lead to uncertainty in determining areas with poor local response in tumors. Another possible explanation for non specific USPIO accumulation in tumors is the enhanced permeation and retention (EPR effect). Nanoparticles are known to passively diffuse and accumulate at sites with excessively leaky vasculature, such as in tumors, regardless of the fact that tumors are undergoing apoptosis or not. A parameter that will require further investigation in future work is the time needed to observe the washout of the USIO-E3 from the tumor in order to determine the frequency of injection of the contrast agent for chronic monitoring of cell death.

DW-MRI is completely non invasive and relies on intrinsic contrast mechanisms rather than the injection of a contrast agent. This characteristic allows a daily monitoring of tumor response to treatment. DW-MRI has been used in the past and has been proven to be a good predictive value for therapy outcome in patients (41). In our tumor model, CP induced a substantial increase in ADC_w 24 hours after treatment, reflecting an increase in extracellular water fraction, while the changes in ADC_w were not significantly different after 5-FU treatment and ADC_w even decreased in ET- and untreated tumors. The increase in ADC_w could be the result of changes in cell membrane permeability, cell shrinkage, cell death or

vasogenic edema. It is difficult to separate shrinkage and necrosis from apoptosis (42). In our study, the increase in ADC_w is likely correlated to the induction of apoptosis as the level of necrosis remained unchanged after treatment.

In vivo MRS can be used to measure the metabolite content of tumors in living animals or patients, repeatedly and non-invasively. Elevation of the choline peak is thought to represent membrane phospholipid synthesis and therefore reflects cellular proliferation and cell density occurring during progression of cancer. In necrotic tumors, release of glycerophosphocholine due to cell membrane degradation contributes to increased total choline (tCho) concentrations. The usefulness of choline ¹H MRS in evaluating response to treatment has been reported for different tumor types in patients (41,43,44). In the present investigation, mean choline to water ratios were higher for control tumors than for treated tumors, although this difference was not significant. When analyzing each mouse individually, we did not find a systematic decrease in the choline peak after efficient treatment. The standard deviation for choline levels was quite large, especially in treated tumors. In our study, the amount of choline-related compounds was assessed with the ratio method by using the unsuppressed water peak as internal standard. However, this method has its limitations because the tumor water content is highly variable. An additional critical factor is the timing used in the present study. We cannot exclude that a longer follow-up after treatment could possibly show a more distinct decrease in choline peak areas after therapy. Seierstad et al. (45) reported similar results 24 hours after irradiation of HT29 xenografts and explained the increase of choline metabolites by a break-down of cell membrane constituting lipids. Another explanation found in literature was the fact that during necrosis almost all metabolites, including choline, increase (46). Finally, another limitation of ¹H single voxel MRS is the lack of spatial resolution of this method applied over a quite heterogeneous tissue. Multi voxel imaging would be a better method for future evaluation in order to take into account the heterogeneity of the tumor. Finally, even though choline was identified as a surrogate marker for some classic cytotoxic therapies and for drugs with specific molecular targets such as HIF-1α, choline kinase, or PI3K (47), unexpected opposite effects on tCho were observed following inhibition of HSP90 or HDAC, which are two broad-spectrum inhibitors. tCho changes may therefore not necessarily mirror the changes associated with the downstream signalling pathways of specific inhibitors (47). This illustrates the complexity of interpretation of the choline peak as a marker of response to treatment that is not likely to be 'universal' for all therapies.

When comparing the change in parameters of CP-treated mice relative to the controls, the so-called dynamic range, there was a difference of 37% for DW-MRI, 19% for choline spectroscopy and 11% for T₂-weighted MRI. Even though the dynamic range was the lowest for cell death detection by USPIO-E3 particles, this technique was more reliable than choline spectroscopy because differences were significant. Our MRS results study showed rather large variations which makes it impossible to be conclusive. In our study, ADC_w was thereby found to be the most sensitive and reliable early marker of tumor response after chemotherapy treatment. It may be interesting to combine two or three protocols in one single MR session to gain insights into the complex tumor response. Yet no single technique is completely reliable, each of these techniques has its own limitations. An interesting perspective could be to determine the value of each marker of response at different time points, as the dynamic range could vary over the time after starting the cytotoxic treatment.

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FIGURES

Figure 1

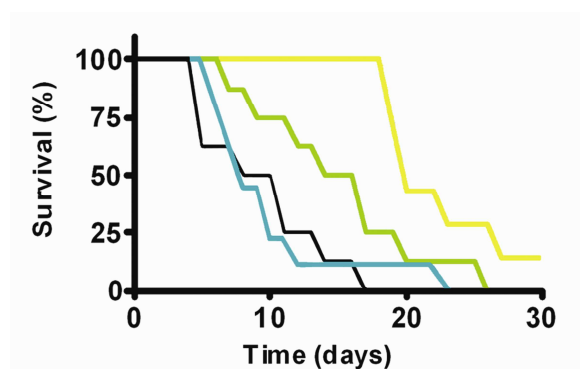


Fig. 1: Survival curve of mice (n=8-9/group) bearing untreated TLT tumors (black line) or mice bearing tumors either treated with cyclophosphamide (yellow line), 5-fluorouracil (green line) or etoposide (blue line). The treatment was started at a tumor size of 8 ± 0.5 mm.

Figure 2

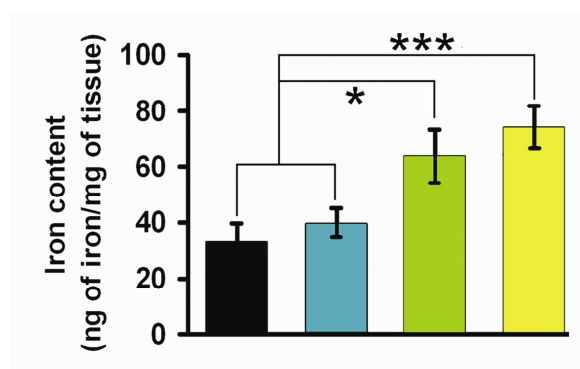


Fig. 2: Iron accumulation (expressed in ng of iron per mg of tumor tissue) in hepatocarcinomas (n=10-11/group) as measured ex vivo by X-band EPR. EPR spectra of the different tumors were recorded 24 hours after i.p. injection of the chemotherapeutic treatment and 3 hours after i.v. injection of the contrast agent USPIO-E3. Yellow bars represent animals injected with cyclophosphamide, green bars tumors treated with 5-fluorouracil and blue bars etoposide treatment. Untreated tumors served as control (black bars)

