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Synthesis and liposome encapsulation of a novel ^{18}F-conjugate of ω-conotoxin GVIA for the potential imaging of *N*-type Ca^{2+} channels in the brain by positron emission tomography

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Summary

ω-Conotoxin GVIA is a potent, irreversible antagonist of *N*-type voltage gated Ca^{2+} channels. A radiofluorinated analogue of GVIA could be useful in assessing regional synaptic density of the brain, *in vivo*, using positron emission tomography. *N*-hydroxy succinimidyl 4-[^{18}F]fluorobenzoate was employed to site-specifically label GVIA, preserving native binding affinity. The tracer was characterized with MALDI-TOF mass spectrometry and colorimetric protein assay. Radiochemical decay-corrected yield of the lysine-24 labeled analogue of [^{18}F]GVIA was 5%. Specific activity of this species was determined to be 1.2×10^5 Ci/mmol. Encapsulation of the tracer in sulfatide containing liposomes, a potential method for enhancing blood–brain penetrance, was accomplished with 40% efficiency. Copyright © 2006 John Wiley & Sons, Ltd.

Key Words: fluorine-18; ω-conotoxin GVIA; peptide labeling; PET brain imaging; liposome

Introduction

N-type Ca^{2+} channels are key members of a family of voltage-gated perisynaptic ion channel found throughout the nervous system. The current mediated by voltage-gated calcium channels (VGCC) initiates a cascade of protein–protein interactions, which ultimately result in exocytosis of vesicular transmitter during neurotransmission. Structurally, *N*-type channels are

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pentameric transmembrane proteins. The large, pore-forming $\alpha 1_B$ subunit is the target of ω-conotoxins GVIA and MVIIA.[1–4] Quantification of *N*-type Ca^{2+} channels by positron emission tomography (PET), using radiofluorinated bioconjugates of ω-conotoxins, could serve as an accurate marker for regional synaptic density of the brain *in vivo*. Furthermore, investigations regarding variations in regional synaptic density stemming from age, memory/learning, or neurodegenerative disease may be possible using such tracers. To this end, we describe here the radiochemical synthesis of a biologically active conjugate of ω-conotoxin GVIA ([^{18}F]GVIA) with yields and labeling conditions suitable for use of the ^{18}F-labeled product in acquiring PET data.

The best characterized ω-conotoxin, GVIA, is an approximately 3kD, 27-residue peptide isolated from the venom of the marine cone snail, *Conus geographus*. Containing three disulfide bonds (1–16, 8–19, and 15–26 linkage) as well as three hydoxyproline residues, the secondary structure of GVIA is constrained and resistant to denaturation under non-reducing conditions.[2,4] Site-specific acetylation studies have shown that residues near the *N*-terminus, such as Cys-1 and Lys-2, are critical for GVIA's binding affinity and specificity for *N*-type VGCC, while residues further along, such as Lys-24, can be chemically modified with little to no effect on binding activity.[1] Therefore, site-specific labeling strategies are important for preserving biological activity of these compounds.

This study compares the efficacy of two well-known approaches for site-specific fluorination in terms of their application to small hydrophilic peptides such as GVIA. The first pathway directly utilizes fluorobenzoic acid (FBA), in presence of diethyl cyanophosphonate and diisopropyl ethylamine, to label GVIA under anhydrous reaction conditions. The second strategy relies on the compound *N*-succinimidyl 4-fluorobenzoate (SFB), which is more difficult and time consuming to prepare than FBA, but has the advantage of being stable in aqueous media, facilitating labeling. Both strategies begin from an identical triflate precursor (**1**), which after fluorination and acid-mediated hydrolysis yield FBA.

SFB is an effective labeling agent for targeting the α- and ε-NH_2 groups associated with the *N*-termini and Lys residues, respectively, of proteins under mildly basic conditions (pH 8.6). SFB labels free amino groups via a trans-amidification reaction resulting in the covalent addition of a [^{18}F]fluorobenzoyl moiety. Furthermore, SFB shows virtually no reactivity towards guanidino or alcohol moieties, and is stable and reactive in aqueous media, unlike FBA. Thus, to develop a synthetic scheme for a biologically active radiofluorinated analogue of GVIA (denoted as K24-FGVIA) we relied on the chemical reactivity and specificity of SFB to target lysine residues. Subsequently, the desired Lys-24 labeled radioanologue was separated from

byproducts and reaction components by reverse-phase HPLC (RP-HPLC) to ensure radiochemical purity of the tracer.

Finally, in developing a peptide based tracer, the blood–brain barrier poses a significant obstacle. To enhance the pharmacokinetic and biodistribution properties of K24-[^{18}F]GVIA, we have adapted a protocol for encapsulation of the radiotracer into large, lipid-based vesicles known as liposomes. It has been previously demonstrated that liposomal incorporation of brain specific fatty acid esters can increase penetrance of peptide ligands.[5,6] In the adult mammalian brain, sulfatide (cerebroside 3-sulfate) is mainly found in myelin sheaths, and accounts for approximately 3.8% of total lipid weight. We have utilized a variety of liposome, containing 15% (mole fraction) sulfatide, in an attempt to maximize cerebral uptake.[5]

Imaging regional synaptic density, through the use of an *N*-type VGCC antagonist and PET, may bring important insights into the dynamic macroscopic organization of the brain. However, to use a peptide tracer, such as K24-FGVIA, three issues must be considered: (1) development of a labeling strategy consistent with site-specific requirements of the protein; (2) purification of biologically active radioanalogues from other isoforms via RP-HPLC; (3) increasing blood–brain penetrance of the peptide tracer to yield viable PET data. This study presents the development of such an approach, based on the native binding properties of ω-conotoxin GVIA.

