[13]C-labeled indolequinone-DTPA-Gd conjugate for NMR probing cytochrome:P450 reductase-mediated one-electron reduction

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Abstract

We designed and synthesized a new class of ^{13}C-labeled NMR probe, **^{13}C-IQ-Gd**, to monitor one-electron reductions by cytochrome:P450 (**CYP450**) reductase under hypoxic conditions. ^{13}C-IQ-Gd consisted of a Gd^{3+}-diethylene triamine pentaacetic acid (**DTPA**) complex unit and an indolequinone (^{13}**C-IQ**) unit bearing a ^{13}C-labeled methoxy group. The ^{13}C-NMR signal of **^{13}C-IQ-Gd** was suppressed because of the intramolecular paramagnetic effect of Gd^{3+}, whereas enzymatic reduction mediated by **CYP450** reducatase under hypoxic conditions yielded an intensed ^{13}C-NMR signal due to enzymatic activation of the IQ unit followed by release of the **DTPA-Gd** unit from **^{13}C-IQ-Gd**. This ^{13}C-NMR spectral change allowed the monitoring of **CYP450** reducatase-mediated one-electron reduction.

Keywords

Cytochrome:P450 (**CYP450**) reductase catalyzes one-electron reduction of various substrates with the aid of cofactors such as β-NADPH. **CYP450** reductase has been identified to play key functions in liver detoxification, biological synthesis of steroid hormones, metabolism of fatty acids and drug activation in malignant tumor cells.[1,2] In particular, the enzyme operates effectively in tumor hypoxia that characterizes various types of solid tumor tissues.[3] Under hypoxic conditions, **CYP450** reductase activates certain drugs and molecular probes, which allows the expression of their inherent functions.[4,5] In this context, one-electron reduction mediated by **CYP450** reductase has attracted considerable attention for designing hypoxia-targeting drugs and probes.

Recently, highly sensitive and sophisticated ^{13}C NMR/MRI techniques such as multiple-resonance[6] and hyperpolarized NMR have been developed.[7] In this association, various types of ^{13}C-labeled compounds have been thitherto reported for application to highly sensitive ^{13}C-NMR analysis of complicated biological systems due to low level of natural ^{13}C abundance.[8]

The present study aims at developing a new class of ^{13}C-NMR probe to monitor **CYP450** reductase-mediated one-electron reduction. We designed and synthesized a

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conjugate (**^{13}C-IQ-Gd**) consisting of a Gd^{3+}-diethylene triamine pentaacetic acid (**DTPA**) complex unit[9] and a ^{13}C-labeled indolequinone (**^{13}C-IQ**) unit that undergoes one-electron reduction by **CYP450** reductase (Figure 1). **^{13}C-IQ-Gd** alone showed no apparent signal in the ^{13}C-NMR spectrum because of the intramolecular paramagnetic effect of Gd^{3+},[10] whereas the **^{13}C-IQ** unit was activated upon treatment with one-electron reducing **CYP450** reductase to be separated from the **Gd-DTPA** unit and thereby showed intense ^{13}C-NMR signal.

(Figure 1)

The synthesis of **^{13}C-IQ-Gd** is outlined in Scheme 1. The hydroxyl group of **1** was methylated by means of ^{13}C-labeled methyl iodide, and following nitration and reduction gave aminoindole (**3**). The resulting **3** was converted into indolequinone (**^{13}C-IQ-OH**) via treatment with LiAlH$_4$ and Fremy's salt, and was then coupled with DTPA to give **^{13}C-IQ-DTPA**.[12] Finally, **^{13}C-IQ-DTPA** was coordinated with Gd^{3+} to give **^{13}C-IQ-Gd**.[13] The NMR probe **^{13}C-IQ-Gd** thus obtained was water soluble up to 4

mM. We confirmed that metal coordination resulted in a broadening and weakening in ^{13}C-NMR signal at 57 ppm of the original **^{13}C-IQ-DTPA** (Figure 2B) because of the paramagnetic effect of Gd^{3+}.

