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Affinity identification of δ-opioid receptors using latex nanoparticles

Makoto Hasegawa,[a] Hiroshi Ohno,[b] Hiroshi Tanaka,[b] Mamoru Hatakeyama,[a] Haruma Kawaguchi,[c] Takashi Takahashi[b,*] and Hiroshi Handa[a,*]

[a]*Frontier Collaborative Research Center and Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuda, Yokohama 226-8501, Japan*
[b]*Department of Applied Chemistry, Tokyo Institute of Technology, Meguro, Tokyo 152-8552, Japan*
[c]*Faculty of Science and Technology, Keio University, Hiyoshi, Yokohama 223-8552, Japan*

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Abstract—Three types of latex nanoparticles carrying naltrindole (NTI) derivatives were synthesized as probes for the affinity isolation of their binding proteins including the δ-opioid receptor. The effect of the attachment of NTI to different positions on the linker was investigated. Only latex nanoparticles in which the NTI derivative was linked through the phenol group were useful for isolating the recombinant δ-opioid receptor solubilized from CHO cell membrane. These latex nanoparticles could be a useful tool for investigations of the pharmacological activity of NTI.

Morphine is a well-known analgesic agent with agonistic properties at μ-opioid receptors, which are located on cell surfaces in the nervous system. Various derivatives that contain the morphine skeleton have been synthesized and have been used as selective antagonists of opioid receptors. Naltrindole (NTI),[1] which contains both a morphine and an indole skeleton, shows a strong and selective antagonist activity against δ-opioid receptors, and is widely used as a pharmacological tool in studies of the role of the δ-opioid system. The *tert*-amine and phenol groups in NTI are important pharmacophores that are responsible for binding to the opioid receptor. Previous studies indicate that the indole moiety in NTI induces binding selectivity to the δ-opioid receptor. In addition, several reports have shown that NTI has various biological activities, that are not related to signal transduction through the activation of the opioid receptors. Hence, the identification of novel receptors that are targeted by NTI could lead to the discovery of new drug candidates.

The affinity concentration[2] of proteins that bind small molecules would help in the identification of their receptor proteins. In a previous study, we reported that latex nanoparticles[3] composed of a styrene–glycidyl methacrylate co-polymer were effective for affinity chromatography and were successfully used in the identification of several candidate receptors of biologically active small molecules from cell lysates using latex nanoparticles.[4] The polyglycidyl surface shows relatively little non-specific adsorption of proteins. This suggests that latex nanoparticles bearing NTI derivatives could eventually be attractive and effective chemical probes for the selective concentration of not only soluble proteins, but also membrane-associated proteins such as opioid receptors. Herein, we describe the synthesis of latex nanoparticles bearing NTI and their use in the selective concentration of a seven-transmembrane receptor.[5]

We designed solid-supported naltrindole derivatives **2**–**4** in which a linker is attached to the latex nanoparticles at three different positions; phenol, *tert*-amine and indole benzene ring as shown in (Scheme 1). An optimally attached linker position could permit target proteins to be concentrated. Our strategy for the synthesis of particles **2**–**4** involves the coupling of naltrindole derivatives **5**–**7** bearing a carboxylic acid in the terminal of the linker with an amino group on the latex nanoparticles **8**, followed by capping the remaining amines with acetyl groups. Capping would be effective for reducing the non-specific binding of proteins to the nanoparticles.

Keywords: Affinity isolation; Naltrindole; Nanoparticles; Opioid receptor.
* Corresponding authors. Tel.: +81 3 5734 2120; fax: +81 3 5734 2884 (T.T.); tel.: +81 45 924 5872; fax: +81 45 924 5145 (H.H.); e-mail addresses: ttak@apc.titech.ac.jp; hhanda@bio.titech.ac.jp

