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## GLINT

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### D4.4 Assessment of intracellular energy substrate levels upon native and methylated glucose injections

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<b>CO</b>	Confidential, only for members of the consortium (including the Commission Services)	
<b>CI</b>	Classified, as referred to in Commission Decision 2001/844/EC	

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## 1 Version log

Version	Date	Released by	Nature of Change
V1.0	01/12/2016	G. Navon	First version
V1.1	06/12/2016	K. Krischak	Format changes, spell check
V1.2	01/02/2017	M. Kim	TAU and UZH reports merged
V1.3	13/02/2017	M. Kim	Final version

## 2 Introduction

In order to address deliverable D4.4,  $^{13}\text{C}$  and  $^{31}\text{P}$  NMR spectroscopy experiments were performed on extracts of tumors following the administration of the 3OMG. The findings presented in this report enabled us to answer the following questions:

1. Whether 3OMG accumulated in the tumors and whether it penetrates the BBB and accumulated in the brain.
2. Whether 3OMG undergoes metabolism and if metabolic products can be found.
3. Whether the penetration of 3OMG changed the status of metabolic profile of the tumor and the brain.

In addition, the effect of 3OMG on glucose consumption was assessed separately in cultured astrocytes.

### 3 Methodology and Approach

#### Orthotropic mammary fat pad implantation

BALB/C female mice were purchased and housed in the breeding facility of Tel Aviv University. Orthotropic xenograft tumors were induced in the mice by injecting 4T<sub>1</sub> cells (10<sup>6</sup>/100µl cells) into the lower left mammary gland of 6-8-week-old female mice (17–22 grams, Envigo, Israel) using a 27-gauge needle. The tumors were allowed to grow for 10–14 days reaching an average tumor volume of 5±2 mm<sup>3</sup>. All experiments with animal models were carried out in compliance with the principles of the Israel National Research Council (NRC) and were approved by Tel Aviv University institutional animal care and use committee (IACUC) (01-15-057).

#### Preparation of tumor extracts

The tumors were surgically excised and immediately weighed and immersed in liquid nitrogen. The frozen tumors were homogenized with a tissue homogenizer, using the methanol/chloroform/water extraction method<sup>16</sup> with a volume ratio of 2/2/1.8, respectively. After centrifugation (−4°C, 4000 × g, 12 min), only the upper aqueous phase was kept for analysis. The samples were dried gently by evaporator, frozen at −80°C, and lyophilized to dryness for 24 h. Each sample was dissolved in 0.4 ml H<sub>2</sub>O and 0.1 ml D<sub>2</sub>O (99.98%, Biolab, Israel), adjusted to pH of 7.4 and inserted into a 5-mm tube for <sup>13</sup>C NMR. 0.01% concentration of sodium azide was added to avoid microbacterial growth.

#### NMR spectroscopy

<sup>13</sup>C NMR spectra were recorded at 125.7 MHz in 5 mm tubes using a 500 MHz AVANCE3. Acquisition parameters were as follows (for the methanol/chloroform/water extracts): spectral width 16 KHz, data size 32 K, pulse width 5 us (45° flip angle), relaxation delay 2 s, acquisition of 1 s.

#### Intracellular glucose measurements in cultured astrocytes

The cells were infected with an adenovirus encoding the FRET glucose nanosensor FLIPΔ6. Astrocytes expressing FLIPΔ6 were excited at 430 nm for 0.2-0.8 sec and the emissions of Citrine and CFP were collected. The Citrine/CFP fluorescence ratio was translated to glucose concentration based on a standardized calibration procedure. Two different concentrations of PU

extracellular 3OMG were tested: 1 mM and 10 mM. Glucose consumption was measured using Cytochalasin B, a mycotoxin that inhibits glucose transporters.