Results and discussion

This study compared two alternate routes of synthesis for a lysine-24 fluoroconjugate of GVIA, from a common triflate precursor, with yields and preparative conditions suitable for neuro-PET studies.

Pentamethyl 4-(trimethylammonium trifluoromethanesulfonate) benzoate (**1**) was synthesized in two steps.[7–10] The coupling of pentamethylbenzyl chloride to 4-(*N*,*N*-dimethylamino) benzoic acid, in the presence of pyridine, to form pentamethylbenzyl 4-(*N*,*N*-dimethylamino) benzoate, had a yield of 63%. Next, methylation of the dimethylamino group to form **1** was 37% efficient (Figure 1(a)). Similarly, the esterification of 4-fluorobenzoic acid with disuccinimidyl carbonate to form *N*-succinimidyl 4-fluorobenzoate yielded 76% of the final purified product (Figure 1(b)).

Site-specific conjugation of GVIA with [^{19}F] SFB proceeded to completion in 30 min. The HPLC elution profile of the reaction mixture showed four major components with native GVIA having a molecular weight of 3037, and conjugated isoforms of GVIA exhibiting an increase in mass of 122, per added fluorobenzoyl group. Thus, analysis of the four fractions via MALDI-TOF MS revealed the first peak to be native GVIA ($M + m/z = 3036.76$), while the next two signals were isomeric mono-conjugates ($M + m/z = 3158.9$).

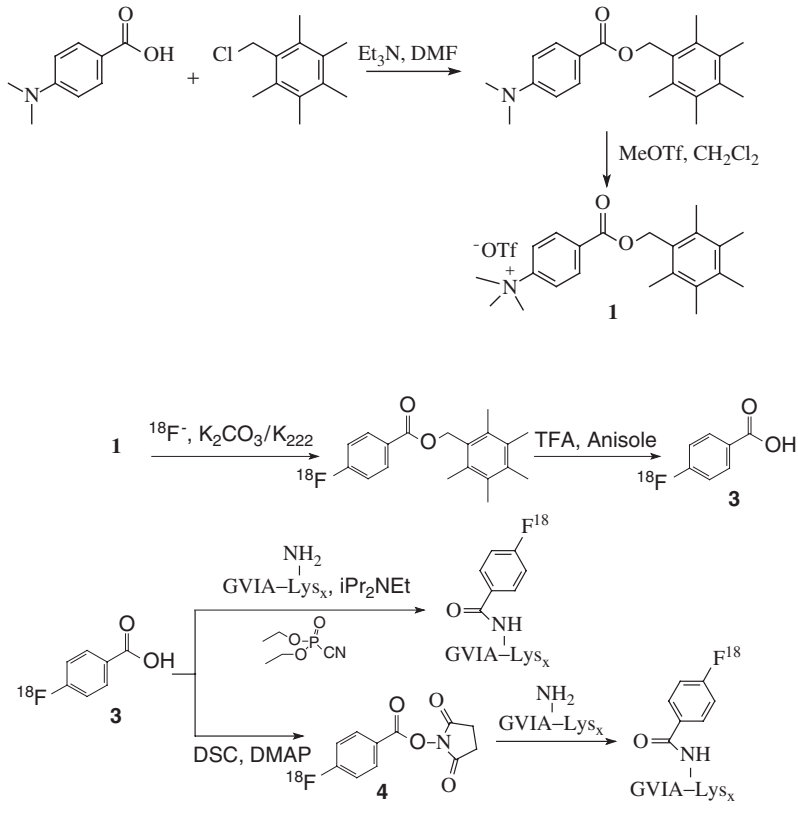

Figure 1. Two step preparation of radiochemical precursor, pentamethyl 4-(trimethylammonium trifluoromethanesulfonate) benzoate (PMBTf), and subsequent radiofluorination of ω-conotoxin GVIA via aqueous and anhydrous pathways

The final peak was di-conjugated GVIA ($M + m/z = 3280.9$), presumably labeled at each of the two lysine sites (Figure 3).

Similar experiments with FBA showed virtually no conjugation of GVIA after 2 h. The basis for such complete reaction failure was the chemical incompatibility of GVIA with the labeling reagents. FBA was only slightly soluble in water (0.4 g/100 ml), while diethyl cyanophosphonate and diisopropyl ethylamine were insoluble. Conversely, GVIA was unstable and insoluble in aqueous buffers containing more than 40% (v/v) acetonitrile.[1] Therefore, the lack of a mutually compatible medium for GVIA and the anhydrous labeling agents prevented the FBA-mediated direct conjugation of GVIA.

