

¹¹C-MCG: Synthesis, Uptake Selectivity, and Primate PET of a Probe for Glutamate Carboxypeptidase II (NAALADase)

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Abstract

Imaging of glutamate carboxypeptidase II (GCP II), also known as *N*-acetylated α -linked L-amino dipeptidase (NAALADase), may enable study of glutamatergic transmission, prostate cancer, and tumor neovasculature in vivo. Our goal was to develop a probe for GCP II for use with positron emission tomography (PET). Radiosynthesis of ¹¹C-MeCys-C(O)-Glu or ¹¹C-(S)-2-[3-((R)-1-carboxy-2-methylsulfanyl-ethyl)-ureido]-pentanedioic acid (¹¹C-MCG), an asymmetric urea and potent ($K_i = 1.9$ nM) inhibitor of GCP II, was performed by C-11 methylation of the free thiol. Biodistribution of ¹¹C-MCG was assayed in mice, and quantitative PET was performed in a baboon. ¹¹C-MCG was obtained in 16% radiochemical yield at the end of synthesis with specific radioactivities over 167 GBq/mmol (4000 Ci/mmol) within 30 min after the end of bombardment. At 30 min postinjection, ¹¹C-MCG showed $33.0 \pm 5.1\%$, $0.4 \pm 0.1\%$, and $1.1 \pm 0.2\%$ ID/g in mouse kidney (target tissue), muscle, and blood, respectively. Little radioactivity gained access to the brain. Blockade with unlabeled MCG or 2-(phosphonomethyl)pentanedioic acid (PMPA), another potent inhibitor of GCP II, provided sevenfold and threefold reductions, respectively, in binding to target tissue. For PET, distribution volumes (DVs) were 1.38 then 0.87 pre- and postblocker (PMPA). Little metabolism of ¹¹C-MCG occurred in the mouse or baboon. These results suggest that ¹¹C-MCG may be useful for imaging GCP II in the periphery.

Mol Imaging (2002) **1**, 96–101.

Keywords: Glutamate carboxypeptidase, NAALADase, urea-based inhibitor, ¹¹C-MCG, PET.

Introduction

In the brain, the metalloprotease, glutamate carboxypeptidase II (GCP II; EC 3.4.17.21), cleaves *N*-acetyl-aspartyl-glutamate (NAAG) to *N*-acetyl-aspartate (NAA) and glutamate [1]. The roles of GCP II in the brain are to terminate the neurotransmitter activity of NAAG and to produce glutamate that is then free to act at its various receptor subtypes. In addition to those central functions, GCP II possesses 87% sequence homology with the prostate-specific membrane antigen (PSMA) [2]. Although some differences exist in substrate specificity and cellular localization, namely, GCP II has only a membrane bound form, whereas PSMA is found both in cell membranes and within cytosol, the two enzymes

are remarkably similar pharmacologically [3]. PSMA was initially detected by the prostate-specific monoclonal antibody (mAb) 7E11-C5 raised against the human prostate cancer cell line LNCaP [4]. PSMA is highly expressed in benign prostatic epithelium, prostatic intraepithelial neoplasia, and prostatic adenocarcinoma with evidence suggesting greatest expression in high-grade and hormone-insensitive cancers [5]. Furthermore, in a study employing five different anti-PSMA mAbs, PSMA was localized to the neovasculature of a wide variety of malignant neoplasms, including those outside of the genitourinary system [5].

Jackson designed the GCP II inhibitor 2-(phosphonomethyl)pentanedioic acid (PMPA) with a view to inhibit excess glutamate production (excitotoxicity), which has been suggested to underlie the neuropathology of stroke and possibly other hyperglutamatergic states [6]. Slusher et al. [7] showed that PMPA is a potent and selective inhibitor of GCP II, and that it is neuroprotective in a rodent model of ischemia. Because of the important functions of GCP II, we sought to produce a positron-emitting substrate of that enzyme in order to measure its activity in vivo in a variety of conditions using positron emission tomography (PET). Furthermore, because of the similarity between GCP II and PSMA, we anticipate that an effective imaging agent for GCP II may also enable imaging of PSMA. PMPA, however, is not readily amenable to synthesis in positron-emitting form, so we sought an alternative but similar substrate that may be a suitable precursor.

