Published in final edited form as:

Nucl Med Biol. 2012 July ; 39(5): 697–701. doi:10.1016/j.nucmedbio.2011.12.003.

A fast chemo-enzymatic synthesis of [^{11}C]-N⁵,N¹⁰-methylenetetrahydrofolate as a potential PET tracer for proliferating cells*

Muhammad Saeed^a, Timothy J. Tewson^b, Colbin E. Erdahl^b, and Amnon Kohen^{a,*}

^aDepartment of Chemistry, University of Iowa, IA 52242 USA

^bDepartment of Radiology, University of Iowa, IA 52242 USA

Abstract

Introduction—Thymidylate synthase (TSase) and folate receptors (FRs) are well developed targets of cancer therapy. Discovery of a simple and fast method for the conversion of $^{11}\text{CH}_3\text{I}$ to [^{11}C]-formaldehyde ($^{11}\text{CH}_2\text{O}$) encouraged us to label the co-factor of this enzyme. Preliminary studies conducted on cell lines have demonstrated a preferential uptake of [^{11}C]-N⁵,N¹⁰-methylene-5,6,7,8-tetrahydrofolate ($^{11}\text{CH}_2\text{H}_4\text{folate}$) by cancerous cell vs. normal cells from the same organ [1] pointing out $^{11}\text{CH}_2\text{H}_4\text{folate}$ as a PET tracer for cancer imaging. Herein we report the synthesis of $^{11}\text{CH}_2\text{H}_4\text{folate}$, which may serve as a potential positron emission tomography (PET) tracer.

Methods—In a remotely controlled module, $^{11}\text{CH}_3\text{I}$ was bubbled into a reaction vial containing trimethylamine N-oxide (TMAO) in DMF and heated to 70 °C for 2 min. $^{11}\text{CH}_2\text{O}$ formed after the completion of reaction was then mixed with a solution of freshly prepared tetrahydrofolate (H_4folate) by using a fast chemo-enzymatic approach to accomplish synthesis of $^{11}\text{CH}_2\text{H}_4\text{folate}$. Purification of the product was carried out by loading the crude reaction mixture on a SAX cartridge, washing with water to remove unbound impurities and finally eluting with a saline solution.

Results—The synthesis and purification of $^{11}\text{CH}_2\text{H}_4\text{folate}$ was completed within 5 min. HPLC analysis of the product after SAX purification indicate more than 90% of the radioactivity that was retained on the SAX cartridge was in $^{11}\text{CH}_2\text{H}_4\text{folate}$ with minor (<10%) radioactivity due to unreacted $^{11}\text{CH}_2\text{O}$.

Conclusion—We present a fast (~5 min) synthesis and purification of $^{11}\text{CH}_2\text{H}_4\text{folate}$, as a potential PET tracer. The final product is received in physiological compatible buffer (100 mM sodium phosphate, pH 7.0 containing 500 mM NaCl) and ready for use *in vivo*.

Keywords

Cancer; PET imaging; radiotracer; folate receptors; thymidylate synthase; carbon

*This work was supported by a pilot grant from the Institute of Clinical and Translational Science, and a Biological Science Funding Program grant, University of Iowa.

Corresponding author: A. Kohen, Department of Chemistry, University of Iowa, IA 52242 amnon-kohen@uiowa.edu, Phone: (319)-335-0234.

1. Introduction

Positron emission tomography (PET) is a non-invasive imaging technology developed to use tracers labeled with short-lived positron emitting radioisotopes as molecular probes to image and measure biochemical processes *in vivo*. In spite of the fact that ^{18}F -labeled metabolic substrates gained much attention due to their favorable half-lives relative to ^{11}C (120 min and 20.4 min, respectively), they are never natural substrates, i.e., these are chemically modified and commonly have an inhibitory role along the metabolic pathway. Metabolites labeled with ^{11}C , however, do not cause a perturbation of the metabolic pathway under study. The incorporation of ^{11}C into small molecules has been important to the success of PET for *in vivo* molecular imaging and drug research and development [2]. The short half-life ($t_{1/2} = 20.38$ min), allows for repeated studies within a short time span, but requires rapid chemical syntheses and purifications [3].