Prior to radiochemical development of an [^{18}F]GVIA conjugate, the target site of each of the mono-labeled species must be determined to ensure that at

least one isomer is predicted to be bio-active. To this end we employed a trypsin-digest protocol to verify which HPLC fraction corresponded to the Lys-2 and Lys-24 isomer, respectively.[1] The procedure was based on the native proteolytic activity of trypsin for peptides containing basic residues such as lysine and arginine. Trypsin cleaves peptides by hydrolysis on the amino side of a basic residue. Figure 2 is a schematic representation of trypsin's predicted activity on native, mono- and di-conjugated GVIA. Depending on which lysine residue(s) is labeled, thus interrupting tryptic cleavage at that site, the corresponding GVIA fragment will have a unique molecular weight. Therefore, by digesting each of the labeled species of GVIA with trypsin, purifying the major fragment via HPLC, and using MALDI-TOF MS to determine molecular weight, it was possible to ascertain the labeling site(s) of the conjugates. The predicted fragment mass of each GVIA species was as follows: native GVIA (2935), K2-FGVIA (3040), K24-FGVIA (3214), K2 + 24-F_2GVIA (3299). Experimentally determined M + fragment masses for each eluting fraction correlates well with predicted values, and are were as follows: 12.5 min [$m/z = 2934.6$], 13.5 min [$m/z = 3039.6$], 18.5 min [$m/z = 3213.1$], and 20.1 min [$m/z = 3297.6$]. Thus, native GVIA elutes at 12.5 min,

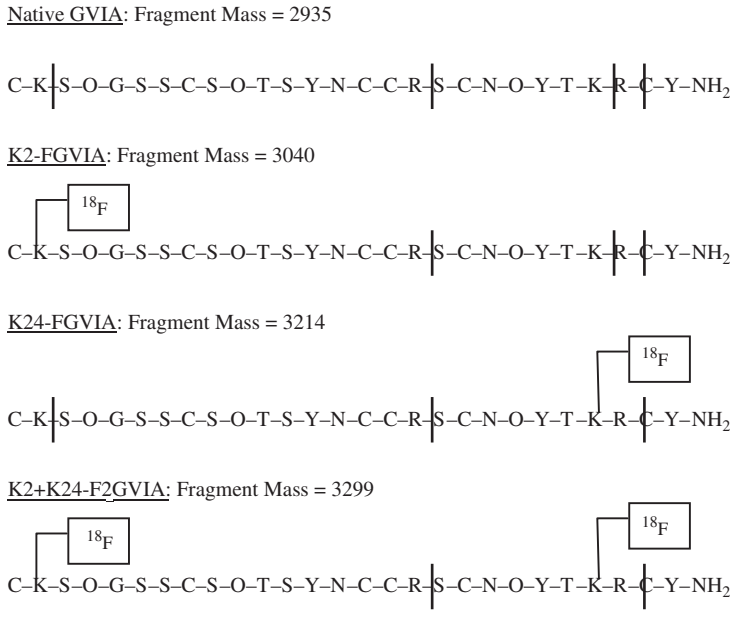

Figure 2. Graphic representation of expected fragment masses from trypsin mediated digestion of each isomer of GVIA. Solid vertical bars illustrate trypsin cleavage sites, while labeling site(s) are depicted by square boxes inscribed with '^{18}F.'

Table 1. Results of HPLC and MALDI-TOF MS analyses of GVIA cold labeling reaction with SFB

Species	Retention time (min)	Predicted mass	Experimental mass (M+)	Predicted fragment mass	Experimental fragment mass (M+)
GVIA	12.8	3037	3036.8	2935	2934.6
K2-FGVIA	13.5	3159	3158.9	3040	3039.9
K24-FGVIA	18.5	3159	3158.9	3214	3213.1
K2+24-F_2GVIA	20.1	3281	3280.1	3299	3297.6