Figure 3

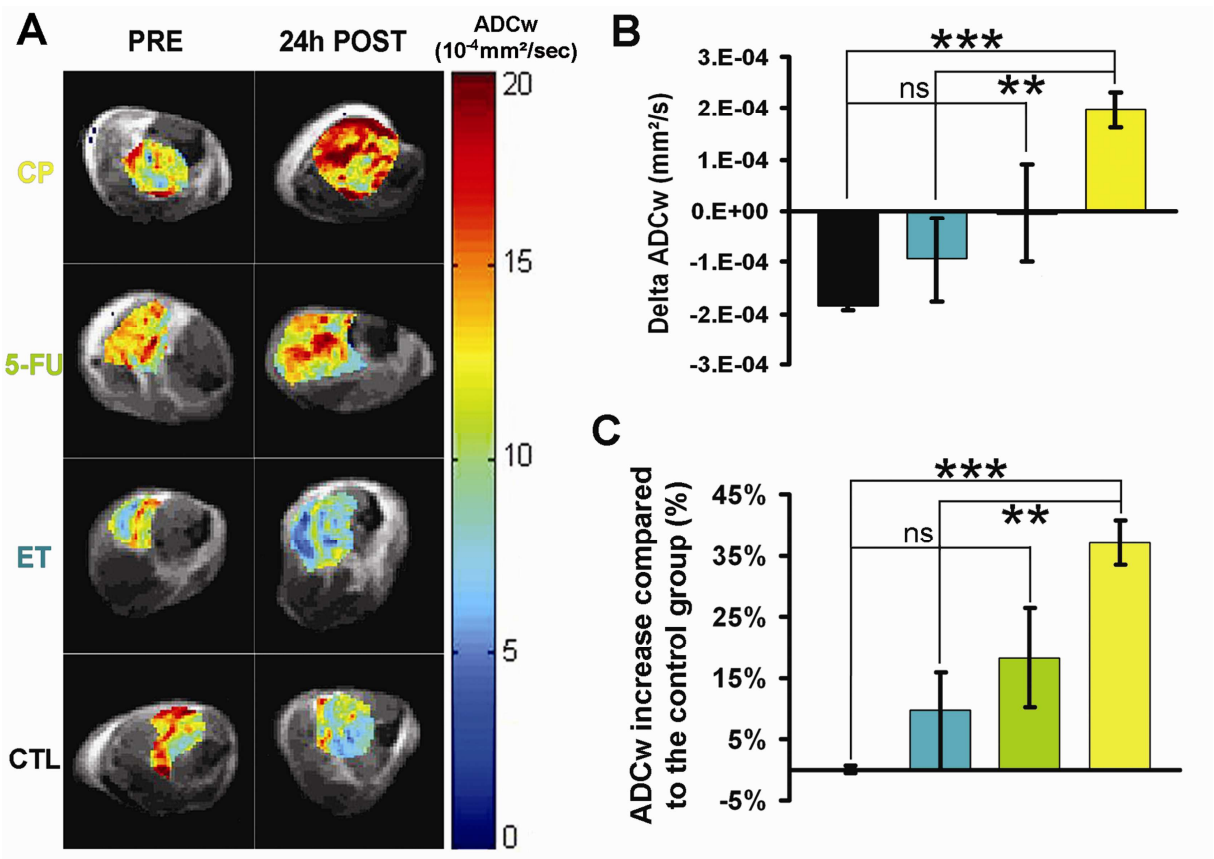


Fig. 3: A: Twenty-four hours after injection of the cytotoxic drug, axial T₂-weighted MR images of murine tumors (n=4.5/group) obtained before and 3 hours after USPIO-E3 administration. B: Three hours after USPIO-E3 injection, mean negative contrast enhancement values were obtained from regions of interest that encompassed the entire tumor region on T₂-weighted MR images. Signal loss of the CP (yellow bars), 5-FU (green bars) and ET group (blue bars) was expressed as percentage of the pre-contrast signal. C: Signal loss expressed as percentage compared to untreated control tumors. D: Shortening of T₂ relaxation times in treated and control tumors after injection of the contrast material. The graph shows mean ΔT_2 measurements, with $\Delta T_2 = T_2$ precontrast - T₂ 3 hours post contrast, expressed as percentage of the ΔT_2 of control mice. (CP: yellow bars, 5-FU: green bars and ET: blue bars).

Figure 4

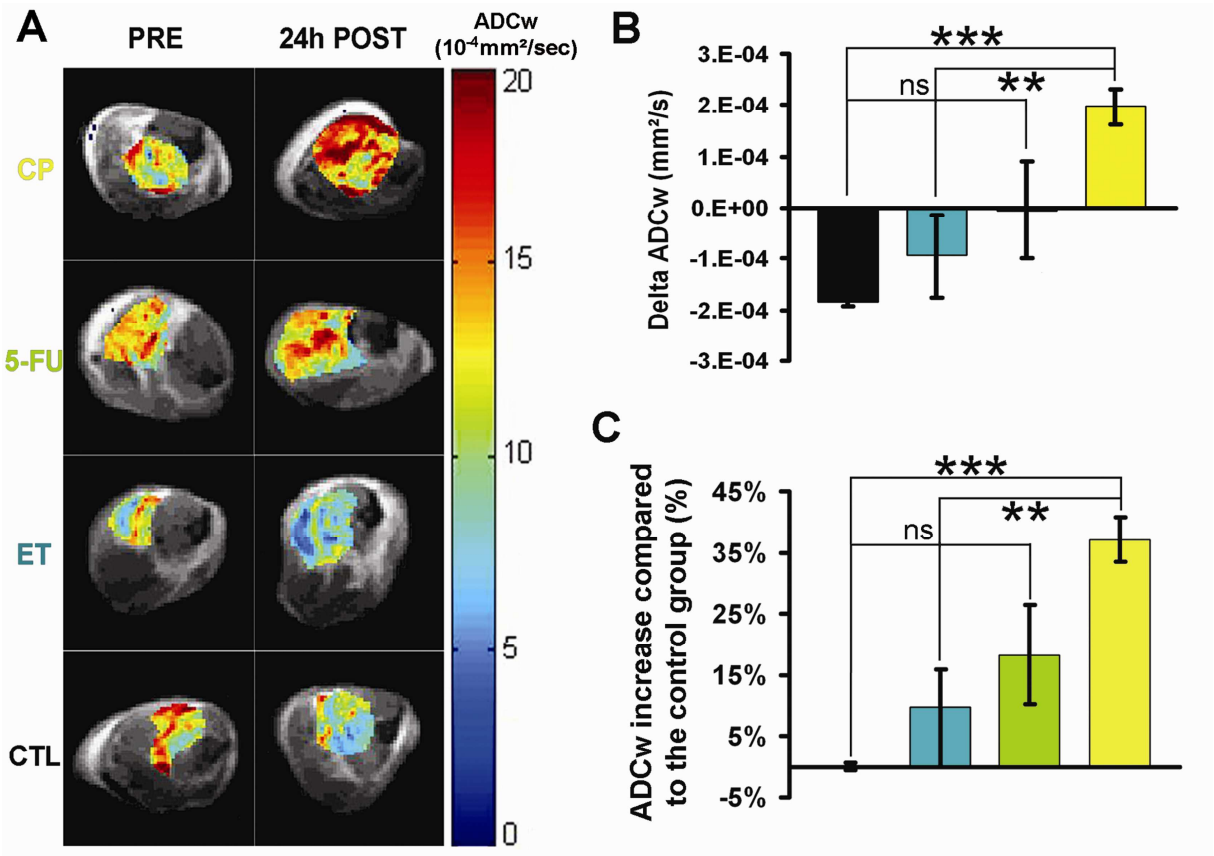


Fig. 4: A: ADCw maps of tumor-bearing mice before and 24 hours after injection of the treatment. Each image represents an axial DW image (b-value=0) of the mouse leg with the tumor area transformed as ADCw map. B: Delta of the average tumor ADCw observed pre- and 24 hours post-treatment. C: Tumor ADCw value expressed as percentage of the ADCw obtained for of untreated control tumors. (CP: yellow bars, 5-FU: green bars and ET: blue bars).

