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Research Article

Synthesis and characterization of 4,6-dichloroindole-based radioligands for imaging the glycine site of the NMDA ion channel

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Summary

To provide effective PET or SPECT ligands for the glycine binding site of the NMDA ion channel, we have synthesized and characterized *in vitro* four substituted derivatives of the potent glycine site antagonist 3-[2-[(phenylamino)carbonyl]ethenyl]-4,6-dichloroindole-2-carboxylic acid (Ki = 3.0 nM). These new ligands contain groups amenable to labeling with C-11 for PET, or I-123 for SPECT. *In vitro* analysis of these compounds revealed that placement of a methoxy group at either the *ortho* or *para* position of the phenylaminocarbonyl group significantly reduced receptor affinity (Ki = 74.0 ± 8.1 and 26.5 ± 4.9 nM, respectively), as did placement of an iodine at the *para* position (Ki = 60.4 ± 8.2 nM). However, the *meta*-methoxy derivative (**4b**) maintained high affinity (Ki = 4.8 ± 0.9 nM) for the glycine site and was therefore labeled with carbon-11 by reacting the corresponding desmethyl derivative with [^{11}C]methyl iodide. Radiochemical yields of 14 ± 10% (EOS), and high specific activity (1.2 ± 0.5 Ci/μmol (EOS, $n = 7$)) were realized, and the product was

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prepared in a sterile saline solution suitable for *in vivo* use.

Key Words: NMDA; glutamate; glycine; PET; radioligand; carbon-11

Introduction

The *N*-methyl-D-aspartate (NMDA) ion channel, a major site of action for glutamate, is known to play a central role in many biological functions including neuroprotection, neurodegeneration, long-term potentiation, and cognition.[1–6] Alterations in normal NMDA receptor composition, densities and function have been implicated in the pathophysiology of certain neurological and neuropsychiatric disorders such as Parkinson's Disease, Huntington's Chorea, schizophrenia, alcoholism and stroke.[6–11] Stimulation of the different binding sites located on or within the NMDA ion channel has been shown to modulate the expression of behavior in different ways.[12–17] In addition to a site of binding for L-glutamate, there exists a second site that binds Mg^{2+} and phencyclidine (PCP) that mediates ion flux through the strychnine-insensitive glycine binding site, a polyamine binding site and a Zn^{2+} binding site. The glycine, glutamate and polyamine sites are located outside the ion channel, while the Zn^{2+} and PCP sites are found within. Glycine is a known co-agonist acting of the NMDA receptor as its presence is required to allow channel activation by glutamate and NMDA. Antagonists of the glycine site are reported to be neuroprotective against focal ischemia and trauma, anxiolytic, anti-epileptic, anti-convulsant, anti-psychomimetic, and able to effect improvements in cognition and memory processes. [6, 8, 18, 19] In line with this, radiotracers suitable for imaging the glycine site of the NMDA ion channel would facilitate a means for assessing the status of this site to provide important biochemical information relevant to the study of these disorders.

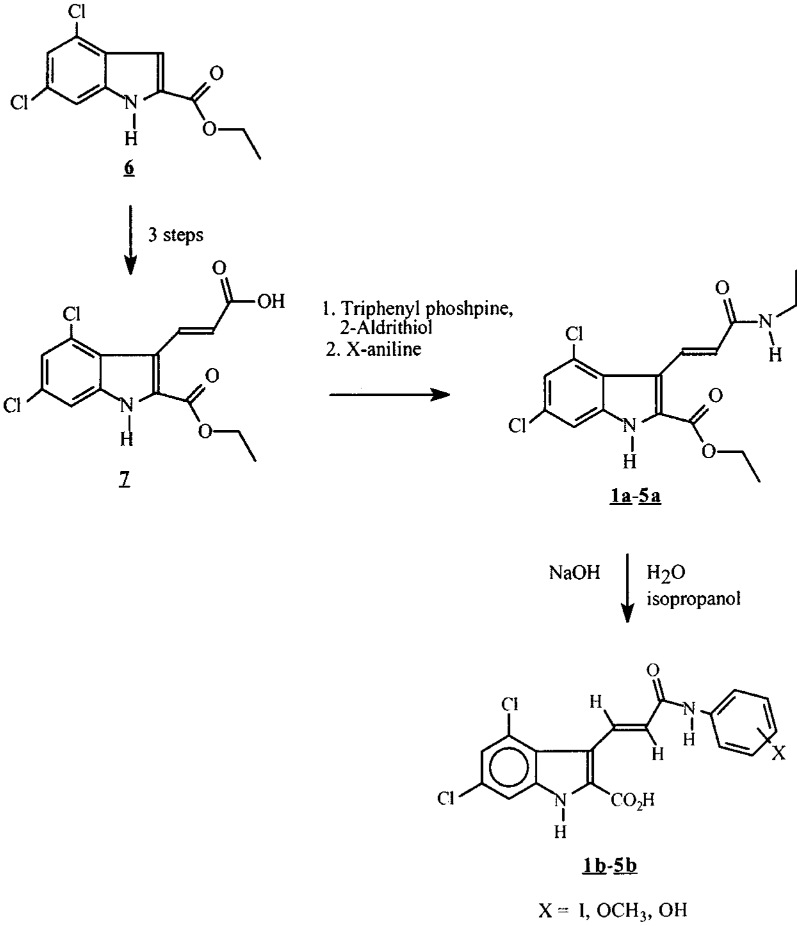
To date the development of effective PET ligands for imaging the glycine site has proven difficult. Past efforts include the high affinity 3-substituted-4-hydroxyquinolin-2-ones such as [^{11}C]L703,717[20,21], [^{123}I]7-Bromo-5-iodo-4-oxo-1,4-dihidroquinoline-2-carboxylic acid and [^{123}I]7-chloro-5-iodo-4-oxo-1,4-dihidroquinoline-2-carboxylic acid.[22] However, to date these tracers have not been proven effective for *in vivo* imaging, and the development of radioligands for the NMDA/

