Note

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Synthesis of tritium-labeled levetiracetam ((2*S*)-2-(2-oxopyrrolidin-1-yl)butanamide) with high specific activity

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A method for the preparation of [^{3}H]levetiracetam with a high specific activity of 98 Ci/mmol (3.6 TBq/mmol) is described. The radioligand proved to be highly useful for the labeling of specific levetiracetam binding sites in rat brain membrane preparations.

Keywords: levetiracetam; tritium labeling; high specific activity; SV2A protein; epilepsy

Introduction

Levetiracetam (**1**; (2*S*)-2-(2-oxopyrrolidin-1-yl)butanamide, Keppra®) is a pyrrolidone drug widely used in antiepileptic therapy. Since its approval in the USA in 1999, it has become one of the most successful therapeutics of the newer classes of antiepileptic drugs (AED). Several beneficial properties including potent antiepileptogenic effects, absence of severe adverse effects, and a low potential for drug interactions[1,2] have indicated that levetiracetam (LEV) acts via a novel, unique mechanism of action, which is distinct from that of conventional AEDs. In 2004, it was discovered that LEV binds to the synaptic vesicle protein SV2A.[3] However, until today it is still unknown how the interaction of LEV with the SV2A protein is translated into its antiepileptic properties. Furthermore, interaction with other yet unknown targets may contribute to LEV's pharmacologic effects.

To study the interaction between drugs and their protein targets, radioligands have been highly useful. LEV has previously been prepared in ^{3}H-labeled form ([^{3}H]LEV) with a specific activity of 36.6 Ci/mmol (1.35 TBq/mmol), which was used for studying LEV binding to SV2A protein in native rat tissues.[4] This radioligand, however, was not ideal for our planned biological investigations because of its relatively low affinity for the SV2A protein (~1 μM) combined with its moderate specific activity. Since the SV2A protein has a very high abundance (expression levels of, e.g., ~9 pmol/mg of protein in rat brain membrane preparations),[4] further potential target proteins (e.g. membrane receptors) that typically show at least 10-fold lower expression levels can hardly be detected with this radioligand. Derivatives of LEV, [^{3}H]ucb 30889 ((2*S*)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide, K_D = 62 nM, 32 Ci/mmol, 1.2 TBq/mmol)[5] and, very recently, [^{3}H]brivaracetam (2*S*-2-[(4*R*)-2-oxo-4-propylpyrrolidin-1-yl] butanamide, K_D = 80 nM, 8 Ci/mmol, 0.3 TBq/mmol),[6] have also been prepared as tritium-labeled radioligands.

In the present study, we aimed at obtaining a tritiated form of the clinically applied drug of this important chemical class of AEDs, [^{3}H]LEV, with a significantly higher specific activity as compared with the previously prepared radioligand. Our goal was to provide an improved tool for studying the interaction of LEV with the SV2A protein, including SV2A mutants, as well as further potential targets of the antiepileptic drug LEV (Figure 1).

Results and discussion

The synthesis of [^{3}H]LEV (**7**) with high specific activity was performed as depicted in Scheme 1. A suitable LEV precursor (compound **6**; see Scheme 1) was prepared for radiolabeling, which would allow for the introduction of four tritium atoms by catalytic hydrogenation. Thus, (*S*)-2-aminobutyric acid **2** was reacted with thionyl chloride in methanol to yield the methyl ester **3** in nearly quantitative yield.[7] The methyl ester was then transformed to the corresponding amide **4** in a microwave reaction using ammonia in methanol. Condensation of **4** with mucochloric acid **5** in the presence of sodium triacetoxyborohydride and catalytic amounts of acetic acid in chloroform yielded lactam **6**,[8] which served as precursor molecule for the generation of the radioligand. Compound **6** was custom-labeled by Quotient Bioresearch, UK, by catalytic hydrogenation with $^3H_2$ gas in the presence of palladium on charcoal as a catalyst. Owing to the exchange of the two chlorine atoms as well as a catalytic reduction of the double bond, it was possible to introduce a maximum of four tritium atoms per molecule and to obtain [^{3}H]LEV with a specific activity of 98 Ci/mmol (3.6 TBq/mmol). Thus, the above-mentioned precursor molecule **6** allows the preparation of a radioligand **7** with very high specific activity, almost 3-fold higher than that of the previously published tritiated LEV.[4]