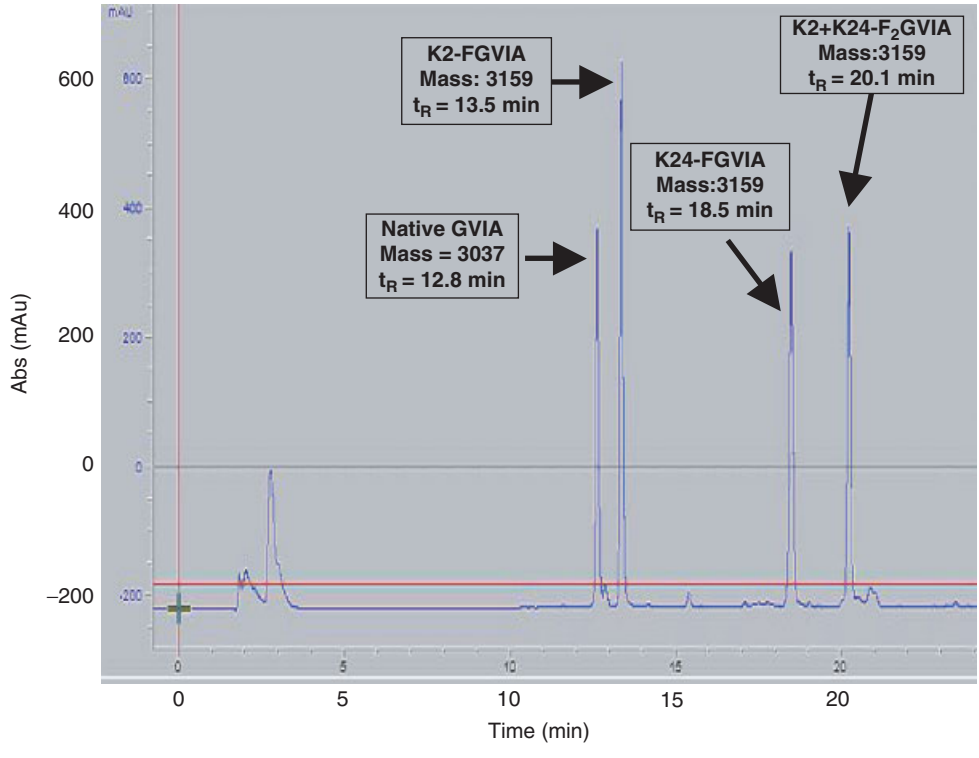

Figure 3. HPLC elution profile of GVIA cold-labeling reaction with SFB. The four major components of the reaction mixture are depicted, along with the identity, mass and retention time of each peak

K2-FGVIA at 13.5 min, K24-FGVIA at 18.5 min, and K2+24-F_2GVIA at 20.1 min (Table 1).

In order to determine the specific activity of K24-[^{18}F]GVIA, an association between absorbance at 215 nm (as measured by an online HPLC spectrophotometer) and molar quantity was made. A method commonly used for

quantifying collagen was adapted for this purpose.[11] The assay relies on a chemical reaction between hydroxyproline, Chloramine-T and *p*-dimethylaminobenzaldehyde to produce a chromogenic compound, whose absorbance is measured at 550 nm. The reaction mixture changes from a translucent yellow to vibrant peach with increasing concentrations of Hyp. Application of native GVIA to the assay yielded a standard curve. Finally, an unknown quantity of K24-FGVIA was assayed and the result divided by its absorption area (mAU × Sec) at 215 nm to give the following linear equation (at μM concentrations):

$$\text{K24-FGVIA (nmol)} = [3.14 \times 10^{-4} \pm 6 \times 10^{-6}] \times [\text{Absorption area at 215 nm}]$$

It was possible to measure the specific activity of K24-[^{18}F]GVIA by collecting the appropriate HPLC fraction, measuring its activity, and dividing by the mass quantity of labeled peptide as determined by the equation above. Thus, we determined the specific activity of K24-^{18}FGVIA to be $1.2\,\text{Ci} \times 10^5$/mmol.

This set the stage for the radiochemical preparation and liposome encapsulation of [^{18}F]GVIA. The aqueous pathway required four radiochemical and two HPLC steps to yield K24-[^{18}F]GVIA with a total preparation time of 165 min (Figure 1(b)). Radiofluorination of **1** and C18 solid phase extraction was 60% efficient. Hydrolysis of the fluorinated ester **2** to form [^{18}F]FBA (**3**), and subsequent coupling to DSC/DMAP yielded > 70% [^{18}F]SFB (**4**), as determined by HPLC. The fractions corresponding to **3** and **4** eluted at 12 and 22 min, respectively (Figure 4). The purified and reconstituted **4** was 90% consumed in the labeling reaction with native GVIA after 30 min. The radioconjugation produced approximately 30% K2-[^{18}F]GVIA, 40% K24-[^{18}F]GVIA, and 10% K2 + 24-[^{18}F]GVIA (Figure 5). The remaining 20% of activity was either hydrolyzed into **3** or remained unused as **4**. The fraction corresponding to K24-[^{18}F]GVIA was isolated and prepared for liposome encapsulation. The procedure required 25 min and was 45% efficient, as determined by size-exclusion chromatography. Non-encapsulated [^{18}F]GVIA was retained by Sephadex-G60 gel well after the encapsulated species eluted, offering virtually complete separation (Figure 6).

The microscale fluoroconjugation of GVIA with SFB and FBA, respectively, was predictive of the radiochemical behavior of each molecule. In examining labeling agent efficacy, and thus choosing the optimal strategy, we had to weigh ease of radiochemical preparation against labeling efficiency and site-specificity. Although the anhydrous labeling method has been shown to be an effective and amine-specific labeling procedure, the insolubility of GVIA in organic media prevented its use in this particular application. On the other hand, SFB is soluble, specific and reactive enough in aqueous buffer to consistently produce two mono-conjugated isomers of GVIA. While the