Figure 5

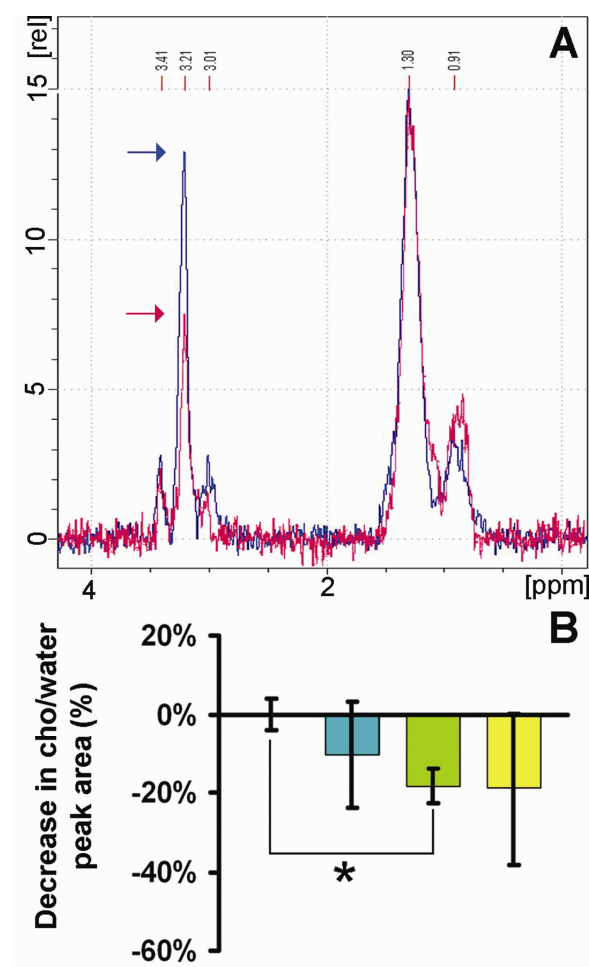


Fig. 5: A: Typical in vivo ¹H MR spectra as obtained from murine TLT tumor before (blue spectrum) and 24 hours after cyclophosphamide treatment (pink spectrum). Besides the total choline peak situated at 3.21 ppm, other peaks displayed on the spectrum were the lipid peaks (0.91 and 1.30 ppm), the creatine peak (3.01 ppm), and the taurine peak (3.41ppm). The presence of creatine and taurine suggests that, at least in part, intact muscle tissue was also present in these spectra. B: Evolution of the choline peak before and 24 hours after tumor treatment. The choline peak area was expressed as choline to water ratio and expressed as percentage of the ratio obtained for control mice. (CP: yellow bars, 5-FU: green bars and ET: blue bars).

Figure 6

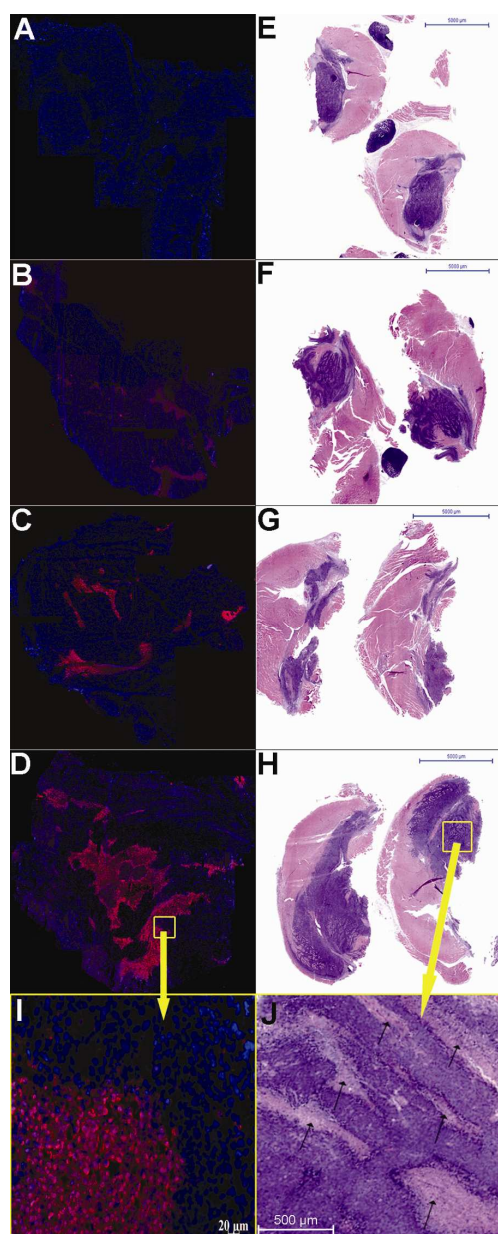


Fig. 6: Representative histological stainings performed on tumor slices. Fluorescent TUNEL stained section from untreated mouse (A) and from tumors 24 hours after etoposide (B), 5-fluorouracil (C) and cyclophosphamide (D) treatment. TUNEL-positive nuclei are stained in red and the nuclear counterstain DAPI in blue (magnification: 50x). H&E stained section from untreated mouse (E) and from mice 24 hours after from tumors 24 hours after etoposide (F), 5-fluorouracil (G) and cyclophosphamide (H) treatment. Only tumor regions were used for quantification of necrosis, the surrounding muscle tissue was not taken into account. Figures I and J represent magnified areas (yellow squares) from CP-treated tumors after TUNEL and H&E staining respectively. Black arrows on figure J indicate necrotic regions of the tumor.

Chapter IV: DISCUSSIONS AND PERSPECTIVES

Imaging of inflammation

The thesis includes the development of new non invasive molecular biomarkers. Although the title of the thesis is the non invasive individual evaluation of tumor response to anti-cancer treatments, biomarkers of inflammation were studied at first in order to familiarize with the approach of molecular imaging and to become acquainted with the techniques used throughout the whole study.