The rates of glucose decrease were measured at the same intracellular glucose level for both cases. The decrease rates are calculated by fitting a linear function, where the slope corresponds to the decrease rate and is converted to the rate of glucose consumption, i.e. hexokinase activity ( $\mu\text{M/s}$ ). All data are expressed as mean plus minus (+/-) standard error of mean (s.e.m.). The use of zero glucose is for calibration purpose and to correct the signal drift.

In vitro assays are a collaboration between UZH and laboratory of L. Felipe Barros, Centro de Estudios Científicos, Valdivia, Chile.

## 4 Results

### *NMR spectroscopy*

$^{13}\text{C}$  and  $^{31}\text{P}$  NMR were used to analyze 3OMG metabolism in  $4\text{T}_1$  orthotopic tumors as well as in brains. Mice were administrated with  $[6-^{13}\text{C}]$  3OMG (1.0 g/kg, PO) and tumors and brains were excised within ~40min after treatment. As is seen from Fig. 1a,b and Fig. 2a-d, the  $^{13}\text{C}$  NMR of the extracts of tumors and brains, respectively, points to a significant peak (63.3ppm) originate from the administrated  $[6-^{13}\text{C}]$ 3OMG. This may serve as an indication to the significant 3OMG CEST effect originates mainly from the intake of 3OMG into the tumors as well as into the brains. The  $^{13}\text{C}$  NMR indicate that there are no other metabolic products besides 3OMG (Fig. 1a,b and Fig.2a-d) as it is a non-metabolized glucose analog that enters the cells via the membrane concentrative sodium dependent glucose transporter and exits the cells via the membrane facilitated diffusional transporter. Hwang et al. [1] have shown by in situ  $^{13}\text{C}$  NMR of implanted murine R1F-1 tumors that while 3OMG entered to the tumors, no other metabolites could be observed.

Examination of  $^{31}\text{P}$  NMR difference spectra (Fig. 3, 4) showed no formation or accumulation of any glucose analog phosphate resonance after 3OMG administration, both in the tumors as well as in the brains. The results obtained by this study correspond with the previous outcomes published by Hwang et al [1]. Additionally, by in situ  $^{31}\text{P}$  NMR Hwang et al. have shown that the intracellular pH dropped by about 0.52 pH units following the i.p. administration of 3OMG, and that without the formation of lactic acid.



