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

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- 23 Title page
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44 Abstract

45 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. 46 Many molecules, employed as excipients for the formulation of finished drug products, are 47 endowed with hydroxyl, amine or amide protons, thus can be exploitable as MRI-CEST contrast 48 agents. Their high safety profiles allow them to be injected at very high doses. Here we investigated 49 the MRI-CEST properties of several excipients (ascorbic acid, sucrose, N-acetyl-D-glucosamine, 50 meglumine and 2-pyrrolidone) and tested them as tumor-detecting agents in two different murine 51 tumor models (breast and melanoma cancers). All the investigated molecules showed remarkable 52 CEST contrast upon i.v. administration in the range 1-3 ppm according to the type of mobile proton 53 groups. A marked increase of CEST contrast was observed in tumor regions up to 30 min post 54 injection. The combination of marked tumor contrast enhancement and lack of toxicity make these 55 56 molecules potential candidates for the diagnosis of tumors within the MRI-CEST approach.

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- 58

59 Keywords

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

61

62 Chemical Compounds studied in this article

- 63 Ascorbic acid (PubChem CID: 54670067);
- 64 Meglumine (PubChem CID: 8567);
- 65 Sucrose (PubChem CID: 5988);
- 66 N-acetyl-D-glucosamine (PubChem CID: 439174);

67 2-Pyrrolidone (PubChem CID: 12025);

68

69 Abbreviations

- 70 MRI: Magnetic Resonance Imaging
- 71 CEST: Chemical Exchange Saturation Transfer
- 72 i.v.: intravenous
- 73

74 **1. Introduction**

75 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, 76 excipients perform multiple functions, besides completing the formulation volume, such as 77 improving bioavailability, administration and acceptance of the treatment by the patient (Loftsson, 78 2015; Narayan, 2011; Wening and Breitkreutz, 2011). Another fundamental characteristic of 79 excipients is their pharmacological and toxicological inactivity that allows them to be used at high 80 doses (Abrantes et al., 2016). Several natural products, simple substances and mixtures are 81 82 commonly used in formulating medicines, with chemical structures that vary from small molecules to polymers. 83

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 91 92 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; 93 94 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 95 al., 2015; Bar-Shir et al., 2014; Cai et al., 2015; Haris et al., 2012; Li et al., 2016; Liu et al., 2016; 96 97 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, 98 2009). However, diamagnetic molecules require high doses to discriminate their contrast from 99 100 direct water saturation and from endogenous magnetization transfer effects, due to the small chemical shift difference from water. 101

These considerations limit the effective use of exogenous molecules as CEST agents to those 102 possessing low in vivo toxicity. According to these considerations, researchers firstly turned their 103 attention to already clinically approved contrast agents, such as iodinated contrast media, exploiting 104 105 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, 106 including perfusion (Anemone et al., 2017; Longo et al., 2016b), acidosis (Chen et al., 2015; Jones 107 108 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). 109

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- 116 CEST properties of several pharmaceutical excipients (sucrose, N-acetyl-D-glucosamine, ascorbic 117 acid, meglumine and 2-pyrrolidone), as novel, biocompatible MRI contrast agents for molecular 118 imaging of tumors. We describe the *in vitro* MRI-CEST contrast enhancing properties and the *in* 119 *vivo* investigation of these molecules in two murine tumor models.

120

121 **2.** Methods

122 2.1 Materials

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

125

126 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 CESTweighted images from -10 to +10 ppm with increments of 0.1 ppm around the water resonance.

134

135 *2.3 Cell lines for xenograft tumor models*

136 TS/A cells, derived from a metastasizing mouse cell line, originated from a mammary 137 adenocarcinoma which arose spontaneously in a BALB/c female, were grown in RPMI 1640 138 medium supplemented with 10% fetal bovine serum (FBS), 100U/mL Penicillin with 100 μ g/mL 139 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 μ g/ml penicillin and 100 μ g /ml streptomycin. B16-F10 cells were obtained from American Type Culture Collection (ATCC).

143

144 2.4 Animal experiments

6-old-week female BALB/c mice (n=5 for each molecule) were inoculated subcutaneously with 2.5 145 $\times 10^5$ TS/A cells in 100 µL of PBS on both flanks and 6-old-week male C57BL/6 mice (n=5 for 146 each molecule) were inoculated subcutaneously with 3×10^5 B16-f10 cell in 100 µL of PBS on both 147 flanks. BALB/c and C57BL/6 mice (Charles River Laboratories Italia S.r.l., Calco Italia) were 148 149 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 150 (EU2010/63). All in vivo studies were conducted according to approved procedures of the 151 Institutional Animal Care and Use Committee of the University of Torino. 152

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.

158

159 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 B₁ $= 1.5 \mu$ T 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, 171 Inc, Natick, MA). The Z-spectra were interpolated, on a voxel-by-voxel basis, by smoothing 172 173 splines, B₀-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 (Δ ST%) 174 were calculated by subtracting the ST contrast after each molecules injection from the ST contrast 175 before the injection on a per voxel basis. Extravasation fraction of each molecule was calculated as 176 the percentage of pixels showing a Δ ST% above the threshold (2%) in the manually-defined tumor 177 178 region of interest.

