EXCI-CEST: Exploiting pharmaceutical excipients as MRI-CEST contrast agents for tumor imaging

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Title page

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Abstract

Chemical Exchange Saturation Transfer (CEST) approach is a novel tool within magnetic resonance imaging (MRI) that allows visualization of molecules possessing exchangeable protons with water. Many molecules, employed as excipients for the formulation of finished drug products, are endowed with hydroxyl, amine or amide protons, thus can be exploitable as MRI-CEST contrast agents. Their high safety profiles allow them to be injected at very high doses. Here we investigated the MRI-CEST properties of several excipients (ascorbic acid, sucrose, N-acetyl-D-glucosamine, meglumine and 2-pyrrolidone) and tested them as tumor-detecting agents in two different murine tumor models (breast and melanoma cancers). All the investigated molecules showed remarkable CEST contrast upon i.v. administration in the range 1-3 ppm according to the type of mobile proton groups. A marked increase of CEST contrast was observed in tumor regions up to 30 min post injection. The combination of marked tumor contrast enhancement and lack of toxicity make these molecules potential candidates for the diagnosis of tumors within the MRI-CEST approach.

Keywords

Excipients, MRI, CEST, tumor, imaging, chemical exchange saturation transfer

Chemical Compounds studied in this article

Ascorbic acid (PubChem CID: 54670067);

Meglumine (PubChem CID: 8567);

Sucrose (PubChem CID: 5988);

N-acetyl-D-glucosamine (PubChem CID: 439174);

2-Pyrrolidone (PubChem CID: 12025);

Abbreviations

MRI: Magnetic Resonance Imaging

CEST: Chemical Exchange Saturation Transfer

i.v.: intravenous

1. Introduction

Medicines could not be made without the use of pharmaceutical excipients that contribute notably to guarantee efficacy and safety of the final pharmaceutical product (Casas et al., 2015). Moreover, excipients perform multiple functions, besides completing the formulation volume, such as improving bioavailability, administration and acceptance of the treatment by the patient (Loftsson, 2015; Narayan, 2011; Wening and Breitkreutz, 2011). Another fundamental characteristic of excipients is their pharmacological and toxicological inactivity that allows them to be used at high doses (Abrantes et al., 2016). Several natural products, simple substances and mixtures are commonly used in formulating medicines, with chemical structures that vary from small molecules to polymers.

Interestingly, most, if not all of these molecules, possess exchangeable protons (hydroxyl, amine, amide groups) that can be potentially detected by chemical exchange saturation transfer (CEST) magnetic resonance imaging (MRI) (van Zijl and Yadav, 2011; Vinogradov et al., 2013). This technique enables the indirect visualization of molecules via magnetization transfer between exchangeable protons and bulk water protons. By applying a selective radiofrequency irradiation to the mobile protons, the induced saturation is transferred to the bulk water protons, thus inducing a reduction of the water signal (Liu et al., 2013). Several natural molecules and polymers (glucose,

glycogen, glycosaminoglycan, sialic acid, gelatin) have already been exploited as MRI-CEST contrast agents, since these molecules have precedence of use with human exposure (Chan et al., 2012; Jin et al., 2017; Liang et al., 2015; Ling et al., 2008; Shinar et al., 2014; van Zijl et al., 2007; Walker-Samuel et al., 2013). Also metabolites, drugs and polypeptides/proteins have been investigated to demonstrate their capability to generate contrast within this approach (Bar-Shir et al., 2015; Bar-Shir et al., 2014; Cai et al., 2015; Haris et al., 2012; Li et al., 2016; Liu et al., 2016; Longo et al., 2014a; McMahon et al., 2008; Zaiss et al., 2013). Moreover, several diamagnetic CEST agents have been proposed as exogenous probes for tumor imaging (Geraldes and Laurent, 2009). However, diamagnetic molecules require high doses to discriminate their contrast from direct water saturation and from endogenous magnetization transfer effects, due to the small chemical shift difference from water.

