Hyperpolarized $^{13}$C Magnetic Resonance detection of Carboxypeptidase G2 activity

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Abstract

Carboxypeptidase G2 is a bacterial enzyme that is currently employed in a range of targeted cancer chemotherapy strategies such as gene directed enzyme prodrug therapy (GDEPT). Employing Dynamic Nuclear Polarization (DNP) and natural abundance $^{13}$C magnetic resonance spectroscopy (MRS) we have observed the CPG2 mediated conversion of a novel hyperpolarized reporter probe 3,5-difluorobenzoyl-L-glutamic acid (3,5-DFBGlu) to 3,5-difluorobenzoic acid (3,5-DFBA) and L-glutamic acid (L-Glu) in vitro. Isotopic labeling of the relevant nuclei with $^{13}$C in 3,5-DFBGlu or related molecules may enable these experiments to be translated in vivo and generate metabolic maps of CPG2 activity.

Key Words: hyperpolarized $^{13}$C, dynamic nuclear polarization, gene therapy, carboxypeptidase G2
Introduction

New frontiers in metabolic imaging have recently been realised *in vivo* through a combination magnetic resonance spectroscopy (MRS) and novel hyperpolarization techniques employing Dynamic Nuclear Polarisation (DNP). The significant enhancement of the MR signal by more than a factor 10,000 has transformed insensitive techniques such as $^{13}$C and $^{15}$N MRS into versatile strategies that afford a window on the dynamics of endogenous enzymatic processes by generating high spatial resolution and real-time maps of the metabolism of hyperpolarized substrates non invasively (1,2). These techniques have primarily focused on hyperpolarized $^{13}$C pyruvate as a substrate due to its favourable relaxation characteristics and central role in cellular energy metabolism (3,4). Other hyperpolarized imaging reporters have emerged in recent years to probe a range of key endogenous metabolic reactions (5-8), as well as further applications such as the measurement of pH *in vivo* (9).

The bacterial enzyme carboxypeptidase G2 (CPG2) and other exogenous enzymes have been utilized in promising targeted chemotherapeutic strategies to activate selectively non toxic prodrugs into cytotoxic drugs in the tumor (10). Several strategies have been developed to target the enzyme to the tumor including the use of CPG2–antibody conjugates in antibody-directed enzyme prodrug therapy (ADEPT) (11), viral vectors that carry the gene encoding for CPG2 in gene-directed enzyme prodrug therapy (GDEPT) (12), and more recently the use of bacteria engineered to express CPG2 (13). These therapeutic strategies would benefit from robust imaging strategies that afford high spatial and temporal resolution images of the biodistribution of CPG2 activity.
We have focused our work on developing MR imaging reporters for CPG2 (EC 3.4.17.11), which is a Zn$^{2+}$-dependent exopeptidase that activates relatively non-toxic prodrugs into activated DNA alkylating agents by removing their glutamate moiety. 3,5-difluorobenzoyl-L-glutamic acid (3,5-DFBGlu) is a reporter probe that can be used to detect CPG2 activity in vivo using $^{19}$F magnetic resonance spectroscopy (MRS), utilizing a 1.4 ppm chemical shift difference associated with the CPG2-mediated conversion of 3,5-DFBGlu to 3,5-DFBA and L-glutamic acid (14), Figure 2. In this study we have investigated the magnetic resonance properties of the relevant $^{13}$C nuclei (natural abundance 1%) in 3,5-DFBGlu and whether they have the prerequisites properties for a molecular imaging approach employing dynamic nuclear polarization. We hereby demonstrate, i) the ability to hyperpolarize efficiently the parent compound 3,5-DFBGlu and its cleaved products, ii) significant $^{13}$C chemical shift changes upon CPG2-mediated cleavage to 3,5-DFBA and glutamic acid, iii) long longitudinal relaxation times $T_1$ of a number of $^{13}$C nuclei in 3,5-DFBGlu and the resulting cleaved compounds, offering a sufficient imaging window to enable us to observe metabolic conversion by CPG2. With suitable isotopic labeling the methodology could potentially be translated to probe CPG2 activity in vivo using hyperpolarized $^{13}$C MRS. These results might be extended to other exogenous enzymes implicated in gene therapy strategies.
Methods and Results

Natural abundance $^{13}$C Hyperpolarisation

$^{13}$-DFBGlu was synthesized according to the literature (15). Substrates were prepared for hyperpolarization by separately dissolving 5 mg of $^{13}$-DFBGlu, $^{3,5}$-DFBA or glutamic acid in solutions containing 3mg of free radical OX63 with a glassing agent composed of 2:1 DMSO: H$_2$O and polarized for 3 hours in a HyperSense® DNP polarizer (Oxford Instruments Molecular Biotools, Abingdon, UK) at low temperature (1.4 K) with microwave irradiation at 94 GHz. The polarized samples were rapidly dissolved in 4ml of a solution of 100mM TrisHCl, 260 µM ZnCl$_2$, 1 mM EDTA and transferred to a 10 mm tube containing 200 µl of 100mM TrisHCl with ZnCl$_2$ (6 mM final concentration) and placed in a 11.7T Bruker Avance spectrometer (Bruker Biospin, Ettlingen, Germany). The pH of the dissolution solution was adjusted with 10M NaOH (Sigma-Aldrich, Gillingham, UK) to provide a pH = 7.4 of to the final solution. A series of $^{13}$C NMR spectra were subsequently acquired every 3 s using a 10° pulse-and-acquire sequence (1 transient, 64k time domain points, a 19kHz spectral width, acquisition time 1.7s, T = 303K).

