Published in final edited form as:

Mol Imaging Biol. 2014 August ; 16(4): 459–468. doi:10.1007/s11307-014-0720-9.

In vitro Characterization of Uptake Mechanism of L-[methyl-³H]-methionine in Hepatocellular Carcinoma

Yu Kuang^{1,3}, Fangjing Wang², David J. Corn¹, Haibin Tian¹, and Zhenghong Lee^{1,2,4}

¹Department of Radiology, University Hospitals Case Medical Center, Cleveland, OH, 44106, USA

²Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH, 44106, USA

³Medical Physics Program, University of Nevada, Las Vegas, Las Vegas, NV, 89154, USA

⁴Nuclear Medicine Division, Department of Radiology, University Hospitals Case Medical Center, 11100 Euclid Ave., Cleveland, OH, 44106, USA

Abstract

Purpose—Methionine (Met) could be a useful imaging biomarker for the diagnosis of hepatocellular carcinoma (HCC), as demonstrated by PET imaging with L-[methyl-¹¹C]-Met. In HCC cells, protein synthesis mainly contributes to radiopharmaceutical uptake. In contrast, lipid synthesis via the phosphatidylethanolamine (PE) methylation pathway is the major metabolic route of L-[methyl-¹¹C]-Met in normal hepatocytes, which contributes to the background contrast observed in PET images. However, the mechanisms of amino acid transport and the roles of the two key enzymes, methionine adenosyltransferase (MAT) and phosphatidylethanolamine N-methyltransferase (PEMT), are not yet completely understood. The aim of this study was to investigate the roles of the amino acid transporters and these two key enzymes in the uptake of L-[methyl-¹¹C]-Met in HCC cells.

Procedures—A well-differentiated woodchuck HCC cell line, WCH17, was used for the study. The amino acid transporter of WCH17 cells was assayed to investigate the Met transport process in HCC. WCH17 cells were treated with 5 mM S-adenosylmethionine (SAM) for 8, 16, 24, and 48 h to downregulate MAT2A gene expression. Control or SAM-treated WCH17 cells were pulsed with L-[methyl-³H]-Met for 5 min and chased with cold media to mimic the rapid blood clearance of radiolabeled Met (pulse-chase experiment). In parallel, WCH17 cells were transfected with a mouse liver PEMT2 expression vector, and the pulse-chase experiment was performed to investigate the uptake of the radiolabeled Met in HCC cells. The water-soluble, protein, and lipid phases from the total uptake were subsequently extracted and measured, respectively.

Results—Met was transported into HCC cells via a facilitative transport process, which was characterized as system L and ASC-like, Na⁺ dependent, and low affinity with partial energy dependence. The total uptake of L-[methyl-³H]-Met was decreased in HCC cells with SAM

Correspondence to: Zhenghong Lee; zhenghong.lee@case.edu.
Fangjing Wang and David J. Corn have equally contributed to this study.

Conflict of Interest. The authors declare that they have no conflict of interest.

treatment. This reduction pattern followed that of MAT2A expression (the duration of SAM treatment). The incorporated ^3H was mostly distributed in the protein phase and, to a lesser degree, in the lipid phase via PE methylation pathway in HCC cells with SAM treatment. The downregulated MAT2A expression led to the decreased uptake in protein and water-soluble phases. In addition, an increased uptake in the lipid phase was observed in WCH17 cells transfected with PEMT2 expression vector.

Conclusions—The amino acid transport processes may be responsible for the rapid accumulation of radiolabeled Met after the intravenous injection of tracers for the imaging of HCC. Upregulated MAT2A expression and impaired PEMT2 activities in HCC are associated with the specific metabolic pattern of L-[methyl- ^{11}C]-Met detected by PET.

Keywords

Hepatocellular carcinoma; Radiolabeled methionine uptake; Methionine adenosyltransferase; S-adenosylmethionine; S-adenosylhomocysteine; Phosphatidylethanol-amine N-methyltransferase; Amino acid transporter

Introduction

Abnormal tumor cell metabolism and molecular mechanisms are closely interrelated [1]. Malignant transformation in hepatocellular carcinoma (HCC) induced by various oncogenes or loss of tumor-suppressor genes may result in quantitative and qualitative alterations of radiolabeled methionine (Met) uptake and metabolism. The tumor environment may also cause specific changes of cellular metabolism that affect the uptake of Met.

