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Conditions for ^{13}C NMR Detection of 2-Hydroxyglutarate in Tissue Extracts from IDH-Mutated Gliomas

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Abstract

^{13}C NMR spectroscopy of extracts from patient tumor samples provides rich information about metabolism. However, in IDH-mutant gliomas ^{13}C labeling is obscured in glutamate and glutamine by the oncometabolite, 2-hydroxyglutaric acid (2HG), prompting development of a simple method to resolve the metabolites. J-coupled multiplets in 2HG were similar to glutamate

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and glutamine and could be clearly resolved at pH 6. A cryogenically-cooled ^{13}C probe but not J-resolved heteronuclear single quantum coherence spectroscopy significantly improved detection of 2HG. These methods enable the monitoring of ^{13}C - ^{13}C spin-spin couplings in 2HG expressing IDH mutant gliomas.

Keywords

2-Hydroxyglutarate; cryo probe; ^{13}C NMR; IDH-mutant gliomas

Gain-of-function mutations in isocitrate dehydrogenases 1 and 2 (IDH1 and IDH2) catalyze conversion of α -ketoglutarate to 2-hydroxyglutarate (2HG) and accumulation of 2HG to supraphysiological concentration in a wide range of cancer subtypes including gliomas [1-5]. 2HG may play a role in malignant transformation [1], and inhibitors of IDH1/2 which prevent 2HG production are already in clinical trials for acute myelogenous leukemia, gliomas, and other solid tumors [6]. Consequently there is intense interest in understanding the IDH pathway, the metabolic impact of elevated 2HG, and changes that occur as a result of IDH inhibition. Since the carbon backbone of 2HG arises from the citric acid cycle, multiple pathways could influence net 2HG synthesis. Methods for determining 2HG concentration in patient tumors by ^1H MR spectroscopy (MRS) have been developed [3-5], although ^1H MRS provides no information about the metabolic pathways involved in 2HG production. ^{13}C MR spectroscopy in patient tumors *in vivo* is limited by low sensitivity [7].

An alternative approach for studying 2HG is analysis of IDH-mutant tumor samples *ex vivo* after metabolism of ^{13}C -enriched nutrients. We have previously shown that ^{13}C -enriched glucose and acetate can be infused safely in patients undergoing surgical resection of a brain tumor [8,9] and analysis of tumor biopsies obtained during surgery by high-resolution NMR spectroscopy provides a wealth of information regarding active metabolic pathways. As a consequence of rapid exchange with α -ketoglutarate, the ^{13}C NMR spectrum of glutamate and glutamine provides direct information about the labeling patterns in the citric acid cycle [8-10]. The ^{13}C NMR chemical shifts, ^1H chemical shifts, and ^1H - ^1H coupling constants of 2HG are known [11,4], but the ^{13}C - ^{13}C coupling constants of 2HG have not been reported. The purpose of this study was to determine all homo- and heteronuclear J couplings of 2HG, improve spectral resolution of 2HG and the overlapping metabolites, and explore methods to improve sensitivity of 2HG detection.

[U- $^{13}\text{C}_5$]2HG was prepared as described in the supplemental section. Solutions containing (in mM) glutamate (50), glutamine (50), lactate (5) and 2HG (150) in D_2O were prepared. Lactate was added for internal chemical shift referencing because it is easily detected in tumor extracts. Structures of 2HG/Glutamate/Glutamine were shown in Figure S1 (supplemental section).