These considerations limit the effective use of exogenous molecules as CEST agents to those possessing low *in vivo* toxicity. According to these considerations, researchers firstly turned their attention to already clinically approved contrast agents, such as iodinated contrast media, exploiting their high safety profile and FDA approval (Aime et al., 2005b; Longo et al., 2011). Consequently, radiographic agents have been exploited for assessing tumor microenvironment properties, including perfusion (Anemone et al., 2017; Longo et al., 2016b), acidosis (Chen et al., 2015; Jones et al., 2015; Longo et al., 2016a; Longo et al., 2014b; Sun et al., 2014) and for assessing renal functionality (Longo et al., 2013; Longo et al., 2017).

Pharmaceutical excipients have attracted our interest since them can be used at very high dose due to their well-known safety profiles. In addition, excipients do not have any pharmacological effects, in contrast to active pharmaceutical ingredients. Ideally, a MRI-CEST contrast agent should possess good solubility and high safety profile, it should accumulate enough in the region of interest to produce contrast; afterwards, it should be excreted through the kidneys without long-term accumulation (Aime et al., 2005a; Sherry et al., 2009). The present investigation reports the MRI-

CEST properties of several pharmaceutical excipients (sucrose, N-acetyl-D-glucosamine, ascorbic acid, meglumine and 2-pyrrolidone), as novel, biocompatible MRI contrast agents for molecular imaging of tumors. We describe the *in vitro* MRI-CEST contrast enhancing properties and the *in vivo* investigation of these molecules in two murine tumor models.

2. Methods

2.1 Materials

All chemicals (Sucrose, N-acetyl-D-glucosamine, Meglumine, 2-pyrrolidone, Ascorbic acid) were purchased from Sigma-Aldrich (Sigma Aldrich, Milan, Italy).

2.2 In vitro MRI CEST acquisition

Phantoms containing vials of phosphate buffer solution of Sucrose, N-acetyl-D-glucosamine, Meglumine, 2-pyrrolidone or ascorbic acid were prepared at a concentration of 30mM and titrated over a range 6-7.4 pH units. CEST-MRI experiments were performed on a vertical 7 T MRI scanner Bruker Avance 300 (Bruker, Ettlingen, Germany) using a fast spin-echo sequence with centric encoding after presaturation pulses varying in power (1.5, 2.0, 3.0 and 6.0 μT) for 5 or 7s at 37°C. A modified RARE sequence including a magnetization transfer module was used to acquire CEST-weighted images from -10 to +10 ppm with increments of 0.1 ppm around the water resonance.

2.3 Cell lines for xenograft tumor models

TS/A cells, derived from a metastasizing mouse cell line, originated from a mammary adenocarcinoma which arose spontaneously in a BALB/c female, were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100U/mL Penicillin with 100 μg/mL Streptomycin (Pen/Strep) and 2mM L-Glutamine (Nanni et al., 1983). B16-F10 cells, an

established murine melanoma cell line, were cultured in DMEM supplemented with 10% FBS, 100 µg/ml penicillin and 100 µg /ml streptomycin. B16-F10 cells were obtained from American Type Culture Collection (ATCC).

2.4 Animal experiments

6-old-week female BALB/c mice (n=5 for each molecule) were inoculated subcutaneously with 2.5 × 10^5 TS/A cells in 100 µL of PBS on both flanks and 6-old-week male C57BL/6 mice (n=5 for each molecule) were inoculated subcutaneously with 3×10^5 B16-f10 cell in 100 µL of PBS on both flanks. BALB/c and C57BL/6 mice (Charles River Laboratories Italia S.r.l., Calco Italia) were maintained under specific pathogen free conditions in the animal facility of the Molecular Biotechnology Center, University of Turin, and treated in accordance with the EU guidelines (EU2010/63). All in vivo studies were conducted according to approved procedures of the Institutional Animal Care and Use Committee of the University of Torino.

Before imaging, mice were anaesthetized with a mixture of tiletamine/zolazepam (Zoletil 100; Vibac, Milan, Italy) 20mg/kg and xylazine (Rompun; Bayer, Milan, Italy) 5mg/kg and during the acquisition their breath rate was monitored throughout in vivo MRI experiments using a respiratory probe. Cannulation of the lateral tail vein with a catheter was exploited for intravenous injection of the investigated molecules.