Our previous study [2] demonstrated that the major metabolic fates of L-[methyl- ^{11}C]-Met in HCC cells are protein synthesis and, to a lesser degree, lipid synthesis via the phosphatidylethanolamine (PE) methylation pathway (Fig. 1.). However, in the PE methylation pathway, the conversion from the water-soluble phase to lipid phase occurred slowly even when protein synthesis was blocked [2], suggesting that phosphatidylethanolamine-N-methyltransferase (PEMT)-mediated conversion of S-adenosylmethionine (SAM) to lipids is highly regulated in HCC cells. In contrast, lipid synthesis was the predominant metabolism in primary hepatocytes, and lipids (phosphatidylcholine (PC), phosphatidylmonomethylethanolamine (PMME), and phosphatidyl dimethylethanolamine (PDME)) contributed to the background contrast shown in the PET images of HCC using L-[methyl- ^{11}C]-Met as probe [2].

These findings suggest that the amino acid transporter and two key enzymes of the Met metabolic process, methionine adenosyltransferase (MAT) and PEMT, may influence the incorporation patterns of L-[methyl- ^{11}C]-Met into tumor cells. However, the mechanisms of these intracellular factors responsible for the radiolabeled Met uptake in HCC cells are not yet completely understood. A thorough investigation of how these factors function in the various metabolic fates of Met will help to better understand the intracellular handling of radiolabeled Met in HCC cells and facilitate the development of new diagnostic and therapeutic strategies.

The aim of this study was to investigate the mechanisms of amino acid transporters and the two key enzymes of PE methylation pathway, MAT and PEMT, in the uptake of L-[methyl-¹¹C]-Met in HCC cells. A well-differentiated HCC cell line WCH17 was used. First, to investigate the rate-limiting steps of Met uptake, we performed the in vitro assay of the Met transport process in WCH17 cells. Then, SAM was used to inhibit the expression of MAT2A in WCH17 cells. The MAT-specific uptake and retention of L-[methyl-³H]-Met in WCH17 cells extracted from the total uptake were measured. Finally, WCH17 cells were transfected with a plasmid expressing mouse liver PEMT2 (mPEMT2) expression to identify the role of PEMT in the L-[methyl-³H]-Met uptake in HCC cells.

Materials and Methods

Materials

All chemical reagents used were obtained from Sigma Chemicals (St. Louis, MO), unless stated otherwise. WCH17 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). L-[methyl-³H]-Met (specific activity 2.96 GBq/ mmol) was obtained from American Radiochemical Inc. (St. Louis, MO). Penicillin streptomycin, Dulbecco's Modified Eagle's Medi-um (DMEM), and Hank's balanced salt solution (HBSS) were obtained from Invitrogen Co. (Carlsbad, CA). All organic solvents were purchased from Fisher Scientific (Pittsburgh, PA).

L-amino Acid Transporter Assay in WCH17 Cells In vitro

The L-amino acid transporter assay followed a procedure described previously with modification [3, 4]. In Brief, WCH17 cells were cultured (3×10^5 cells/24-well plate) in 1-ml DMEM supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin streptomycin solution in a 10 % CO₂-humidified atmosphere at 37 °C. The assay was initiated with the replacement of culture medium with uptake medium (20-mM HEPES/Tris buffer, pH 7.4, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl₂, 1 mM MgCl₂, and 10 mM glucose). Cells were incubated with L-Met at concentrations ranging from 5 to 2,000 μM (containing 1.54 KBq L-[methyl-¹⁴C]-Met per well) at 37 °C for 1 min. The uptake medium was removed, and the cells were washed three times with ice-cold phosphate-buffered saline (PBS) and scraped for scintillation counting. To examine the transporter's dependence on sodium, experiments were repeated using HBSS uptake medium containing 141 mM choline chloride instead of 141 mM NaCl.

Experiments were also performed with specific competitive inhibitors of three amino acid transporter systems (2-amino-2-norbornanecarboxylic acid (BCH) for system L, α-(methylamino)isobutyric acid (MeAIB) for system A, and serine for system ASC (a transport system with a strong preference for alanine, serine, and cysteine [5])); each inhibitor was administered at concentrations of 1.5 and 15 μM, respectively. The effects of the metabolic inhibitors ouabain and 2,4-dinitrophenol on the Met transporter were also investigated; each metabolic inhibitor was administered at a concentration of 0.1 and 1 mM, respectively. Ouabain was used to determine the sensitivity of Met transport rates to the Na⁺/K⁺-dependent adenosine triphosphatase (ATPase), while the oxidative phosphorylation

uncoupler 2,4-dinitrophenol was used to inhibit the production of cellular adenosine triphosphate (ATP).