Folate receptors (FRs), especially folate receptor- α (FR- α) and thymidylate synthase (TSase, E.C. 2.1.1.45) have been exploited as targets for cancer therapy and imaging. The FR, a tumor associated glycosylphosphatidylinositol anchored protein, can actively internalize bound folates via receptor-mediated endocytosis [4–6]. TSase, on the other hand, catalyzes the only *de novo* biosynthesis of the DNA building block T, i.e., 2'-deoxythymidine-5'-monophosphate (dTMP) in human [7–9]. It is found to be over-expressed in several human cancers [10–14]. Consequently, TSase has been a target for fluoropyrimidine-based drugs such as 5-fluorouracil. Furthermore, intrinsic tumor TSase values are also thought to be of importance in predicting the response to chemotherapy and survival in patients. Patients with low tumor TSase expression have a significantly better outcome than those with high TSase expression [15].

TSase catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to dTMP. The cofactor (*R*)- $\text{N}^5, \text{N}^{10}$ -methylene-5,6,7,8-tetrahydrofolate ($\text{CH}_2\text{H}_4\text{folate}$) first donates a methylene group to form (*S*)-5,6,7,8-tetrahydrofolate (H_4folate), then a hydride to produce dTMP and 7,8-dihydrofolate (H_2folate) [7, 8, 16]. Its mechanism and multiple steps chemical conversions have been carefully examined, in part by the use of $^{14}\text{CH}_2\text{H}_4\text{folate}$ [8, 17, 18]. We envisioned that by radiolabeling the methylene moiety of $\text{CH}_2\text{H}_4\text{folate}$ with ^{11}C -radionuclide, this radioactive methylene can be transferred by the TSase catalyzed reaction to the DNA building block dTMP as shown in Fig. 1. Preliminary *in vitro* studies compared the up-take of $^{14}\text{CH}_2\text{H}_4\text{folate}$ by various human cancer cell lines vs. their normal counterparts. The findings indicated that indeed the cancerous cells were significantly labeled over the normal ones. Furthermore, the fast proliferating cells also over expressed FR and TSase enzyme, which reflected the higher uptake of the radiotracer [1].

In this communication, we describe the synthesis of $^{11}\text{CH}_2\text{H}_4\text{folate}$ by combining two fast and high yielding methods, i.e., a simple and fast method for the synthesis of [^{11}C]-formaldehyde ($^{11}\text{CH}_2\text{O}$), reported by Hooker et al [3], with an equally efficient chemo-enzymatic method for the preparation of $\text{CH}_2\text{H}_4\text{folate}$ from folic acid as reported earlier by our laboratory [16, 19].

2. Material and Methods

2.1. Reagents and instrumentation

All chemicals, unless otherwise noted, were acquired from Sigma-Aldrich (St. Louis, MO, USA) and were used as received without further purification. H₄folate was prepared by following the procedure reported previously from our laboratory [16, 19]. All water employed was ultra-pure (>18.2 MO cm at 25 °C, Milli-Q, Millipore, Billerica, MA, USA), and was purged with argon gas to remove dissolved oxygen. All instruments were calibrated. Activity measurements were made using a Biodex Atom 300 Dose Calibrator (Biodex Medical Systems, Shirley, NY, USA). HPLC was performed using a C-18 reversed phase column and elution was followed by UV detector and a flow through radioactivity detector. Column was initially eluted isocratically for one min at 1% methanol in 50 mM sodium phosphate buffer, pH 7.8 and then linearly changed to 20% methanol in 50 mM sodium phosphate buffer, pH 7.8 in 15 min at a flow rate of 1 mL/min.