Targeting of E-selectin, a protein expressed on endothelial cells during inflammation, has been realized using a mimetic molecule of its natural ligand sLe^x, which has been grafted onto PEGylated USPIO particles. This biomarker has previously been tested in an in vitro model of HUVECs, over-expressing inflammatory adhesion molecules after stimulation by TNF α (Boutry 2006). To assess USPIO binding to E-selectin in vivo, T₂-weighted MRI was combined to highly sensitive EPR quantification. USPIOs are used as negative CAs in MRI and they can also be detected by EPR due to their unpaired electrons. The EPR method is much more sensitive than MRI and we were therefore able to quantify the iron content in tumors by EPR after establishment of a calibration curve. The spectral height is directly proportional to the amount of USPIOs, without any confounding noise. Using these agents we can combine both complementary techniques in order to assess the amount and the localization (mapping) of USPIOs. This association is innovative and to our knowledge only one study has reported in vivo detection of iron oxide particles by L-band EPR, but without any correlation to MRI (Fujii 2002).

We first assessed biodistribution of USPIOs in vivo. To this end, grafted and ungrafted PEGylated particles were intravenously administered to mice and USPIO concentration was measured ex vivo in tissue homogenates (liver and spleen) and in blood samples by highly sensitive X-band EPR (detection limit: 1-2 ng of iron). As already extensively described, the major part of the injected particles accumulated in the liver and spleen, despite PEGylation. About 70% of the injected particles accumulated in these two organs of the RES. We also assessed the kinetics of several commercial or previously commercialized preparations and we concluded that particle size plays an important role in plasma circulation time. One hour after intravenous injection only 1% of the initially injected dose of ferumoxides (80-150 nm) and 3% of ferumcarbotran (60 nm) remained in the blood, whereas for the ultrasmall ferumoxtran-10 particles (20-40 nm) 5% were measured. As we wanted to increase the chances of attaining the targeted region, particles of small size were chosen. Particles of small size have increased blood circulation time by decreasing macrophagic capture. Moreover, the

particles were PEGylated to further decrease particle opsonisation in the blood by steric repulsion. After grafting PEG chains onto the particles, the iron oxide concentration measured in the blood one hour after intravenous injection was four times higher than for unpegylated ferumoxtran-10 particles. Even though our PEGylated particles have a similar size compared to ferumoxtran-10, about 17% of the dose initially injected was measured in the blood one hour after injection. 24 hours after injection of the contrast probe, particles were completely cleared from blood.

Localized inflammation in murine muscles was induced by injection of Freund's complete adjuvant, a substance used to enhance immune response to vaccines. The timing we chose after induction of inflammation was 24 hours because the up-regulation of E-selectin lasts from 4 to 30 hours after induction of inflammation (Leeuwenberg 1992). By combining EPR and MRI, we demonstrated that E-selectin-targeted USPIOs accumulated two times more in inflamed muscles than ungrafted control particles, whereas healthy muscle only took up very few particles. We observed a non specific accumulation of control USPIOs in inflamed muscles. This has numerous explanations such as increased vasodilatation and permeability occurring during inflammation, as well as non specific uptake by macrophages present at the inflammation site. Although we provided histological proof that inflammation was induced in the muscle, histological confirmation of E-selectin expression in the inflamed region is missing. It would be very useful to have a better understanding of the distribution and the timing of E-selectin expression. A co-localization of both, E-selectin and USPIO accumulation could be interesting to perform in future experiments.

Tumor cell death

The main part of the work was dedicated to magnetic probes recognizing phosphatidylserine by EPR and MRI. PS is exposed on tumor cells during treatment-induced apoptosis. As detected by phage display, the E3 hexapeptide (TLVSSL) used in this study is able to target the PS molecule with high affinity (Laumonier 2006). A library of bacteriophages, carrying several copies of aleatory hexapeptides on their surface, was exposed to apoptotic and healthy livers. After selection of the peptide with the highest affinity for the PS target, competition studies with AnxV were carried out on PS-coated ELISA plates. This peptide in question was grafted to our USPIO particles. Contrastophores were prepared from nanoparticles carrying carboxylate groups on their surface (Port 2004), on which the targeted peptide and aminoPEG chains were covalently bound. As control we used ungrafted USPIOs

and particles coupled to a scrambled peptide containing the same amino acids but arranged in a different order.

Flow cytometry analysis showed that 90% of staurosporine-treated TLT tumor cells bound to FITC-labeled AnxV, whereas the nuclear stain propidium iodide was excluded from cells. This experiment demonstrated that PS exposure is higher on apoptotic cells than on untreated control cells. At the beginning of our study, healthy and apoptotic TLT tumor cells were incubated for 2 hours with the three different types of USPIOs. Measurements by X-band EPR showed that USPIO-E3 accumulation in apoptotic cells was higher than in healthy cells and also higher compared to the accumulation of control particles. We cannot affirm with certitude if increased concentrations of USPIO-E3 were due to specific membrane binding or to internalization, since it has been reported in the literature that cancer cells are able to endocytose SPIOs in a non specific manner (Heyn 2006). Furthermore, most of the endogenous ligands of PS, such as AnxV, were shown to be internalized after targeting PS on the cellular surface (Kenis 2004). In control cells, it is difficult to dissociate cellular uptake from specific binding, but as USPIO-E3 accumulation in apoptotic cells was higher, we assume that at least this increased fraction is due to specific PS-binding. To determine USPIO localization more precisely, electron microscopy should be carried out.

Thereafter, we took a step forward and 3 hours after injection we measured the particle accumulation in irradiated TLT tumors by EPR and MRI. Results were compared to the measurements obtained in untreated tumors. 24 hours after radiotherapy, USPIO-E3 showed higher accumulation in irradiated tumors than ungrafted and scrambled particles. Even when the USPIO-E3 concentration was twice as high in irradiated tumors as in control tumors, there was a non specific accumulation of USPIO-E3 and control USPIOs in untreated tumors. This fact presents the major limitation of the use of USPIO-E3. Zhao et al. reported that both the PS-targeted C₂A-SPIO and the correspondent control SPIOs produced a decrease by 10-15% on T₂-weighted MR images, but for C₂A-SPIO the decrease was more persistent in relatively well defined areas of the tumor (Zhao 2001). This example shows that other investigators encountered the same problems of non specific accumulation of iron oxide particles in tumors.

Timing is a critical factor in this whole approach. Apoptosis occurs rapidly and apoptotic cells are removed quickly by phagocytosis. The time between trigger exposure and commitment to apoptosis depends heavily on cell type, trigger type, and environmental conditions. Little is known on exact time frames for anti-tumor therapy imaging. In apoptosis,

PS exposure is first localized (after 12-14 hours) and later generalized (~24 hours) (Shi 2007). Therefore, we chose a timing of 24 hours after tumor irradiation to visualize PS exposure.

