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Direct Monitoring of γ-Glutamyl Transpeptidase Activity In Vivo Using a Hyperpolarized ^{13}C-Labeled Molecular Probe

Tatsuya Nishihara[+], Hikari A. I. Yoshihara[+], Hiroshi Nonaka, Yoichi Takakusagi, Fuminori Hyodo, Kazuhiro Ichikawa, Emine Can, Jessica A. M. Bastiaansen, Yuhei Takado, Arnaud Comment, and Shinsuke Sando**

Abstract: *The γ-glutamyl transpeptidase (GGT) enzyme plays a central role in glutathione homeostasis. Direct detection of GGT activity could provide critical information for the diagnosis of several pathologies. We propose a new molecular probe, γ-Glu-[1-^{13}C]Gly, for monitoring GGT activity in vivo by hyperpolarized (HP) ^{13}C magnetic resonance (MR). The properties of γ-Glu-[1-^{13}C]Gly are suitable for in vivo HP ^{13}C metabolic analysis since the chemical shift between γ-Glu-[1-^{13}C]Gly and its metabolic product, [1-^{13}C]Gly, is large (4.3 ppm) and the T_1 of both compounds is relatively long (30 s and 45 s, respectively, in H_2O at 9.4 T). We also demonstrate that γ-Glu-[1-^{13}C]Gly is highly sensitive to in vivo modulation of GGT activity induced by the inhibitor acivicin.*

The enzyme γ-glutamyl transpeptidase (GGT) is a cell-surface enzyme that plays an essential role in glutathione (GSH) homeostasis.[1] It is widely expressed on the apical surface of epithelial-cell lining ducts, most prominently in the proximal tubules of the kidney and liver biliary ducts, and is also expressed on leukocytes and astrocytes.[2]

GGT catalyzes the cleavage of the γ-peptide bond between the glutamyl and cysteinyl residues of GSH and related molecules, either hydrolyzing the bond or ligating the free α-amine of an amino acid or peptide (Figure 1a). Elevated serum GGT levels are a general marker of diseases affecting the liver.[2b] GGT has been implicated in the development and progression of malignant tumors, where its elevated expression increases the availability of cysteine for intracellular GSH production, limiting damage from oxidative stress and facilitating GSH-dependent drug resistance mechanisms.[3] It is often overexpressed in a number of different types of cancer, including those arising from tissues that do not normally express much GGT, such as soft tissue sarcoma and ovarian adenocarcinoma.[3]

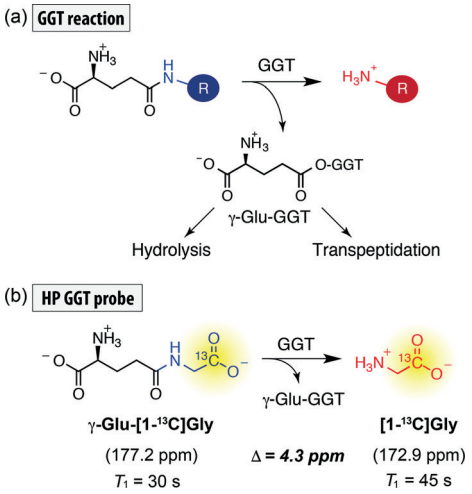

Figure 1. a) Outline of the GGT reaction. b) The designed molecular probe, γ-Glu-[1-^{13}C]Gly.

GGT may therefore serve as a tumor biomarker and its presence on the cell surface is particularly attractive for imaging applications. Recently, reagents capable of detecting the presence of GGT in vivo using fluorescence or radioactivity have been described.[4] GGT requires substrates bearing a γ-glutamyl moiety but accommodates a range of substituents at the γ-position (Figure 1a),[5,6] providing a strategy for the design of molecular probes targeting GGT. A fluorogenic probe activated upon cleavage by GGT may be useful to identify GGT-expressing tumors during surgical resection.[4a] This particular application avoids the problem of tissue opacity often encountered with fluorescent probes. Conversely, GSH derivatives bearing chelated ^{99m}Tc or ^{68}Ga radioisotopes have been used to image GGT-expressing

