Metabolite profiling using ^{1}H-HRMAS NMR spectroscopy of the response to methionine stress in a malignant melanoma model

S. Guénin[1], A. Demidem[1], J. C. Madelmont[1], and D. Morvan[1,2]
[1]Inserm 484, Clermont-Ferrand, France, [2]Centre Jean Perrin, Clermont-Ferrand, France

Abstract
L-methionine (Met) deprivation has antitumor effects, and was shown to act synergistically with conventional chemotherapy. To get more insight into the mechanism of Met deprivation and interaction with chemotherapy, metabolite profiling by ^{1}H NMR spectroscopy of the response to Met deprivation was performed in a malignant melanoma model *in vitro*. It is shown that Met deprivation alters methylations and phospholipid metabolism, and that these disorders are reversible upon return in standard medium.

Introduction
L-methionine (Met) deprivation (or Met stress) may be used in association with conventional chemotherapy to improve the efficacy of the anticancer treatment (1). Met is an essential amino acid, the major fate of which is protein synthesis. Conversion of Met into S-adenosyl-Met is a prerequisite for other important Met fates: polyamine, taurine and glutathione biosynthesis and transmethylation reactions. The main methylated acceptors are phosphatidylcholine (PtC) through the activity of phosphatidylethanolamine-N-methyltransferase (PEMT) and creatine (Cr) (2). Transcriptomics of Met stress response of tumors has been documented (1), but little is known about the effects of Met stress on tumor metabolism. Metabolite profiling by ^{1}H NMR spectroscopy may help to identify metabolic pathways involved in Met stress response, and better understand the synergy with chemotherapy.

Methods
For Met stress, B16 melanoma cells were grown in Met-deficient RPMI 1640 medium mixed with 10% dialyzed foetal calf serum (Met(-)). For the standard medium culture, the RPMI 1640 medium was completed with 100 µM Met (Met(+)). Met(-) cells were grown in Met-deficient medium for 4 days, then returned to Met(+) medium. Their recovery (Rec) was followed for 6 days. Cell cycle repartition was measured by flow cytometry. Cell protein content was measured using Coomassie Blue at 595 nm. Met concentration was determined by RP-HPLC. PEMT activity was measured by a radioisotope technique. ^{1}H-NMR spectroscopy analysis was performed at 500 MHz (Bruker, Germany) using a HRMAS coil. Intact cell pellets were set into a 50-µL rotor and spun at 4 kHz. Two NMR sequences were performed on each sample: a water signal-suppressed, fully relaxed saturation recovery sequence (duration 6:20 min) and a 2D TOCSY sequence (duration 1:40 hour) with parameters as previously reported in ref (3). All experiments were performed in triplicate. Derivatives of interest included polyunsaturated fatty acids (PUF), taurine (Tau), total glutathione (GSx), total creatine (Cr+PCr= tCr), glycine (Gly), phosphoethanolamine (PE), phosphocholine (PC), glycerophosphocholine (GPC), and phosphatidylcholine (PtC).

Results
Met(+) cells grew exponentially. In contrast, Met(-) cells either died or arrested proliferation by accumulating in the G_1 phase of the cell cycle (> 80%) (Fig 1). In Met(-) cells, protein content was decreased by 35% ($p < 0.05$, Met(-) *vs* Met(+)), and Met concentration was decreased by 95%. After 2 days of reinfusion in Met(+) medium, Rec cells grew again with similar growth rate, cell cycle distribution, protein concentration and Met concentration than Met(+) cells. Typical ^{1}H-NMR spectra of Met(+), Met(-) and Rec cells are shown in Fig 2. In comparison with Met(+) cells, Met(-) cells showed a decrease in tCr, Tau and GSx, and an increase in Gly, PE, PC, GPC and PUF. The latter 3 metabolites suggested hydrolysis of PtC. However the PtC pool was not decreased (Fig 2, inserts). This prompted us to test the PEMT activity that was found to be increased in Met(-) cells (+90%, $p < 0.01$, Met(-) *vs* Met(+), Fig 3). Decrease in polyamine content was found on TOCSY spectra of Met(-) cells. After 2 days of reinfusion in Met(+) medium, Rec cells showed similar metabolic profile than Met(+) cells (Fig 2 and 3).