2.5 In vivo MRI CEST acquisition and analysis

A Bruker 7T Avance 300 MRI scanner (Bruker Biospin, Ettlingen, Germany) equipped with a 30 mm 1H quadrature coil was used to scan mammary adenocarcinoma (TS/A cell line) and melanoma (B16-f10 cell line) tumor bearing mice 15 days post-inoculation. After the scout image acquisition, T_{2w} anatomical images were acquired with a Fast Spin Echo sequence and the same geometry was

used for the following CEST experiments. CEST images were acquired with a single shot FSE sequence with centric encoding (TR: 6000 ms, TE: 4.0 ms) after a CW-RF presaturation pulse of B_1 = 1.5 μT x 5s from a single axial slice with high in-plane resolution of 234 μm (FOV 3 cm, MTX 96, zero filled to 128, slice thickness 1.5mm) with 55 frequency offsets unevenly spaced in the range ±10 ppm. Each investigated molecule was administrated intravenously at the dose of 1.2 g/kg b.w. with a single bolus of 100 μL followed by continuous infusion at a rate of 500 μL/h and CEST images were acquired before and every 10 minutes up to 30 minutes.

CEST images were analyzed using homemade scripts implemented in MATLAB (The Mathworks, Inc, Natick, MA). The Z-spectra were interpolated, on a voxel-by-voxel basis, by smoothing splines, B_0-shift corrected and saturation transfer efficiency (ST%) was measured by punctual analysis at 1.2 ppm (Terreno et al., 2009). For in vivo images, difference contrast maps (ΔST%) were calculated by subtracting the ST contrast after each molecules injection from the ST contrast before the injection on a per voxel basis. Extravasation fraction of each molecule was calculated as the percentage of pixels showing a ΔST% above the threshold (2%) in the manually-defined tumor region of interest.

2.6 Statistical analysis

Calculations were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA) software package; data are presented as mean ± SD unless otherwise stated. Statistical significance was established at $P < 0.05$.

3. Results

3.1 In vitro characterization of CEST properties

The investigated molecules differ for the types of mobile protons, i.e. belonging to the hydroxyl, amide and amine groups, and for the number of exchanging protons (Figure 1). The ability to yield CEST contrast in the MR images is shown in Figure 2, where the contrast efficiency (ST effect) is plotted as a function of the chemical shift. Sucrose, ascorbic acid, meglumine and N-acetyl-D-glucosamine show CEST contrast peaking at ca. 0.7-1.2 ppm, due to the presence of hydroxyl protons. 2-Pyrrolidone showed a small CEST contrast at 0.7 ppm downfield from water, due to the presence of a cyclic amide (lactam) group. Both meglumine and N-acetyl-D-glucosamine showed, in addition to the less shifted hydroxyl protons, a second broad CEST contrast peak between 2 and 3.5 ppm, due to amine and amide protons, respectively. As shown in Figure 2, when keeping constant the saturation field strength (1.5 μT x 7s), the CEST contrast showed a significant dependence with pH. The hydroxyl mobile protons of sucrose showed higher CEST contrast values at lower pH values (Figure 3A). Conversely, the hydroxyl protons of N-acetyl-D-glucosamine, meglumine and ascorbic acid showed a steady increase of the CEST contrast at high pH values, at all the investigated saturation field strengths (1.5 – 6 μT, Figures 3B, D and E). The CEST contrast of 2-pyrrolidone was almost independent from pH (Figure 3C). Sucrose, meglumine, ascorbic acid and N-acetyl-D-glucosamine showed in vitro a ST efficiency close to 1% per 1 mM concentration (irradiation pulse of 1.5 μT x 5s, pH 7, T=37°C; Figure 3F). The highest CEST contrast efficiency observed for sucrose (1.5 % ST per 1 mM concentration) likely depends on the large number of mobile protons (8 –OH protons), whereas 2-pyrrolidone, having only one exchanging proton, showed the lowest CEST contrast.