(Scheme 1)

Upon incubation of **^{13}C-IQ-Gd** with **CYP450** reductase and its cofactor β-NADPH in hypoxic aqueous solution[14] the reaction was monitored by ^{13}C-NMR. As shown in Figure 2C, a new signal appeared at 63 ppm after the enzymatic treatment for 1 h. This response is consistent with the present molecular design. **^{13}C-IQ-Gd** was activated by enzymatic reduction to release the DTPA-Gd unit, resulting in the appearance of a ^{13}C NMR signal along with a decreased extent of intramolecular paramagnetic effect due to separation of the ^{13}C-labeled IQ unit from the **DTPA-Gd** unit. In contrast, aerobic enzymatic reduction of **^{13}C-IQ-Gd** by **CYP450** reductase yielded no apparent signal (Figure 2D). These results indicate that the enzymatic reduction of **^{13}C-IQ-Gd** occurred in a hypoxia-selective manner, as monitored by ^{13}C-NMR.

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(Figure 2)

For further characterization of this enzymatic reduction, we performed a similar enzymatic reaction of **[13]C-IQ-OH** without bearing a **DTPA-Gd** unit that showed a distinct signal at 57 ppm in ^{13}C-NMR (Figure 3A) in contrast to **[13]C-IQ-Gd**. After treatment with **CYP450** reductase in the presence of β-NADPH for 1 h in hypoxic phosphate buffer, we observed appearance of a new ^{13}C-NMR signal at 63 ppm (Figure 3B),[15] which is identical with the signal resulted from **[13]C-IQ-Gd** upon similar enzymatic treatment. These results are a strong indication that the **[13]C-IQ** unit was activated by **CYP450** reductase to be released from **[13]C-IQ-Gd** or **[13]C-IQ-OH** during the enzymatic reaction. Recent studies on the activation mechanism of **IQ** derivatives under reduction conditions demonstrated that **IQ** was converted to an electrophilic intermediate **4**, which was trapped by ambient nucleophiles (Figure 4).[16] In this context, one of the possible reaction products that yielded a characteristic ^{13}C-NMR signal at 63 ppm is a **[13]C-IQ**-protein adduct. We therefore attempted to identify the reaction

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products. However, the product characterization was unsuccessful, because purification

of the reaction product could not be achieved.

(Figure 3)

(Figure 4)

An attempt was also made to feature the selective activation of **^{13}C-IQ-Gd** by **CYP450**

reductase.[17] We compared enzymatic activity toward **^{13}C-IQ-Gd** of **CYP450** reductase

with five other oxidases and reductases such as glucose dehydrogenase, alcohol

dehydrogenase, peroxidase, nitrate reductase, and flavin reductase. In contrast to

CYP450 reductase (see Figure 2C), incubation of **^{13}C-IQ-Gd** with the other enzymes

failed to give signal in ^{13}C NMR (Figure S1). Thus, it is most likely that **^{13}C-IQ-Gd**

undergoes one-electron reduction exclusively by **CYP450** reductase.

In summary, we designed and synthesized a new class of ^{13}C-labeled NMR probe for

monitoring of enzymatic one-electron reduction by **CYP450** reductase. The reduction of

^{13}C-IQ-Gd, which consisted of a ^{13}C-labeled **IQ** unit and a **DTPA-Gd** unit, was

monitored by ^{13}C-NMR. Hypoxia-selective activation of **^{13}C-IQ-Gd** induced appearance of a ^{13}C-NMR signal of the ^{13}C-methoxy group in the **IQ** unit, which was attributable to a decreased extent of the intramolecular paramagnetic effect of Gd^{3+}. In view of these properties, **^{13}C-IQ-Gd** is a promising candidate for an NMR probe for the presence of **CYP450** reductase.