glycine site remains problematic. High lipophilicity, excessive acidic character, and binding to the warfarin binding site on human serum albumin are reportedly the major factors contributing to high plasma protein binding and low brain uptake.[23] This has been reported for radiotracers, as well as for glycine site antagonists developed as potential therapeutic agents. A detailed study has been undertaken to probe the relationships between lipophilicity, plasma protein binding and *in vivo* activity of 76 different glycine site antagonists.[23] A parabolic relationship was found between lipophilicity and *in vivo* activity, and suitable $\log P$ values for optimum *in vivo* potency occurred between 2.0 and 3.0, with a maximum at 2.39. Also, compounds which possess an excess number of acidic functional groups exhibited higher degrees of binding to plasma proteins.

In an effort to provide effective ligands for imaging the glycine site of the NMDA receptor, we considered the 5,7-dichloroindole-2-carboxylate class of compounds. Of the ligands reported thus far, 3-(2-((phenylamino)carbonyl)ethenyl)-4,6-dichloroindol-2-carboxylic acid, GV150526A, is one of most potent glycine site antagonists when administered intravenously (i.v.).[24–26] This compound inhibits NMDA induced convulsions in mice with an ED_{50} of 0.06 mg/kg (i.v.). The high *in vivo* potency seemed a reasonable indication that this ligand may adequately cross the blood/brain barrier. Other advantages are its high affinity ($Ki = 1\text{–}3\,nM$) and reasonable lipophilicity ($\log P = 2.24$).[23] However, there is no direct method to quickly label GV150526A with PET isotopes using standard procedures. Published structure/affinity relationships for glycine site ligands reveal that a general region exists around the phenyl ring where there is bulk tolerance.[25] We hypothesized that substituents amenable to direct labeling with PET or SPECT isotopes could best be placed in this region to hopefully provide ligands with preserved high affinity for the glycine site.

Results and discussion

Four compounds were synthesized and evaluated *in vitro* to identify candidate radiotracers that can be labeled with either C-11 or I-123 using established methods (Scheme 1). (E)-3-(2'-carboxyethenyl)-4,6-dichloroindole-2-carboxylic acid 7 was prepared in 3 steps from the known indole 6 according to the methods of Di Fabio *et al.*[24] Amidation of 7 was accomplished by a modification of the method



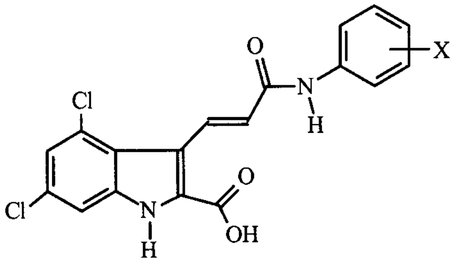
Scheme 1. Synthesis of the target compounds 1b–5b

described by Di Fabio *et al.*[24] Included was activation of the carboxylic acid group through the formation of the corresponding 2-pyridyl thioester intermediate, followed by the addition of the appropriately substituted aniline to provide the ethyl esters (**1a**–**5a**). Hydrolysis of the ester with sodium hydroxide in isopropanol afforded the target compounds **1b**–**4b** and the *meta* phenol precursor **5b** in good yield (62–89%). By established methods, *in vitro* assays were subsequently performed to determine the inhibition constants (Ki values) of the new ligands.[27,28] It was found that placement of a methoxy group at either the *ortho* or *para* position of the phenyl ring significantly reduced receptor affinity (Ki = 74.0 ± 8.1 and 26.5 ± 4.9 nM, respectively), as did placement of an iodine at the *para* position (Ki = 60.4 ± 8.2 nM)