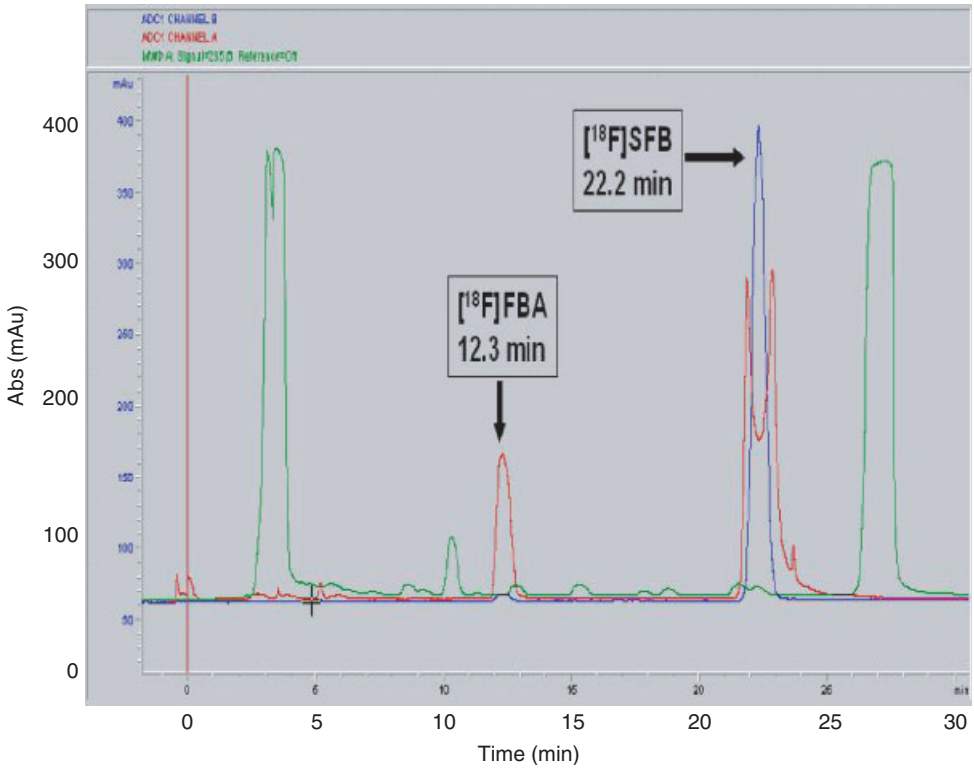

Figure 4. HPLC elution profile of the radiochemical synthesis of ^{18}F-SFB from ^{18}F-FBA. Depicted are traces from the high sensitivity γ detector (red), low sensitivity γ detector (blue), and UV absorption (green) at 235.6 nm

aqueous pathway will require longer radiochemical preparation than the direct anhydrous approach, the lack of reactivity exhibited by FBA makes its use impractical. Thus, we have chosen to develop the aqueous radiochemical strategy to site-specifically label GVIA.

Quantification of regional synaptic density by PET may offer fresh insight to the pathology of neurodegenerative disease, neurodynamics of aging, and the macroscopic plasticity involved in learning/memory. This study presents the radiochemical synthesis of a biologically active radiofluorinated analogue of ω-conotoxin GVIA, a natural antagonist of *N*-type channel. The potency of GVIA to bind to channels, and inhibit neurotransmission, is already being exploited by the pharmaceutical industry for analgesia. We describe here a four-step aqueous labeling strategy and one-step encapsulation protocol which provides practical means for preparing a radiofluorinated GVIA analogue potentially useful for exploring distribution of *N*-type calcium channels with PET.

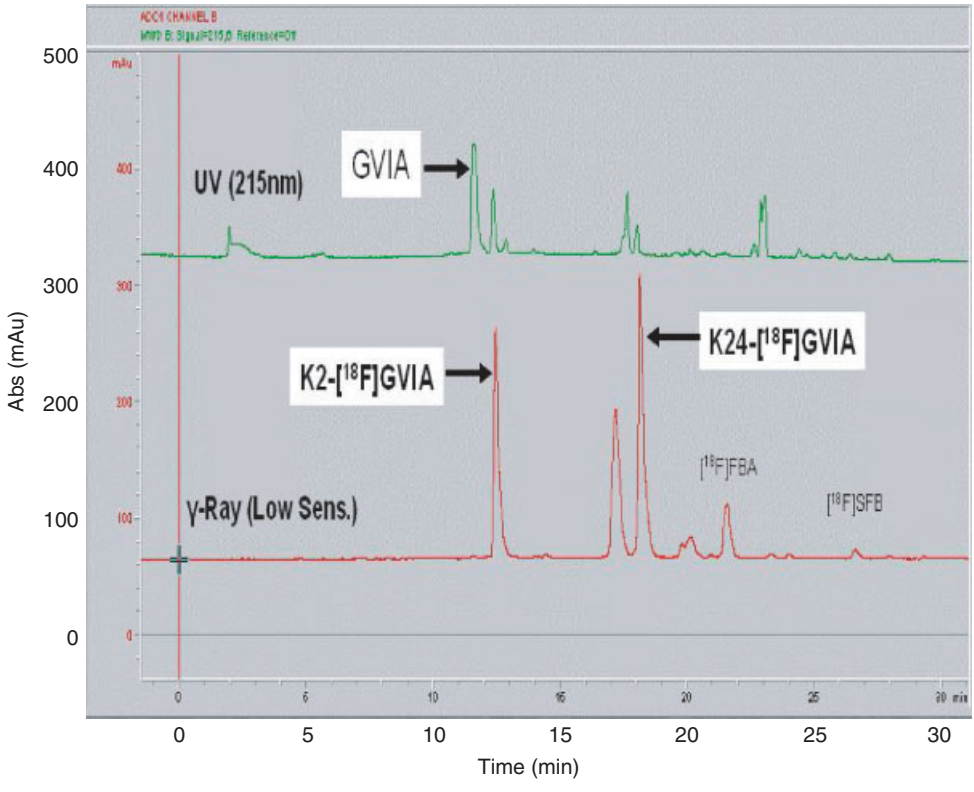

Figure 5. HPLC elution profile of the site-specific radiochemical labeling of GVIA with ^{18}F-SFB. UV (green) and γ-ray (red) traces are displayed simultaneously. Corresponding gamma peaks appear directly below their respective UV peaks