Kozikowski et al. [8] recently synthesized a series of inhibitors of GCP II that maintain a structural motif similar to that of the phosphinic bis-dicarboxylic acid

Abbreviations: GCP II, glutamate carboxypeptidase II; NAALADase, *N*-acetylated α -linked L-amino dipeptidase; ¹¹C-MCG, ¹¹C-MeCys-C(O)-Glu or ¹¹C-(S)-2-[3-((R)-1-carboxy-2-methylsulfanyl-ethyl)-ureido]-pentanedioic acid; PSMA, prostate-specific membrane antigen; PMPA, 2-(phosphonomethyl)pentanedioic acid.

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Received 10 March 2002; Accepted 18 March 2002.

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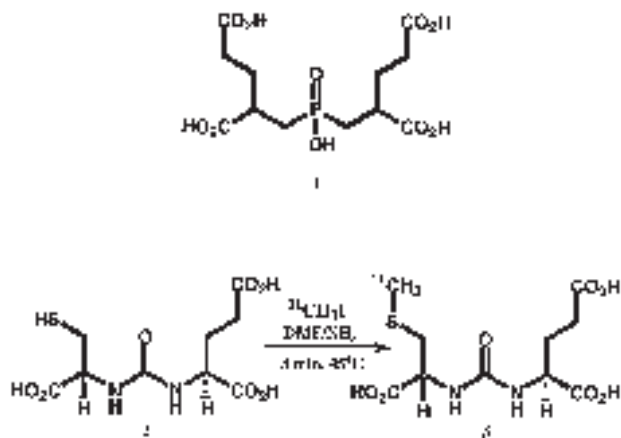


Figure 1. Phosphinic bis-dicarboxylic acid (**1**), after which the urea-based inhibitors were patterned, and synthesis of ¹¹C-MCG (**3**) from desmethyl precursor (**2**).

(**1**; Figure 1), a potent inhibitor of GCP II, but have the central CH₂P(O)(OH)CH₂ replaced with a urea group (Figure 1). One compound of that series, Cys–C(O)–Glu, is amenable to labeling with C-11. Herein, we describe the radiosynthesis, mouse biodistribution, and initial PET study performed in a baboon of ¹¹C–MeCys–C(O)–Glu or ¹¹C-(S)-2-[3-((R)-1-carboxy-2-methylsulfanyl-ethyl)-ureido]-pentanedioic acid (¹¹C-MCG) (*K_i* = 1.9 nM) (**3**; Figure 1), the C-11 methylated analog of Cys–C(O)–Glu. Because of the relatively low concentration of GCP II in rodent prostate, we used the kidney as a surrogate target organ for ¹¹C-MCG in that species, as well as in the baboon [1,3].

Materials and Methods

Chemistry

N,N-Dimethylformamide (DMF) was distilled under reduced pressure from BaO. High-performance liquid chromatography (HPLC) equipment consisted of model 7126 injectors (Rheodyne, Rohnert, CA), model 590 EF pumps (Waters, Milford, MA), a model 440 ultraviolet (UV) absorbance detector (214 nm) (Waters), and a 5.08-cm (2 in.) NaI(Tl) crystal scintillation detector (model 276; Ortec, Oak Ridge, TN). Model 3390A integrators (Hewlett-Packard, Andover, MA) and a Dynamax system (Rainin Instrument, Woburn, MA) were used to record and analyze HPLC chromatograms. Semipreparative (10 × 250 mm) and analytical (4.6 × 250 mm) reverse-phase HPLC columns (C-18 Luna, Phenomenex, Torrance, CA) were used for purification and quality control, respectively, of the radiotracer. The *S*-desmethyl precursor of ¹¹C-MCG [8] (1 mg) (**2**; Figure 1) was dissolved in 0.1 mL of DMF. To that was added 0.1 mL of a DMF/NH₃ solution (freshly prepared by bubbling