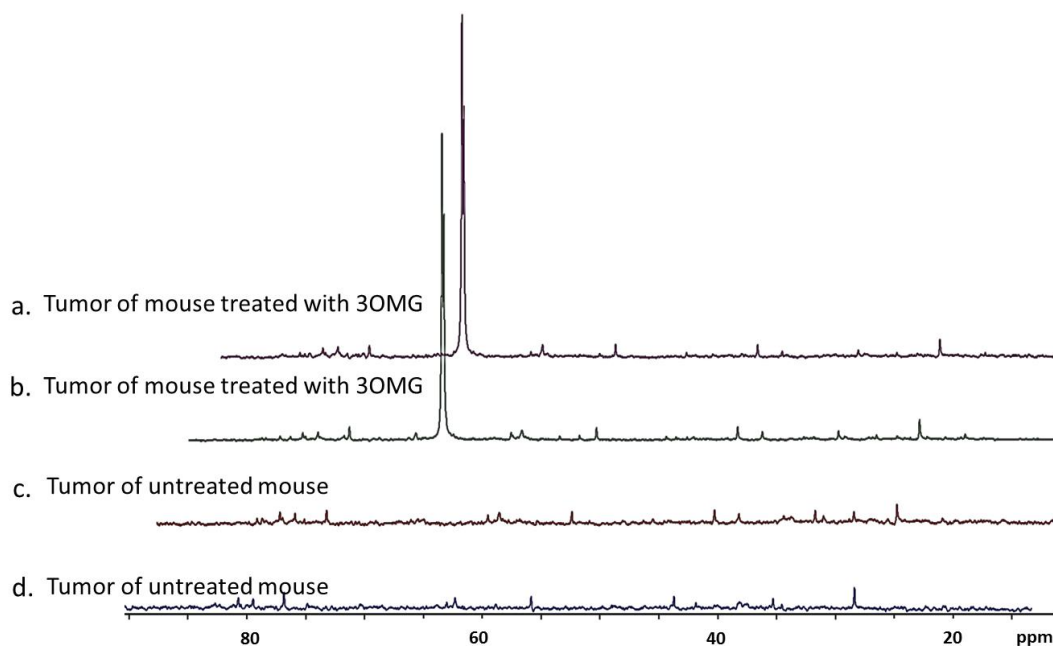


Figure 1:  $^1\text{H}$ -decoupled  $^{13}\text{C}$  NMR spectra of metabolites extracted from tumors of mice bearing  $4\text{T}_1$  model. a and b are extracts from tumors of mice administrated with  $[6\text{-}^{13}\text{C}]$  3OMG (1.0 g/kg, PO). c and d are extracts from control tumors (without treatment). Each spectrum corresponds to an overnight data accumulation and represents a single specific tumor of a mouse. The resonance of  $[6\text{-}^{13}\text{C}]$  3OMG is shown at 63.3 ppm in spectra a and b, respectively. The peaks were referenced to DSS (0 ppm).

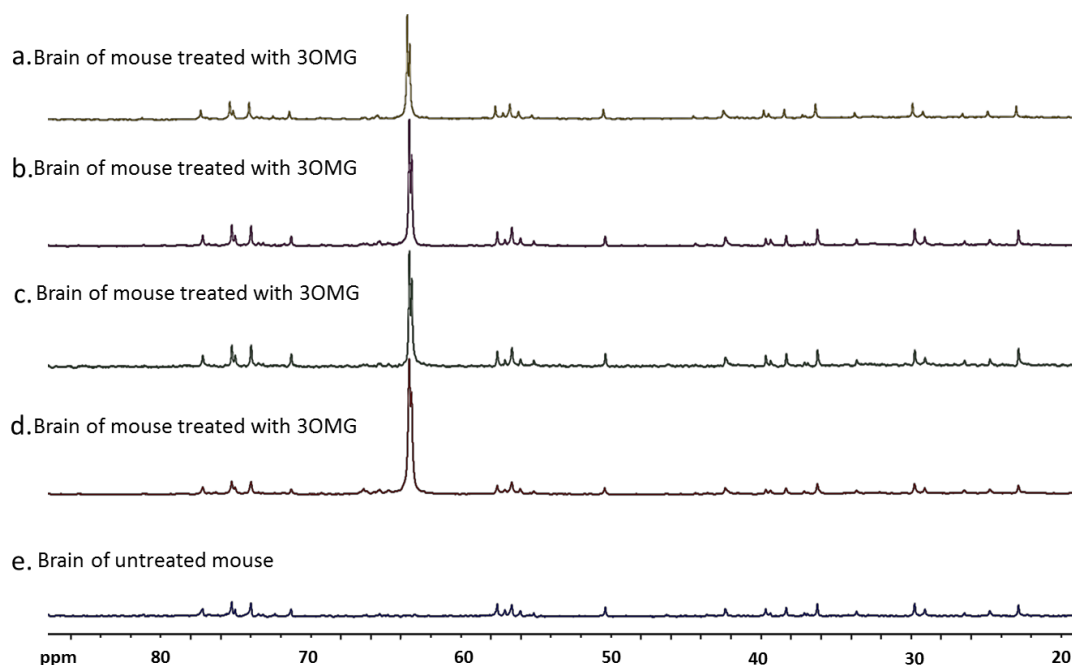


Figure 2:  $^1\text{H}$ -decoupled  $^{13}\text{C}$  NMR spectra of metabolites extracted from brains of mice bearing  $4\text{T}_1$  model. a-d are extracts from brains of mice administrated with  $[6\text{-}^{13}\text{C}]$  3OMG (1.0 g/kg, PO). e is extract from control brain

(without treatment). Each spectrum corresponds to an overnight data accumulation and represents a single specific tumor of a mouse. The resonance of  $[6-^{13}\text{C}]$  3OMG is shown at 63.3 ppm in spectra a-d, respectively. The peaks were referenced to DSS (0 ppm).