From these transporter assays, the uptake rate was determined by fitting the nonlinear least-square curve [6]:

$$\text{Uptake rate (nmole/mg protein/min)} = \frac{V_{\max} [S]}{K_M + [S]} \quad (1)$$

where [S] (μM) is the Met concentration, V_{max} (nmol/mg protein/ min) and K_M (μM) are the Michaelis-Menten constants for maximal transport velocity and concentration at half-maximal velocity, respectively.

WCH17 Cells Treatment with SAM

SAM and its metabolite methylthioadenosine (MTA) can be used to inhibit growth of hepatoma cells via lowering the expression of MAT2A [7–9]. The optimized experimental conditions were recommended by Dr. Shelly C. Lu from the University of Southern California [9]. Before treatment with SAM, WCH17 cells were cultured in a medium with 0.1 % FBS and incubated overnight. WCH17 cells were then cultured in a medium without serum and treated with 5 mmol/l SAM or vehicle controls for 8, 16, and 24 h. The cell viability of WCH17 treated with SAM was evaluated with a CellTiter-Blue Cell Viability Assay kit by following the vendor's protocol (Promega Co. Madison, WI). Caspase-3/7 activities in SAM-treated WCH17 cells were also assayed using a Caspase-Glo® 3/7 Assay Systems kit according to the vendor's protocol (Promega Co. Madison, WI).

DNA Flow Cytometry

WCH17 cells (5×10^5) were cultured in T25 culture flasks for 24 h and then treated with 5 mM SAM for 8, 16, 24, 48, and 72 h, respectively. Nontreated WCH17 cells were used as control. After incubation, the cells were trypsinized, washed with PBS, and centrifuged at $1,200 \times g$ at 4 °C for 5 min. The resultant pellet was washed with 2 ml of 1 % BSA/PBS and centrifuged again at $1,200 \times g$ at 4 °C for 5 min to remove supernatant. The pellet was resuspended in 100 μl 0.9 % NaCl and fixed overnight in 1 ml 100 % methanol at –20 °C. Fixed cells were washed twice with 1 % BSA/PBS (containing 50 % Triton X-100 solution for the second washing), resuspended in 0.5 ml of 1 % BSA/PBS (containing 0.05 mg/ml RNase A) at room temperature for 30 min. DNA-binding propidium iodide (PI) solution (100 μl, 10 mg/ml in PBS) was added into cell suspensions, and 0.4-ml sample was analyzed with an EPICS-XL MCL flow cytometer (Beckman Coulter, Miami, FL). Cell cycle, sub-G1, and apoptotic cells were analyzed using FlowJo software.

Pulse-Chase Study

To mimic in vivo conditions, pulse-chase experiments described previously were used to track the transient change of the intracellular metabolites derived from radiolabeled Met [2]. Pulse-chase experiments were conducted on WCH17 cells treated with SAM or with vehicle control for 8 and 16 h. The intracellular distributions of radioactivity in water-soluble, lipid-

soluble, DNA, RNA, and protein phases at different chase periods (0, 10, 25, 40, and 55 min) were determined as reported previously [2, 10].

Establishment of Hepatoma Cell Lines that Express Liver PEMT2

To investigate the role of PEMT2 in the metabolism of L-[methyl-³H]-Met in HCC, WCH17 cells were transfected with a plasmid expressing mPEMT2 (a kind gift from Dr. Dennis E Vance at University of Alberta) [11]. The plasmid were constructed by fusing the mPEMT2 complementary DNA (cDNA) to the 3' end of the constitutive cytomegalovirus (CMV) promoter. The plasmid was extracted from the filter paper sent from the laboratory of Dr. Vance by rehydrating a punched circle in TE buffer for 1 h with occasional vortexing. The extracted DNA was transformed into a competent cell (DH5a), and 250 µl of transformed competent cells was plated onto a prewarmed LA plate and allowed to incubate overnight at 37 °C. Selected colonies were grown in 5–10-ml LB medium for 8–12 h until the solution became turbid. DNA was extracted using a QIAprep® Spin Miniprep Kit (Qiagen, Valencia, CA), and the DNA sequence was verified by enzymatic digestion with Xho1/Xba1 followed by electrophoresis, which generated an ~600-bp insert. DNA purified with a Qiagen plasmid Giga kit (Qiagen, Valencia, CA) was used to transfect cells. DNA concentration was measured by using a UV spectrophotometer (Beckman Du® 640B, Brea, CA), and its purity was analyzed by calculating the ratio of the absorbance values at 260 and 280 nm. Before transfection, 1×10^6 WCH17 cells were seeded in a 75-cm² culture flask. The next day, cells were transfected with the plasmid DNA using lipofectamine 2000 (Invitrogen Co., Carlsbad, CA) following the vendor's protocol. The cells were harvested on day 3 for subsequent analysis.