3.2 In vivo CEST detection in tumor murine models

Two cancer xenograft models, TS/A (highly metastatic mouse breast cancer cells) and B16 (mouse melanoma cancer cells) were used for *in vivo* experiments. CEST agents were administered at the same dose by intravenous injection through the tail vein. A pronounced increase in the CEST

contrast in both TS/A and B16 tumor models for all the investigated molecules, with an average ΔST increase in the range 2- 6% in comparison to the pre-contrast ST values was observed (Figure 4). Sucrose showed a slightly higher CEST contrast in the B16 model (ΔST=4.1 ±0.7%) in respect to TS/A tumors (ΔST=2.7 ±0.5%) at all the investigated time points (Figure 4A). N-Acetyl-D-glucosamine slightly raised the CEST effect from the baseline with no difference between the two tumor models (ΔST=2.1 ±0.5% and 2.5 ±0.5% for B16 and TS/A, respectively, Figure 4B). The CEST signal of meglumine increased by 4.1 ±1.0% for the B16 tumors and by 2.5 ±0.4% for the TS/A tumors (Figure 4C), at 10 min post injection ($P<0.05$). Ascorbic acid showed the highest enhancement in TS/A tumors (ΔST=5.4 ±1.1%) in comparison to the B16 model (ΔST=2.7 ±0.7%), with statistically significant difference already 10 min after the i.v. administration ($P<0.05$, Figure 4D). For all the investigated molecules the CEST contrast measured in tumors persisted up to 30 min after the administration. Representative CEST contrast maps overimposed on anatomical images show the differential enhancement pattern among the investigated excipients within the investigated two murine tumor models (Figure 5). In particular, ΔST images showed a marked increase in CEST contrast in B16 and TS/A tumors for meglumine and ascorbic acid, respectively. The percentage of the tumor pixels where the CEST effect was detectable (ΔST higher than 2%) is dependent on both the molecules and the tumor model (Figure 6). In particular, CEST contrast increase was higher for the B16 model than for the TS/A one. Sucrose was detected in 40-60% of the tumor area according to the tumor model, whereas all the other molecules showed a detection fraction lower than 50% of the whole tumor volume.

4. Discussion

This study demonstrated that several excipients can be exploited as MRI-CEST contrast agents for tumor detection in mice. A moderate to marked increase in CEST contrast in the tumor region was detected up to 30 min following intravenous administration, according to the exploited agents or the

investigated tumor models. Meglumine and ascorbic acid yielded the highest contrast enhancement (ΔST>5%) in B16 and TS/A models, respectively (Figure 4). Conversely, lower CEST enhancements were measured for sucrose and N-acetyl-D-glucosamine. The potential of glucose and its derivatives to act as MRI-CEST agents for tumor imaging has already been demonstrated (Chan et al., 2012; Walker-Samuel et al., 2013; Xu et al., 2015). However, for glucose, the main drawback was associated to its rapid metabolism once entered in the tumor cells, with consequent reduction of CEST contrast capabilities. For this reason, glucose analogs, such as 2-deoxy-glucose (2DG) and 3-oxy-methyl-gluose (3OMG) have been proposed as they showed superior contrast efficiency owing to the reduced metabolic conversion in the case of 2DG (Nasrallah et al., 2013; Rivlin et al., 2013) or to the lack of metabolic transformation in the case of 3OMG (Rivlin et al., 2014). As a consequence, such derivatives provide an improved and long-lasting CEST contrast in mice carrying xenograft tumors. On the other hand, the safety of these compounds has still to be demonstrated, since the high concentrations (> 1-1.5 g/kg b.w.) required to generate enough CEST contrast might limit their use to laboratory animals. An analogous limitation may be envisaged for the recently reported CEST agents based on salicylic acid (a metabolite of aspirin), although their very large chemical shift difference (unusual for diamagnetic CEST agents) holds promise for applications at lower magnetic fields (Lesniak et al., 2016; Yang et al., 2013). Conversely, the herein investigated excipients hold a very high safety profile, considering that they are used at high dosages to provide suitable formulations for drugs.