2.2. Kinetics of the formation of CH₂H₄folate

In order to optimize the conditions for synthesis of ¹¹CH₂H₄folate, we determined the kinetics of the formation of CH₂H₄folate by using relatively stable ¹⁴C-nuclide. Thus, ¹⁴CH₃I (5 µl, 0.5 µCi, 10 nmol) was dissolved in DMF (300 µl) in an air tight vial. After adding 4 mg of trimethylamineN-oxide (TMAO), the reaction contents were heated at 70 °C for a time point (5, 30, 60, 90, and 120 sec) and then mixed with a solution of freshly prepared H₄folate. The product was purified on a SAX cartridge as described below and analyzed by HPLC. The yield (percentage conversion from ¹⁴CH₃I) of ¹⁴CH₂H₄folate (Fig. 2) formed at the specific time point was quantified from a calibration curve developed by running five serial dilutions (1000 µg/ml to 1µg/ml) of standard ¹⁴CH₂H₄folate.

2.2. Radiosynthesis

The synthesis of ¹¹CH₂H₄folate was carried out on the remotely controlled module that is schematically presented in Fig. 3. Following preparation of ¹¹CO₂ (~ 1 Ci at 10 Ci/µmol) at the cyclotron target, ¹¹CH₃I was produced using PETtrace MeI Microlab (GE Medical Systems, Milwaukee, WI, USA). The ¹¹CH₃I was pushed from the ¹¹CH₃I synthesis module using helium carrier gas and was bubbled directly into the reaction vial containing a suspension of TMAO (4 mg) in DMF (300 µl) at -20°C. Exhaust was vented to an exhaust bag to collect any escaped radioactivity. After the collection of ¹¹CH₃I was completed, the temperature of the reaction vial was raised to 70°C within 30 sec, and kept there for 2 min to allow complete conversion of ¹¹CH₃I to ¹¹CH₂O. At this stage, the temperature was lowered to 25°C, and H₂O was introduced into the reaction vial to produce an aqueous solution of ¹¹CH₂O, which was then transported to the intermediate vial containing H₄folate. H₄folate was prepared from folic acid by reducing it first with dithionite to dihydrofolate (H₂folate) [20] and then converting H₂folate enzymatically to H₄folate by dihydrofolate reductase (DHFR) [16, 19]. After letting the mixture stand for a minute, the mixture was loaded onto a SAX cartridge, preconditioned with methanol (2 mL) and de-ionized water (5 mL), and allowed to pass through under a mild pressure (5–10 psi). The pass-through was collected in a waste vial. The cartridge was then washed with H₂O (2 mL). H₂O was pushed (using a syringe) through the SAX cartridge. The washings were also collected in the waste

vial, and the contents of the waste vial were analyzed, at the end of washing, by placing it in a dose calibrator to count any radioactivity. The $^{11}\text{CH}_2\text{H}_4\text{folate}$ was eluted from the cartridge, with an elution buffer (100 mM sodium phosphate, pH 7.4, containing 500 mM NaCl and 1% ascorbic acid), which was pushed through the cartridge using a syringe, and the product was collected in the product vial.

3. Results and Discussion

3.1. Optimization of the Reaction

To assess the kinetics of the conversion of $^{11}\text{CH}_3\text{I}$ to $^{11}\text{CH}_2\text{H}_4\text{folate}$, we performed synthesis of $^{14}\text{CH}_2\text{H}_4\text{folate}$ by using commercially available $^{14}\text{CH}_3\text{I}$ as starting material. The reaction mixture containing $^{14}\text{CH}_3\text{I}$ and TMAO were heated at 70°C for 5, 30, 60, 90 and 120 sec, mixed with a solution of H_4folate , and then analyzed immediately on HPLC equipped with a radioactivity flow detector, after purification on a SAX cartridge. As shown in Fig. 2, about 70% of the reaction was completed within 60 sec. In the next min, the yield of the product $^{14}\text{CH}_2\text{H}_4\text{folate}$ was observed up to 80%, and there was no substantial increase in the yield after that, which is in accordance with ref [3]. Therefore, we chose 2 min reaction time for the conversion of $^{11}\text{CH}_3\text{I}$ to $^{11}\text{CH}_2\text{O}$ when synthesizing of $^{11}\text{CH}_2\text{H}_4\text{folate}$.