Experimental

Materials

ω-Conotoxin GVIA was purchased from Peptides International, Inc., Louisville, KY. Trypsin (Gold) mass spectrometry grade was acquired from Promega Co., Madison, WI. HPLC was performed using an Agilent 1100 series apparatus with an online degasser, UV/Vis spectrophotometer, and gamma detector from Agilent Technologies, Inc. Palo Alto, CA. HPLC eluates were monitored for their UV absorbance and radioactive content by connecting the outlet of the UV photometer to a NaI detector. The recorded data was processed by Agilent ChemStation software. All other chemicals were purchased from Sigma-Aldrich Co. or Fisher Scientific International without further purification.

*Synthesis of pentamethyl 4-(trimethylammonium trifluoromethanesulfonate) benzoate (**1**)*

A solution of 4-(*N*,*N*-dimethylamino) benzoic acid (570 mg, 3.4 mmol), pentamethylbenzoyl chloride (650 mg, 3.3 mmol), and triethylamine (0.46 ml,

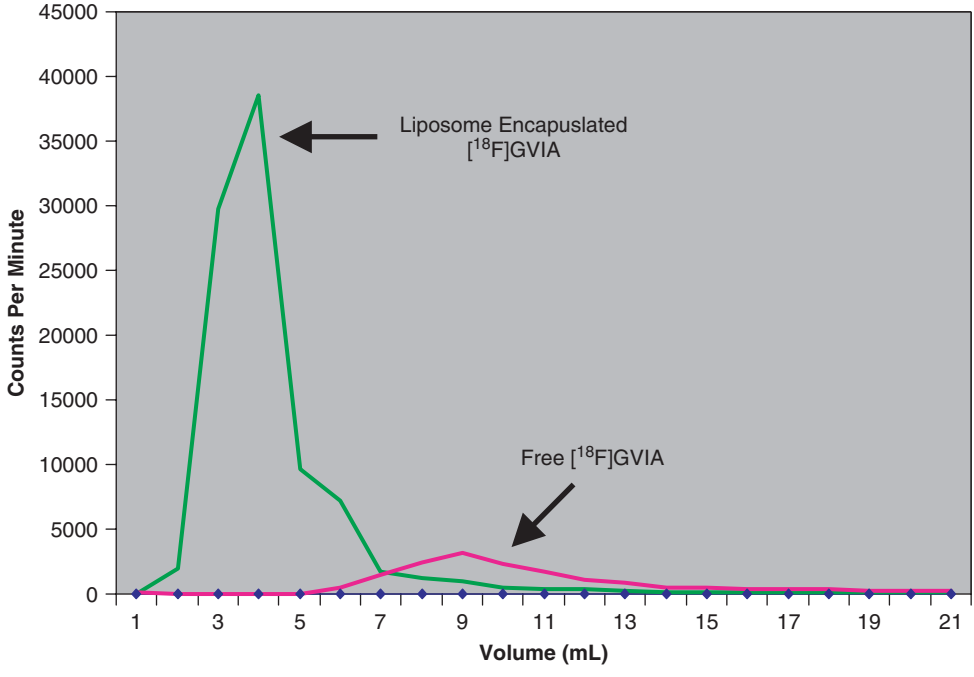

Figure 6. Gel filtration elution profile of liposome encapsulated and non-encapsulated ^{18}F-GVIA

3.3 mmol) in *N*,*N*-dimethylformamide (15 ml) was stirred overnight at room temperature, and subsequently poured into a solution of 10% $NaHCO_3$ (80 ml). The resulting white precipitate was filtered off and dried under vacuum. The crude product was then recrystallized from ethyl acetate to afford pentamethyl 4-(*N*,*N*-dimethylamino)benzoate (680 mg, 63%): ^{1}H-NMR ($CDCl_3$) δ 2.2–2.4 (3s, 15H), 3.01 (s, 6H), 5.42 (s, 2H), 6.60 (d, 2H), 7.90 (d, 2H). Pentamethyl 4-(*N*,*N*-dimethylamino)benzoate (255 mg, 0.75 mmol) was then dissolved in anhydrous methylene chloride (5 ml). Trifluoromethanesulfonate (90 µl, 0.8 mmol) was added and the reaction was stirred overnight. 10–20% hexanes by volume is added to the reaction mixture and the resulting solution was set into a −20°C freezer overnight. Compound **1** was collected by filtration as a fine, white needle-like crystal (140 mg, 37%): ^{1}H-NMR ($CDCl_3$) δ 2.1–2.3 (m, 15H), 2.75 (s, 3H), 3.8 (s, 9H), 5.5 (s, 2H), 7.8–8.2 (m, 3H).

Synthesis of succinimidyl 4-fluorobenzoate (SFB)

A solution of 4-fluorobenzoic acid (140 mg, 1 mmol), disuccinimidyl carbonate (256 mg, 1 mmol), and pyridine (81 µl, 1 mmol) in anhydrous acetonitrile (1 ml) was stirred for 90 min at room temperature. The crude product was

evaporated and applied to a silica gel column and eluted with 50:50 ethyl acetate:hexanes. Fractions containing SFB, as assessed by thin-layer chromatography, were pooled and evaporated under reduced pressure to yield white crystals (181 mg, 76%): ^{1}H-NMR ($CDCl_3$) δ 2.91 (s, 4H), 7.19 (m, 2H), 8.18 (m, 2H).