Acknowledgement

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12. *^{13}C-IQ-DTPA*: Red solid: MP 122–125 °C.; ^{1}H NMR (300 MHz, CD$_3$OD) δ 1.95 (s, 8H), 2.24 (s, 3H), 3.30–3.35 (10H), 3.76 (d, *J* = 150 Hz, 3H), 3.85 (s, 3H), 4.66 (s, 2H), 5.64 (s, 1H); ^{13}C NMR (67.8 MHz, DMSO-d_6) δ 9.3, 32.0, 54–58 (strong), 106.5, 120.6, 122.3, 127.6, 137.3, 159.2, 170.8, 172.6, 173.6, 176.6, 177.5, 178.1; FABMS: m/e 634 [(M+Na)$^+$]; HRMS calcd for C$_{25}$^{13}CH$_{34}$N$_4$NaO$_{13}$$^+$ [(M+Na)$^+$] 634.2048, found 630.2048.

13. *^{13}C-IQ-Gd*: Orange oil: ESI-MS m/z 765.5 (calcd for [M-H]$^-$ 765.1) Purity was confirmed by analytical HPLC.

14. Bioreduction by **CYP450** reductase. To establish hypoxia, a solution of NADPH:cytochrome P450 reductase (final concentration: 0.13 μM) and β-NADPH (final concentration: 2 mM) in 5 mM phosphate buffer (pH 7.4) was purged with argon for 10 min at 37 °C. To the resulting solution was added **^{13}C-IQ-Gd** (final concentration: 2 mM) and incubated at 37 °C for 1 h. The ^{13}C-NMR spectra were then measured (acquisition time: 2048 times). A control aerobic sample solution was incubated and analyzed in a similar manner. Experiment with **^{13}C-IQ-OH** was conducted in a similar manner in the presence of MeCN (final concentration: 10%).

15. Although a small amount of **CYP450** reductase (0.13 μM) was used for the reduction of **^{13}C-IQ-OH** (2 mM), the signal of reduction product of **^{13}C-IQ-OH**

was distinctly observed in the NMR spectra as shown in Figure 3. This result strongly indicates that the catalytic reduction of indolequinone derivatives by **CYP450** reductase occurred in the present system.

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17. Bioreduction by other enzymes. Five Enzymes (Glucose dehydrogenase (from microorganism) PQQ- 0.1 mg/mL, alcohol dehydrogenase (from yeast) 0.25 mg/mL, peroxidase (from horseradish) 0.1 mg/mL, nitrate reductase (cytochrome) 0.005 unit, and flavin reductase (recombinant) 1 μg/mL) were used in this study.

