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Monitoring of Biological One-Electron Reduction by ^{19}F NMR Using Hypoxia Selective Activation of an ^{19}F-Labeled Indolequinone Derivative

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Intracellular reductases have been closely linked with activation of certain drugs and probes in the tumor-specific microenvironments.[1] Among various enzymes, reductases that catalyze one-electron reduction are involved in the selective activation of functional compounds or materials under hypoxia,[2] a well-known pathophysiological characteristic of solid tumors.[3] Such an enzymatic one-electron reduction has been recognized to be a useful reaction applicable to the design of a tumor hypoxia targeting and imaging strategy. Thus, further quantitative insights into the features of this reaction are important.

Here we demonstrate probing of the biological one-electron reduction of a fluorine (F)-labeled indolequinone (IQ) derivative by ^{19}F NMR that gave us straightforward molecular information even under complicated biological reduction conditions due to low concentrations of endogenous F atoms and the absence of interference with proton signals.[4−6] The family of IQ compounds is well characterized to be superior substrates for several reductases expressed in tumor cells and readily undergoes enzymatic one-electron reduction under hypoxic conditions. Consequently, IQ derivatives have been employed to develop bioreductive prodrugs and imaging probes targeting tumor hypoxia[7,8] that are efficiently activated by endogenous reductase to release a given functional component selectively under hypoxic conditions. We prepared an ^{19}F NMR signal supplier (IQ-F) consisting of a hypoxia-sensitive IQ parent unit and a nonafluoro-*tert*-butyl group and monitored the change in ^{19}F chemical shift during the bioreduction. One-electron reduction of IQ-F by isolated or intracellular reductase under hypoxic conditions released the nonafluoro-*tert*-butyl alcohol (F-OH) constituent. We observed a new ^{19}F signal due to the resultant F-OH at a characteristic chemical shift, which differed from that of IQ-F. In contrast, the release of F-OH was efficiently suppressed upon addition of O_2 and thereby the corresponding signal failed to appear. Kinetic studies indicated that O_2 prevented to a lesser extent the binding of IQ-F to reductase but decreased the rate of the net reaction due to oxidation of a semiquinone anion radical intermediate generated during the course of the one-electron reduction into the parent IQ-F. In addition, the present reaction could be monitored by chemical shift selective fast spin echo (FSE),[9] leading to visualization of the hypoxia-selective reduction by signal intensity using MR imaging.

IQ-F was synthesized by coupling 3-hydroxymethyl-5-methoxy-1,2-dimethylindole-4,7-dione with F-OH (Scheme S1). We conducted the enzymatic reduction of IQ-F in an Ar-purged aqueous acetonitrile solution by means of NADPH:cytochrome P450 reductase, which catalyzes the one-electron reduction of quinone derivatives to semiquinone anion radicals.[7a] We incubated IQ-F with NADPH:cytochrome P450 reductase and its cofactor β-NADPH under hypoxic conditions. Figure 1 shows the reaction of IQ-F monitored by ^{19}F NMR. The appearance of a single new signal at −73.6 ppm during hypoxic treatment is attributable to the formation of F-OH, as confirmed by reference to authentic sample, while the IQ-F starting compound almost completely disappeared (Figure 1B). These results clearly indicate that IQ-F is activated to release the corresponding alcohol F-OH by enzymatic reduction, thereby causing a change in the ^{19}F NMR spectra. In contrast, upon enzymatic treatment under aerobic conditions, a substantial amount of IQ-F remained to produce a negligible signal attributable to F-OH (Figure 1C). Thus, enzymatic reduction of IQ-F occurred in a hypoxia-selective manner, as can be monitored by ^{19}F NMR.[10]

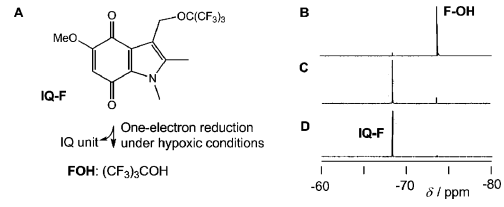

Figure 1. (A) Bioreduction of IQ-F under hypoxic conditions to release F-OH. (B, C, D) One-electron reduction of IQ-F monitored by ^{19}F NMR. IQ-F (0.93 mM) was incubated with NADPH:cytochrome P450 reductase (11.4 μg) and β-NADPH (2 mM) at 37 °C in phosphate buffer; (B) incubated for 15 min under hypoxic conditions; (C) incubated for 15 min under aerobic conditions; and (D) before incubation.

The steady-state kinetic parameters, V_{max} and K_m, for the release of F-OH were derived from a Lineweaver−Burk plot[11] of the integration values evaluated in the ^{19}F NMR spectra (Figure S1). The V_{max} value for F-OH formation under aerobic conditions (0.20 ± 0.04 pmol min^{-1}) was considerably lower than that under hypoxic conditions (3.1 ± 0.2 pmol min^{-1}), while the K_m value obtained from hypoxic treatment (0.41 ± 0.04 mM) was similar to that from aerobic treatment (0.59 ± 0.16 mM). Thus, binding of IQ-F to reductase was slightly affected by the oxygen concentration, while the net reaction rate was dramatically reduced in the presence of O_2. These characteristics are consistent with the conventional mechanism hitherto proposed for IQ

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derivatives. A semiquinone anion radical intermediate is generated under both hypoxic and aerobic conditions by enzymatic one-electron reduction of IQ. The resulting intermediate is subject to oxidization by O_2 to regenerate the original IQ along with the formation of $O_2^{-\bullet}$ under aerobic conditions, leading to significant suppression of the net reaction. The enzymatic activation of IQ-F, which leads to an NMR signal change, is likely to occur substantially under hypoxic conditions.