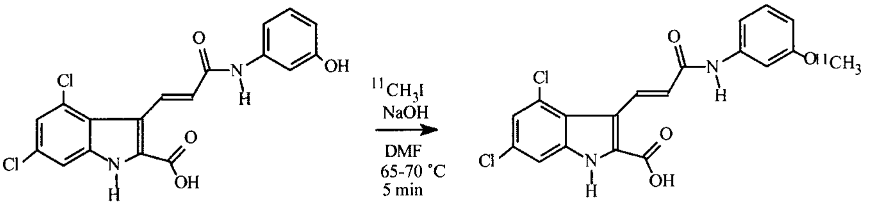
(Table 1). However, the *meta*-methoxy derivative (**4b**) maintained high affinity (Ki = 4.8 ± 0.9 nM) for the glycine site and was therefore considered a good candidate for labeling with C-11 for further *in vitro* and *in vivo* testing.

The desired tracer [^{11}C]-**4b** was synthesized by methylation of the corresponding 3-hydroxyphenyl derivative using [^{11}C]methyl iodide in dimethylformamide in the presence of sodium hydroxide. This mixture was heated at 65–70°C for 5 min, and purified by a reversed phase semi-preparative HPLC method as described in the experimental section. Radiochemical yields of 14–20% (EOS) and high specific activity (2.2 ± 0.5 Ci/μmol EOS) were realized (Scheme 2). The radiochemical purity was > 95% and up to 69 mCi of product was obtained after

Table 1. *In vitro* inhibition constants for 2-carboxyindole derivatives



	X	Ki (nM)	n
1b	*p*-OCH3	26.5 ± 4.9	3
2b	*o*-OCH3	74.0 ± 8.1	4
3b	*p*-I	60.4 ± 8.2	3
4b	*m*-OCH3	4.8 ± 0.9	3



5b

[^{11}C]-**4b**

Scheme 2. Radiosynthesis of [^{11}C]-4b

HPLC purification. The fractions containing [^{11}C]-**4b** were diluted with de-ionized water (100 ml) and this solution was passed through a C-18 Sep-Pak. This procedure is performed to remove the acetonitrile and buffer from the product, and to facilitate its concentration. The radioactivity was well retained, and the radiotracer was eluted from the Sep-Pak with 1–1.5 ml absolute ethanol. The ethanol solution was diluted with sterile saline to provide approximately 200 µCi/100 µl, and the preparation was passed through a sterile 0.22 µm filter into a sterile vial. This formulation is suitable for *in vivo* use.

Materials and methods

General

Proton NMR spectra were recorded on a Brucker 400 MHz FT-NMR spectrometer (Department of Chemistry, Columbia University). Chemical shifts were recorded in ppm (δ) from an internal tetramethylsilane standard in either dichloromethane-d_2 or methylsulfoxide-d_6 and coupling constants (J) are reported in Hz. Chromatographic purification of unlabeled compounds was performed using silica gel (Aldrich, 70-230 mesh, ASTM) using the solvent systems indicated in the text. For mixed solvent systems, the ratios are given with respect to volumes. All reagents were purchased from commercial sources and were used without further purification. (E)-3-(2’-Carboxyethenyl)-4,6-dichloroindole-2-carboxylic acid **6** was prepared using reported methods.[27,28] [^{11}C]Methyl iodide is synthesized routinely at Columbia University Radioligand Laboratory using established methods. HPLC analysis of the radioligand was performed using a Waters.

515 HPLC pump, a Waters PDA UV detector, and a Bicron Flow-Scint radiation detector. The column used was a reverse-phase base-deactivated column (Phemomenex, ODS, analytical: 4 × 250 mm^2, 5 µm particle size; semipreparative: 10 × 250 mm^2, 10 µm particle size) and the mobile phases and flow rates used are indicated in the text below.