3.2. Synthesis of $^{11}\text{CH}_2\text{H}_4\text{folate}$ on a remotely controlled module

The synthesis of $^{11}\text{CH}_2\text{H}_4\text{folate}$ begins with a nuclear reaction [$^{14}\text{N}(\text{p},\alpha)^{11}\text{C}$] using 17 MeV protons and a target gas that contains traces of oxygen to produce $^{11}\text{CO}_2$. $^{11}\text{CO}_2$ was reduced to $^{11}\text{CH}_4$ with hydrogen over a nickel catalyst and then converted to $^{11}\text{CH}_3\text{I}$ by reaction with iodine at $\sim 700^\circ\text{C}$ using PETtrace MeI Microlab (GE Medical Systems, Milwaukee, WI, USA).

In order to remotely control the synthesis and purification of $^{11}\text{CH}_2\text{H}_4\text{folate}$ (specific radioactivity of 10–30 Ci/mmol), we developed a synthesis module (Fig. 3). The synthetic and operational procedure is described in detail at item 2.2 above. In short, the module consisted of a reaction vial to synthesize $^{11}\text{CH}_2\text{O}$ and an intermediate vial for mixing H_4folate with the $^{11}\text{CH}_2\text{O}$. Both vials were secured with airtight caps. The reaction vial was placed snugly on a rapid heating-cooling thermoelectric micro-plate. For reagents and carrier gas, the reaction vial cap was supplied with airtight tubing ports, one each for incoming $^{11}\text{CH}_3\text{I}$ and H_2O supply, and one for the carrier nitrogen gas. The module was placed inside a lead-lined hot cell with the temperature controller outside. Thus, oxidation of $^{11}\text{CH}_3\text{I}$ with TMAO to $^{11}\text{CH}_2\text{O}$ was conducted by the fast and high-yield method developed by Hooker and co-workers[3]. As illustrated in Fig. 4, we coupled this reaction with a fast (< 1 min) chemo-enzymatic synthesis of $\text{CH}_2\text{H}_4\text{folate}$ from H_2folate reported earlier from our laboratory [16, 19].

The product collected was then analyzed by HPLC equipped with both UV absorbance and radioactivity detectors (Fig. 5). The results indicated that most of the impurities (DMF, TMAO etc) were removed during washing step and radioactive loss from the cartridge, probably as unreacted $^{11}\text{CH}_2\text{O}$, accounts for <10% of the original radioactivity trapped in the reaction vial. The product, $^{11}\text{CH}_2\text{H}_4\text{folate}$ was cleanly eluted with 100 mM sodium phosphate, pH 7.4 containing 500 mM NaCl and 1% ascorbic acid and HPLC radiogram,

which indicated that this product accounts for >95% of the radioactivity with trace amounts of $^{11}\text{CH}_2\text{O}$ (Fig. 5). The UV trace indicated that H_4folate (major peak, ~12 nmol/injection; 3 μmol total, 0.6 mM) and $\text{CH}_2\text{H}_4\text{folate}$ (minor peak, ~1.1 nmol/injection; 0.3 μmol total, 0.06 mM) were the only two products. Since H_4folate is highly unstable in aqueous solutions and undergoes oxidation/decomposition to the products that do not compete with $\text{CH}_2\text{H}_4\text{folate}$ (e.g., para- aminobenzoic glutamate, and biopterine), its presence in the formulation is not likely to compete for the folate receptors for uptake. Moreover, it is a non-toxic natural derivative of folic acid ($\text{LD}_{50} = 10 \text{ g/Kg}$; mouse), and due to the short life of ^{11}C , we chose not to purify this mixture further and use it as is in future *in vivo* experiments. Additionally, the elution buffer (100 mM sodium phosphate, 500 mM NaCl, pH 7.4) is a physiologically appropriate for *in vivo* studies.