The accumulation of USPIO-E3 in tumors was correlated to histological slices stained by TUNEL or H&E to quantify apoptosis and necrosis respectively. Apoptotic fraction in tumors increased from 1 to 10% after X-ray irradiation, whereas the level of necrotic cells was 23% at baseline and did not change after treatment. According to the literature, only a minor proportion of cells immediately die by apoptosis after irradiation, most of them are still able to undergo one or more mitosis. In the meantime DNA is allowed to be repaired and it will be seen later on if the cell survives or fails to repair the damages. If the cell is not able to complete mitosis, mitotic catastrophe will be initiated, which eventually leads to late cell death (van der Kogel 2009).

No feature seems to be unique to apoptotic cell death, except maybe for DNA laddering. For this reason, any method claiming to exclusively image apoptosis should be interpreted with caution (van der Kogel 2009). The same is true for PS; which is actually exposed in almost all forms of cell death. Since every kind of cell death will contribute to tumor destruction, it does not matter which type of cell death exactly occurs. Anyway, the different cell death pathways cannot necessarily be imaged separately, as they often cross over into each other.

After these first promising results, the same biomarker was tested for its ability to discern different responses to treatment. For this purpose different tumor types with different responses to radiotherapy were used in order to see if different levels of radiation-induced cell death are detectable. Therefore, radiosensitive EL4 lymphoma and TLT hepatocarcinoma models, and a highly resistant FsaII fibrosarcoma were implanted intramuscularly into both mouse legs. The right tumor-bearing leg was irradiated, whereas the left remained untreated to provide a control for each animal. After irradiation, accumulation of targeted USPIOs was followed by T₂-weighted MRI. The signal difference between pre- and post-contrast images induced by USPIO-E3 was the highest for the irradiated lymphoma that is also the most radiosensitive. MR results were in agreement with data obtained by EPR and histology. The latter was obtained due to FITC-labeled USPIO-E3 particles. We observed that, although no difference in USPIO-E3 accumulation was seen between irradiated and control FsaII tumors, the basal level of USPIO-E3 accumulated inside the tumor was very high. First we assumed that, despite their radioresistance, the spontaneous level of apoptosis is higher for these tumors than for EL4 and TLT tumors. Meyn et al. reported that occurrence of apoptosis in untreated tumors can vary widely. In their study, spontaneous levels of apoptosis ranged from

0 to 9.5%, depending on tumor type (Meyn 1997). Other investigators already reported a relation between high apoptotic index and tumor malignancy (Boersma 2005). Contradictorily, other groups correlated high apoptotic levels in tumors at baseline with positive treatment outcome, as visualized by tumor regrowth delay (Meyn 1993, Milross 1996). Moreover, we found a study in the literature, where only 0.3% of FSaII cells were stained positive for apoptosis at baseline (Schimming 1999) compared to 6% of EL4 lymphoma cells (Saito 1999). Therefore, high spontaneous levels of apoptosis in FSaII cannot be the correct explanation for increased USPIO-E3 accumulation pre-treatment. Maybe differences result from the use of different tumor models or different mouse strains, meaning that differences in control tumors could also be caused by differences in the pharmacokinetic profile, in tumor perfusion or in permeability. To exclude this factor, experiments with untargeted control particles should have been carried out. But the important finding of this study was that USPIO-E3 uptake increased after irradiation of radiosensitive TLT and EL4 models and that this increase was not due to treatment-related increase of permeability and vasodilation, as excluded in the previous study using control particles. We wondered if the lack of apoptosis induction in the radioresistant FSaII model was linked to mutated p53. The tumor suppressor gene p53, frequently mutated in treatment-resistant cancers, controls the genome integrity and the cell cycle and treatment-induced apoptosis may activate the p53 pathway. However, in the literature it was suggested that the radiosensitive lymphoma EL4, as well as the resistant fibrosarcoma FSaII, have wild-type p53 proteins (Schimming 1999, Truksa 2003,).

The next step of the work was to test the efficacy of different chemotherapies. Survival curves were established to have a first idea of the impact of the different agents on TLT tumors. They showed that cyclophosphamide was the most efficient drug, followed by 5-fluorouracil, whereas the etoposide curve was almost similar to the one of the control tumors, at least at the doses used in this study. As assessed by EPR and by a 9.4T MR system, the accumulation of PS-targeted particles in chemotherapeutically treated tumors followed the same order as the survival curves, which means that the most efficient treatments have caused the highest USPIO-E3 accumulation inside the treated tumors. Here as well, a high non specific accumulation of USPIOs in control tumors was observed. It should be emphasized that EPR and MRI could already predict tumor behaviour 24 hours after the start of the therapy, which is much earlier than if we waited for the outcome of survival curves, which took about one month in our study. In parallel experiments, our cell death recognizing contrast probe was compared to other well-known MR techniques of tumor response

evaluation: diffusion-weighted MRI and proton spectroscopy of choline. Diffusion imaging, reflecting cellularity, provides a functional diffusion map, in which each image pixel represents the spatially localized ADC. The present study confirmed: the more efficient a therapy was, the higher was the diffusion coefficient. According to the literature, choline-containing metabolites should decrease in tumors following some conventional chemotherapy and most of the recent targeted therapies. For relative measurements, the ratio of the choline peak area to the water peak area was calculated. Even if we could see a tendency, results of our spectroscopy experiment were too variable to rely on this technique. Comparing the different MR modalities, we noticed that DWI provides the highest dynamic range, meaning the biggest change induced in tumors after cytotoxic treatment.

According to our histological quantification, the apoptotic fraction in TLT tumors increased from 0.5 to 26% 24 hours after injection of the most efficient chemotherapeutic drug, whereas the high initial level of necrosis (20%) did not change after injection of the different drugs. These findings evidenced that apoptosis is the major form of cell death induced by our chemotherapeutic agents. When the same tumor model was irradiated, apoptotic fraction was only about 10%, meaning that cyclophosphamide treatment at the dose used in our study had a greater capacity to induce apoptosis in TLT tumors than X-rays at a dose of 10Gy. Zhao et al. treated EL4 lymphomas with etoposide/cyclophosphamide, apoptosis increased from 4% to 32% and distribution of apoptotic cells was shown to be heterogeneous. They saw no evidence of necrosis, so they concluded that apoptosis was the predominant mode of cell death (Zhao 2001). In another publication, 20-30% of apoptosis in EL4 cells treated by chemotherapy was found too (Krishnan 2008). These results are comparable with apoptosis occurring in human tumors after treatment (Symmans 2000). Not all chemotherapeutic treatments mostly induce apoptosis. The major form of cell death can vary with cell type and cytotoxic drug. For instance, experiments on 15 mouse tumor models demonstrated that docetaxel is a potent inducer of cell lysis compared to paclitaxel, which mainly causes apoptosis (Schimming 1999). Tumor response to radiation therapy only results in modest levels of apoptosis in most tumor cell lines (Brown 2005, Gudkov 2003). On the other hand, tumors treated with DNA-damaging therapy such as irradiation undergo cell death via mitotic catastrophe (Seluanov 2001).