Incubation of mPEMT2 Transfected WCH17 Cells with ³H-SAM

PEMT2 expression was confirmed in WCH17 cells by incubating the cells with 10 ml of HBSS containing 74 KBq of ³H-SAM (American Radiolabeled Chemicals, Inc., St. Louis, MO) at 37 °C for 60 min. The total intracellular radioactive lipids (³H-PC, ³H-PMME, ³H-PDME) incorporated from ³H-SAM were extracted and measured based on the procedure reported previously [2].

Pulse-Chase Study on WCH17 Cells Transfected with mPEMT2

WCH17 cells transfected with mPEMT2 or transfected with vehicles were treated or untreated with 500 µg/ml cycloheximide, a protein synthesis inhibitor, for 2 h [12–15]. Cells were then used in pulse-chase experiments with L-[methyl-³H]-Met as the procedure described above. The lipid-soluble phase incorporated from L-[methyl-³H]-Met was extracted based on the procedure reported previously [2].

Statistical Analysis

Each in vitro experiment was carried out in triplicate, and the results are reported as average ± standard deviation. The data were compared using one-way analysis of variance (ANOVA) or ANOVA on ranks, when appropriate. All pairwise multiple comparison procedures used Tukey's honestly significant difference test. Differences were regarded as statistically significant for $p < 0.05$.

Results

Transport of Methionine in HCC Cells

In our imaging study (unpublished data), following intrave-nous injection of L-[methyl- ^{11}C]-Met in vivo, HCC regions in the woodchuck model displayed a rapid increase in tracer uptake compared with the surrounding hepatic tissues. This finding suggested that the amino acid transporter systems in the regions of HCC could be a rate-limiting step of L-[methyl- ^{11}C]-Met uptake, thus motivating the specific characterization of Met transport in HCC cells. The L-amino acid transporter in WCH17 cells had a low estimated affinity for the uptake of L-Met ($K_M = 723.1 \pm 130.1 \mu\text{M}$) when sodium was present; however, in the absence of sodium, the K_M increased to $2326 \pm 528.9 \mu\text{M}$, suggesting that the uptake mechanism in WCH17 cells was sodium dependent (Fig. 2a vs b). Moreover, the facilitative Met transport pattern seems to change in the absence of sodium. There were no significant differences in $V_{\max}$ value between culture media that contained sodium and sodium-free culture media.

To characterize the specificity of competitive inhibitors of the radiolabeled L-Met transport system, BCH, MeAIB, and serine were used to inhibit the main transport systems L, A, and ASC, respectively. It appeared that most L-Met uptake in HCC cells was driven by systems L and ASC. Inhibition by BCH significantly reduced the uptake of Met by 50 % (1.5 μM BCH) and 80 % (15 μM BCH) ($p < 0.05$; Fig. 2c). MeAIB had no significant effect on the uptake of L-Met, which excluded system A-type transport from having a significant role on L-Met uptake in HCC cells. Serine also caused a significant reduction in L-Met uptake ($p < 0.05$).

The inhibitory effects of ouabain (0.1 and 1 mM) and of 2,4-dinitrophenol (0.1 and 1 mM) on L-amino acid transport in WCH17 cells were also evaluated (Fig. 2c). Both ouabain and 2,4-dinitrophenol exhibited a significant impact on L-amino acid transport (all $p < 0.05$). Moreover, a significant difference existed between the inhibitory effects of low and high concentrations of 2,4-dinitrophenol ($p < 0.05$). In addition, a reduction in Na^+/K^+ -dependent ATPase activity by ouabain also resulted in a concentration-dependent decrease in Met uptake and metabolism.

Cell Viability and Caspase-3/7 Activities in SAM Treated WCH17 Cells

Figure 3a shows the viability of WCH17 cells treated with SAM. The number of viable WCH17 cells significantly decreased after the 16-h treatment with 0.5 mM SAM ($p < 0.05$). Caspase-3/7 activities in WCH17 cells treated with SAM were significantly increased after the 24-h treatment (Fig. 3b), suggesting that more apoptotic WCH17 cells started to stimulate caspase-3/7 activities.