The binding properties of the radioligand were investigated in radioligand binding assays using rat brain cortical membrane

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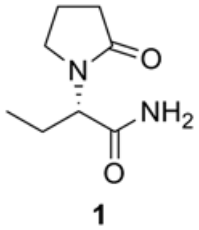
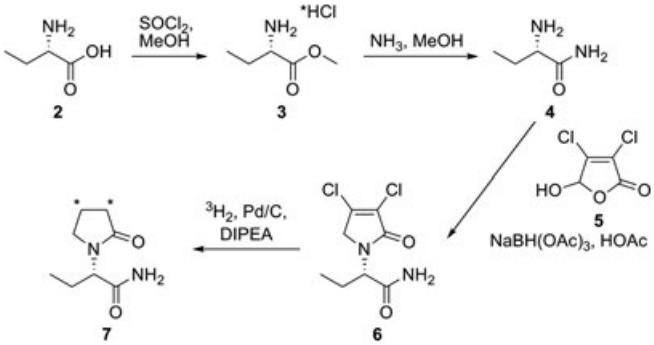


Figure 1. Structure of levetiracetam.



Scheme 1. Synthesis of [^{3}H]levetiracetam.

preparations (Figures 2–4). Nonspecific binding was determined in the presence of a high concentration of unlabeled levetiracetam (1 mM). The obtained results showed that the binding was concentration as well as protein dependent. A protein concentration of 200 μg/assay and a radioligand concentration of 10 nM yielded the best results, affording a specific binding of ~85% (Figure 2). In saturation binding experiments, the radioligand showed saturable binding, with a K_D value of 1.12 ± 0.12 μM and a B_{max} value of 3.8 ± 0.1 pmol/mg protein (Figures 3 and 4), compatible with previously published results.[4]

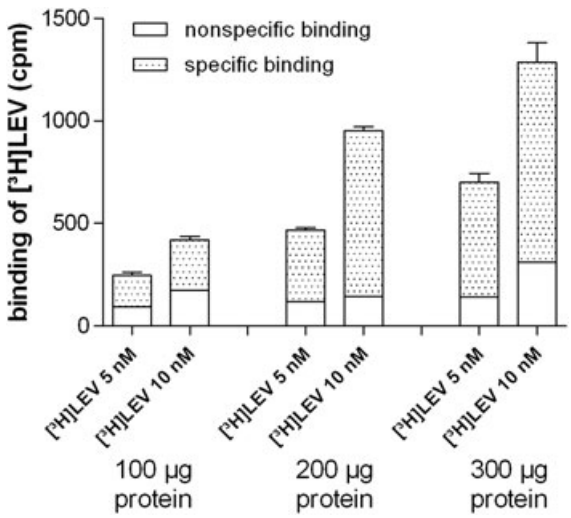


Figure 2. Nonspecific and specific binding (cpm) of [^{3}H]levetiracetam in rat brain cortical membrane preparations: 100, 200, or 300 μg of protein was incubated for 120 min at 4°C with [^{3}H]levetiracetam (5 and 10 nM). Nonspecific binding (open bars) was determined in the presence of unlabeled levetiracetam (1 mM). Specific binding (dotted bars) was obtained by subtraction of nonspecific binding from total binding, which was determined in the absence of unlabeled levetiracetam.