4. Conclusions

An expeditious preparation of $^{11}\text{CH}_2\text{H}_4\text{folate}$, ready in physiological solution, was developed by combining two fast and high yield methods i.e., a fast conversion of cyclotron generated $^{11}\text{CO}_2$ to $^{11}\text{CH}_3\text{I}$, and its conversion to $^{11}\text{CH}_2\text{O}$ [3], which is then reacted with chemo-enzymatically prepared H_4folate [16, 19]. The feasibility of its use in PET imaging of cancer was assessed using a longer lived isotope (^{14}C) in cell cultures as described elsewhere [1]. Those experiments also revealed a contribution of over-expressed FR- α and TSase to the time dependent uptake and retention of radioactivity. This “double phenotype selection” of the radiotracer by two phenotypic gene expression typical to numerous cancerous cells suggests that $^{11}\text{CH}_2\text{H}_4\text{folate}$ may be exploited in PET technology as a valuable addition in PET probe armamentarium. It is also interesting to indicate that Hooker et al [3] suggested that formaldehyde could be used for Mannich-type condensations, which is indeed the critical step in the methylene transfer catalyzed by TSase *in vivo*. Finally, with very minor modification in the current procedure, the oxidized or reduced derivatives of $^{11}\text{CH}_2\text{H}_4\text{folate}$ can be prepared. These include [^{11}C]-leucovorin (folinic acid) and 5- [^{11}C]-methyl- H_4folate ($^{11}\text{CH}_3\text{H}_4\text{folate}$), which are both natural metabolites that play a role in cell biology and cancer therapy. Thus, the current preparation of $^{11}\text{CH}_2\text{H}_4\text{folate}$ may open the door for the synthesis of a new class of ^{11}C -labeled one-carbon carriers as metabolites for different PET applications.

References

1. Saeed M, Sheff D, Kohen A. Novel positron emission tomography tracer distinguishes normal from cancerous cells. *J Biol Chem.* 2011; 286:33872–8. [PubMed: 21832075]
2. Phelps ME. Positron emission tomography provides molecular imaging of biological processes. *Proc Natl Acad Sci U S A.* 2000; 97:9226–33. [PubMed: 10922074]
3. Hooker JM, Schonberger M, Schieferstein H, Fowler JS. A simple, rapid method for the preparation of [^{11}C]formaldehyde. *Angew Chem Int Ed Engl.* 2008; 47:5989–92. [PubMed: 18604787]
4. Theti DS, Jackman AL. The role of alpha-folate receptor-mediated transport in the antitumor activity of antifolate drugs. *Clin Cancer Res.* 2004; 10:1080–9. [PubMed: 14871988]
5. Kamen BA, Smith AK. A review of folate receptor alpha cycling and 5-methyltetrahydrofolate accumulation with an emphasis on cell models in vitro. *Adv Drug Deliv Rev.* 2004; 56:1085–97. [PubMed: 15094208]
6. Sabharanjak S, Mayor S. Folate receptor endocytosis and trafficking. *Adv Drug Deliv Rev.* 2004; 56:1099–109. [PubMed: 15094209]