Supplementary Material

Supplementaly Material is available. ^{13}C-NMR spectra of **[13]C-IQ-Gd** treated by five enzymes.
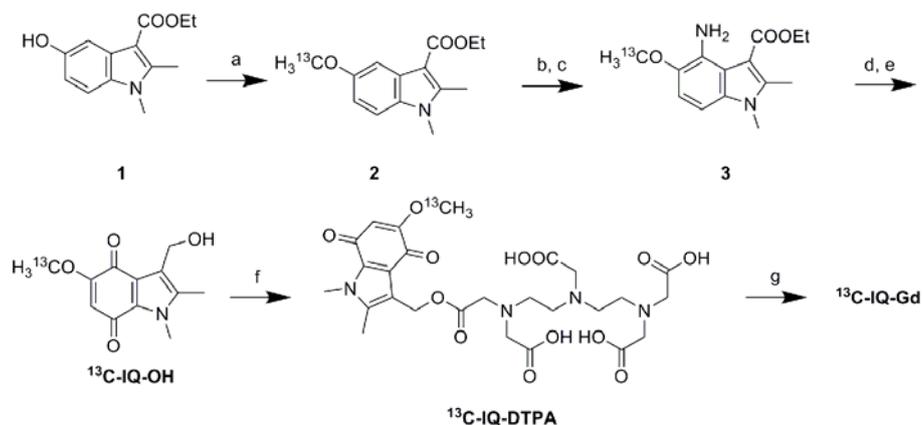
Scheme 1. Reagents: (a) NaH, $^{13}CH_3I$, 64%; (b) HNO_3, AcOH, 78%; (c) Sn, HCl, EtOH, 76%; (d) $LiAlH_4$, THF; (e) Fremy's salt, Me_2CO, NaH_2PO_4, water, 77% in 2 steps; (f) DTPA, TATU, DMAP, DIPEA, DMF, 77%; (g) $GdCl_3$, $NaHCO_3$, water, 94%.
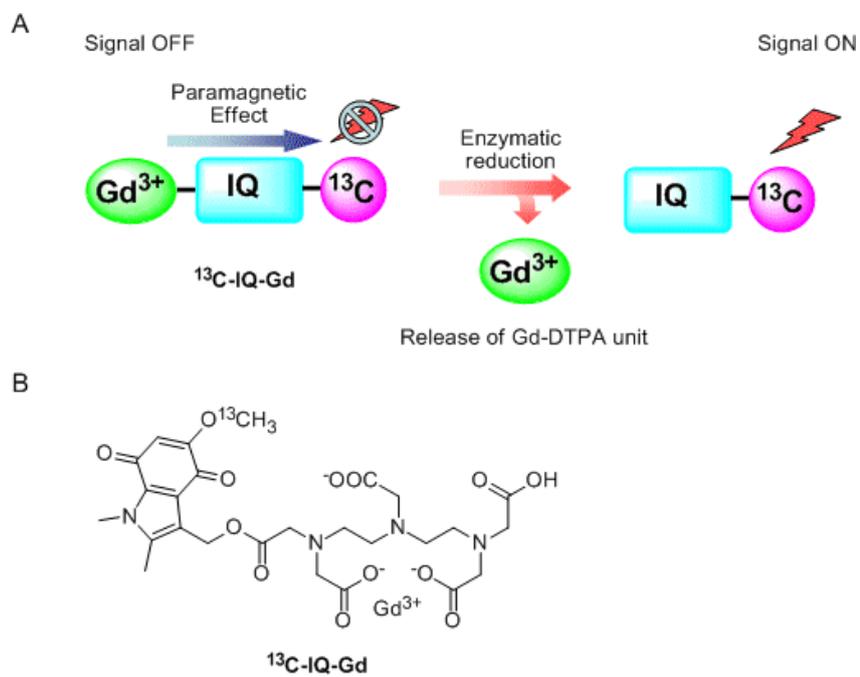
Figure 1. (A) Molecular design of **[13]C-IQ-Gd** as a probe for one-electron reduction by **CYP450** reductase. (B) Chemical Structure of **[13]C-IQ-Gd**.
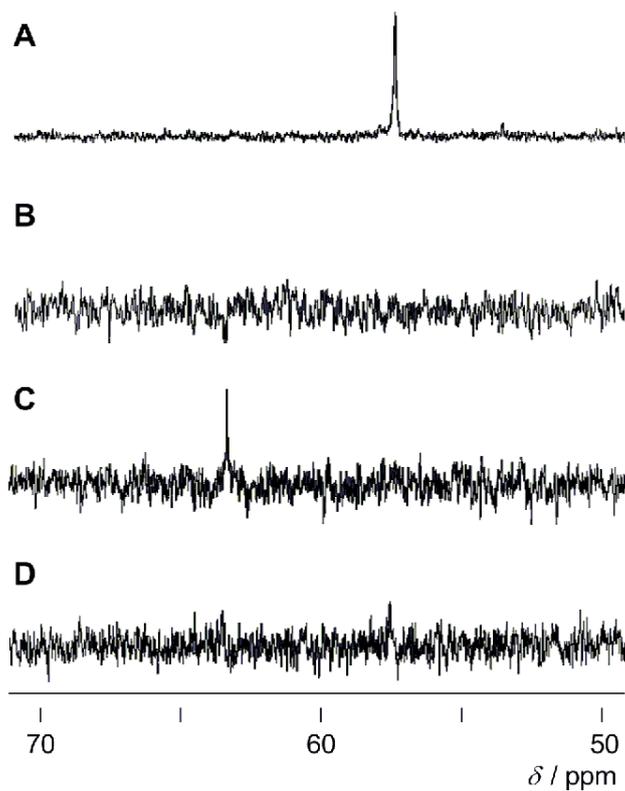