of anhydrous ammonia at approximately 50 mL/min in 10 mL of DMF for 5 min) followed by 0.05 mL of water. The precursor solution, contained in a 1-mL septum-sealed vial, was cooled in a 20°C bath and ¹¹C-iodomethane prepared from a MeI MicroLab module (GE, Milwaukee, WI) and GE PETtrace cyclotron was bubbled into the vial. The reaction vessel was subsequently heated in a 45°C bath for 60 sec before quenching the reaction with 0.6 mL of HPLC buffer (6/94/0.075 acetonitrile/water/trifluoroacetic acid) and 0.05 mL of 20% trifluoroacetic acid. The contents of the reaction vessel were injected onto a HPLC column using the above mobile phase at flow rate of 10 mL/min and UV detector at 214 nm. The radioproduct (*t_R* = 8.1 min) was well separated from thiol precursor (*t_R* = 2.5 min) and was remotely collected. Rotary evaporation of the solvent (80°C under vacuum) was followed by formulation of the radiotracer in 0.9% sterile saline (7 mL) and sterile filtration (Acrodisc 0.2 μm, 25 mm HT Tuffryn filter, PALL Gelman Laboratories, Ann Arbor, MI) into a 10-mL sterile, evacuated dose vial. For specific radioactivity determination, a 0.1-mL aliquot of ¹¹C-MCG (typically approximately 3 mCi) was assayed for radioactivity and injected onto an analytical HPLC column using a mobile phase of 10/90 acetonitrile/0.01 M phosphoric acid at 2 mL/min. After determination of the specific radioactivity of ¹¹C-MCG, 3 mL of 8.4% sterile, sodium bicarbonate was added to the radiotracer to bring the pH of the final formulation to approximately 7. (It was found that addition of the bicarbonate solution prior to removal of an aliquot for specific radioactivity determination resulted in an undesired shortening of the retention time of ¹¹C-MCG and a noisier UV baseline.)

Animal Studies

Rodent In Vivo Biodistribution Studies of ¹¹C-MCG. All animal studies were approved by the Animal Care and Use Committee of the Johns Hopkins University. Male CD-1 mice (Charles River, Wilmington, MA) weighing between 20 and 25 g were used and received an injection of 3.7 MBq (100 μCi) of ¹¹C-MCG through the tail vein. That amounted to, at a maximum, 0.27 μg/kg. For kinetic studies, mice were killed by cervical dislocation at 5, 15, 30, 60, and 120 min after injection of radiotracer in 200 μL of saline vehicle. The brains were removed and placed on ice, and the cerebellum, olfactory bulb, hypothalamus, hippocampus, striatum, parietal cortex, brainstem, and thalamus were harvested. Kidneys, blood, fat, muscle, small intestine, and prostate were also harvested. The tissue samples were weighed, and their radioactivity content was determined in an

automated γ counter (1282 Compugamma CS; Pharmacia/LKB Nuclear, Gaithersburg, MD). Aliquots of the injected tracer were counted along with the samples and served as standards for the calculation of percentage injected dose per gram of tissue (% ID/g). To assess binding specificity, groups of three mice each were pretreated with the high-affinity GCP II inhibitor PMPA [6] at doses of 1, 10, and 100 mg/kg in 200 μ L of saline vehicle 5 min prior to ¹¹C-MCG injection. In an additional binding specificity study, animals were pretreated similarly with unlabeled MCG standard at doses of 5, 50, 100, 500, and 1000 μ g/kg before ¹¹C-MCG injection.

Metabolism Studies of ¹¹C-MCG. At different times after injection of ¹¹C-MCG into mice, blood and kidneys were collected to determine the rate of metabolism of the radiotracer. Heparinized blood (0.2–0.3 mL) was diluted to 0.9 mL with cold 0.9% saline and acidified to 0.5 N by the rapid addition of 0.1 mL of 5 N perchloric acid. Following 5 min on ice, the precipitate was removed by centrifugation to yield an acid-soluble supernatant that was analyzed by HPLC. Similarly, an acid extract of mouse kidney was obtained from an initial homogenate of two kidneys in 0.8 mL of cold water.