Cell Cycle Distribution of WCH17 Cells in 5-mM SAM-Treated WCH17 Cells

To determine the effect of SAM on apoptosis, WCH17 cells treated with 5 mM SAM at different time periods were examined by flow cytometry (Fig. 4). The apoptosis induced by SAM in cells is followed time dependent. The data show that the sub-G1 peak (apoptotic peak) after the 8-h treatment was significantly higher than that of control cells.

L-[methyl-³H]-Met uptake in 5-mM SAM-treated WCH17 cells

Downregulation of MAT by the 5-mM SAM treatment in WCH17 cells resulted in a reduced total uptake of L-[methyl-³H]-Met with time in WCH17 cells (Fig. 5). Two major metabolic pathways in HCC cells, both the water-soluble phase (PE methylation pathway via MAT) and protein phase, show reduced radioactivity over time (Fig. 5). The lipid-soluble phase produced from SAM was negligible. Meanwhile, longer time exposure of 5 mM SAM to WCH17 cells also reduced L-[methyl-³H]-Met uptake. The change in cell cycle distribution after the 8 and 16-h treatment of 5 mM SAM was correlated with the reduced uptake of L-[methyl-³H]-Met.

Expressed PEMT2 Converts ³H-SAM to ³H-lipids

The DNA sequence of the PEMT2 expression plasmid was verified by enzymatic digestion with Xho1/Xba1 followed by electrophoresis, which generated an ~600-bp insert (Fig. 6a). The expression of PEMT2 in WCH17 cells was validated via incubating the cells with the substrate of PEMT2 and ³H-SAM (Fig. 6b). The rat hepatocytes showed a strong endogenous PEMT activity, significantly converting ³H-SAM to ³H-lipids ($p<0.05$; Fig. 6b). WCH17 cells transfected by vehicles without the mPEMT2 cDNA insert (control) had negligible PEMT activity. The WCH17 cells transfected with the mPEMT2 expression vector showed a significantly higher PEMT activity compared with the control ($p<0.05$; Fig. 6b).

Effect of PEMT2 on the L-[methyl-³H]-Met Metabolism

The WCH17 cells transfected with the mPEMT2 expression vector produced a significantly higher amount of ³H-lipids (PC, PMME, PDME) than the cells transfected with the empty vectors ($p<0.05$; Fig. 7). In addition, a significantly higher amount of ³H-lipids in the WCH17 cells transfected with the mPEMT2 expression vector was generated after following treatment with cycloheximide (blocking protein synthesis) as compared with cells that were not treated with cycloheximide ($p<0.05$).

Discussion

Amino acid transport is generally enhanced in malignant transformation [16–18]. Translocation across the hepatocyte plasma membrane could be the rate-limiting step in the metabolism of L-[methyl-¹⁴C]-Met. Two types of Na⁺-independent systems for neutral amino acid transport exist in hepatocytes [19]. System L1 is a high affinity, low capacity agency with estimated K_m values of less than 200 μ M, whereas system L2 is a low affinity, high capacity system with estimated K_m values between 2 and 5 mM [19]. In this study, the K_m value of the L-amino acid transporter for L-Met uptake in WCH17 cells fell between those for system L1 and system L2, in addition to being Na⁺-dependent. Moreover, the Met transport processes in WCH17 cells can be inhibited by two Na⁺/K⁺-dependent ATPase-related inhibitors, ouabain, and 2,4-dinitrophenol. The combined evidence suggested that Met transport in WCH17 cells might be partially energy dependent. And, in HCC cells, uptake of L-Met was found to be mediated mainly by systems L and ASC.

MAT and PEMT are two key enzymes responsible for the PE methylation pathway in HCC cells (Fig. 1). After transportation into the cells, Met can either be incorporated in protein synthesis or be converted to SAM with the catalysis of MAT. SAM can further enter PE methylation pathway, with the catalysis of PEMT. SAM reacts with PE to form S-adenosylhomocysteine (SAH), PMME, and PDME; SAH can decompose back to Met, while PMME and PDME can immediately convert to PC. Abnormal MAT and PEMT expression in HCC cells may partly suggest a different mechanism of L-[methyl-³H]-Met uptake in HCC cells compared with normal hepatocytes.