Figure 2 (a)-(c) show hyperpolarized $^{13}$C spectra of $^{3,5}$-DFBGlu, $^{3,5}$-DFBA and L-Glu, respectively, corresponding to the first spectrum from the above time series. The $^{13}$C signal enhancement of the carbonyl groups of the parent compound $^{3,5}$-DFBGlu was estimated to be 5000 with respect to the thermal equilibrium value at 11.7T yielding a polarization of the order of 5%. It is interesting to note in Figure 2(c) that whilst efficient polarization of the side chain carbonyl was achieved in L-Glu, no signal was observed for the amide carbonyl. The relaxation times T$_1$, table 1, were measured for the relevant $^{13}$C
nuclei from the decay of the hyperpolarized signal with time and corrected for the loss of polarization from the 10° RF pulse (16). Relaxation times were of the order 5-10s for 3,5-DFBGlu and of the order 20s for the daughter carbonyl carbons in 3,5-DFBA. The $^{13}$C chemical shift differences between parent and daughter compounds are also presented in table 1. The largest chemical shift change (4.8 ppm) is observed for the amide carbonyl closest to the site of CPG2 mediated cleavage.

$^{13}$C MRS detection of CPG2 activity using hyperpolarised 3,5-DFBGlu

Cleavage by CPG2 was carried out by adding hyperpolarized 3,5-DFBGlu to a solution containing the enzyme. 3,5-DFBGlu was polarized as described above and transferred to a solution of 100 mM Tris HCl, pH 7.4 containing 3 (results not shown) or 10 units (see below) of the commercially available CPG2 (EC 3.4.17.11, Sigma-Aldrich, UK) (1 unit converts 1 µmol.min$^{-1}$ of methotrexate at 30 °C, and 2 µmol.min$^{-1}$ of 3,5-DFBGlu). Figure 3(a) shows a time series of spectra recorded every 3s with acquisition parameters as previously described with hyperpolarized DFBGlu added to 10 units of enzyme. The decay of the hyperpolarized signal from the parent compound resulting from relaxation processes and metabolic conversion is clearly evident, coupled with the appearance of the metabolic products of carbons 1' and 2' of 3,5-DFBA and one of the carbonyls e' of L-Glu. Consistent with our inability to detect the polarized amide carbonyl signal of L-Glu this peak is also missing from the enzyme reaction despite the same carbonyl being efficiently polarized in the parent compound. Figure 3(b) shows the peak integral of the parent carbonyl 1 and the product 1' as a function of time.
**19F detection of CPG2 activity using 3,5-DFBGlu**

We have also measured the activity of CPG2 using the same enzyme solution from the DNP experiment and employing a standard 19F MRS assay. For this purpose 3 units of enzyme was used (cleaves 6 μmol 3,5-DFBGlu.min⁻¹). 1H-decoupled 19F spectra were recorded using a 90 degree pulse-and-acquire sequence at 30 °C and 470 MHz (1 transient, 32k time data points, 4.6s repetition time). Each spectrum was apodized with a 5 Hz exponential line-broadening function. Figure 4(a), bottom spectrum, shows the 19F spectrum after the DNP experiment demonstrating a single resonance from 3,5-DFBA and indicating that complete conversion has occurred. Note the comparable S/N ratio of the 19F spectrum (100% natural abundance) recorded with a 90° pulse compared to the hyperpolarized 13C (1% natural abundance) spectra recorded previously with a 10° pulse. Following addition of an extra 126 μmol of 3,5-DFBGlu to the solution a time series of 19F spectra was recorded, Figure 4(b), demonstrating conversion of DFBGlu to DFBA. From these data the linear change can be observed in the substrates as a function of time, consistent with first order enzyme kinetics from which the kinetic parameters can be derived. CPG2 activity was estimated by linear regression of the first 50 points (r² = 0.9993, p < 0.0001) to be equal to 6.25 μmol 3,5-DFBGlu cleaved .min⁻¹ and is in agreement with the amount of enzyme present.