Possible explanations for non specific accumulation of USPIOs in tumors

For irradiated tumors, this phenomenon could be explained by an increase in blood flow and vascular permeability (Crokart 2005, Cron 2005) or recruitment of macrophages occurring after X-rays. However, as the non specific accumulation also occurs after chemotherapy, in radioresistant tumor models, or even in untreated tumors, the non specific accumulation does not seem to be related to the treatment but to the tumor itself. Possible reasons could be the macrophage presence at tumor sites and, as already discussed earlier, the fact that tumor cells can take up iron oxide particles in a non specific manner (Heyn 2006).

As already mentioned in the introduction, the enhanced permeation and retention effect (EPR) is also an important component, since the leaky tumor vasculature allows passage of nanoparticles. The increased permeability can also be seen as an advantage since particles have easy access to tumors and tend to accumulate there naturally because intact endothelium impedes transendothelial passage to other tissues. The disadvantage is a slow tumor clearance of unbound particles because of slow venous return and low lymphatic drainage. In many preclinical and clinical studies, SPIOs without any targeting moieties have shown to passively accumulate in cancerous lesions and have therefore been used to detect tumors. The mechanisms of this passive targeting include non targeted cellular uptake, enhanced retention in leaky tumor vasculature and accumulation in recruited macrophages (Iyer 2006).

Last but not least, a significant contribution to non specific particle accumulation in tumors is probably made by the high basal level of necrosis in untreated tumors, as shown on our H&E-stained tumor slices. Since PS becomes accessible in necrotic cells after membrane disintegration, PS can be easily targeted by the E3 peptide and as necrosis is always accompanied by inflammation, on-site macrophages will inevitably engulf USPIO particles.

Comparison of the different MR methods used in the course of the doctorate

The value of PS-targeted USPIOs was compared to other MR biomarkers of early treatment response. We used diffusion imaging, studying the random microscopic motion of water protons, and proton spectroscopy, particularly measuring the total choline peak.

Our new biomarker showed enhanced accumulation in radiosensitive tumor models after irradiation and also permitted to point out efficient chemotherapeutic agents 24 hours after injection of the treatment. Unfortunately, non specific accumulation of the contrast probe

in untreated tumors could impede its potential use in the clinic. We first worked on a 4.7T magnet and then switched to a 9.4T system, on which we observed that magnetic susceptibility effects highly increased at higher field. Besides an advantageous increase in signal loss for the apoptotic tumors, the negative signal enhancement was also increased for the non specific accumulation in control tumors. At 4.7T the signal loss in TLT control tumors was about 30%, whereas at high field it was even about 75% in untreated tumors.

DWI allows longitudinal studies by relying completely on intrinsic contrast. Measurements of water ADC are independent of the magnetic field, but the application of diffusion-weighted MRI relies on strong magnetic field gradients and is therefore sensitive to motion artifacts. DWI becomes challenging at the abdominal level because of respiration movements (Le Bihan 2004). Because of this sensitivity to macroscopic movements, fast imaging techniques (echo-planar imaging) are used, but fast sequences are sensitive to susceptibility effects. With slower sequences ECG-triggering will be necessary. DWI presented the highest dynamic range in our study. It presents the advantage of allowing a longitudinal follow up, but it is an indirect measurement of cell death. What do we really measure? The distinction between apoptosis, necrosis, tumor shrinkage, fibrosis, edema, and other processes that can occur with therapy may be difficult. A decrease in ADC value does not necessarily mean that radiotherapy was unsuccessful; irradiation can cause replacement of tumor tissue by fibrotic cells (Stone 2003). Besides apoptosis, explanations for increased ADC after tumor irradiation are necrosis or edema (Hein 2003, Seierstad 2007). Contradictorily, it has also been reported that low ADC values may be seen in radiation necrosis, possibly reflecting early necrosis with abundant polymorphonuclear leukocytes (Asao 2005). However, appearance of necrosis and fibrosis is associated with successful therapy intervention. Nevertheless, ADC is currently considered as one of the surrogate markers of tumor response following anti-cancer therapy in many translational and clinical studies (Jain 2010, Koh 2009, Thoeny 2010).

The prime advantage of MR spectroscopy lies in its ability to probe tumors non invasively during longitudinal studies. However, as MRS is not a sensitive technique, it seems likely that tests for markers of tumor response will rely on more sensitive methods. To increase signal-to-noise ratios, multiple measures have to be acquired, which increases acquisition time. One of the problems with spectroscopic sampling of tumors is the presence of metabolic heterogeneity, and as single-voxel spectroscopy only analyses a small volume, chemical shift imaging is more appropriate for tumor studies. The more a tumor shrinks after treatment, the more challenging it is to quantify metabolites, since a reduced tumor area is left

for measurements. By introducing higher magnetic fields and parallel imaging, these disadvantages could be reduced, but not solved (Barker 2001). In our study, the choline peak area did not give us concluding results, but we only tested one single time point (24 hours) after chemotherapeutic treatment. Testing several time points after initiation of therapy could have offered valuable clues about the evolution of the choline-containing metabolites after treatment. We expressed the total choline peak area as ratio to the unsuppressed water peak, but this method is not optimal because the water content in background tumor tissues is variable. Therapy-resulting edema may change the water content in tumors, which means that the unsuppressed water may be inappropriate as reference peak. Moreover, literature about the choline peak is quite confusing. Although in most studies a decrease in choline peak after treatment was observed, others reported an increase preceding the decrease. The choline level will increase when cell membrane-constituting lipids are broken down or damaged (Rutkowski 2003). Seierstad et al. suggested that the increase in choline observed 24 hours after therapy could result from breakdown of the cell membrane (Seierstad 2007). In their study, a decrease in choline/water peak was only demonstrated at 3 and 6 days post-treatment. Concerning the behaviour of choline-containing metabolites during necrosis, Strauss et al. reported an increase of almost all metabolites, under which choline, as observed by proton spectroscopy (Strauss 2008). Since in our study occurrence of necrosis was demonstrated by histology (20% of the total tumor area), this fact could counteract a decrease of the total choline peak. However, another group observed decreasing choline levels in a CWR22 xenograft model 24 hours after X-ray irradiation (Dyke 2003). Gillies et al. suggested that changes in the total choline peak may depend on the cell cycle arrest, which generally occurs after 24 hours or later (Gillies 2005). If this is true, spectra acquisitions within the first 24 hours, as used in most studies, should be extended. Perhaps choline measurements are better suited for targeted anti-cancer therapies. Cytostatic agents stop tumor cell proliferation, avoiding further increase of the choline peak, without any confounding choline liberation stemming from cell membrane breakdown as it is the case for cytotoxic agents (Beloueche-Babari 2010).