To obtain a ^{13}C -enriched tumor sample, a patient with an IDH1 (R132H) glioblastoma was infused intravenously with [U- ^{13}C]glucose (bolus of 8 g of [U- ^{13}C]glucose over 10 min, followed by 8 g/h of [U- ^{13}C]glucose continuous infusion for 2 Hours) during surgical resection of the tumor under an Institutional Review Board Approved protocol at University of Texas Southwestern Medical Center. Tumor sampling and processing for ^{13}C -NMR have

been previously described [8,9]. ^1H decoupled ^{13}C NMR spectra were acquired from authentic solutions and tumor extracts using a Varian (Agilent Technologies, Walnut Creek, CA) 14.1 T spectrometer equipped with a 3-mm broadband probe using 1.5 s acquisition time, 1.5 s delay, flip angle of 45° , and a spectral width of 32 KHz. The number of transients was 2000-4000 for phantoms and 20,000 for the tissue sample. ^1H decoupling was achieved using WALTZ-16. Free induction decays were zero-filled and multiplied by a weighting function of 0.5 Hz. All data were processed using ACD (Advanced Chemistry Development, Toronto, Canada).

J-resolved heteronuclear single quantum coherence spectroscopy (JHSQC) was performed on the same instrument equipped with a 5-mm proton detect gradient probe [12,13]. ^1H decoupled ^{13}C NMR of the tissue extract in a spinning 3 mm tube was performed using a Bruker Avance 14.1 T spectrometer equipped with a 10-mm broadband cryogenically-cooled probe using the acquisition parameters described above (Bruker Biospin, Billerica, MA).

The ^{13}C chemical shifts of the five carbons of 2HG, pH 10 in D_2O , referenced to tert-butanol (CH_3 , $\delta = 30.29$ ppm) were 183.5 (C5), 181.8 (C1), 72.6 (C2), 34.4 (C4), and 31.6 (C3). The various one-bond and multiple bond ^{13}C - ^{13}C J coupling constants (in Hz) were measured from ^{13}C NMR and JHSQC at pH 6 (results were not different at pH 7). ^{13}C - ^{13}C couplings were: J_{12} (54.7), J_{23} (36.5), J_{34} (34.8), J_{45} (51.6), J_{14} (2.5), and J_{25} (4.1) (Table S1: supplemental section). The ^1H - ^{13}C couplings were: $J_{\text{C}2\text{H}2}$ (144), $J_{\text{C}4\text{H}4}$ (127), $J_{\text{C}5\text{H}4}$ (4.50), $J_{\text{C}1\text{H}2}$ (3.50), $J_{\text{C}2\text{H}3}$ (3.70), $J_{\text{C}4\text{H}3}$ (4.0). These coupling constants are not substantially different from glutamate or glutamine.

Analysis of the tumor extract at pH 7 revealed that the carbon 4 signal of 2HG completely overlaps with the carbon 4 signal of glutamate (~ 34.2 ppm, Figure 1A and 1C). Similarly, the carbon 3 signal of 2-HG partially overlaps with the carbon 4 signal multiplets from glutamine (~ 31.5 ppm, Figure 2C). Consequently assignment of these chemical shifts is difficult in the tumor extracts. We next examined the ^{13}C chemical shifts of 2HG at pH 6, 7 and 8, in D_2O referenced to lactate C3 at 20.8 ppm and obtained the following chemical shifts (respectively, in ppm): C1 (181.97, 181.97, 181.96); C2 (72.76, 72.79, 72.80); C3 (31.68, 31.74, 31.75); C4 (34.09, 34.22, 34.25); and C5 (183.39, 183.59, 183.60) (Table S2: supplemental section). The ^{13}C chemical shifts of C1, C2 and C3 carbons of 2HG were relatively insensitive to pH in the range 6 – 8 but C4 and C5 exhibited a small upfield shift (0.16 and 0.21 ppm, respectively). At pH ~ 5 , unacceptable line broadening was observed (data not shown).

Two methods were tested to determine whether sensitivity could be improved relative to direct ^{13}C NMR spectroscopy at 14.1T using a 3 mm probe. JHSQC of solutions revealed, as expected, resolution of glutamate, glutamine and 2HG in the ^1H (F2) dimension (Figure S2: supplemental section). However, ^{13}C -enriched 2HG could not be detected from the tumor extract by JHSQC. In contrast, the cryogenically-cooled direct-detect probe provided $\sim 1.6\times$ improved sensitivity. Dynamic Nuclear Polarization (DNP) NMR methods can be used to enhance ^{13}C sensitivity both in-vivo and ex-vivo (14,15). However, short ^{13}C T_1

values of protonated carbons of these molecules would make this method technically challenging.

