Novel nitroimdazolyl derivatives and its reduction products reveal hypoxia in cultures of C6 cells using ^{1}H HR MAS

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Introduction: Hypoxia is known to play a central role in oncologic processes (1). In different tumors, a variety of studies reported a direct link between low tumoral oxygenation and tumor aggressiveness, poor outcome (2,3) and resistance to different therapies (4,5). Consequently, several methods have been proposed to measure tumour oxygenation (6). In particular, a number of nitroimidazole derivatives have been used in combination with either optical methods (EF5, pimonidazole) or nuclear medicine approaches (SR-4554, 18F-MISO) (7). This approach is based on the selective reduction and trapping of these derivatives in hypoxic regions *in vivo* (8). To our knowledge, this approach had not been previously used in combination with ^{1}H Magnetic Resonance Methods to measure oxygen tension. To this end, we synthesized and tested a new family of nitroimidazolyl derivatives, comparing the results obtained with those derived from the well known hypoxia marker pimonidazole.

Aim: The development of a new methodology for measuring oxygen concentration by ^{1}H-Magnetic Resonance Spectroscopy Imaging (MRSI) using nitroimidazolyl derivatives.

Materials and Methods: Nitroimidazolyl derivative **1** (figure **1a**) was synthesized by Michael addition of the corresponding nitromidazol to the appropriate acceptor. The Xantine/Xantine oxidase and NADPH:cytochrome P450 reductase enzymatic systems were used to investigate the *in vitro* reduction of derivatives **1** and pimonidazole (**1b**) under normoxic (21% O_2) or hypoxic (1% O_2) conditions in aqueous media. The UV absorbance peaks of the nitroimidazolyl compounds (300 – 330nm) (Figure 1b) and the magnetic resonances of the proton in the imidazole ring were used to follow the time course of the reaction and quantify the reduction rate. C6 cells were grown to confluence in DMEM medium. The medium was then replaced and cells were pre-incubated for 24h under the same oxygen concentrations used later. Then, DMEM medium containing the nitroimidazolyl derivative (2.5 mM) was added and cells were incubated under the different oxygen concentrations used in the pre-incubation period (0%, 1%, 2.5% and 21%) for different times (3, 6 and 12 hours). At the end of the incubation, the cells were harvested and a High Resolution Magic Spinning (HRMAS) ^{1}H NMR spectrum (Figure 1c) of the cell pellet was obtained (500.13 MHz, 4ºC, 5000 rpm).

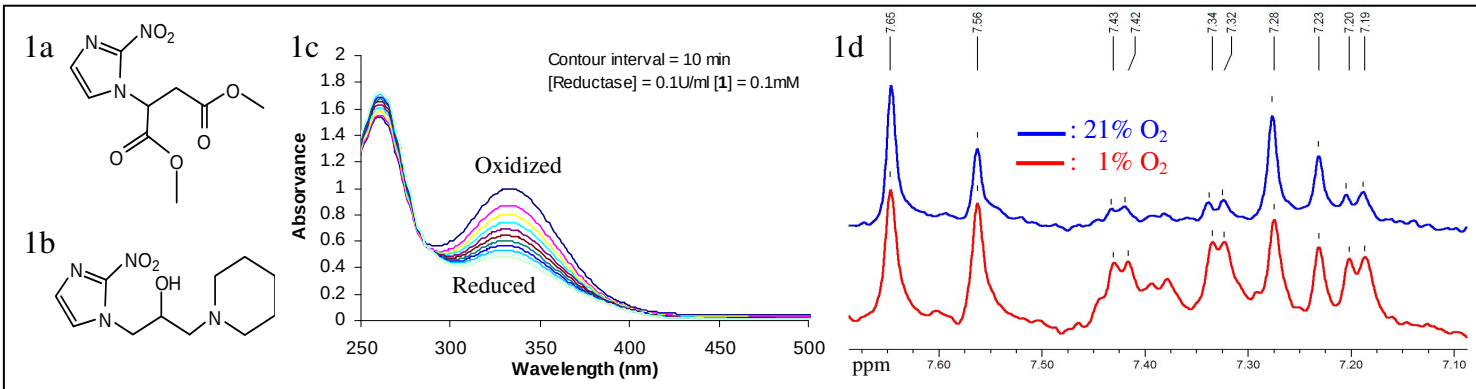

Figure 1. a) Chemical structure of compound 1 and b) pimonidazole. c) Reduction of **1** in the presence of NADPH:Cytochrome P450 reductase followed by UV. d) ^{1}H-NMR spectra of C6 cell pellets containing the nitroimidazol derivative **1** under different O_2 conditions after 12 hours of incubation.

Results: Significant reduction of the compound **1** (Figure 1a) was obtained with the NADPH:cytochrome P450 reductase system under anoxyc conditions, indicating an adequate redox potential. The ^{1}H HRMAS spectra of the cell pellets (Figure 1c) show in the aromatic region, the H4 (7.65 ppm) and H5 (7.28 ppm) resonances from diester **1**, as well as a series of new signals (7.56-730 and 7.23-7.19 ppm) corresponding to reductive species derived from **1**, appearing downfield of the oxydated compound. The area of these new signals is significantly smaller than in normoxic than in hypoxic cells, revealing its accumulation during hypoxia. Similar results were found with pimonidazole, a commercial hypoxia probe.

Conclusions: We report the *in vitro* reduction and *in vivo* metabolism of a novel family of nitroimidazoles as pO_2 indicators for ^{1}H-MRSI as validated with pimonidazole, a commercial derivative. In our hands, cytochrome P450 reductase appears to be the major enzyme involved in reduction of these nitroimidazolyl derivatives under anaerobic conditions. The incubation of C6 cells with these probes resulted in the intracellular production of the corresponding reductive species. This reaction is oxygen level dependent and its evolution can be monitored using ^{1}H High Resolution Magic Angle Spinning Spectroscopy and potentially in vivo using ^{1}H MRSI.

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