Synthesis

General synthesis of Aryl Amides. To a solution of (E)-3-(2′-carboxyethenyl)-4,6-dichloroindole-2-carboxylic acid in DMF were added 2,2′-dipyridyl disulfide and triphenylphosphine at room temperature under an N_2 atmosphere. The solution was stirred for 2 h, then the appropriately substituted aniline was added. The temperature was increased to 85°C, and stirring continued for another 20 h. Water was added to effect precipitation, and the resulting precipitate was collected and dried to obtain the desired ethyl esters **1a**–**5a** in good yield (62–89%).

3-[2-[(4-methoxyphenylamino)carbonyl]ethenyl]-4,6-dichloroindole-2-carboxylic acid ethyl ester, ***1a***. mp = 242–243°C; ^{1}H-NMR: 1.23. (t, 3 H), 4.1 (q, 2 H), 6.33 (d, 1 H, $J = 15.0$), 7.2–7.7 (m, 7 H), 10.18 (s, 1 H), 12.3 (br s, 1 H).

3-[2-[(2-methoxyphenylamino)carbonyl]ethenyl]-4,6-dichloroindole-2-carboxylic acid ethyl ester, ***2a***. mp = 250–253°C; ^{1}H-NMR: 1.3. (t, 3 H), 3.85 (s, 3 H), 4.2 (q, 2 H), 6.8–7.7 (m, 7 H), 8.55 (d, 1 H, $J = 15.0$), 10.2 (s, 1 H), 12.3 (br s, 1 H).

3-[2-[(4-iodophenylamino)carbonyl]ethenyl]-4,6-dichloroindole-2-carboxylic acid ethyl ester, ***3a***. mp = 231–235°C; ^{1}H-NMR: 1.3. (t, 3 H), 3.85 (s, 3 H), 4.2 (q, 2 H), 6.8–7.7 (m, 7 H), 8.55 (d, 1 H, $J = 15.0$), 10.2 (s, 1 H), 12.3 (br s, 1 H).

3-[2-[(3-methoxyphenylamino)carbonyl]ethenyl]-4,6-dichloroindole-2-carboxylic acid ethyl ester, ***4a***. mp = 257–260°C; ^{1}H-NMR: 1.3. (t, 3 H), 3.85 (s, 3 H), 4.2 (q, 2 H), 6.8–7.7 (m, 7 H), 8.55 (d, 1 H, J = 15.0), 10.2 (s, 1 H), 12.3 (br s, 1 H).

3-[2-[(3-hydroxyphenylamino)carbonyl]ethenyl]-4,6-dichloroindole-2-carboxylic acid ethyl ester, ***5a***. mp = 279–281°C; ^{1}H-NMR: 1.3. (t, 3 H), 4.2 (q, 2 H), 6.5–7.6 (m, 8 H), 8.55 (d, 1 H, $J = 15.0$), 10.2 (s, 1 H), 12.3 (br s, 1 H).

General Procedure for the basic hydrolysis of ethyl esters. The 2-indole carboxylic acid ethyl esters were dissolved in isopropanol and heated at reflux for 6 h in the presence of sodium hydroxide (1 equivalent). The solution was cooled, and water was added to effect precipitation. The alcohol was evaporated *in vacuo*, and the precipitate filtered and washed with water to provide the target compounds **1b**–**5b** in high yield (91–98%).

3-[2-[(4-methoxyphenylamino)carbonyl]ethenyl]-4,6-dichloroindole-2-carboxylic acid, ***1b****. mp = 220–223°C; ^{1}H-NMR: 3.85. (s, 3 H), 6.8–7.5 (m, 7 H), 8.75 (d, 1 H, J = 15.0); MS m/z: 403.*

3-[2-[(2-methoxyphenylamino)carbonyl]ethenyl]-4,6-dichloroindole-2-carboxylic acid, ***2b****. mp = > 230°C; ^{1}H-NMR: 3.85. (s, 3 H), 6.8–7.5 (m, 7 H), 8.75 (d, 1 H, J = 15.0); MS m/z: 403.*

3-[2-[(4-iodophenylamino)carbonyl]ethenyl]-4,6-dichloroindole-2-carboxylic acid, ***3b****. mp = > 250°C; ^{1}H-NMR: 6.12. (d, 1 H, J = 16 Hz), 7.0–7.6 (m, 6 H), 8.6 (d, 1 H, J = 16.0); MS m/z: 499.*