These studies confirmed that, in spite of the wide chemical shift dispersion in ^{13}C NMR spectra, 2HG overlaps glutamate and glutamine in tissue extracts. Protonation of either the amino or carboxylate group generally favors an upfield shift of the nearby carbons, with of the β -carbon usually experiencing larger up-field than the α -carbon [16-18]. The pKa values of glutamate carboxylates are 2.19 and 4.25 while the protonation constants for dicarboxylic acids without an amino group are typically $\text{pKa1} \sim 3$ and $\text{pKa2} \sim 5$ to 7 [17,18]. Consequently it was not surprising to find a small but adequate upfield shift of 2HG relative to glutamate with changing pH from 7 to 6. Importantly, line shape was not adversely affected. Resolution of 2HG from glutamate and glutamine was achieved in the tumor extract from a patient with a glioblastoma. The presence of ^{13}C - ^{13}C multiplets in 2HG demonstrates metabolism of the infused glucose to 2HG.

^{13}C NMR spectroscopy of aqueous extracts from biopsies of human malignancies offers a simple method to investigate metabolism. Since stable isotopes are used, these approaches are easy to integrate into the clinical workflow of the operating room. Detection of ^{13}C in product molecules by mass spectrometry is attractive because of high sensitivity, but ^{13}C NMR provides detailed information, resulting from the chemical shift and J coupling, about the distribution of ^{13}C in product molecules which is difficult to access by mass spectrometry [8, 9]. Consequently, ^{13}C NMR offers substantial advantages if resolution and sensitivity can be optimized. Although JHSQC provided excellent spectra of 2HG in solution, we were unable to detect ^{13}C -enriched 2HG from tumor samples. However, the cryogenically cooled probe provided improved sensitivity in spite of the poor filling factor. An optimized cooled probe would dramatically improve sensitivity for monitoring 2HG.

In summary, infusion of ^{13}C -enriched substrates followed by biopsy and ^{13}C NMR spectroscopy of tumor extracts is an increasingly attractive approach for analysis of tumor metabolism in patients. The ability to resolve the ^{13}C - ^{13}C multiplets in 2HG, glutamate and glutamine is highly valuable to understanding the role of IDH mutations in tumor cells and the impact of inhibiting production of 2HG by specific IDH inhibitors that are currently in clinical trials. Analysis of IDH mutated tumors at pH 6 and ^1H -decoupled ^{13}C spectra in a cryogenically-cooled probe provides optimal spectra to achieve this goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Effect of pH on the ^{13}C NMR spectrum of C4 region of glutamate and 2HG at ~ 34.2 ppm. The carbon 4 region of unenriched glutamate (50 mM) and 2-hydroxyglutarate (150 mM) at pH ~ 7 is shown in panel A. At pH 6, a small upfield shift of 2HG relative to glutamate observed (panel B). The ^{13}C NMR spectrum of the tumor extract at pH 7 is shown in panel C and the spectrum at pH 6 is shown in panel D. Multiplets due to J_{45} in 2HG are resolved.

Figure 2.

Effect of pH on the ^{13}C NMR spectrum of C4 region of glutamine and C3 region of 2HG at ~ 31.5 ppm. The carbon 4 region of unenriched glutamine (50 mM) and carbon 3 region of unenriched 2-hydroxyglutarate (150 mM) at pH ~ 7 is shown in panel A. At pH 6, a small upfield shift of 2HG relative to glutamine observed (panel B). The ^{13}C NMR spectrum of the tumor extract at pH 7 is shown in panel C and the spectrum at pH 6 is shown in panel D. peaks labeled as “*” are unassigned.