[*] Dr. T. Nishihara,[+] Dr. H. Nonaka, Prof. Dr. S. Sando
Department of Chemistry and Biotechnology
Graduate School of Engineering, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)
E-mail: ssando@chembio.t.u-tokyo.ac.jp
Dr. H. A. I. Yoshihara,[+] E. Can, Dr. J. A. M. Bastiaansen, Dr. Y. Takado, Prof. Dr. A. Comment
Institute of Physics of Biological Systems
École Polytechnique Fédérale de Lausanne
1015 Lausanne (Switzerland)
E-mail: arnaud.comment@epfl.ch
Dr. Y. Takakusagi, Prof. Dr. K. Ichikawa
Incubation Center for Advanced Medical Science, Kyushu University
3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582 (Japan)
Dr. F. Hyodo, Prof. Dr. K. Ichikawa
Innovation Center for Medical Redox Navigation, Kyushu University
3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582 (Japan)
Dr. Y. Takado
Current address: Department of Functional Brain Imaging Research, Brain and Mind Research Team, National Institutes for Quantum and Radiological Science and Technology
4-9-1 Anagawa, Chiba 263-8555 (Japan)
[+] These authors contributed equally to this work.

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xenografts in vivo,[4b] but this type of probe does not directly detect GGT activity. Therefore, despite the clear interest in non-invasively obtaining real-time information on GGT activity in vivo, no imaging modality can currently offer an adequate way to do so.

Dissolution dynamic nuclear polarization (DNP) is a versatile method to hyperpolarize nuclear spins.[7] It can temporarily increase the signal from NMR-active nuclei by as much as 4 or 5 orders of magnitude, which has enabled the detection of metabolic transformations in real-time with high sensitivity using NMR spectroscopy and imaging.[8] However, because of constraints related to the relatively short time window available for hyperpolarized (HP) MR measurements, the design of HP molecular probes remains a challenge[9] and only a limited number have been successfully used for in vivo applications.[10] Herein, we report the design and preparation of an HP molecular probe targeting GGT activity, γ-Glu-[1-^{13}C]Gly (Figure 1b). It is shown that this probe can monitor real-time GGT activity in vivo.

γ-Glu-[1-^{13}C]Gly (Figure 1b) was designed to probe GGT activity following three essential requirements of HP molecular probes. First, the HP reporter nuclear spins must relax slowly such that a sufficiently large signal can be detected over the course of the experiment. The lifetime of the HP state is directly correlated with the longitudinal relaxation time (T_1), and ^{13}C reporter nuclei relax slowly and have long T_1 values when placed in small molecules at sites without adjacent ^{1}H nuclei. The HP glycine carboxylate,[11] for instance, has reported T_1 values of 40 to 50 s.[12] Second, an HP molecular probe must provide a functional readout, such as a change in chemical shift. The ^{13}C chemical shifts of amino acid carboxylates are sensitive to the ionization state of the α-nitrogen.[13] The glycine carboxylate ^{13}C chemical shift changes by 9.1 ppm when pH is decreased from 12.9 to 6.0 (Supporting Information, Figure S1, α-amino group pK_a = 9.75[14]). Free [1-^{13}C]Gly released from γ-Glu-[1-^{13}C]Gly is in the ammonium form at physiological pH. Therefore, the cleavage of the γ-glutamyl amide bond by GGT was expected to produce a distinct ^{13}C signal.[15] Third, for in vivo applications, an HP molecular probe should not be toxic when infused at millimolar concentrations. γ-Glu-Gly is found endogenously at low levels[16] and was not anticipated to be toxic.