The acid extracts were loaded onto a 4.6 $\times$ 250 mm Prodigy ODS-3 column (Phenomenex) eluted with 10% acetonitrile in 50 mM sodium phosphate buffer pH 2.4 at a flow rate of 2 mL/min. Radioactivity was measured by a dual BGO flow detector, and the chromatograph analyzed by Laura software (Bioscan, Washington, DC). ¹¹C-MCG eluted after 4.0 min with a minor earlier eluting product at 2.5 min.

Baboon PET Study of ¹¹C-MCG. A dynamic PET study of the renal cortical uptake and clearance of ¹¹C-MCG was performed in an adult male baboon (*Papio anubis*; body weight, approximately 30 kg). Before each study, two intravenous catheters and a single arterial catheter were placed for infusion of anesthesia, injection of radiotracer, and sampling of arterial blood, respectively. The animal was initially anesthetized intramuscularly with 8–10 mg/kg alfadolone and alfaxalone acetate (Saffan; Pitman-Moore, Middlesex, UK) and was intubated. Anesthesia was maintained throughout the study by a continuous intravenous infusion drip of 6–9 mg/kg/h Saffan. The animal was secured to the PET bed using an individually fitted thermoplastic mask. Pulse, blood pressure, and oxygen saturation were monitored continuously during the studies. Blood oxygen saturation was always maintained above 85%. After the animal was positioned in the PET scanner, transmission scanning was performed with a 370 MBq (10 mCi)

⁶⁸Ga source to allow for attenuation correction. PET scanning was started immediately after intravenous injection of 370 MBq (10 mCi) of high-specific-activity ¹¹C-MCG (corresponding, at a maximum, to 0.02 μ g/kg). Thirty-five simultaneous, contiguous (18 direct planes, 17 cross planes, z -axis = 14.45 cm), sequential, quantitative tomographic slices of the brain were obtained with a GE Advance PET tomograph (General Electric Medical Systems, Milwaukee, WI) in the high-resolution mode (4.25–5.00 mm full width at half maximum within the slice) over a 90-min period. The animal was positioned so that the renal cortex was in the field of view. Approximately 30 arterial blood samples (for radioassay and protein binding) were obtained over 90 min. To correct the input function for unmetabolized ¹¹C-MCG, arterial samples were also obtained at 10, 20, 30, 45, 60, 75, and 90 min.

PET images were reconstructed from the raw data using a two-dimensional OSEM algorithm. Images were corrected for attenuation and decay and were scaled to the same maximum. A region of interest was chosen over the left lower pole renal cortex and time–activity curves (TACs) were generated. To assess binding specificity, 2 mg/kg of PMPA (in 6 mL of saline) was administered intravenously 10 min prior to injection of ¹¹C-MCG at the end of the first 90 min scan. Static images obtained over 10 min were performed before and after blocker (Figure 3).

Tracer Kinetic Modeling

A one-tissue, three-parameter (K_1 = influx, k_2 = efflux, DV = distribution volume) model was applied to the TACs and to the metabolite-corrected renal uptake curves to describe tracer kinetics with DV ($= K_1/k_2$ in mL/mL) used as an index of receptor density. The effect of blocking with PMPA was evaluated by changes in the DV and calculated as $100 \times (DV_{\text{baseline}} - DV_{\text{blocker}})/DV_{\text{baseline}}$. The model was fit to the PET data using nonlinear least squares minimization [9].

Statistics

ANOVA, which was used in rodent radiotracer uptake studies, was performed with StatView SE + Graphic software, version 1.03 (SAS Institute, Cary, NC). For the Student's t test, $p < .01$ was considered to indicate statistical significance.