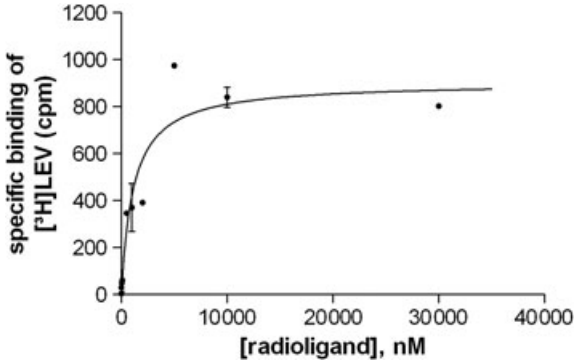


Figure 3. Specific binding of [^{3}H]levetiracetam obtained in saturation binding experiments using rat brain cortical membrane preparations. Incubation was performed for 120 min at 4°C using 200 μg of protein per vial. For this experiment, the radioligand was diluted with unlabeled levetiracetam (isotopic dilution). The graph shows the mean curve of two individual experiments performed in triplicates. A K_D value of 1.12 ± 0.12 μM and a B_{max} value of 3.8 ± 0.1 pmol/mg protein were calculated.

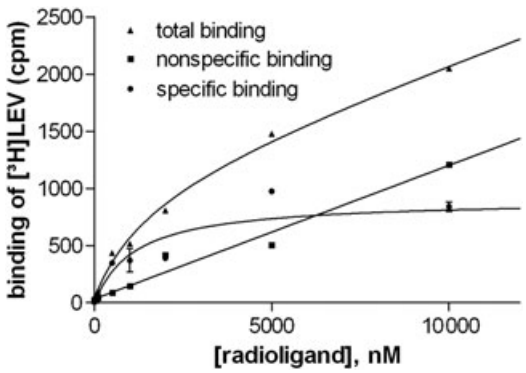


Figure 4. Total, nonspecific, and specific binding (cpm) of [^{3}H]levetiracetam obtained in saturation binding experiments using rat brain cortical membrane preparations. Incubation conditions were as described in Figure 3. The graphs show the mean curves of two individual experiments performed in triplicates.

Experimental

General

All solvents and reagents were obtained from commercial sources and not further purified unless indicated otherwise. The microwave reaction was performed in a Discover microwave (CEM GmbH, Kamp-Lintfort).

Analysis of unlabeled compounds. Analytical thin layer chromatography (TLC) was performed on silica-coated aluminum plates containing a fluorescent indicator (*Merck* silica gel 60 F_{254}), and detection was achieved with UV light (254 nm) and by spraying with Ninhydrin[9] reagent. Low-resolution mass spectra were obtained on an API 2000 mass spectrometer (electrospray ionization, Applied Biosystems, Darmstadt, Germany) coupled to an HPLC system (Agilent 1100) using the following procedure: Compounds were dissolved in methanol (0.5 mg/ml), and a 10-μl sample of this solution was injected into the HPLC system containing a Phenomenex Luna C18 column (50 mm × 2.00 mm, particle size 3 μm). It was chromatographed using a gradient of water/methanol from 90:10 to 0:100 in 30 min. The gradient was started after 10 min; the flow rate was 250 μl/min. UV absorption was detected using a diode array detector (from 190 to 900 nm), and purity was determined at 254 nm. NMR

spectra were recorded on a Bruker Avance 500 spectrometer (^{1}H: 500 MHz, ^{13}C: 125 MHz) at room temperature. Spectra were recorded in $CDCl_3$, and the remaining protons of the deuterated solvent were used as an internal standard (^{1}H: δ (ppm) $CDCl_3$: 7.24 and ^{13}C: δ (ppm) $CDCl_3$: 77.0). Coupling constants are given in hertz (Hz), and chemical shifts in parts per million (ppm). Spin multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad).

Analysis of the radioligand **7**. Analysis was performed by Quotient Bioresearch, UK, by HPLC, using a Luna C18 column (150 × 4.6 mm, particle size 3 μm) applying a gradient of water/methanol from 90:10 to 0:100 in the presence of ammonium acetate (2 mM) within 20 min with a flow rate of 500 μl/min, and by mass spectrometry. Chemical purity was detected at 205 nm.

