Reduced glutathione rather than oxygen concentration determines the reduction rate of nitroimizadol probes used as hypoxia markers.

J. Pacheco-Torres[1,2], P. Ballesteros[2], P. Lopez-Larrubia[1], and S. Cerdan[1]
[1]Biomedical Research Institute "Alberto Sols" - CSIC/UAM, Madrid, Spain, [2]Laboratory of Organic Synthesis and Molecular Imaging, UNED, Madrid, Spain

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). Nitroimidazolyl derivatives (EF5, Pimonidzole, 18F-MISO) have been extensively used as molecular markers of hypoxia in combination with different techniques as immunohistochemistry (2), PET (3) or MRI (4). 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 (5). A key issue to be solved in order to fully understand the activity of these markers is the mechanism of reduction. However the details of the reduction mechanism and its rate determining steps have remained elusive through the years. Here we investigate 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 (Sigma) to investigate *in vitro* the reduction of pimonidazole, a commercially available hypoxia probes, and NIMAC, a novel hypoxia-sensitive probe 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 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 next twelve hours. The spectra obtained were analyzed using MestReNova software.

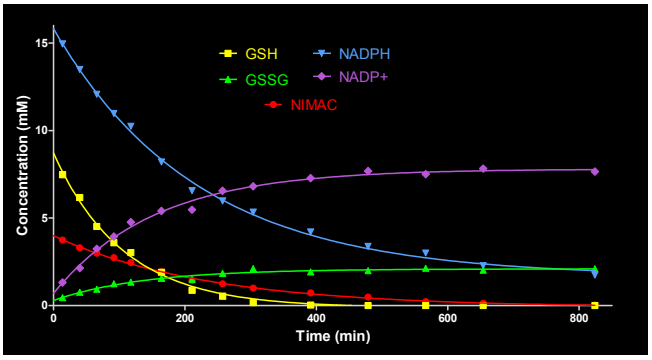

Fig 1. Monoexponential decays of NIMAC, NADPH and GSH resonances and monoexponential increase of $NADP^+$ and GSSG resonances with time after addition of Cytochrome P450 reductase.

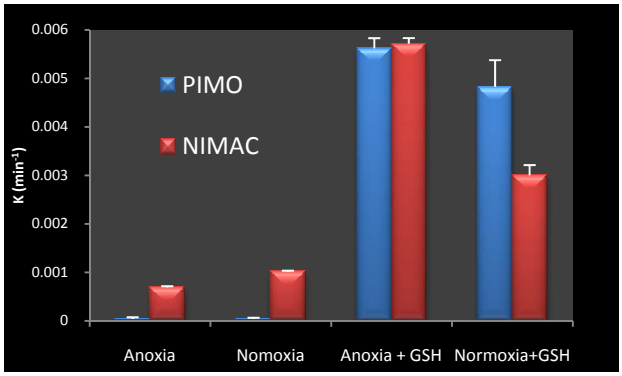

Fig 2. First order rate constants (K) for the reduction of Pimonidazole (blue) and NIMAC (red) under different conditions.

Results: The H4 and H5 resonances of the nitroimidazole ring from all probes were clearly detected in the corresponding spectra, as well as a plethora of new resonances derived from the reduction products, downfield of the resonances from the parental compound (Fig. 1). The disappearance of the hypoxic marker and GSH resonances, as well as the appearance of corresponding $NADP^+$ and GSSG appeared to follow first order kinetics with rate constant K (Fig 1). Notably, the oxygen content in the solution was found not to have a significant effect in the reduction rate of these compounds, as indicated in the rate constants depicted in Fig 2. However, the presence of reduced glutathione increased spectacularly the reduction rate of all compounds, from 5 to 100 times, minimizing the impact of the oxygen content. In the presence of reduced glutathione, the reduction of one mol of nitroimidazolyl derivative is accomplished with the oxidation of two mol of NADPH and one mol of GSH, rendering a 5 electron reduction stoichiometry (Fig 3).

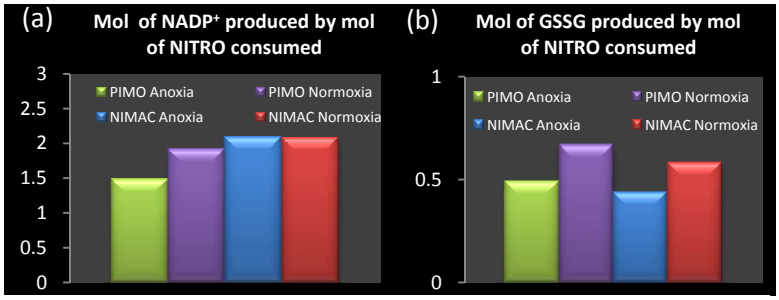

Fig 3. Stoichiometry of nitroimidazolyl derivatives reduction. Production of $NADP^+$ (a) and GSSG (b) per molecule of nitroimidazole consumed.

Conclusions: It has been previously thought that the main factor determining the reduction rate of nitroimidazolyl derivatives was the local oxygen tension. In contrast, our results show that it is the intracellular redox state, rather than the oxygen tension, what determines the reduction rate of these compounds. Since the GSH dependent increases in reduction rate occur in all compounds investigated (although to different extents), present findings suggest that GSH dependence is a general mechanism in nitroimidazolyl reduction in hypoxic zones. It was also demonstrated for the first time that reduction of this type of compounds required the concomitant oxidation of 2 molecules of NADPH to $NADP^+$ and 1 molecule of GSH to GSSG per molecule of nitroimidazole reduced.

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