Results

Radiochemical Synthesis

Facile radiosynthesis of ¹¹C-MCG was effected by treatment of the corresponding desmethyl precursor

Table 1. Biodistribution of ¹¹C-MCG in Male CD-1 Mice

Tissue	% ID/g ± SD (N = 4)				
	5 min	15 min	30 min	60 min	120 min
Blood	6.19 ± 0.94	3.27 ± 0.50	1.09 ± 0.22	0.25 ± 0.01	0.09 ± 0.03
Heart	2.43 ± 0.38	1.10 ± 0.10	0.38 ± 0.09	0.13 ± 0.00	0.07 ± 0.06
Liver	1.42 ± 0.20	0.87 ± 0.05	0.50 ± 0.09	0.30 ± 0.02	0.07 ± 0.03
Kidneys	60.94 ± 6.93	54.15 ± 3.69	32.99 ± 5.14	11.70 ± 1.99	0.22 ± 0.03
Muscle	2.23 ± 0.37	1.08 ± 0.48	0.45 ± 0.13	0.25 ± 0.22	0.09 ± 0.04
Fat	1.17 ± 0.93	0.75 ± 0.27	0.41 ± 0.16	0.12 ± 0.04	0.06 ± 0.05
Small intestine	1.10 ± 0.41	0.70 ± 0.10	0.43 ± 0.12	0.23 ± 0.05	0.12 ± 0.02

Each mouse received approximately 3.7 MBq [100 µCi (0.27 µg/kg)] of ¹¹C-MCG. Data are means ± SD.

with ¹¹C-iodomethane as depicted in Figure 1. A carrier peak for ¹¹C-MCG ($t_R = 3.9$ min) was not readily detected at the 214-nm wavelength. The analytical HPLC conditions can detect MCG at 20 nmol. Based on that detection limit, a minimum specific radioactivity of ¹¹C-MCG of 167 GBq/µmol (4000 Ci/mmol) at end-of-synthesis was derived. In all likelihood, specific radioactivities for ¹¹C-MCG are much higher based on our extensive preparation of other C-11 methylated radiotracers under similar reaction conditions. Radiochemical yield based on starting ¹¹C-iodomethane was calculated to be 16% ($n = 6$) and radiochemical purity was >97%. The time of synthesis including formulation was approximately 30 min (from the end of bombardment).

Mouse Biodistribution and Metabolism Studies

Regional uptake at 5, 15, 30, 60, and 120 min for ¹¹C-MCG in mouse organs is presented in Table 1. Radiotracer concentration was highest in the target

organ, the kidneys, and showed prompt washout, that is, within the time course of the study. Kidney/blood and kidney/muscle ratios were 30 and 73, respectively, at 30 min after injection. Prostate uptake was $1.55 \pm 1.01\%$ ID/g at 30 min ($n = 3$). Little activity gained access to the brain, with <0.1% ID/g in the cerebellum, hippocampus, or cortex and only $0.12 \pm 0.03\%$ ID/g in the brainstem at 30 min postinjection. Figure 2 depicts the significant [inset: $p < .0001$ and $p = .0002$ in the case of low-specific-activity (LSA) MCG and PMPA, respectively] blocking of radiotracer uptake when mice were pretreated with either an excess of unlabeled MCG (up to 1 mg/kg) or PMPA (1 mg/kg) (inset), indicating target binding specificity. An approximately sixfold reduction in uptake was demonstrated for either MCG or PMPA.

Metabolites were determined in vivo at 5, 15, 30, and 60 min and showed at most 9.2% metabolism in plasma at 60 min ($n = 2$) and 10.4% metabolism in kidney ($n = 2$). The 30-min time points ($n = 2$) showed 3.5% and 2.0% metabolism for plasma and kidney, respectively.

Baboon PET

When ¹¹C-MCG was administered to a male baboon, there was prominent uptake within the renal cortex, a peripheral site of GCP II in the primate (Figure 3). Pretreatment of the animal with 2 mg/kg PMPA showed a decrease in renal cortical radiotracer uptake as demonstrated in Figure 3, in the TACs (Figure 4) and by a 37% reduction in the DV (from 1.38 to 0.87 mL/mL).