Conjugation of ω-conotoxin GVIA via succinimidyl 4-fluorobenzoate

To a 50 µl solution of GVIA (5 nmol) in borate buffer (50 mM, pH 8.3), 100 µl of deionized water and 5 µl of SFB (5 nmol in CH_3CN) were added.[12,13] The reaction was allowed to sit for 30 min, after which 800 µl of 0.1 M NaCl, pH 2.4, was added. RP-HPLC was used to isolate and purify conjugated species. The reaction mixture was injected onto an Agilent Zorbax Rx-C8 Column (4.6 µm, 150 mm × 5 mm), and eluted at 1 ml/min under a gradient ranging from 99:1 to 21:79 0.1 M NaCl pH 2.4: acetonitrile in 50 min. Eluates were visualized at 215 nm and fractions corresponding to the different conjugated species were collected for further analysis.

Conjugation of ω-conotoxin GVIA via 4-fluorobenzoic acid

To 100 µl of GVIA solution (10 nmol) in deionized water, FBA (1.4 µg, 10 nmol) solution in 110 µl of acetonitrile, cyanophosphonate (1 mg, 6.1 µmol) solution in 50 µl acetonitrile, and 10 µl iPr_2NEt were added. The reaction was allowed to sit for 30 min, after which 800 µl of 0.1 M NaCl (pH 2.4) was added. The reaction mixture was then injected onto HPLC, using the conditions described above.

Identification of conjugated species and determination of labeling site(s)

The HPLC fractions were lyophilized and reconstituted with 50 µl borate buffer (50 mM, pH 8.3). Each solution was then analyzed by MALDI-TOF mass spectrometry, using α-cyano cinnamic acid as matrix, to determine the presence of native, mono- or poly-conjugated species of GVIA. Those fractions corresponding to an isoform of GVIA were further assayed to investigate site(s) of conjugation.

Labeling site determination was performed using a protocol similar to that developed by Lampe *et al.* in their acetylation studies of GVIA.[1] Briefly, 100 µg of trypsin was reconstituted with 100 µl of buffer (20 mM $CaCl_2$, 100 mM *Tris*, pH 7.8), yielding a solution with a specific activity of 15 units/µl. About 2 µl of trypsin solution was added to each GVIA-associated HPLC fraction, which was previously reconstituted in 48 µl of the previous buffer. The solution was vortexed and incubated for 20 h at 37°C. After incubation, the major digest fragment of each species was isolated via RP-HPLC, dried and reconstituted in 50 µl of water. Thereafter, each fraction was subjected to

MALDI-TOF MS, including a control sample of pure trypsin. The resulting mass was compared to previously predicted values of fragment mass, based on the hydrolytic reactivity of trypsin towards GVIA (Figure 2).

Calculating the specific activity (mAU/nmol) of K24-FGVIA

Specific activity was determined by a chromogenic protein assay, exclusive for peptides containing hydroxyproline residues.[12] First, a series of standard solutions containing 0–5 μg of GVIA (in increments of 0.5 μg) were prepared in water. Each solution was dried under reduced pressure, reconstituted in 600 μl of 6 M HCl, and transferred to individual test tubes. The tubes were sealed and refluxed at 110°C for 16 h to ensure complete hydrolysis, and dried under reduced pressure. Second, the assay buffer was prepared containing anhydrous citric acid (4.57 g, 23.8 mmol), 1.2 ml glacial acetic acid, sodium acetate trihydrate (12.0 g, 146 mmol), sodium hydroxide (3.4 g, 85.0 mmol), and 100 μl toluene in deionized water (1 l). Each tube of dried hydrosylate was reconstituted in 2 ml of assay buffer. The standards were then treated with 1 ml of chloramine-T reagent (1.41 g chloramine-T, 26 ml *n*-propanol, and 53.3 ml assay buffer per 100 ml of solution), and allowed to stand at room temperature for 20 min. A 1 ml aliquot of a solution containing *p*-dimethylaminobenzaldehyde (15 g, 101 mmol), *n*-propanol (60 ml), and 26 ml perchloric acid (40%) was added to each tube. The tubes were placed in a water bath at 60°C for 15 min, and immediately transferred to a new water bath at room temperature for 5 min. Absorption measurements were made at 550 nm for each standard solution, and a curve was formulated. To correlate this standard curve with absorption values at 215 nm for K24-FGVIA, the conjugate was purified by RP-HPLC, the peak area at 215 nm (mAU × Sec) recorded, and the hydroxyproline assay applied. The resulting molar amount of K24-FGVIA, as determined by the Hyp-assay, was divided by the absorption area at 215 nm to yield a conversion factor between area and mass quantity.