γ-Glu-[1-^{13}C]Gly fulfills the requirements of an HP molecular probe. First, the ^{13}C T_1 values of both γ-Glu-[1-^{13}C]Gly and [1-^{13}C]Gly are relatively long (30 s and 45 s in H_2O, respectively, at 9.4 T and 25 °C, Figure 1b). Second, as shown in Figure 1b, the ^{13}C chemical shift difference of 4.3 ppm between γ-Glu-[1-^{13}C]Gly and [1-^{13}C]Gly at physiological pH is sufficient to easily distinguish the two signals at the lower spectral resolutions typically encountered in vivo. Third, it has a low cellular toxicity in a WST assay, even after 24 h of exposure to a probe concentration of 10 mM (Supporting Information, Figure S2). Furthermore, it can be easily prepared in high yield in two steps from affordable, commercially available reagents (Supporting Information, Scheme S1).

The ability of the γ-Glu-[1-^{13}C]Gly to detect GGT in vitro was tested using ^{13}C NMR after dissolution DNP hyperpolarization (enhancement factor in solution = 6.7×10^3, P_{13C} = 5.4 %, T = 298 K, B_0 = 9.4 T, 1.5 h polarization, stacked spectra versus inset in Figure 2a). Using low flip-angle pulses,

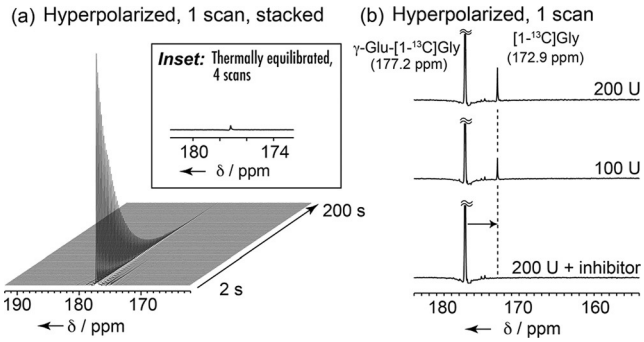

Figure 2. a) Single-scan ^{13}C NMR spectra of HP γ-Glu-[1-^{13}C]Gly (12 mM) stacked from ca. 2–200 s (every 2 s, 100 times, 5° pulse angle). Inset: thermally equilibrated γ-Glu-[1-^{13}C]Gly (12 mM, 4 scans, 90° pulse angle). b) Single-scan ^{13}C NMR spectra (30° pulse angle) of γ-Glu-[1-^{13}C]Gly (2.0 mM) with various amounts of GGT (100 or 200 U) in PBS (pH 7.4, 5.1 mL) containing glycylglycine (3.9 mM) and acivicin as GGT inhibitor (0 or 1.0 mM).

the HP ^{13}C NMR signal was detectable for at least 200 s (Figure 2a). When mixed with commercially available GGT, the γ-Glu-[1-^{13}C]Gly (177.2 ppm) produced a new ^{13}C NMR signal 4.3 ppm upfield, at 172.9 ppm, which was assigned to the expected product, [1-^{13}C]Gly (Figure 2b). The observed [1-^{13}C]Gly-to-total ^{13}C signal ratio was proportional to the measured GGT activity (Supporting Information, Figure S3). Conversely, no [1-^{13}C]Gly was detected in the presence of acivicin, a GGT inhibitor (Figure 2b).

With its abundant expression of GGT,[1b] the kidney is a good source of the enzyme and a likely candidate for functional imaging of its activity. As with the experiments using purified GGT, ^{13}C NMR measurements of HP γ-Glu-[1-^{13}C]Gly immediately after being mixed with kidney homogenate showed the production of a [1-^{13}C]Gly signal at 172.9 ppm (left spectra in Figure 3a). Similarly, mixing the HP γ-Glu-[1-^{13}C]Gly with kidney homogenate from rats treated with acivicin resulted in a highly attenuated [1-^{13}C]Gly signal (right spectra in Figure 3a), and a chromogenic assay revealed a 96 % reduction in GGT activity (Figure 3b). These combined results strongly indicate that γ-Glu-[1-^{13}C]Gly conversion resulted from GGT activity and that endogenous GGT activity in the kidney can be detected using HP γ-Glu-[1-^{13}C]Gly.