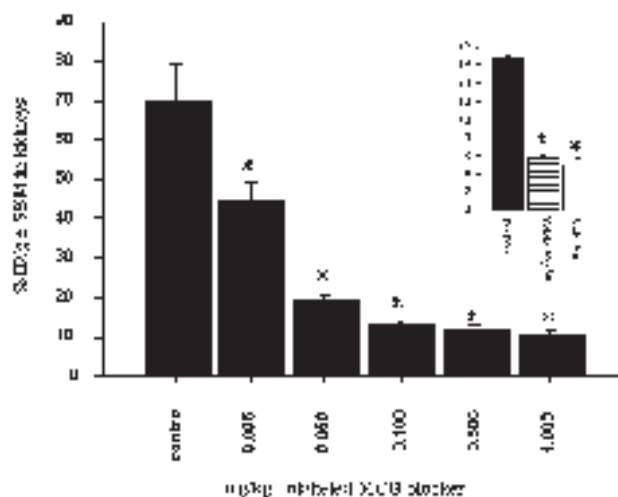


Figure 2. Binding specificity of ¹¹C-MCG to mouse kidney. Note decreasing ¹¹C-MCG uptake (up to approximately sevenfold) with increasing concentration of unlabeled MCG blocker. Uptake is expressed in percentage of injected dose per gram of tissue. The inset reveals blocking with PMPA, another high-affinity inhibitor of GCP II. Sacrifice time was 30 min in each experiment. LSA = low specific activity. Statistical significance is indicated by an asterisk over the error bar ($p < .01$).

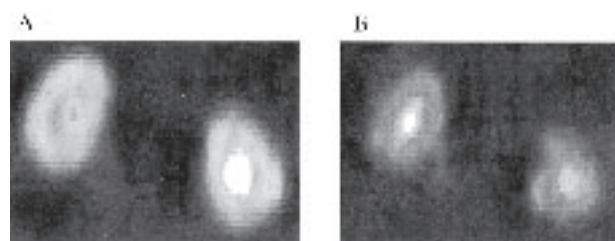


Figure 3. Static baboon renal PET images obtained before (A) and after (B) administration of blocker (2 mg/kg PMPA). Note decrease in cortical radioactivity after blocker administration.

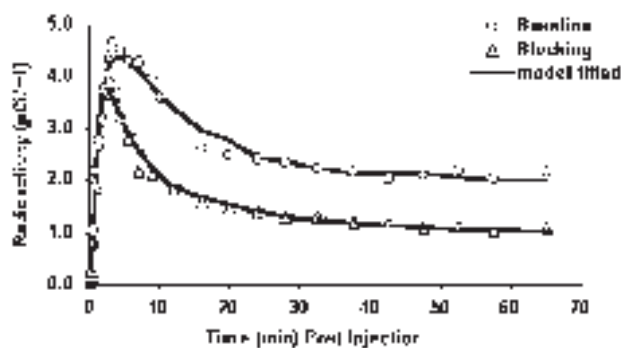


Figure 4. Baboon renal TAC before and after blocker (2 mg/kg PMPA). Note the decrease in renal cortical radioactivity after administration of blocker.

At baseline, peak metabolism of ¹¹C-MCG was 9.0% at 90 min after injection. Administration of blocker (2 mg/kg PMPA) 10 min prior to tracer injection decreased ¹¹C-MCG metabolism, which showed a peak value of 4.0% at 90 min after injection.

Discussion

GCP II and PSMA are very similar enzymes, such that an imaging probe for GCP II may serve useful to image PSMA. PSMA is expressed in a variety of normal and malignant tissues in and outside the central nervous system (CNS) [10]. Immunohistochemistry using the anti-PSMA antibody 7E11-C5 has shown PSMA to have a fairly restricted pattern of expression in human tissues, with the highest levels of activity demonstrated in a subset of proximal renal tubules, prostate epithelium, and within the duodenum and colon [5]. An immunocytochemical study that focused on the brain distribution of GCP II revealed staining of areas previously noted to contain immunoreactivity for NAAG, the natural substrate for GCP II [11]. Those areas included the basal ganglia, hippocampus, substantia nigra, among others, and included regions that did not demonstrate NAAG immunoreactivity. A study that employed ³H-NAAG demonstrated a 14-fold elevation of PSMA in human prostate cancer relative to normal prostate tissue [12]. PSMA expression is highest in high-grade and hormone-refractory disease [5]. Using a panel of anti-PSMA antibodies, PSMA immunoreactivity has been demonstrated in tumor-associated neovasculature in a host of tumors, including breast, colon, and lung [5]. Because of the distribution and variety of functions for GCP II, an imaging agent that could quantify GCP II activity would find utility in studying presynaptic glutamatergic transmission and may prove useful in prostate cancer diagnosis and monitoring, and in monitoring tumor neoangiogenesis.