To better understand the function of IQ-F in living cells, we also assessed the one-electron reduction of IQ-F in a human cell line of lung carcinoma A549 cells that express NADPH:cytochrome P450 reductase in high amounts.[12,13] A549 cells were cultured in the presence of IQ-F for 12 h under hypoxic or aerobic conditions. The culture medium and the cell lysate were individually harvested and subjected to an NMR study. In a similar manner to the treatment of IQ-F with isolated reductase, a signal originating form the formation of F-OH was observed in the medium obtained upon incubation under hypoxic conditions, as shown in Figure 2A. We also confirmed that a weak F-OH signal was produced upon aerobic treatment. In a control experiment, no signal was observed in the corresponding cell lysates independent of oxygen concentrations (Figure S2). Therefore IQ-F most likely penetrates into living cells where it is activated to release F-OH by intracellular reductases in a hypoxic environment, while it is promptly eliminated from cells due to the small size of the molecule, resulting in the low concentration of the intracellular F-signal supplier, which was below the detection limit.

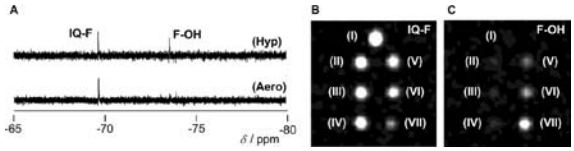

Figure 2. (A) One-electron reduction of IQ-F in A549 cells. A549 cells were incubated with IQ-F (355 μM) for 12 h under hypoxic (Hyp) or aerobic (Aero) conditions. The medium obtained from the reaction sample was subjected to an ^{19}F NMR study. (B,C) ^{19}F MR images of IQ-F incubated with cell lysate of A549 for 0 (I), 6 (II and V), 12 (III and VI), and 24 h (IV and VII) under aerobic (II, III, and IV) or hypoxic (V, VI, and VII) conditions: (B) IQ-F signal selected image; (C) F-OH signal selected image.

To confirm whether the reduction can occur within cells, we further studied the reaction of IQ-F upon treatment with cell lysate. An aqueous solution of IQ-F containing 6% CD_3CN was incubated at 37 °C under hypoxic or aerobic conditions with the lysate of A549 cells. We observed that the ^{19}F NMR signal of F-OH increased in a time-dependent manner only during treatment under hypoxic conditions (Figure S3), indicating that hypoxia-selective one-electron reduction of IQ-F by intracellular reductase is responsible for an exclusive NMR signal change.

In light of the above reaction characteristics of IQ-F, attempts were also made to monitor the reaction of IQ-F by means of MR imaging, a technique widely employed in medical diagnosis.[14] We employed ^{19}F chemical shift selected imaging[9] for probing of the one-electron reduction of IQ-F. IQ-F was incubated under hypoxic and aerobic conditions with the lysate of A549 cells. As shown in Figure 2B and 2C, ^{19}F images of IQ-F and F-OH were obtained individually and simultaneously by FSE with a chemical shift selective pulse.[15] Upon aerobic treatment, an intense IQ-F signal remained, while no F-OH signal was detected even after prolonged incubation. It is striking that the F-OH signal increased with increasing time upon hypoxic treatment, along with a concomitant decrease in IQ-F signals. In accord with the evidence that incubation of IQ-F in a buffer resulted in a similar ^{19}F image as in the sample incubated under aerobic conditions (Figure S5), the hypoxia-selective one-electron reduction of IQ-F could be clearly monitored by ^{19}F MR imaging.

In conclusion, the hypoxia-selective one-electron reduction of IQ-F, which consists of a hypoxia-sensitive IQ parent unit and an ^{19}F signal-transmitting molecular unit of nonafluoro-*tert*-butyl group, was characterized by ^{19}F NMR. During monitoring of the biological reduction of IQ-F, hypoxia-selective activation occurred to induce a chemical shift change of ^{19}F signals attributable to the reductive formation of F-OH. The disappearance of IQ-F to form F-OH could be imaged simultaneously by ^{19}F FSE, thus visualizing the occurrence of the enzymatic one-electron reduction in a hypoxia-selective manner by means of MR imaging.

The one-electron reduction of IQ derivatives has been widely used for hypoxia targeting and imaging. The IQ-F activation system could be applicable to MR imaging of tumor hypoxia. Optimization of the chemical structure of IQ-F derivatives to increase water solubility and intracellular retention and characterization of their pharmacokinetic profiles are now in progress.

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Supporting Information Available: Detail of synthetic protocol and enzymatic reduction of IQ-F. This material is available free of charge via the Internet at http://pubs.acs.org.

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