Reduced Glutathion concentration limits the reduction rate of nitoimidazol derivatives *in vitro*

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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). Nitroimidazolyl derivatives (EF5, Pimonidzole, 18F-MISO) have been extensively used as molecular markers of hypoxia in combination with different techniques as immunohistochemistry (3), PET (4) or MRI (5). 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 (6). A key issue to be solved in order to fully understand the activity of these markers is the mechanism of reduction and the nature of the complexes formed between reduced nitroimidazolyl derivatives and the cellular components. However the precise mechanism of the reduction and its rate determining steps as well as the nature of the adducts formed, remained elusive. Here we investigate the kinetics study of *in vitro* reduction of commercially available, as well as newly synthesized nitromidazole based hypoxia probes.

Subjects and Methods: We used purified NADPH:cytochrome P450 reductase (Sigma) to investigate in vitro the reduction of pimonidazole and misonidazole, two commercially available hypoxia probes, and NIMAC, a novel hypoxia-sensitive probe synthesized by us. Phosphate buffered (75 mM, pH= 7.7, 37 ^{0}C, 10% D_2O) reaction mixtures containing the probes, NADPH and reduced glutathione (where appropriate), were prepared in 5 mm NMR tubes using TSP 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 (p/3 pulse, 32K data table, 6s total cycle time, 128 acquisitions) during the next twelve hours. The spectra obtained were analyzed using Mest-Rec software.

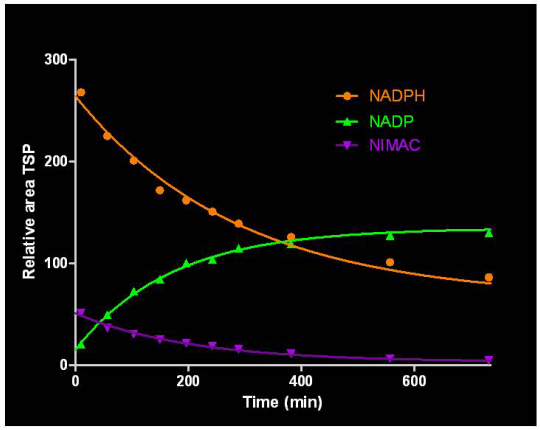

Figure 1. Monoexponential decays of the NADPH and NIMAC resonances and monoexponential increase of NADP resonances with time after addition of Cytochrome P450 reductase.

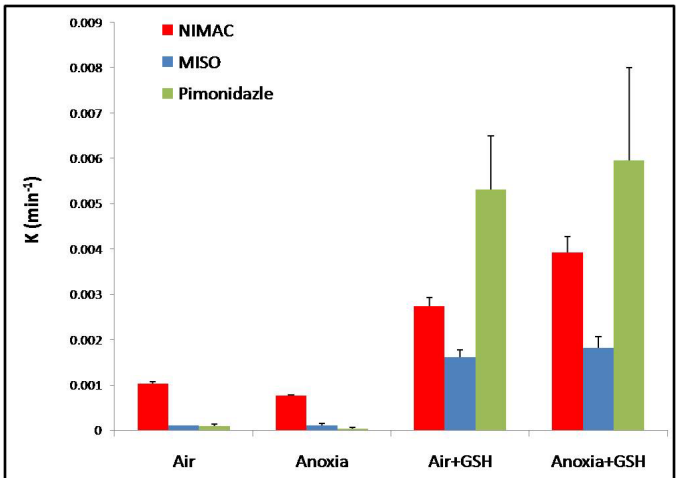

Figure 2. Reduction rate constants of NIMAC (red), Misonidazole (blue) and Pimonidazole (green) under different conditions.

Results: The H4 and H5 resonances of the nitroimidazol 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 probe. The disappearance of the hypoxic marker resonances appeared to follow first order kinetics with rate constant k, as depicted in Figure 1. Notably, the fractional 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 Figure 2. However, the presence of reduced glutathione increased spectacularly the reduction rate of all compounds, from 5 to 100 times, regardless of the oxygen content.

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 the reduced glutathione concentration and the associated NADP/NADPH redox state, may determine the reduction rate of these compounds in vivo and in vitro. 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 nitroimidazolil reduction in hypoxic zones.

[1] Vaupel P, Mayer A, et al. *Adv.Exp.Med.Biol.* **2005**; 566: 333-342. [2] *Int. J. Radiat. Biol.*, **2006;** 82:699-757 [3] Ljungkvist AS, Bussink J, et al. *Radiat.Res.* **2007**; 167(2):127-145. [4] Foo SS, Abbott DF, et al. *Mol.Imaging Biol.* 2004; **6** (5):291-305. [5] Robinson SP, Griffiths JR. *Philos.Trans.R.Soc.Lond B Biol.Sci.* **2004**; 359(1446):987-996. [6] Varghese AJ, Gulyas S, et al. *Cancer Res.* **1976**; 36 (10):3761-3765.