mAb imaging and therapy for prostate cancer based on agents that bind either intra- or extracellular domains of PSMA has been reported and includes Prostascint, a clinical agent that utilizes single photon emission computed tomography (SPECT) [13–15]. An agent that uses PET might be expected to have superior pharmacokinetics, due to a smaller molecular weight with consequently less nonspecific binding, and provide superior image resolution to one based on SPECT. That—the homology between GCP II and PSMA, the ease of synthesis of the precursor **2** to MCG, and radiosynthesis of MCG (Figure 1)—provided the rationale for this study.

Because the rodent prostate does not demonstrate significant PSMA activity [3] or [³H]PMPA uptake [1], we used the kidney as a surrogate organ for the in vivo ¹¹C-MCG uptake studies. The high level of ¹¹C-MCG uptake, prompt washout during the 90 min study (Table 1), and significant preblockade with unlabeled MCG or PMPA (Figure 2), another high-affinity inhibitor of GCP II, suggest that ¹¹C-MCG may be a site-selective imaging agent for GCP II. An element of nonspecific binding of ¹¹C-MCG may be present, however, as there is no ¹¹C-MCG uptake with blockade. That could be due to several factors, including the fact that the route of excretion of MCG is renal, so ¹¹C-MCG, which is not bound to GCP II, is also included in the “blocked” kidney, and that ¹¹C-MCG may be a substrate for other enzymes and nonspecific transporters present in kidney, although at much lower affinity. The kidney has urea and glutamate transporters [16,17], each of which could be a target of ¹¹C-MCG and may be blocked in a dose-dependent manner. If so, they may contribute significantly to the blockade depicted in Figure 2. Further studies are necessary to uncover the GCP II-specific *vs.* transporter binding activity of ¹¹C-MCG in the kidney. ¹¹C-MCG also displayed salutary metabolic characteristics for an enzyme-based radiopharmaceutical, i.e., little metabolism either in plasma or in the target organ. That will facilitate tracer kinetic modeling used for quantification of enzyme activity.

We performed one primate PET study with ¹¹C-MCG. In that study, blocking of ¹¹C-MCG uptake was demonstrated when the animal was pretreated with a low dose (2 mg/kg) of PMPA, a previously determined safe dose to administer to primates (Pomper, unpublished results) (Figure 3). Because of renal excretion of ¹¹C-MCG, less than complete blockade of radiotracer was demonstrated in the baboon renal cortex. The concentration of GCP II has not been determined in the primate renal cortex, and its relative concentration to that in the mouse

kidney or to prostate is unknown. However, GCP II activity is present in the human renal cortex [5,10]. As in the mouse, little metabolism of ¹¹C-MCG was demonstrated in the primate plasma.

Brain uptake of ¹¹C-MCG was disappointingly low, suggesting that ¹¹C-MCG will not be a useful probe of brain GCP II activity. That is due to its hydrophilicity (logP = −.235) and the lack of a suitable transport mechanism that is active within the time scale of a typical PET study (90 min).

In conclusion, we describe ¹¹C-MCG as the first small molecule imaging probe for GCP II. The ease of the synthesis of the asymmetric urea precursor to MCG promises fruitful investigation of several analogs. With analogs of improved lipophilicity, we intend to study GCP II in the CNS, as well as in the periphery. Analogs labeled with radionuclides amenable to SPECT, such as iodine-123, are currently being pursued, the precursors of which may also be modified to be used for therapy (with iodine-125/131).

Acknowledgments

We thank Paige Rauseo and Paul Clark for excellent technical assistance, Dr. Hayden Ravert for specific activity calculations and radiosynthetic expertise, and Guilford Pharmaceuticals (Baltimore, MD) for providing PMPA. Financial support was provided by CA92871 (MGP) and NS42672 (APK).

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