Towards clinical use

Besides morphological imaging, there are some serum markers (alphafetoprotein, lactate dehydrogenase, cancer antigen 125, PSA...) that are useful in monitoring therapeutic response in specific cancer types. However, for the majority of the common tumors, there

presently is no serum marker that can assist with response monitoring acutely after therapy; moreover they do not allow response assessment in situ (Berglund 2002). Multimodality imaging techniques are now combining different anatomical and functional methods. Modalities used to monitor therapeutic effect should ideally be fast, robust, and hardly invasive. Biologically significant processes, such as apoptosis, should be quantified in real-time and tumor heterogeneity should also be reflected. In general, the major challenge is to standardize these new techniques worldwide. Study populations are often small and most reports do not evaluate reproducibility, which has to be done for translation into routine clinical practice.

Besides monitoring of currently used therapies, molecular imaging techniques are an interesting tool to evaluate the efficiency of experimental drugs. It will save time and money during preclinical and clinical stages (Rehman 2005). Development of new drugs is a succession of long and expensive experimental stages. Years elapse between drug discovery and drug commercialization and only one of five thousand newly discovered drugs will actually be launched on the market. Overall survival and reductions in tumor size are the endpoint by which most Phase III studies are evaluated, but it requires a too long follow-up period.

DW-MRI has already been implemented in most of the clinical systems and is routinely used in research studies. MRS on the other hand, is still an experimental tool as it requires specific hardware and software to be implemented on clinical systems. The magnetic fields used for our MR studies (4.7T and 9.4T) were higher than the fields currently used in the clinic (1.5T or 3T). Technical evolution pursues an increase in magnetic field intensity to obtain a better signal-to-noise ratio and a diminution of acquisition time, but consequences of an increased field are more wide-ranging than a simple gain in signal-to-noise ratio (Hoa 2007). Signal intensity increases proportionally to the square of the magnetic field amplitude, whereas noise increases linearly to the field magnitude. The resulting signal-to-noise ratio thus follows a linear relation with the field intensity. Chemical shift resolution of the different molecules is also proportional to the field and therefore high field MRI is advantageous for MRS. Also magnetic susceptibility effects (T_2 effect) increase proportionally to the magnetic field, which can be disadvantaging (image distortion and signal loss) or advantaging for certain types of acquisitions (after SPIO/USPIO administration). When working with clinical MR systems, signal loss after administration of our contrast probe will be less important, but the non specific signal diminution in untreated tumors too, as observed when comparing results obtained at 4.7T and 9.4T. Also important to mention is that the magnetic field

intensity influences relaxation times of tissues, which requires an adjustment of the MR sequences.

The reported protocols and timings of post-treatment MR examinations vary a lot. Further work is required to determine the most effective post-therapeutic window. Variability of the optimal timing with regard to the cancer type and treatment protocol emphasizes the need to determine the best time for administration of our contrast probe and for imaging of therapeutic response. For optimal timing, one should also consider that biodistribution of USPIOs is different in mice and in humans.

Human cancer is complex and differs from mouse cancer in its pathogenesis. It is clear that useful information can be obtained from the simpler experimental models before investing in extensive clinical development studies, given that the agents that are not efficient in these models are unlikely to be useful in humans. In these models, tumor cells are injected subcutaneously or intramuscularly and tumor growth can be followed up easily. Therefore, tumor microenvironment and perfusion are different in experimental models and other parameters like tracer kinetics can also differ in clinical situations.

The administered dose of USPIO particles in mice was 137 $\mu\text{molFe/kg}$, which was about three times higher than the appropriate dose for human use in lymph node imaging (Sinerem[®]: 45 $\mu\text{molFe/kg}$). This can be explained by the fact that a high dose is needed to saturate the uptake by macrophages of the RES, but as USPIOs have shown a very high therapeutic index (1:150), no toxic side-effects are feared at this dose.

The main pitfall for progression of our biomarker towards clinical use is the natural accumulation of nanoparticles in tumors. How shall we distinguish if the observed signal loss in treated tumors is due to recognition of PS or simply due to non specific accumulation? It will be difficult to delineate treatment-resistant areas under these conditions. A solution could consist in combining molecular PS imaging with other biomarkers of therapeutic response. This is likely to increase the predictive value for decision making, but accumulating examination methods is expensive and cumbersome for the patient. Despite the huge non specific accumulation, we did not assess the tumor wash-out of our biomarker. At first, one should measure the wash out of unbound particles to obtain an optimal target-to-background signal. Then, one should also measure the tissue clearance of the PS-bound probes because this information could be valuable for longitudinal studies. This is a huge advantage of non invasive DWI and MRS, where endogenous contrast allows post-treatment follow-up as often as needed.

Perspectives

Summing-up table for the perspectives of this study:

Possible applications of our PS-targeted biomarker
Extension of its use as treatment monitoring tool to prognosis and prediction
Applicability to other pathologies with apoptotic context (early detection, monitoring...)
Possibility to target anti-cancer drugs (combining diagnostic and therapy)
How to increase the affinity for tumor cell death or how to reduce non specific accumulation
Use of better suited targeting molecules (phages, combination of several peptides...)
Modification of grafted PEG molecules or reduction in macrophages capture
Decrease of the administered dose of USPIO-E3
Replacement of the contrast probe (Gd, radiolabels...)
Comparison of different methods used to assess tumor treatment outcome
Comparing different methods for detecting cell death
Other techniques used to evaluate treatment response

Possible applications of our PS-targeted biomarker

We evaluated USPIO-E3 particles as biomarker for evaluation of anti-cancer treatment outcome immediately after onset of the therapy (24 hours). As it was suggested in the literature that high apoptotic levels at baseline correlate well with response to treatment our marker could also be used as prognostic and predicting marker even before initiation of the therapy (Meyn 1993). The term “prognosis” defines the chances to succeed in therapy in general, whereas “prediction” is related to a specific treatment. Such experiments have already been performed using PS-targeting ^{99m}Tc -AnxV to determine pre-treatment levels of apoptosis and to correlate them with later treatment response (Kartachova 2004, Kartachova 2007).

Besides its use in cancer, monitoring of death-inducing or death-preventing therapies could be another application of the PS-targeted contrast agent. Deficient or excessive activation of apoptotic pathways is responsible for a large number of pathologies. Excessive apoptosis contributes to neurodegenerative diseases (Friedlander 2003, Honig 2000), autoimmune diseases (Lorenz 2000), graft rejection (Martinez 1999), and consequences of ischemia/reperfusion also have an apoptotic component (Gujral 2001, Kohli 1999). Non

invasive imaging of apoptotic cells would help to diagnose diverse diseases, as well as to follow-up their therapy. In this context, PS seems to be an interesting marker.

In the context of cancer, anti-tumor therapies kill tumor cells, but also cells of normal tissues, which contributes to the toxicity associated with treatment. A severe side effect of anti-cancer therapy is chemotherapy-induced enteropathy (CIE), which is characterized by unpredictable severity and large inter-patient variability (Levin 2009). Early detection and monitoring of CIE by biomarkers of apoptosis may therefore help to prevent enteropathy, to add specific damage-reducing treatments, or to select an alternative anti-cancer therapy. The same is true for severe cardiotoxicity induced by the doxorubicin treatment that is commonly used in a wide range of cancers (Bennink 2004).