Preparation of K[^{18}F]F-K_{222} complex

[^{18}F] fluoride ion, in deionized water, was produced using the $^{18}O(p,n)^{18}F$ reaction by bombarding [^{18}O]H_2O in a low volume (300 μl) silver target.[14] The activity, ranging from 5.6 to 6.7 GBq, was transferred to a solution containing 30 μl of 0.2 M K_2CO_3 and 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]-hexacosane (4.5 mg, 6 nmol) in 50 μl of acetonitrile. Water was removed via azeotropic evaporation (4 × 200 μl) of anhydrous acetonitrile under an argon stream at 150°C.

*Preparation of 4-[^{18}F]fluorobenzoic acid ([^{18}F]FBA, **3**)*

The preparation of **3** was modified from the protocols described by Lee and Gangloff *et al.*[7,15] The azeotropically dried ^{18}F-ion was reconstituted with a

120 µl solution of DMSO containing **1** (3 mg, 6 µmol). The mixture was vigorously vortexed and heated to 150°C for 15 min. The reaction vial was cooled and the contents poured into water (30 ml), and subsequently passed through a C18 Sep Pak. Compound **2** was then eluted with 5 ml of ether, and dried over a short Na_2SO_4 column. Approximately 5 µl of anisole was added to the ether, which was then evaporated under an argon stream. Once the ether had been evaporated, 200 µl of trifluoroacetic acid (TFA) was added and allowed to sit at room temperature for 5 min. The TFA was removed under an argon stream at room temperature to yield **3**. Typically, 2.59 GBq of **3** could be obtained in 65 min starting from 6.29 GBq aliquot of a cyclotron produced [^{18}F]F^- batch (60% decay corrected yield).

Radiofluorniation of GVIA via direct-coupling with [^{18}F]FBA

3 was reconstituted with 100 µl of anhydrous GVIA solution (1 nmol/10 µl water). To this solution 50 µl of cyanophosphonate solution (1 mg/50 µl acetonitrile) and 10 µl iPr_2NEt were added. The reaction vessel was sealed and allowed to sit for 30 min at room temperature. The reaction mixture was analyzed by RP-HPLC using the same chromatographic parameters as described for the [^{19}F]fluoroconjugation studies. No significant amount of radiofluorinated GVIA was achieved.

*Preparation of succinimidyl 4-[^{18}F]fluorobenzoate (^{18}F-SFB, **4**)*

3 was treated with *N*,*N*-succinimidyl carbonate (5 mg, 19.5 µmol) and 4-dimethylaminopyridine (2 mg, 16.4 µmol) in acetonitrile (150 µl) at 90°C for 5 min. Thereafter, 900 µl of water was added, and the reaction mixture was loaded onto a Phenomonex Luna C-18 column (5 µm, 250 mm × 5 mm) and eluted isocratically at 4 ml/min using 35:65:0.1 acetonitrile:water:acetic acid. The eluatess were visualized at 235 nm and activity monitored via an online gamma detector. The HPLC fraction corresponding to **4** was collected around 22 min and diluted to a volume of 30 ml using deionized water. The sample was then applied to a C18 Sep-Pak. The retained activity was eluted with 5 ml of ether and passed through a Na_2SO_4 plug. The ether was evaporated under an argon stream at room temperature. Typically, 1.30 GBq of **4** was prepared from 6.29 GBq of ^{18}F-ion in 120 min (44% decay corrected yield).

*Radiofluorination of GVIA via succinimidyl 4-[^{18}F]fluorobenzoate (**4**)*

To **4** in acetonitrile (35 µl) was added 85 µl of a GVIA solution (3 nmol/10 µl 50 mM borate buffer). The reaction vessel was sealed and allowed to sit for 30 min at room temperature. The reaction mixture was analyzed by RP-HPLC using the same chromatographic parameters as described for the ^{19}F-fluoroconjugation studies. Routinely, 0.11 GBq of K24-[^{18}F]GVIA was obtained from 6.29 GBq of ^{18}F-ion in 165 min (5.0% decay corrected yield).

Preparation of sulfatide containing liposome and K24-FGVIA encapsulation

Sulfatide containing liposomes were prepared essentially according to the method described by Chen and Lee.[5] Briefly, a 7:10:3 molar ratio solution of phosphatidyl choline (PC): cholesterol (CH): sulfatide (SF) was prepared in 2.5 ml of 1:1 methanol:chloroform. The solution was evaporated under reduced pressure, and the residue immediately refrigerated at -20°C until use. The dry film was then reconstituted with 750 µl of ether and 1.5 ml of buffer (5 mM *Tris*, 20 mM NaCl, pH 7.4). The volume of the HPLC fraction corresponding to K24-FGVIA was adjusted to 1 ml and added to the lipid mixture. The solution was vortexed for 5 min, followed by sonication for an additional 5 min. The ether was removed under reduced pressure, and the aqueous suspension applied to a short column (1 cm) of Sephadex G-60 gel for size-exclusion chromatography. The fraction was eluted under pressure and assayed for activity. An encapsulation efficiency of 40% was normally observed.

Conclusion

We have accomplished the radiochemical synthesis of a biologically active conjugate of ω-conotoxin GVIA, with yields and labeling conditions suitable for use of the ^{18}F-lableled product in acquiring PET data. Preclinical studies to assess the pharmacology and biodistribution of both the free and liposome encapsulated form of the radiotracer are currently underway using small animal-dedicated PET to examine K24-[^{18}F]GVIA distribution in rats.

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