Kinetics and mechanism of bioreduction of nitrimidazoles as hypoxia probes

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Introduction: Tumor hypoxia results from the negative balance between the oxygen demands of the tissue and the capacity of the vasculature to deliver satisfactory oxygen provisions. The resulting oxygen deficit has important consequences in the aggressiveness and malignancy of the tumors as well as on their resistance to therapy, endowing the imaging of hypoxia with vital repercussions in tumor prognosis and therapy design (1). 2-Nitroimidazole derivatives (EF5, Pimonidzole, ^{18}F-MISO) have been extensively used as molecular markers of hypoxia in combination with different techniques as immunohistochemistry, PET or ^{19}F-MRI (2). Their use is based on the *in vivo* reduction of the nitro-group and the subsequent trapping of reactive imidazolyl derivatives in regions of low oxygen tension (3). A key issue to be solved in order to fully understand the activity of these markers is to establish precisely the kinetic mechanism for their reduction, an aspect that has remained elusive through the years. Here we report on the kinetics of *in vitro* reduction of commercially available, as well as newly synthesized nitroimidazole based hypoxia probes.

Materials and Methods: We used purified NADPH:cytochrome P450 reductase to investigate *in vitro* the reduction of four different 2-2-nitroimidazole derivatives: Pimonidazole, a Misonidazole analog, NIMAC and NISUCA, two novel hypoxia-sensitive probes synthesized by us. Phosphate buffered (75 mM, pH= 7.7, 37 ºC, 10% D_2O) reaction mixtures containing the probes, NADPH and reduced glutathione (GSH, where appropriate), were prepared in 5 mm NMR tubes using TSP (1 mM) as an internal reference. Anoxic conditions were induced by sealing the NMR tube with a rubber septum and bubbling pure nitrogen for 30 minutes. For normoxic conditions, tubes were left open to the ambient environment during NMR acquisition. The reaction was triggered by the addition of NAPDH:P-450 reductase and the tube quickly placed in a Bruker Avance 11.7 Tesla magnet for high resolution ^{1}H NMR acquisitions ($\pi/2$ pulse, 32K data table, 6s total cycle time, 128 acquisitions) during the following twelve hours. The spectra obtained were analyzed using MestReNova software.

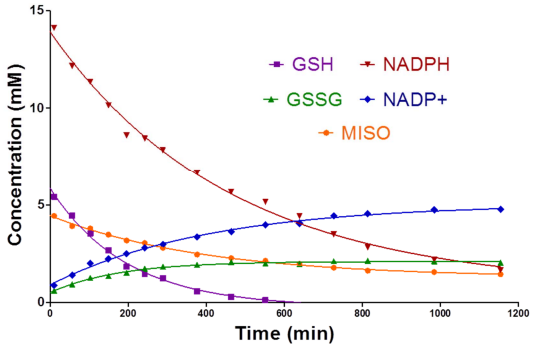

Fig 1. Monoexponential decays of MISO, NADPH and GSH resonances and monoexponential increases of $NADP^+$ and GSSG resonances after addition of NADPH:Cytochrome P450 reductase.

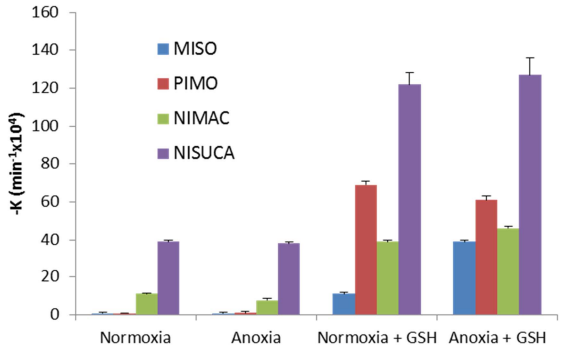

Fig 2. First order rate constants (*k'*) for the reduction of Misonidazole analog (blue), Pimonidazole (red) and NIMAC (green) and NISUCA (purple) under different conditions.

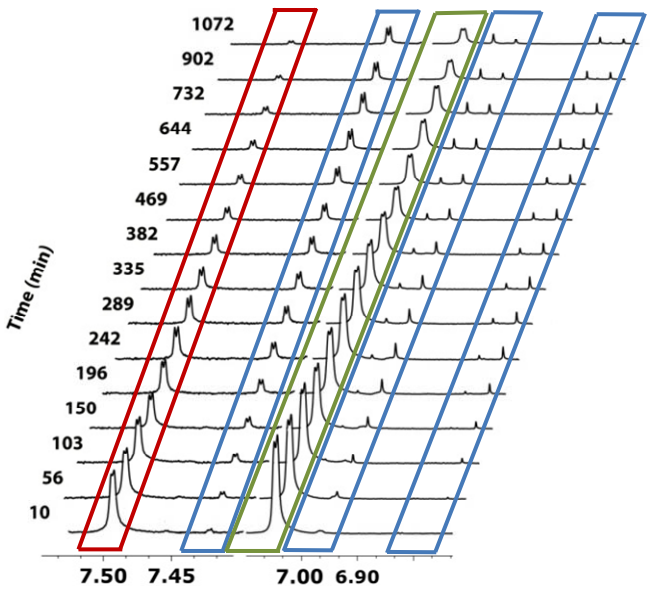

Fig 3. Regions of the ^{1}H NMR spectra of the reaction mixture at various times showing the evolution of the different compounds during the reduction of NIMAC with NADPH:cytochrome P450 reductase under aerobic conditions in presence of GSH. NIMAC (red), NADPH (green) and newly generated signals (blue squares).

Results: The disappearance of the hypoxia markers and GSH resonances, as well as the appearance of corresponding $NADP^+$ and GSSG appeared to follow in all cases first order kinetics (Fig 1) with an apparent rate constant *k´* (Fig 2). Notably, the oxygen content in the solution was found not to have a significant effect in the reduction rate of these compounds, as indicated by the rate constants under normoxia and hypoxia depicted in Fig 2. However, the presence of reduced glutathione increased spectacularly the reduction rate of all compounds, from 5 to 100 times, far exceeding the impact of the changes in oxygen content. When GSH was present in the reduction mixture, a plethora of new resonances appeared downfield of the resonances from the parental compounds (Fig 3). These newly generated signals do not belong to any of the known compounds present in the reduction mixture, representing clearly, intermediates generated during the reduction reaction.

Conclusions: It has been previously thought that the main factor determining the reduction rate of nitroimidazolyl derivatives in tumors was the local oxygen tension. We previously showed that it is the intracellular redox state, rather than the oxygen tension, what determines the reduction rate of these compounds. In this study, we demonstrate that this behavior is common to both commercial and newly synthesized 2-nitroimidazolyl derivatives. Since the GSH dependent increases in reduction rate occur in all compounds investigated, present findings suggest that GSH dependence is a general mechanism for nitroimidazolyl reduction in hypoxic zones. We report also the appearance of new reaction intermediates providing further insight into the mechanism of reduction of these hypoxia markers.

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