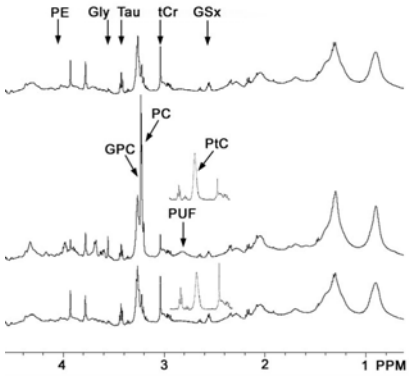

Fig 2: ^{1}H-NMR spectra of the response of B16 melanoma cells to Met stress. From bottom to top: Met(+) cells at day 10; Met(-) cells at day 4; Rec cells at day 10. The latter shows complete metabolic recovery. Inserts: PtC $N(CH_3)_3$ signal at 3.26 ppm after deconvolution of superposing signals (2). Abbreviations: see Methods.

Discussion
Met stress induced in B16 melanoma cells *in vitro*:
1) disorders of transmethylation reactions as attested by a decrease in tCr level and increased activity of PEMT. The latter may aim at compensating for decreased availability of Met for transmethylation of phosphatidylethanolamine. This suggests that the preservation of the PtC pool has priority over that of Cr;
2) a decrease in transsulfuration products that may reflect reduced homocysteine production and transmethylation reactions (Fig 4);
3) an accumulation of Gly consistent with decreased utilization for Cr, GSx and DNA biosyntheses, but sustained production;
4) alterations in PtC metabolism, with increased turnover consistent with cell cycle arrest in the G_1 phase (4). PtC hydrolysis by the phospholipase A pathway releases GPC and PUF (4,5).
In Rec cells, these disorders were fully reversible.
These data may provide novel understanding of the therapeutic synergy of Met stress with chemotherapy. First the association must be simultaneous as attested by rapid recovery upon Met reinfusion. Second, some disorders in response to Met stress are similar to those in response to nitrosourea treatment like decreased GSx levels and increased PtC hydrolysis (3,6). Because GSx is protective against chemotherapy and PtC pool homeostasis is critical for cell survival (4,5), these metabolic alterations may participate to the therapeutic synergy.

References
1- Kokkinakis D.M. Cancer Lett 2006;233:195-207.
2- Morvan D et al. Magn Reson Med 2006;55:984-996.
3- Morvan D et al. Magn Reson Med 2003;49:241-248.
4- Jackowski S. J Biol Chem 1994;269:3858-3867.
5- DeLong C.J. et al. J Biol Chem 1999;274:29683-29688.
6- Demidem A. et al. Int J Cancer 2006;119:992-1004.

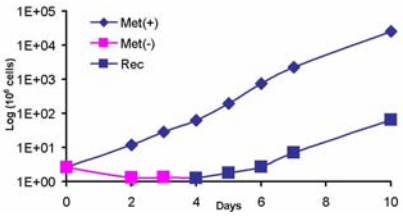

Fig 1: Effect of Met stress on B16 melanoma cell proliferation.

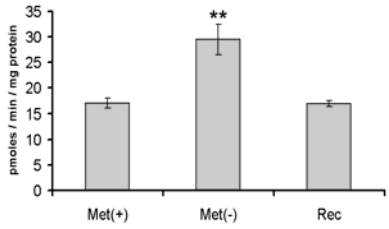

Fig 3: PEMT activity in response to Met stress. **, $p < 0.01$.

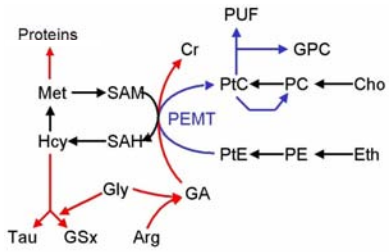

Fig 4: Met metabolism and interpretation of Met stress response. Arg, arginine; Eth, ethanolamine; GA, guanidinoacetate; Hcy, homocysteine; PtE, phosphatidylethanolamine; SAH, S-adenosyl-homocysteine; SAM, S-adenosyl-methionine.
Red, downregulation; blue, activation.