Chemistry

(S)-Methyl 2-aminobutanoate hydrochloride (**3**)

Freshly distilled thionyl chloride (30 mmol) was added dropwise to 10 ml of methanol previously cooled to −20 °C. After addition of **2** (10 mmol), the mixture was stirred at room temperature. The reaction progress was monitored by TLC, and if needed, additional thionyl chloride was added until the reaction was completed, giving an almost quantitative yield. ^{1}H NMR δ ppm 1.08 (t, 3H, J = 7.43, CH_3), 2.07–2.13 (m, 2H, CH_2), 3.78 (s, 3H, OCH_3), 4.09–4.13 (m, 1H, CH), 8.73 (s, 3H, $NH_3^+Cl^-$). ^{13}C NMR δ ppm 9.63 (CH_3), 23.78 (CH_2), 53.04 (OCH_3), 54.40 (CH), 169.77 (C=O).

(S)-2-Aminobutanamide (**4**)

A sample of **3** (1 mmol) was dissolved in 6 ml of ammonia (7 M) in methanol. The solution was stirred under microwave irradiation (70 W) at 100 °C for 240 min. The mixture was evaporated to dryness, and the residue was purified by column chromatography (gradient of dichloromethane/methanol from 100:0 to 70:30, containing 2% aqueous ammonia solution). Pure fractions were combined, evaporated to dryness, and subsequently dissolved in water and lyophilized (yield 77%). ^{1}H NMR δ ppm 0.95 (t, 3H, J = 7.55, CH_3), 1.52–1.60 (m, 1H + 2H, $CH_2 + NH_2$), 1.78–1.87 (m, 1H, CH_2), 3.28–3.30 (m, 1H, CH), 5.79, 7.03 (2 s, 1H each, $CONH_2$). ^{13}C NMR δ ppm 9.94 (CH_3), 27.97 (CH_2), 56.32 (CH), 178.16 (C=O). Obtained data corresponded to published data.[10]

(S)-2-(3,4-Dichloro-2,5-dihydro-2-oxo-1*H*-pyrrol-1-yl)butanamide (**6**)

Mucochloric acid **5** (2 mmol) and compound **4** (2 mmol) were dissolved in a mixture of 10 ml of chloroform and 0.2 ml of acetic acid. After addition of sodium triacetoxyborohydride (3 mmol, 1.5 equiv.), the mixture was stirred at room temperature for several hours. The reaction progress was monitored by TLC. After approximately 20 h, a saturated solution of ammonium chloride (20 ml) was added to the reaction mixture. The product was extracted from the aqueous phase with chloroform (3 × 20 ml). The organic layers were collected and washed with water (20 ml) and subsequently with brine (10 ml). After drying over sodium sulfate, the solvent was evaporated, and the residue purified by column chromatography eluting with cyclohexane/ethyl acetate (1:4). Yield 75% (lit. yield 62%).[8] ^{1}H NMR δ ppm 0.94 (t, 3H, J = 7.43, CH_3), 1.70–1.79, 1.95–2.04 (2 m, each 1H, CH_2), 4.04, 4.34 (AB-system, 2H, J = 18.9, CH_2-N), 4.55–4.59 (m, 1H, CH), 5.42, 6.17 (2 br, each 1H, NH_2). ^{13}C NMR δ ppm 10.42 (CH_3), 22.42 (CH_2), 50.98 (CH_2-N), 56.39 (CH), 124.73 (CCl-CO), 141.37 (CH_2-CCl), 165.00 (CON), 171.40 ($CONH_2$). LC-MS *m/z* 237 ($[M + H]^+$), $[\alpha]_D^{20} = -21.2$ (c = 0.33, $CHCl_3$).