3-[2-[(3-methoxyphenylamino)carbonyl]ethenyl]-4,6-dichloroindole-2-carboxylic acid, ***4b****. mp = 227–230°C; ^{1}H-NMR: 3.9. (s, 3 H), 6.8-7.5 (m, 7 H), 8.75 (d, 1 H, J = 15.0); MS m/z: 403.*

3-[2-[(3-hydroxyphenylamino)carbonyl]ethenyl]-4,6-dichloroindole-2-carboxylic acid, ***5b****. mp = > 230°C; ^{1}H-NMR: 6.5–7. 6 (m, 8 H), 8.55 (d, 1 H, J = 15.0); MS m/z: 391.*

Radiolabeling

[^{11}C]3-[2-[(3-methoxyphenylamino)carbonyl]ethenyl]-4,6-dichloroindole-2-carboxylic acid, [^{11}C]-**4b**.

Into a 1 ml reaction vial was added 2 mg **5b**, 5 μl aqueous sodium hydroxide (5 N) and dimethylformamide (400 μl). The vessel was sealed and [^{11}C]methyl iodide (820–1,430 mCi) was transferred into the solution via a stream of argon. The vessel activity was monitored, and once maximized, the mixture was heated at 65–70°C for 5 min. The crude product was diluted with 1 ml mobile phase, and then injected onto an HPLC column (ammonium acetate (0.1 M, pH = 5)/acetonitrile 35:65 (v/v); flow rate = 12 ml/min). The desired product eluted between 11-13 min, and the eluent was diluted with 100 ml deionized water, passed through a C-18 Sep-Pak. [^{11}C]-**4b** was eluted from the Sep-Pak with ethanol (1.0–1.5 ml) and this solution was subjected to QC analysis to determine overall radiochemical purity, specific activity, and product yield. From this analysis, the desired radiotracer was produced in yields of 14–20% (EOS), specific activities of 2.2 ± 0.5 Ci/μmol (EOS, $n = 4$) and a radiochemical purity > 95%. Synthesis, HPLC purification and initial QC of the ethanol was typically completed within 40–45 min.

To obtain preparations of the radiotracer suitable for use *in vivo*, the ethanol solution was subsequently diluted with saline (0.9% NaCl, sterile) to provide the desired concentration of [^{11}C]-**4b** while maintaining a maximum of 10% ethanol by volume. This final saline solution was passed through a sterile filter (22 μM) into an evacuated sterile to provide approximately 10 mCi [^{11}C]-**4b** per ml solution. The formulation step typically took 5–7 min. The range in the amount of product formulated was 12.3–68 mCi when prepared in this manner. Aliquots were further diluted with sterile saline to provide concentrations of 200 μCi of [^{11}C]-**4b** per 100 μl solution, which is suitable for use in rodent studies.

Ligand binding assay

Once prepared, compounds **1b**–**4b** were evaluated for selective binding to the glycine site through receptor binding assays established using highly purified rat cortex membrane preparations and established in vitro assay conditions.[27,28] The radioligand used was the well characterized glycine site antagonist [^{3}H]MDL 105,519 (KD = 3.7 nM). Briefly, assays were performed in triplicate, and the ability of various concentrations of unlabeled **1b**–**4b** to inhibit specific binding of [^{3}H]MDL 105,519 (3 nM) was determined. Unlabeled ligands were dissolved as 1 mM stock solutions in 10% DMSO in buffer and diluted with binding buffer as appropriate. The presence of 1% DMSO has been reported to not alter the binding of [^{3}H] MDL 105,519.[29] Samples varying in concentration (10^{-12} to 10^{-5} M) of **1b**–**4b** were incubated for 1 h at room temperature in a total volume of 1 ml binding buffer (tris acetate 0.05 M, pH = 7.4) in the presence of membrane (2 mg) and tracer. Samples were filtered using a 48-well cell harvestor (Brandel) and unbound tracer was removed by washing three times (approx. 4 ml per wash) with ice cold buffer. The radioactivity was determined by scintillation counting (Packard TriCarb 1500) after drying the membrane filters at room temperature for 2 h, followed by the addition of scintillation fluid (4 ml, Packard Ultima Gold). The effect of different concentrations of test ligand **1b**–**4b** was examined using non-linear regression analysis (Prism), and the IC50 and Ki values determined.

Conclusion

Four new glycine site ligands have been synthesized and their inhibition constants evaluated *in vitro*. Of these, one ligand, [^{11}C]-**4b**, exhibited high affinity for the glycine site. This compound was radiolabeled with the positron emitting isotope carbon-11 in good yield and high specific activity, and subsequently prepared as a formulation suitable for *in vivo* studies.

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