Figure 2. ^{13}C-NMR spectra of (A) **^{13}C-IQ-DTPA** alone and (B, C, D) **^{13}C-IQ-Gd** upon treatment with **CYP450** reductase (10 μg/mL) in the presence of β-NADPH (2 mM) at 37 °C in phosphate buffer (pH 7.4): (B) before treatment with reductase; (C) treated under hypoxic conditions for 1 h; (D) treated under aerobic conditions for 1 h.
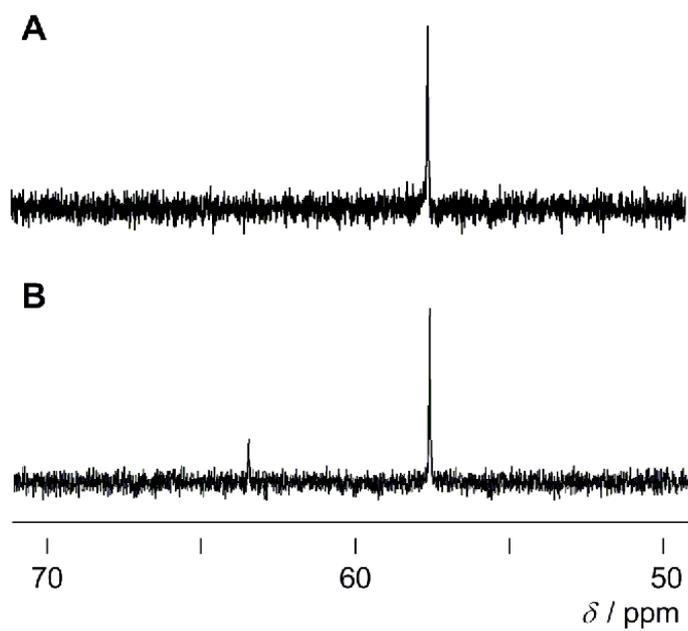
Figure 3. ^{13}C-NMR spectra of **^{13}C-IQ-OH** (2 mM) (A) before and (B) after treatment with CYP450 reductase (10 μg/mL) in the presence of β-NADPH (2 mM) at 37 °C for 1 h in hypoxic phosphate buffer (pH 7.4, 10% MeCN).
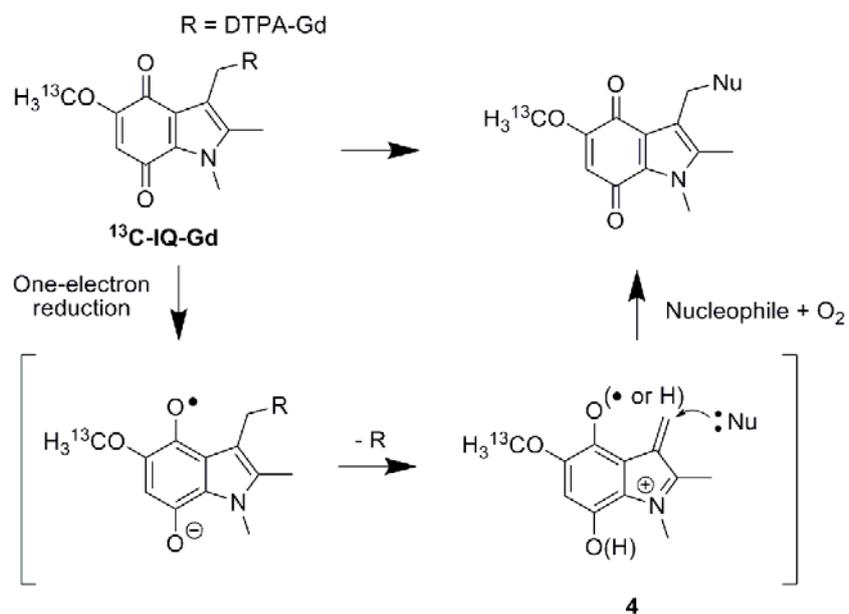
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Figure 4. Plausible activation pathways of ^{13}**C-IQ-Gd** leading to elimination of **DTPA-Gd** unit followed by alkylation of the resulting iminium intermediate.