(S)-[3,3,4,4-^{3}H]2-(2-Oxopyrrolidin-1-yl)butanamide (**7**)

The labeling of **6** with tritium was performed by Quotient Bioresearch using the following procedure: Compound **6** (3 mg) and 10% palladium on charcoal (20 mg) were stirred in ethanol (2 ml) containing *N*,*N*-diisopropylethylamine (100 μl) in the presence of tritium gas (10 Ci) for 4 h. Labile tritium was removed by repeated evaporation to dryness with ethanol. The crude yield was 850 mCi, and the radiochemical purity was 60%. Purification of the radioligand was performed by HPLC (detection at 205 nm), giving a radiochemical purity of 99.7%. The specific activity was determined to be 98 Ci/mmol (3.6 TBq/mmol).

Radioligand binding studies

Radioligand binding studies were performed in analogy to the procedure described by Noyer *et al.*[4] Rat brain cortical membrane preparations (100–300 μg/vial) were incubated for 120 min at 4 °C in a total volume of 500 μl of Tris-HCl buffer solution (50 mM, pH 7.4), containing 2 mM $MgCl_2$ and [^{3}H]LEV (5 and 10 nM, respectively). Nonspecific binding was determined in the presence of unlabeled levetiracetam (1 mM). For saturation experiments, 200 μg of rat brain cortical membrane preparations was incubated with increasing amounts of radioligand, which was diluted with unlabeled levetiracetam (isotopic dilution). Separation of bound from unbound radioligand was achieved by filtration through GF/C glass fiber filters pre-soaked for 30 min in 0.1% aqueous polyethyleneimine solution. The filters were subsequently dried (for 90 min at 50 °C), and the remaining radioactivity was determined by liquid scintillation counting at a counter efficiency of 0.55. Data were analyzed with GraphPad Prism® 5.01 (GraphPad Software, San Diego, CA, USA).

Conclusions

A synthetic route has been devised that allows for the preparation of ^{3}H-labeled levetiracetam with a high specific activity of 98 Ci/mmol (3.6 TBq/mmol), which is nearly 3-fold higher than that of the previously prepared tritiated LEV. The radioligand has been successfully used in initial radioligand binding studies with rat brain cortical membrane preparations and was shown to be a useful tool for the specific labeling of high-affinity binding sites for levetiracetam, which has previously been identified as the vesicular protein SV2A. The described radioligand should be applicable for further investigations on native tissues including human brain samples as well as recombinantly expressed SV2A protein. Moreover, besides determining binding to the SV2A protein, this new radioligand should also be suitable for the detection of potential target proteins of much lower abundance than SV2A, and may thus contribute to getting more insight into the mechanisms of action of LEV.

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References

[1] W. Löscher, D. Hönack, C. Rundfeldt, *J. Pharmacol. Exp. Ther.* **1998**, *284*, 474–479.
[2] S. Shorvon, *Lancet* **2001**, *358*, 1885–1892.
[3] B. A. Lynch, N. Lambeng, K. Nocka, P. Kensel-Hammes, S. M. Bajjalieh, A. Matagne, B. Fuks, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9861–9866.
[4] M. Noyer, M. Gillard, A. Matagne, J.-P. Hénichart, E. Wülfert, *Eur. J. Pharmacol.* **1995**, *286*, 137–146.
[5] M. Gillard, B. Fuks, P. Michel, P. Vertongen, R. Massingham, P. Chatelain, *Eur. J. Pharmacol.* **2003**, *478*, 1–9.
[6] M. Gillard, B. Fuks, K. Leclercq, A. Matagne, *Eur. J. Pharmacol.* **2011**, *664*, 36–44.
[7] M. E. F. Braibante, H. T. S. Braibante, A. F. Morel, C. C. Costa, M. G. Lima, *J. Braz. Chem. Soc.* **2006**, *17*, 184–188.
[8] K. Das Sarma, J. Zhang, Y. Huang, J. G. Davidson, *Eur. J. Org. Chem.* **2006**, *37*, 3730–3737.
[9] V. J. Harding, R. M. MacLean, *J. Biol. Chem.* **1916**, *25*, 337–350.
[10] P. V. R. Acharyulu, C. M. H. Raju, PCT Int. Appl. US 2005/0182262 A1, **2005**.