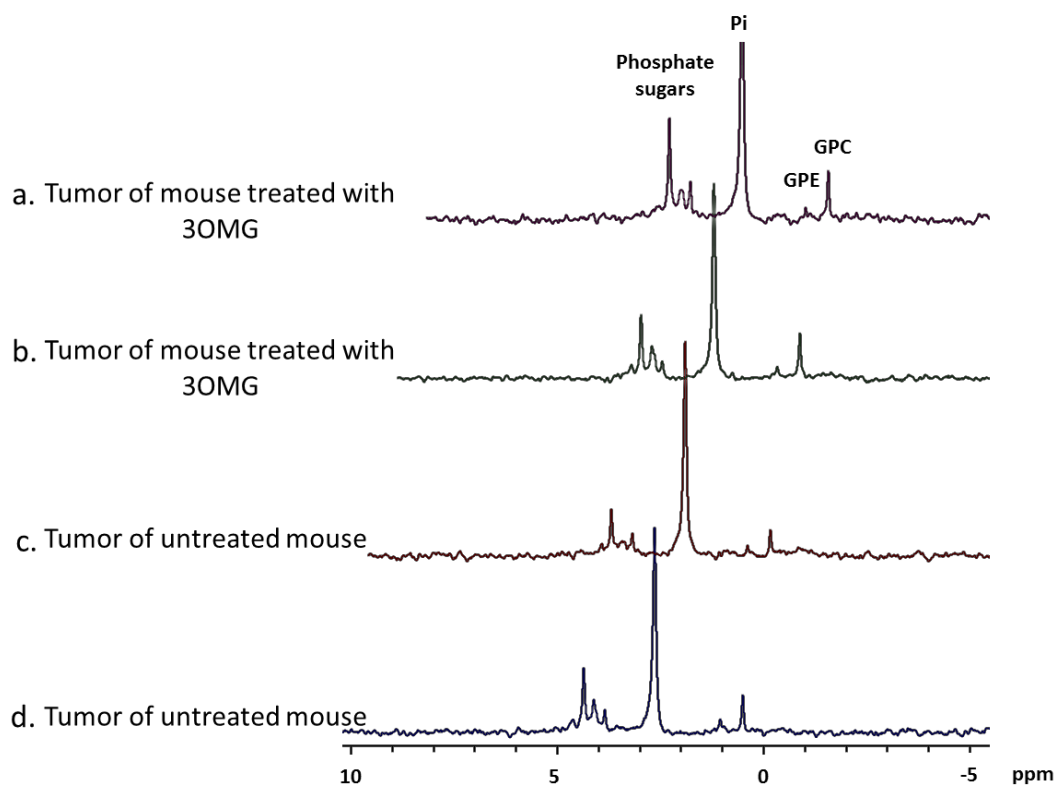


Figure 3:  $^{31}\text{P}$  NMR spectra of metabolites extracted from tumors of mice bearing  $4\text{T}_1$  model. a and b are extracts from tumors of mice administrated with  $[6-^{13}\text{C}]$  3OMG (1.0 g/kg, PO). c and d are extracts from control tumors (without treatment). The peaks were referenced to GPC (0.49 ppm). Each spectrum represents a single specific tumor of a mouse. The peaks were assigned according to previously published data: GPC-glycerphosphocholine; GPE-glycerphosphoethanolamine; Pi- inorganic phosphate.