For in vivo experiments in rats, γ-Glu-[1-^{13}C]Gly was hyperpolarized using the TEMPOL nitroxyl radical (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl), and the resulting ^{13}C polarization at the time of injection was 9 %. A surface coil was placed over the right kidney, liver, heart, or brain, and its correct placement was confirmed by ^{1}H MRI (Inset of Figure 3d and the Supporting Information, Figure S4). Time series of the HP ^{13}C NMR spectra were acquired following the bolus intravenous infusion of γ-Glu-[1-^{13}C]Gly (Figure 3c). In the kidney, the time-dependent

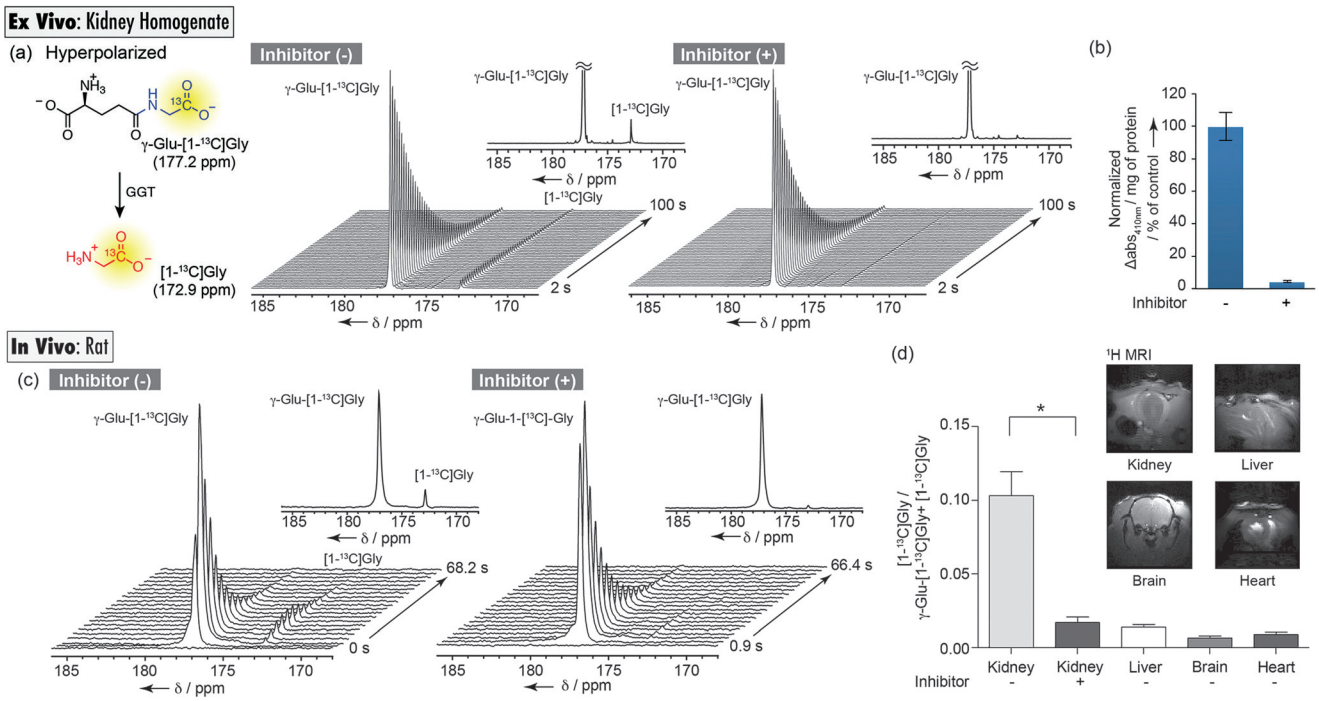

Figure 3. a) GGT sensing by γ-Glu-[1-^{13}C]Gly and time series stack of single-scan ^{13}C NMR spectra of HP γ-Glu-[1-^{13}C]Gly from 2 s to 100 s (repetition time: 2 s, 5° flip angle), acquired after mixing HP γ-Glu-[1-^{13}C]Gly (20 mM) with kidney homogenate from rats treated or untreated with the GGT inhibitor acivicin (0 or 10 mg kg^{-1}). Inset spectra: sum of individual scans (scans 1–100). b) GGT activity in kidney homogenate measured by colorimetric assay using γ-Glu-*p*-nitroanilide. Error bars indicate the SD for three independent experiments. c) Time series of ^{13}C NMR spectra of HP γ-Glu-[1-^{13}C]Gly acquired in vivo with the coil placed over the right kidney (repetition time: ca. 3 s, 30° flip angle) before and after the infusion of acivicin (8 mg kg^{-1}). Inset spectra: sum of individual scans containing HP signal (scans 3–19). d) Ratio of the [1-^{13}C]Gly signal to the sum of the γ-Glu-[1-^{13}C]Gly and [1-^{13}C]Gly signals in each organ. [1-^{13}C]Gly is expressed as a fraction of total HP signal, quantitated by integration of the peaks in the sum of spectra with the HP signal (* $p = 0.026$). Kidney experiments were performed in triplicate; other organs in duplicate, with 1–3 infusions of HP γ-Glu-[1-^{13}C]Gly per rat. Inset: example of observed area (^{1}H MRI). Error bars indicate the SEM.

evolution of a spectral peak corresponding to [1-^{13}C]Gly was readily detected (left spectra in Figure 3c). To test whether the observed conversion of γ-Glu-[1-^{13}C]Gly to [1-^{13}C]Gly in vivo was dependent on GGT activity, acivicin (8 mg kg^{-1}) was infused after the first kidney MR acquisition, and a second HP ^{13}C MR experiment was performed one hour later (right spectra in Figure 3c).