Mammals express three MAT isoenzymes (MATI, MATII, and MATIII), which are the products of MAT1A and MAT2A genes [7]. In human HCC, MAT1A expression is silenced, whereas MAT2A expression is induced [20]. This switch in MAT gene expression provides a growth advantage to the liver cancer cells [8]. Overexpression of MAT2A may be implicated as a predictive marker for early detection and/or treatment assessment of HCC [7]. SAM and its metabolite MTA can be used to inhibit growth of hepatoma cells via lowering the expression of MAT2A [7–9]. In this study, we found that the downregulation of MAT2A expression through SAM treatment led to a corresponding reduction of L-[methyl-³H]-Met uptake in HCC cells. It suggests that MAT2A is involved in the L-[methyl-³H]-Met uptake in HCC cells and the upregulation of MAT2A expression in HCC cells may partially lead to an increase of L-[methyl-³H]-Met uptake. In addition, these findings suggest MAT as a potential therapeutic target for pharmacologic or gene therapeutic interventions. Further study might be needed to evaluate the usefulness of L-[methyl-³H]-Met as a pharmacokinetic marker to predict the HCC response with MAT-targeted treatment.

PEMT is another key enzyme in the PE methylation pathway. An isoform, PEMT2 has a specific role in regulating hepatoma cell division [21]. Vance DE et al. revealed that expression of PEMT2 caused a slower cell division of cultured rat hepatoma cells [21], and PEMT2 is believed to be a liver-specific tumor suppressor [22]. In this study, we used an mPEMT2 to transfect the HCC cells. We found that the expression of PEMT2 causes an increased incorporation of L-[methyl-³H]-Met into PC compared with untreated HCC cells. This indicates that the rate of PC incorporation from L-[methyl-³H]-Met is low in HCC cells because of a lack of PEMT2 activity. In contrast, the predominant metabolism of L-[methyl-³H]-Met in normal hepatocytes exclusively pointed to PC synthesis in our previous study [2]. Therefore, the different PEMT2 activities may be partly attributed to the different metabolic pathways of L-[methyl-³H]-Met between HCC cells and normal hepatocytes.

The major pathway for the biosynthesis of PC in mammals is via the CDP-choline pathway. An alternative pathway that is largely liver specific is the PE methylation pathway, i.e., the conversion of PE to PC catalyzed by PEMT. PC derived from the CDPcholine pathway has an unknown function in DNA replication and cell division [11, 21]. Alternatively, the PC species made via PEMT2 might in some unknown fashion inhibit hepatocyte cell division [11, 21]. From this metabolic context, the switch from PC synthesis to protein synthesis pathway derived from L-[methyl-³H]-Met in WCH17 cells may represent a metabolic adaptation for the carcinogenesis of HCC. This would also provide a surrogate endpoint derived specifically from the L-[methyl-³H]-Met pathway in HCC that could be employed in

PET imaging to detect early, physiologic changes in the HCC tumor and enable switching from ineffective therapies in real time.

An understanding of the properties of HCC cells with L-[methyl- ^{11}C]-Met PET imaging, including specific gene expression and metabolic status, is necessary to develop novel early detection methods and/or assessment methods for treatment efficacy via targeting of the specific genes in the L-[methyl- ^{11}C]-Met pathway such as MAT2A and PEMT2. In this study, we found that the uptake of L-[methyl- ^{11}C]-Met partially reflects MAT2A expression in HCC cells and that predominant metabolism of L-[methyl- ^{11}C]-Met is partially related to the activity of PEMT2 in HCC cells. However, these findings are still limited to in vitro conditions. The intratumoral heterogeneity was not considered in this study. To further understand the PET images of HCC using L-[methyl- ^{11}C]-Met, detailed information, such as the effect of microenvironment on the metabolic fate of L-[methyl- ^{11}C]-Met in vivo and on the levels of MAT2A and PEMT2 in intact tumors, will be obtained in the future study.

Conclusion

Met was transported into HCC cells via a facilitative transport process, which was characterized by systems L and ASC-like pattern, Na^+ dependence, low affinity, and partial energy dependence. The facilitative transport process may contribute to rapid uptake of radiolabeled Met in regions of HCC. Decreased expression of the MAT2A gene resulted in reduction of L-[methyl- ^3H]-Met uptake by the HCC cells. The transfection of WCH17 cells with a mouse PEMT2 expression vector was associated with enhanced PC synthesis incorporated from L-[methyl- ^3H]-Met.