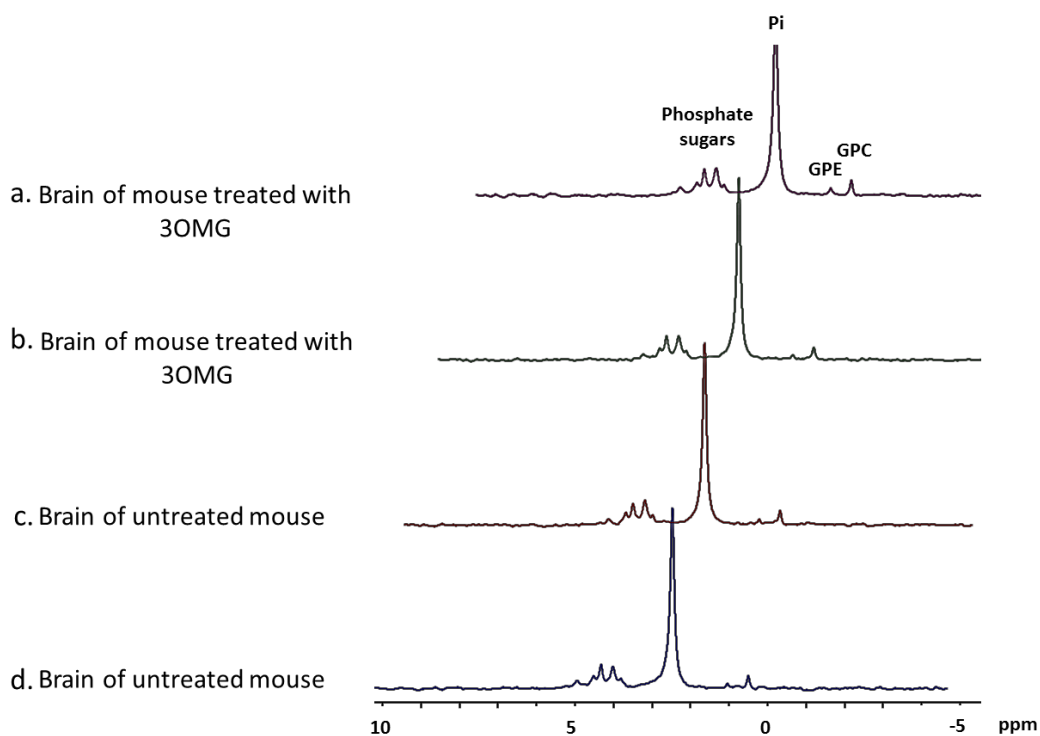


Figure 4:  $^{31}\text{P}$  NMR spectra of metabolites extracted from brains of mice bearing  $4\text{T}_1$  model. a and b are extracts from brains of mice administrated with  $[6\text{-}^{13}\text{C}]$  3OMG (1.0 g/kg, PO). c and d are extracts from control brains (without treatment). The peaks were referenced to GPC (0.49 ppm). Each spectrum represents a single specific tumor of a mouse. The peaks were assigned according to previously published data: GPC- glycerphosphocholine; GPE- glycerphosphoethanolamine; Pi- inorganic phosphate.

*Intracellular glucose measurements in astrocytes*

In the case of 1 mM 3OMG, no significant effect was observed, neither on the glucose consumption relative to basal nor on the intracellular glucose level after 3OMG addition.

In the case of 10mM 3OMG, the difference in glucose consumption at basal and at 10 mM extracellular concentration of 3OMG was not significant (Fig. 5, 6). However, we observed a mean decrease of 0.05 mM in intracellular glucose, most probably related to the competition at the glucose transporter.