[17] After acivicin treatment, substantially less conversion was apparent, and the ratio of [1-^{13}C]Gly to total ^{13}C signal was reduced by 6.4 ± 1.0-fold ($n = 3$, $p = 0.026$, paired t-test, Figure 3d), confirming that most of the conversion was due to GGT. That a small [1-^{13}C]Gly signal is still produced after acivicin treatment in the in vivo and kidney homogenate experiments but not with acivicin-treated purified GGT suggests either incomplete GGT inhibition in the biological milieu or the minor activities of other enzymes in the kidney.

The HP ^{13}C MR experiments performed in the major organs show that the apparent GGT activity correlates with the tissue expression of the enzyme (Figure 3d). The high amount of conversion of γ-Glu-[1-^{13}C]Gly to [1-^{13}C]Gly observed when scanning the kidney results from its high level of GGT expression. The small HP [1-^{13}C]Gly signals detected in the brain, heart, and liver represented less than 2% of the total HP ^{13}C signal (Figure 3d) and were apparent only when the spectra were summed. Some of the apparent liver signal may originate from the kidneys, given their proximity to the liver. Cardiac muscle has very low levels of GGT activity,[18] and we currently cannot exclude that the small [1-^{13}C]Gly signal observed in the heart and brain is generated by the recirculating [1-^{13}C]Gly that is produced and reabsorbed in the kidneys.[19] Conversely, renal reabsorption may account for the persistence of the [1-^{13}C]Gly in the kidney. It is also important to note that γ-Glu-[1-^{13}C]Gly was well tolerated, with no change in the heart rate, blood pressure, or breathing observed upon injection.

In conclusion, we successfully designed γ-Glu-[1-^{13}C]Gly as an effective HP ^{13}C MR probe of GGT activity. It is readily hyperpolarized by dissolution-DNP and fulfills the other important performance requirements of such a probe, including biocompatibility, a long-lasting HP signal, and an easily measured change in ^{13}C chemical shift upon enzymatic cleavage. These features of γ-Glu-[1-^{13}C]Gly for the first time allow the non-invasive, real-time detection of GGT activity in vivo and indicate its potential for application in functional imaging, including the identification of GGT-overexpressing tumors and kidney function. Sensitivity for GGT activity may be improved with more reactive or better polarizing probes, or with optimized formulations for polarization. Because of the medical and biological importance of this cell-surface enzyme, γ-Glu-[1-^{13}C]Gly expands the applicability of HP MR techniques. Further work is now underway in our laboratories.

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