179

180 2.6 Statistical analysis

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

184

185 **3. Results**

186 *3.1 In vitro characterization of CEST properties*

The investigated molecules differ for the types of mobile protons, i.e. belonging to the hydroxyl, 187 188 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 189 plotted as a function of the chemical shift. Sucrose, ascorbic acid, meglumine and N-acetyl-D-190 glucosamine show CEST contrast peaking at ca. 0.7-1.2 ppm, due to the presence of hydroxyl 191 protons. 2-Pyrrolidone showed a small CEST contrast at 0.7 ppm downfield from water, due to the 192 193 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 194 3.5 ppm, due to amine and amide protons, respectively. As shown in Figure 2, when keeping 195 196 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 197 at lower pH values (Figure 3A). Conversely, the hydroxyl protons of N-acetyl-D-glucosamine, 198 meglumine and ascorbic acid showed a steady increase of the CEST contrast at high pH values, at 199 200 all the investigated saturation field strengths $(1.5 - 6 \mu T, Figures 3B, D and E)$. The CEST contrast of 2-pyrrolidone was almost independent from pH (Figure 3C). Sucrose, meglumine, ascorbic acid 201 202 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 203 204 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, 205 showed the lowest CEST contrast. 206

207

208 *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

| 212 | contrast in both TS/A and B16 tumor models for all the investigated molecules, with an average |
|-----|--------------------------------------------------------------------------------------------------------------|
| 213 | Δ ST increase in the range 2- 6% in comparison to the pre-contrast ST values was observed (Figure |
| 214 | 4). Sucrose showed a slightly higher CEST contrast in the B16 model (Δ ST=4.1 ±0.7%) in respect |
| 215 | to TS/A tumors (Δ ST=2.7 ±0.5%) at all the investigated time points (Figure 4A). N-Acetyl-D- |
| 216 | glucosamine slightly raised the CEST effect from the baseline with no difference between the two |
| 217 | tumor models (Δ ST=2.1 ±0.5% and 2.5 ±0.5% for B16 and TS/A, respectively, Figure 4B). The |
| 218 | CEST signal of meglumine increased by 4.1 \pm 1.0% for the B16 tumors and by 2.5 \pm 0.4% for the |
| 219 | TS/A tumors (Figure 4C), at 10 min post injection (P<0.05). Ascorbic acid showed the highest |
| 220 | enhancement in TS/A tumors (Δ ST=5.4 ±1.1%) in comparison to the B16 model (Δ ST=2.7 ±0.7%), |
| 221 | with statistically significant difference already 10 min after the i.v. administration (P<0.05, Figure |
| 222 | 4D). For all the investigated molecules the CEST contrast measured in tumors persisted up to 30 |
| 223 | min after the administration. Representative CEST contrast maps overimposed on anatomical |
| 224 | images show the differential enhancement pattern among the investigated excipients within the |
| 225 | investigated two murine tumor models (Figure 5). In particular, ΔST images showed a marked |
| 226 | increase in CEST contrast in B16 and TS/A tumors for meglumine and ascorbic acid, respectively. |
| 227 | The percentage of the tumor pixels where the CEST effect was detectable (Δ ST higher than 2%) is |
| 228 | dependent on both the molecules and the tumor model (Figure 6). In particular, CEST contrast |
| 229 | increase was higher for the B16 model than for the TS/A one. Sucrose was detected in 40-60% of |
| 230 | the tumor area according to the tumor model, whereas all the other molecules showed a detection |
| 231 | fraction lower than 50% of the whole tumor volume. |

233 **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 237 238 $(\Delta ST > 5\%)$ 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 239 240 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 241 242 drawback was associated to its rapid metabolism once entered in the tumor cells, with consequent 243 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 244 efficiency owing to the reduced metabolic conversion in the case of 2DG (Nasrallah et al., 2013; 245 246 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 247 248 mice carrying xenograft tumors. On the other hand, the safety of these compounds has still to be 249 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 250 251 the recently reported CEST agents based on salicylic acid (a metabolite of aspirin), although their 252 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 253 254 herein investigated excipients hold a very high safety profile, considering that they are used at high 255 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 262 comparison to the other excipients, despite the high in vitro CEST contrast efficiency. Similar 263 findings for N-acetyl-D-glucosamine were observed by Navon group, who investigated the CEST 264 properties of this molecule and of glucosamine as alternatives to glucose analogs (Rivlin and 265 Navon, 2016). In our study, the observed increase in CEST contrast in tumors with N-Acetyl-D-266 Glucosamine was lower ($\Delta ST = 2-3\%$) than what previously reported ($\Delta ST = 6-7\%$), albeit a 267 similar dosage was administered. These observations may be accounted by the use of different 268 tumor cell lines or by the lower saturation pulse power that has been applied in our experimental 269 270 protocol (1.5 μ T vs 2.4 μ T).

271 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 272 only dependent on the accumulation within the extracellular extravascular space, hence reflecting 273 different vascularization properties. As such, this molecule can be considered an extracellular-fluid 274 agent analogous to the clinically approved Gadolinium-based contrast agents (Morana et al., 2013) 275 276 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 277 contrast efficiency. 278

279

280 **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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419 Figure Legends

420 **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-Dglucosamine, 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 $1.5 \,\mu\text{T}$ 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 B₁ levels (saturation power from 1.5 μ T to 6 μ T for 5s, 7T, 37°C) for sucrose (A), N-acetyl Dglucosamine (B), 2-Pyrrolidone (C), meglumine (D) and ascorbic acid (E).

Figure 4. Box-plots showing averaged mean Δ ST increase (calculated as ST post -ST pre injection) in TS/A (grey bars) and B16 (black bar) tumor regions using B₁ = 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 Δ ST 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 B₁ = 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 $B_1 = 1.5 \mu T x 5s$ for B16 (A) and TS/A (B) murine tumors.