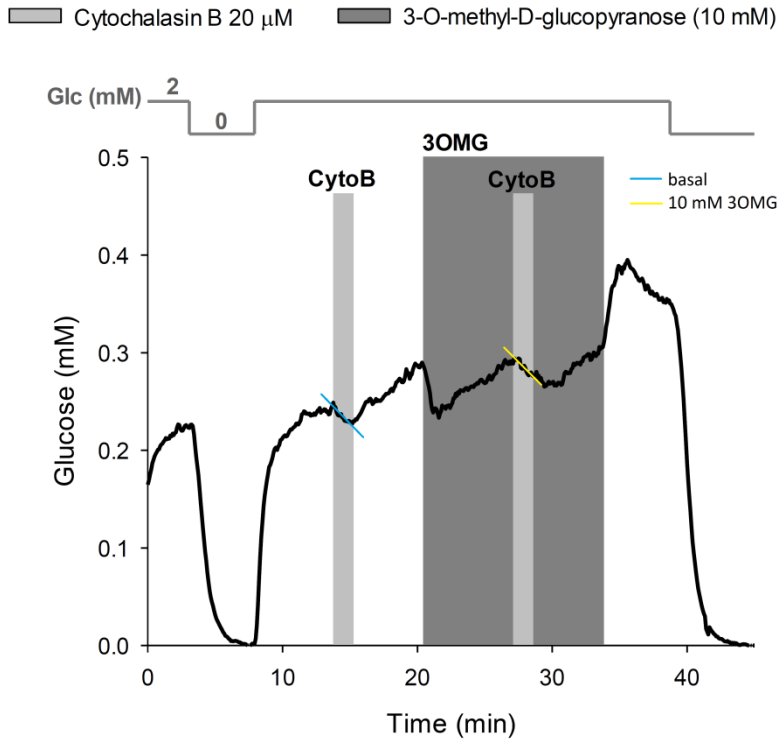


Figure 5: Time course of intracellular glucose concentration of cultured astrocytes upon changes in the extracellular medium

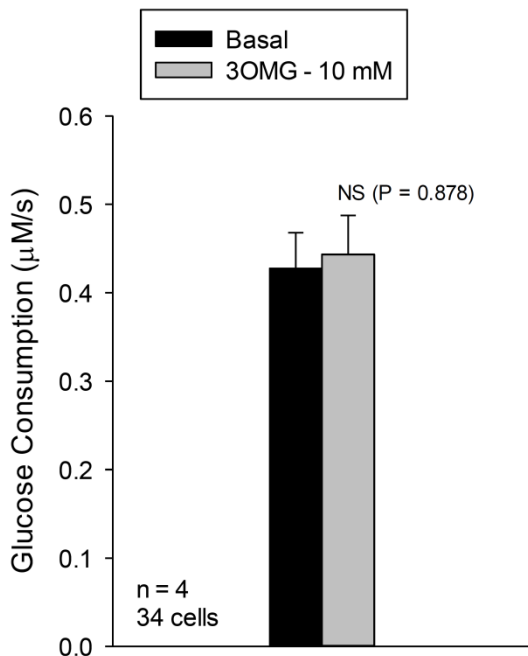


Figure 6: Glucose consumption of cultured astrocytes in basal condition (zero extracellular 3OMG) and in 10 mM of extracellular 3OMG

## 5 Conclusions

$^{13}\text{C}$  NMR of extracts of tumors and the brains of mice following administration of [6- $^{13}\text{C}$ ] 3OMG (1.0 g/kg, PO) indicated the penetration of 3OMG to both tumors and the brains, and no other metabolic product could be observed. This is corroborate the generally excepted 3OMG as "non- metabolizabled" glucose analogue.

$^{31}\text{P}$  NMR indicated no change in the metabolic profile of the tumors and the brains upon the penetration of 3OMG.

Intracellular glucose measurement showed that glucose consumption was not significantly different between basal and 10 mM extracellular concentration of 3OMG.

## 6 References

Hwang, Y.C., Kim, S.-G., Evelhoch, J.L. & Ackerman, J.J. “Nonglycolytic acidification of murine radiation-induced fibrosarcoma 1 tumor via 3-O-methyl-D-glucose monitored by <sup>1</sup>H, <sup>2</sup>H, <sup>13</sup>C, and <sup>31</sup>P nuclear magnetic resonance spectroscopy”. *Cancer research* 52, 1259-1266 (1992).