Acknowledgments

We would like to thank Mr. Corey Levitan for helping with editing the paper. We recognize Dr. Shelly C. Lu (Research Center for Liver Diseases, University of Southern California, Los Angeles, CA) for her advice regarding the MAT inhibition experiment. We would also like to thank Dr. Dennis E Vance at the University of Alberta for the generous gift of mouse liver PEMT2 express plasmid. In addition, we extend a special thank you to Joe Molter, Nathan P. Tenley (Case Western Reserve University Center for Global Health and Disease, Cleveland, OH), and Airat A. Agbetoba (the University of Texas Health Science Center at Houston) for their help with some of the aforementioned experiments. We also thank Ms. Yanling Miao and Dr. Lili Liu (Case Western Reserve University Center for Global Health and Disease, Cleveland, OH) for helping with the flow cytometry experiment. This work was supported by an NIH/NCI R01 grant CA095307.

References

1. Plathow C, Weber WA. Tumor cell metabolism imaging. *J Nucl Med.* 2008; 49(Suppl 2):43S–63S. [PubMed: 18523065]
2. Kuang Y, Wang F, Corn DJ, et al. Metabolism of radiolabeled methionine in hepatocellular carcinoma. *Mol Imaging Biol.* 2014; 16:44–52. [PubMed: 23921714]
3. Langen KJ, Muhlensiepen H, Holschbach M, et al. Transport mechanisms of 3-[^{123}I]iodo-alpha-methyl-L-tyrosine in a human glioma cell line: comparison with [^3H]methyl-L-methionine. *J Nucl Med.* 2000; 41:1250–1255. [PubMed: 10914918]
4. Kuang Y, Salem N, Corn DJ, et al. Transport and metabolism of radiolabeled choline in hepatocellular carcinoma. *Mol Pharm.* 2010; 7:2077–2092. [PubMed: 20698576]
5. Bass R, Hedegaard HB, Dillehay L, et al. The A, ASC, and L systems for the transport of amino acids in Chinese hamster ovary cells (CHO-K1). *J Biol Chem.* 1981; 256:10259–10266. [PubMed: 7287709]

6. Hara T, Bansal A, DeGrado TR. Choline transporter as a novel target for molecular imaging of cancer. *Mol Imaging*. 2006; 5:498–509. [PubMed: 17150162]
7. Lu SC, Ramani K, Ou X, et al. S-adenosylmethionine in the chemoprevention and treatment of hepatocellular carcinoma in a rat model. *Hepatology*. 2009; 50:462–471. [PubMed: 19444874]
8. Yang H, Sadda MR, Li M, et al. S-adenosylmethionine and its metabolite induce apoptosis in HepG2 cells: role of protein phosphatase 1 and Bcl-x(S). *Hepatology*. 2004; 40:221–231. [PubMed: 15239106]
9. Chen H, Xia M, Lin M, et al. Role of methionine adenosyltransferase 2A and S-adenosylmethionine in mitogen-induced growth of human colon cancer cells. *Gastroenterology*. 2007; 133:207–218. [PubMed: 17631143]
10. Ishiwata K, Vaalburg W, Elsinga PH, et al. Comparison of L-[1-¹¹C]methionine and L-methyl-[¹¹C]methionine for measuring in vivo protein synthesis rates with PET. *J Nucl Med*. 1988; 29:1419–1427. [PubMed: 3261334]
11. Cui Z, Houweling M, Vance DE. Expression of phosphatidyl-ethanolamine N-methyltransferase-2 in McArdle-RH7777 hepatoma cells inhibits the CDP-choline pathway for phosphatidylcholine biosynthesis via decreased gene expression of CTP: phosphocholine cytidyltransferase. *Biochem J*. 1995; 312(Pt 3):939–945. [PubMed: 8554542]
12. Maurer U, Charvet C, Wagman AS, et al. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell*. 2006; 21:749–760. [PubMed: 16543145]
13. Ashwell MA, Work TS. Contrasting effects of cycloheximide on mitochondrial protein synthesis “in vivo” and “in vitro”. *Biochem Biophys Res Commun*. 1968; 32:1006–1012. [PubMed: 5701187]
14. Ishiwata K, Enomoto K, Sasaki T, et al. A feasibility study on L-[1-carbon-11]tyrosine and L-[methyl-carbon-11]methionine to assess liver protein synthesis by PET. *J Nucl Med*. 1996; 37:279–285. [PubMed: 8667062]
15. Fu Y, Fang Z, Liang Y, et al. Overexpression of integrin beta1 inhibits proliferation of hepatocellular carcinoma cell SMMC-7721 through preventing Skp2-dependent degradation of p27 via PI3K pathway. *J Cell Biochem*. 2007; 102:704–718. [PubMed: 17407140]
16. Isselbacher KJ. Sugar and amino acid transport by cells in culture—differences between normal and malignant cells. *N Engl J Med*. 1972; 286:929–933. [PubMed: 4335317]
17. Jager PL, Vaalburg W, Pruim J, et al. Radiolabeled amino acids: basic aspects and clinical applications in oncology. *J Nucl Med*. 2001; 42:432–445. [PubMed: 11337520]
18. Busch H, Davis JR, Honig GR, et al. The uptake of a variety of amino acids into nuclear proteins of tumors and other tissues. *Cancer Res*. 1959; 19:1030–1039. [PubMed: 13806375]
19. Kilberg MS. Amino acid transport in isolated rat hepatocytes. *J Membr Biol*. 1982; 69:1–12. [PubMed: 6811749]
20. Li J, Ramani K, Sun Z, et al. Forced expression of methionine adenosyltransferase 1A in human hepatoma cells suppresses in vivo tumorigenicity in mice. *Am J Pathol*. 2010; 176:2456–2466. [PubMed: 20363925]
21. Vance DE, Houweling M, Lee M, Cui Z. Phosphatidylethanolamine methylation and hepatoma cell growth. *Anticancer Res*. 1996; 16:1413–1416. [PubMed: 8694509]
22. Vance DE. Phosphatidylethanolamine N-methyltransferase: unexpected findings from curiosity-driven research. *Eur J Med Res*. 1996; 1:182–188. [PubMed: 9386267]