As our new contrast probe accumulates in tumors, it could also be used for targeted delivery of anti-cancer drugs, reducing unwanted side-effects. Especially for those drugs directed at tumor vasculature as PS is also expressed on living endothelial cells during neoangiogenesis. Tumor vasculature is an interesting target for therapy because of its non malignant character, which means low probability to mutate and to develop drug resistance. Using anti-PS monoclonal antibody as a probe, Ran et al. showed that PS is also a potential target for cancer therapy (Ran 2002).

How to increase the affinity for tumor cell death or how to reduce non specific accumulation

What can be done to increase the sensitivity of our contrast probe and to reduce its non specific accumulation in tumors? Affinity of the E3 peptide for PS was about ~70 times lower than the PS-affinity of AnxV, but it was still situated in the nanomolar range. Unfortunately, when passing from the E3-expressing phage to the E3 peptide, we lost some affinity. Other investigators also reported the same problem which can be explained by the fact that five copies of the same peptide are expressed on the phages' surface (Hong 2008, Thapa 2008). For this reason Segers et al. proposed to directly use the phages as molecular targeting agents (Segers 2007). Using magnetophages instead of peptides-bound particles could increase the affinity for the PS molecule and this way also increase the target-to-background signal. Besides trying to increase the number of grafted E3 peptides, one can also try to graft different targeting molecules on the nanoparticle surface with the aim of recognizing different targets expressed during cell death and thereby increasing the chances of specific accumulation in cell death regions. This multivalent interaction could possibly enhance the affinity to a considerable extent.

By decreasing the administered dose of USPIOs, we might be able to reduce the non specific accumulation. However, the high accumulation in liver and spleen will still be a problem. Several injection doses should be tested to determine the optimal USPIO concentration for imaging tumor cell death.

One can also increase the number of PEG molecules bound to the particle or use PEG chains with higher MW. A study comparing PEGylation with different PEG chain lengths demonstrated that, despite PEG chain addition to particles, the particle size decreased because particle aggregation was reduced due to steric interaction (Luo 2010), and a smaller size means less susceptibility to be captured by the macrophages and a higher chance to reach the tumor. With PEG750, the same MW as used for PEGylation of our CA, only poor steric repulsion was observed. Therefore, using a longer PEG chain to PEGylate our targeted particles could be a possibility to increase plasma circulation and thereby potential tumor accumulation. Another group evaluated the influence of the PEGylation degree on pharmacokinetics. PEGylated dendrimers were prepared at three different molar ratios (4:1, 16:1 and 32:1) yielding conjugation of 4, 12 and 20 PEG molecules per dendrimer respectively (Zhu 2010). Even the slightest degree of PEGylation increased circulation time, reduced liver and spleen uptake and increased tumor uptake, but at a lower degree of PEGylation steric repulsion was not as strong as at higher degrees. Pharmacokinetic and biodistribution properties seem to be more dependent on PEGylation degree than on particle size. But question is: will the targeted peptide still be accessible for PS-binding after all these modifications, or will the PEG molecules shield access to the target molecule.

Another possibility is to eliminate plasma opsonins by injection of decoy particles immediately before injecting the particles of interest. By doing so, the plasma clearance system will already be saturated before injection of the targeted particles (Simberg 2007). Drugs like lovastatin are able to reduce the recycling of macrophage receptors and thereby to diminish internalization of the nanoparticles by macrophages (Rogers 2005). Administration of lovastatin could possibly decrease USPIO capture by macrophages.

The most logical approach would be to use another magnetic reporter such as Gd complexes. Advantages of Gd are the positive contrast enhancement on T₁-weighted images and the small size. One should check the wash out of these agents though, in order to avoid toxic effects like nephrogenic systemic fibrosis that can occur after too slow elimination from tissues. As the relaxivity of Gd is relatively low compared to USPIOs, one has to use macromolecular complexes such as dendrimers or liposomes, which are able to carry a lot of Gd³⁺ ions and can therefore increase the contrast. One has to find a good compromise

between relaxivity and size of the CA. Of course, if labeling methods are not too cumbersome, the E3 peptide can also be radiolabeled by a PET-tracer, involving all advantages and disadvantages of the PET technique.

Comparison of different methods used to assess tumor treatment outcome

First, the markers of tumor response used in this study could be better characterized, meaning that a longitudinal follow-up of the measured parameters (cell death, cellularity, and choline) and a comparison of them over a given time frame could give interesting new insights. An interesting perspective would of course be the comparison of the methods used in this study with ^{18}F -FDG-PET or with proliferation markers like ^{18}F -FLT. Our biomarker of apoptosis could also be used in combination with biomarkers of necrosis (NACAs) to compare maps of apoptosis and necrosis within treated tumors. It could also be interesting to compare tCho measurements by proton MRS with ^{18}F -FCH uptake as measured by PET. This approach is not new. Interrelation between both techniques has already been assessed *in vivo*, but no correlation was found (Kwee 2010, Rommel 2010). An explanation could be the limited ability of MRS to discriminate between the individual choline-containing metabolites *in vivo*. ^{18}F -FCH is a marker of PC synthesis; thus, if we resolve the peak of PC in tumor biopsies, PC concentration may correlate better with ^{18}F -FCH than the total choline peak.

PET enables visualization of changes in glucose and choline metabolism, as well as imaging of apoptosis, in cancer patients after various treatments. However, the PET technology exposes patients to radioactivity, cannot differentiate parent and breakdown products, and is not able to measure endogenous compounds (Payne 2006). Thus, a complimentary tool for metabolic imaging such as MRI/MRS can be useful. Let us take the example of prostate cancer. The serum marker PSA is a very useful marker of prostate cancer, even before the tumor becomes palpable. This method permits to detect 80% of prostate cancers, but unfortunately PSA lacks in specificity. Actually, only one out of three PSA-positive patients has cancer. Another popular surrogate marker, the ^{18}F -FDG, turns out to be less useful in this specific type of tumor since prostate tumor has a low metabolism. ^{11}C -acetate detection by PET is well able to point out all primary prostate tumors, but for bone metastases, bone scintigraphy was superior to PET. On the basis of this example we can see how important it is to combine different methods or to figure out the best method for each specific tumor type. The response evaluation method will also depend on the chosen treatment. Many of the targeted therapies currently being tested are cytostatic rather than

cytotoxic and of course in such a case a non invasive marker of tumor cell death will be pointless.

Combining different modalities is a nice idea, but difficult to translate into the clinic. It will be money- and time-consuming, cumbersome for the patient and complicated to schedule overbooked machines. It could be easier to run several acquisitions during one single MR session, or use multi-tracer PET studies, but then one would not benefit from combining the intrinsic advantages of the different techniques. There is a need for a systematic approach comparing the timing and the dynamic range of different markers to assess the respective value of each, or the added value of combined markers in order to see if a multimodal approach would make sense or not.

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