7. Carreras CW, Santi DV. The catalytic mechanism and structure of thymidylate synthase. *Annu Rev Biochem.* 1995; 64:721–62. [PubMed: 7574499]
8. Hong B, Maley F, Kohen A. Role of Y94 in proton and hydride transfers catalyzed by thymidylate synthase. *Biochemistry.* 2007; 46:14188–97. [PubMed: 17999469]
9. Finer-Moore JS, Santi DV, Stroud RM. Lessons and conclusions from dissecting the mechanism of a bisubstrate enzyme: thymidylate synthase mutagenesis, function, and structure. *Biochemistry.* 2003; 42:248–56. [PubMed: 12525151]
10. Yasumatsu R, Nakashima T, Uryu H, Ayada T, Wakasaki T, Kogo R, et al. Correlations between thymidylate synthase expression and chemosensitivity to 5-fluorouracil, cell proliferation and clinical outcome in head and neck squamous cell carcinoma. *Chemotherapy.* 2009; 55:36–41. [PubMed: 19023200]
11. Shimoda M, Sawada T, Kubota K. Thymidylate synthase and dihydropyrimidine dehydrogenase are upregulated in pancreatic and biliary tract cancers. *Pathobiology.* 2009; 76:193–8. [PubMed: 19571608]
12. Lindebjerg J, Nielsen JN, Hoeffding LD, Bisgaard C, Brandslund I, Jakobsen A. Expression of thymidylate synthase in primary colorectal adenocarcinoma. *Appl Immunohistochem Mol Morphol.* 2006; 14:37–41. [PubMed: 16540728]
13. Yu Z, Sun J, Zhen J, Zhang Q, Yang Q. Thymidylate synthase predicts for clinical outcome in invasive breast cancer. *Histol Histopathol.* 2005; 20:871–8. [PubMed: 15944938]
14. Kiss-Laszlo Z, Nagy B, Thurzo L, Szabo J. Thymidylate synthase gene promoter polymorphism in patients with advanced head and neck cancer treated by radio- and 5-fluorouracil chemotherapy. *Magy Onkol.* 2006; 50:33–7. [PubMed: 16617381]
15. Van Triest B, Peters GJ. Thymidylate synthase: a target for combination therapy and determinant of chemotherapeutic response in colorectal cancer. *Oncology.* 1999; 57:179–94. [PubMed: 10545786]
16. Agrawal N, Mihai C, Kohen A. Microscale synthesis of isotopically labeled R-[6-xH]N5,N10-methylene-5,6,7,8-tetrahydrofolate as a cofactor for thymidylate synthase. *Anal Biochem.* 2004; 328:44–50. [PubMed: 15081906]
17. Agrawal N, Hong B, Mihai C, Kohen A. Vibrationally enhanced hydrogen tunneling in the *Escherichia coli* thymidylate synthase catalyzed reaction. *Biochemistry.* 2004; 43:1998–2006. [PubMed: 14967040]
18. Wang Z, Kohen A. Thymidylate synthase catalyzed H-transfers: two chapters in one tale. *J Am Chem Soc.* 2010; 132:9820–5. [PubMed: 20575541]
19. Hong B, Kohen A. Microscale synthesis of isotopically labeled 6R-N-5, N-10 methylene-5,6,7,8-tetrahydrofolate. *Journal of Labelled Compounds & Radiopharmaceuticals.* 2005; 48:759–69.
20. Blakley RL. Crystalline dihydropteroylglutamic acid. *Nature.* 1960; 188:231–2.

Fig. 1.

The *de novo* synthesis of dTMP. The methylene (CH_2 – in red) is transferred from MTHF to the C5 carbon of the precursor dUMP. Here we describe the labelling of that methylene with ^{11}C for PET imaging.

Fig. 2.
The conversion of $^{14}\text{CH}_2\text{O}$ to ^{14}C -MTHF in DMF at 70°C .

Author Manuscript

Fig. 4.

Synthesis of ^{11}C -MTHF. $^{11}\text{CCH}_2\text{OH}$ is synthesized as described in ref [3], and THF is synthesized by dihydrofolate reductase (DHFR) catalyzed reduction of DHF (pre-prepared from folic acid) and the cofactor NADPH is recycled by isopropanol (iPrOH) reduction of NADP^+ catalyzed by alcohol dehydrogenase (tbADH).

Fig. 5.
HPLC chromatogram of ^{11}C -MTHF. The top panel is the UV chromatogram, and the bottom panel is the radiogram.