In contrast to N-acetyl-D-glucosamine and ascorbic acid that can be metabolized within the body, sucrose (when injected i.v.) and meglumine are rapidly excreted unchanged in the urine, with no evidence for metabolism (Heeg et al., 1977). Clearly, this represents a great advantage in comparison to metabolizable probes that do not accumulated in the extracellular extravascular space of tumors. Furthermore, metabolic products cannot provide enough CEST contrast as the original molecule, with a following decrease of their observed CEST contribution. This may partly explain

the non-optimal CEST contrast in tumors upon N-acetyl-D-glucosamine administration, in comparison to the other excipients, despite the high *in vitro* CEST contrast efficiency. Similar findings for N-acetyl-D-glucosamine were observed by Navon group, who investigated the CEST properties of this molecule and of glucosamine as alternatives to glucose analogs (Rivlin and Navon, 2016). In our study, the observed increase in CEST contrast in tumors with N-Acetyl-D-Glucosamine was lower (ΔST = 2-3%) than what previously reported (ΔST = 6-7%), albeit a similar dosage was administered. These observations may be accounted by the use of different tumor cell lines or by the lower saturation pulse power that has been applied in our experimental protocol (1.5 μT vs 2.4 μT).

Meglumine showed distinct contrast enhancement capabilities between TS/A and B16 tumors. Meglumine is not internalized inside cells, therefore differences in CEST contrast enhancements are only dependent on the accumulation within the extracellular extravascular space, hence reflecting different vascularization properties. As such, this molecule can be considered an extracellular-fluid agent analogous to the clinically approved Gadolinium-based contrast agents (Morana et al., 2013) or to Iodine-containing X-ray systems (Rutten and Prokop, 2007). Thus, meglumine may be an attractive candidate to be used as MRI-CEST contrast agents for tumor imaging with remarkable contrast efficiency.

5. Conclusions

The herein investigated excipients show remarkable MRI-CEST properties as demonstrated by the *in vivo* visualization of tumors in two murine models. The extremely good safety profile of the excipients provides support to the view that these systems may be considered reliable candidates for clinical translation.

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Figure Legends

Figure 1. Chemical structures and molecular weights (g/mol) of the investigated excipients.

Figure 2. CEST contrast curves for the investigated molecules (sucrose, black; N-acetyl-D-glucosamine, red; 2-Pyrrolidone, grey; meglumine, green; ascorbic acid, blue) obtained at concentration of 30 mM at pH 6 (A) and pH 7.4 (B) using a saturation power level of 1.5 µT with duration of 7s at 7T and 37°C.

Figure 3. CEST contrast dependence on pH measured in the range of 6-7.4 pH units at different B_1 levels (saturation power from 1.5 µT to 6 µT for 5s, 7T, 37°C) for sucrose (A), N-acetyl D-glucosamine (B), 2-Pyrrolidone (C), meglumine (D) and ascorbic acid (E).

Figure 4. Box-plots showing averaged mean ΔST increase (calculated as ST post -ST pre injection) in TS/A (grey bars) and B16 (black bar) tumor regions using B_1 = 1.5µTx5s on a 7T MRI scanner. CEST contrast observed after i.v. administration of sucrose (A), N-acetyl-D-glucosamine (B), meglumine (C) and ascorbic acid (D) at a dose of 1.2 g/kg b.w. was observed at 10, 20 and 30 minutes post-injection.

Figure 5. Representative ΔST tumor maps overimposed on anatomical images showing CEST contrast increase for sucrose, N-acetyl-D-glucosamine, meglumine and ascorbic acid 20 min after i.v. administration using B_1 = 1.5 µT x 5s in B16 (top row) and TS/A (bottom row) tumors.

436 **Figure 6.** Box-plot showing extravasation fraction calculated as percentage of pixels with ΔST
437 higher than 2% for sucrose, N-acetyl-D-glucosamine, meglumine and ascorbic acid 20 min after i.v
438 administration using B_1= 1.5 μT x 5s for B16 (A) and TS/A (B) murine tumors.