Fig. 1.

Metabolism of L-[methyl-¹¹C]methionine. Met methionine, SAM S-adenosylmethionine, SAH S-adenosylhomocysteine, PMME phosphatidylmonomethylethanolamine, PDME phosphatidylmethylethanolamine, PE phosphatidylethanolamine, PC phosphatidylcholine, MAT methionine adenosyltransferase, PEMT phosphatidylethanolamine N-methyltransferase, LAT L-amino acid transporter. In the water-soluble phase, the major radiolabeled metabolites include MET and SAM. In the lipid-soluble phase, the major radiolabeled metabolites include PMME, PDME, and PC.

Fig. 2.

Characterization of the L-amino acid transporter in WCH17 cells. **a** Standard culture medium containing 141 mM NaCl. **b** Medium in which sodium was replaced with 141 mM choline chloride. Lines show the fit of the experimental data to the Michaelis-Menten equation (Eq. (1)). **c** Inhibition of L-amino acid transporter by different inhibitors: inhibitors for amino acid transporter system L (BCH), for system A (MeAIB), and for system ASC (L-serine); inhibitor for Na/K-dependent adenosine triphosphatase (ouabain), and inhibitor for cellular adenosine triphosphate (2,4-dinitrophenol). %Uptake equals to the total uptake in that particular inhibition condition divided by the total uptake in the control condition (noninhibition). * $p < 0.05$ versus control. Solid lines indicate significant differences between the two groups ($p < 0.05$).

Fig. 3. Cell viability and Caspase-3/7 activities in 5-mM SAM-treated WCH17 cells. **a** Cell viability assay, WCH17 cells (control) versus treated WCH17 cells. **b** Comparison of caspase-3/7 activity between WCH17 cells (control) and treated WCH17 cells.

Fig. 4. Cell cycle distribution of WCH17 cells and WCH17 cells treated with 5 mM SAM different time period. Similar results were obtained from three independent experiments for each time period. *, **, ♣, ¥ versus control $p < 0.05$.

Fig. 5.

The uptake of L-[methyl-³H]-Met in the WCH17 HCC cells treated with 5 mM SAM for 6 and 18 h, respectively, during pulse and chase period. Chase 0 min means only pulse 5 min no chase. The fraction of water-soluble phase and protein phase was extracted from the total uptake. The radioactivity in the fraction of lipid-soluble phase was negligible. *p<0.05.

Fig. 6. WCH17 cells transfected with mPEMT2 expression vector. a DNA sequence was verified by enzymatic digestion with Xho1/Xba1 followed by electrophoresis, which generated an ~600-bp insert. b The lipid-soluble phase derived from ^3H -SAM uptake in rat hepatocytes, WCH17 cells transfected with vehicles (control), and WCH17 cells transfected with mPEMT2 expression vector. * $p < 0.05$. Chase 0 min means only pulse 5 min no chase.

Fig. 7. The radioactivity distribution in lipid-soluble, water-soluble, and protein phases incorporated from L-[methyl-³H]-Met in WCH17 cells transfected with mPEMT2 expression vector. **a** Without the protein synthesis inhibitor cycloheximide. **b** With protein synthesis inhibitor cycloheximide. Chase 0 min means only pulse 5 min no chase. *p<0.05.