**Discussion**

Generating high temporal and spatial resolution maps of CPG2 activity non-invasively in vivo would be of benefit to all the CPG2–based cancer chemotherapy strategies by assessing the successful targeting of the enzyme to the tumor. In this pilot
investigation we have demonstrated that 3,5-DFBGlu is a hyperpolarized $^{13}\text{C}$ MRS reporter probe of CPG2 activity in vitro. We observe two $^{13}\text{C}$ nuclei with long enough $T_1$ times and CPG2-induced chemical shift changes that encourage the translation of this approach in vivo. This would necessitate isotopic labeling of one of the identified $^{13}\text{C}$ nuclei providing a further 100-fold enhancement of the signal. From these experiments the benzoyl carbonyl I nucleus of 3,5-DFBGlu would seem to be the best candidate for $^{13}\text{C}$ labeling. These results open an avenue for studying the real-time kinetics of exogenous enzymes implicated in therapeutic as well as gene reporter strategies using hyperpolarized MR imaging with the potential for translation in vivo.

Acknowledgements

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Table 1: $^{13}$C $T_1$ values measured from the decay of the hyperpolarized signal and observed chemical shift changes $\Delta \Omega$ (ppm) on CPG2 mediated cleavage.

<table>
<thead>
<tr>
<th></th>
<th>3,5-DFBGlul</th>
<th>3,5-DFBA</th>
<th>L-Glu</th>
<th>$\Delta \Omega$ (ppm)</th>
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<tr>
<td>$^{13}$C$_1$</td>
<td>6.5 ± 0.5s</td>
<td>$^{13}$C$_1'$</td>
<td>22.9 ± 1.6s</td>
<td>4.8</td>
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<tr>
<td>$^{13}$C$_2$</td>
<td>5.7 ± 0.4s</td>
<td>$^{13}$C$_2'$</td>
<td>18.8 ± 1.7s</td>
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<tr>
<td>$^{13}$C$_a$</td>
<td>9.3 ± 0.4s</td>
<td>$^{13}$C$_a'$</td>
<td>Not detected</td>
<td>-</td>
</tr>
<tr>
<td>$^{13}$Ce</td>
<td>7.5 ± 0.4s</td>
<td>$^{13}$Ce'</td>
<td>11.3 ± 0.6s</td>
<td>-0.4</td>
</tr>
</tbody>
</table>
Figure Captions

Figure 1. CPG2 mediated cleavage of 3,5-DFBGlu to form the products 3,5-DFBA and L-glutamic acid.

Figure 2. Hyperpolarized $^{13}$C MR spectra of 5mg each of (a) 3,5-DFBGlu, (b) 3,5-DFBA and (c) L-Glu.

Figure 3. (a) Serial $^{13}$C MR spectra recorded every 3s following addition of hyperpolarized 3,5-DFBGlu to 10 units of CPG2. (b) Integrals of the parent peak 1 and its metabolic product 1'.

Figure 4. $^{19}$F MR assay of CPG2 activity under DNP hyperpolarization conditions. (a) $^1$H-decoupled $^{19}$F spectrum of the solution following the DNP experiment acquired at 470 MHz, showing the presence of a single $^{19}$F resonance assigned to 3,5-DFBA. (b) Stacked $^{19}$F spectra showing the dynamic changes in the $^{19}$F resonances of 3,5-DFBGlu and 3,5-DFBA after adding an extra 126 μmol of 3,5-DFBGlu to the solution. Each $^{19}$F MRS spectrum was acquired at 37 °C in 9 seconds using a 90° pulse-and-acquire sequence (1 transient, repetition time 4.6 s, 32k time domain points. (c) Integral of the $^{19}$F peaks as a function of times showing the change in the quantity of 3,5-DFBGlu and 3,5-DFBA from which the kinetic parameters may be derived.
References


Figure 1. CPG2 mediated cleavage of 3,5-DFBGlut to form the products 3,5-DFBA and L-glutamic acid.

137x51mm (600 x 600 DPI)
Figure 2. Hyperpolarized $^{13}$C MR spectra of Smg each of (a) 3,5-DFBGlu, (b) 3,5-DFBA and (c) L-Glu.
Figure 3. (a) Serial $^{13}$C MR spectra recorded every 3s following addition of hyperpolarized 3,5-DFBGlu to 10 units of CPG2. (b) Integrals of the parent peak 1 and its metabolic product 1'.

136x160mm (600 x 600 DPI)
Figure 4. $^{19}$F MR assay of CPG2 activity under DNP hyperpolarization conditions. (a) $^1$H-decoupled $^{19}$F spectrum of the solution following the DNP experiment acquired at 470 MHz, showing the presence of a single $^{19}$F resonance assigned to 3,5-DFBA. (b) Stacked $^{19}$F spectra showing the dynamic changes in the $^{19}$F resonances of 3,5-DFBGlu and 3,5-DFBA after adding an extra 126 µmol of 3,5-DFBGlu to the solution. Each $^{19}$F MRS spectrum was acquired at 37 °C in 9 seconds using a 90° pulse-and-acquire sequence (1 transient, repetition time 4.6 s, 32k time domain points. (c) Integral of the $^{19}$F peaks as a function of times showing the change in the quantity of 3,5-DFBGlu and 3,5-DFBA from which the kinetic parameters may be derived.