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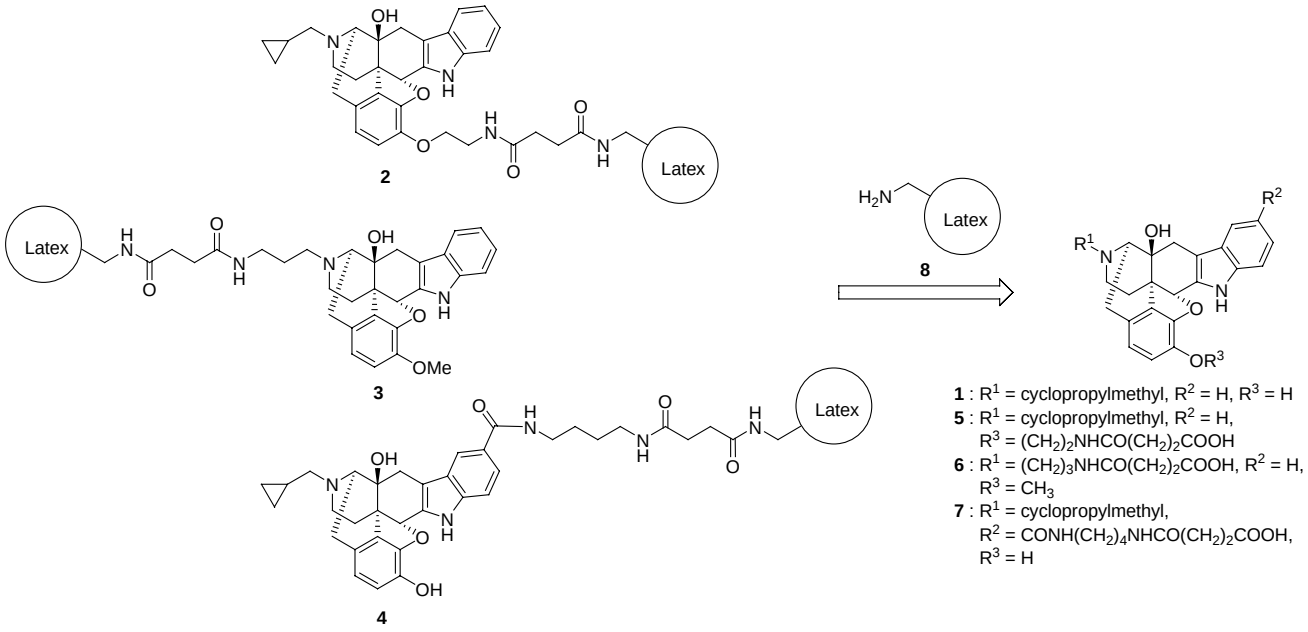
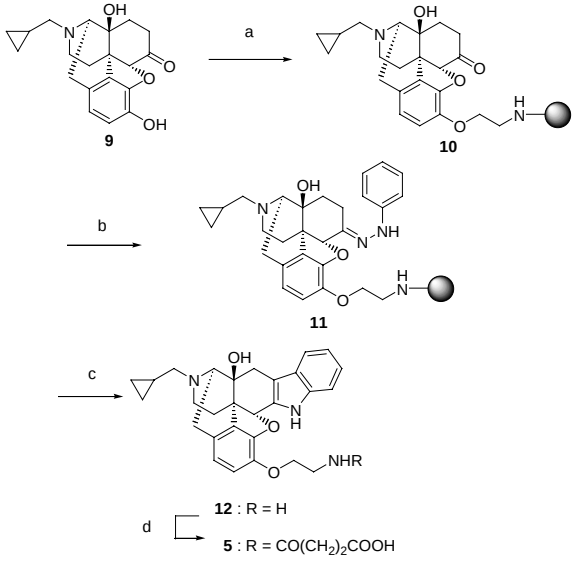


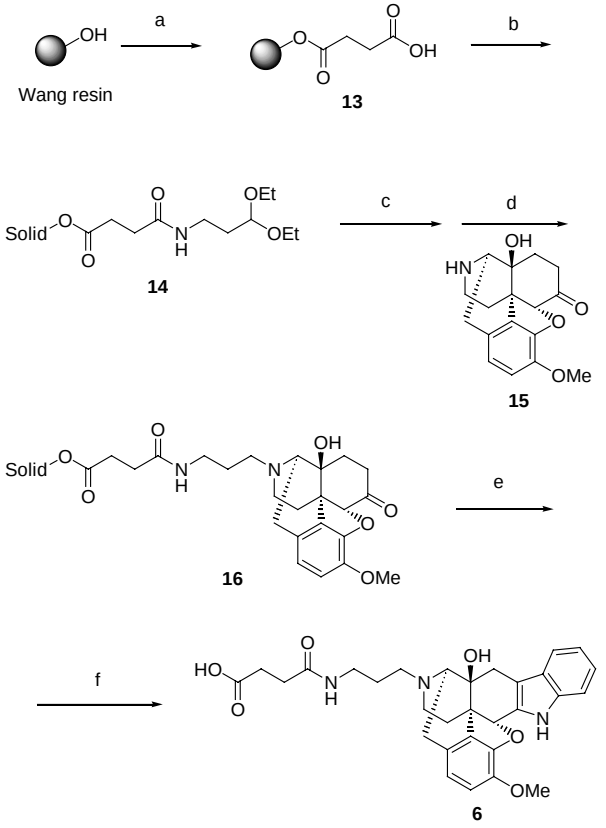
Scheme 1. Strategy for the synthesis of three types of latex nanoparticles containing NTI derivatives.

The preparation of the naltrindole derivative **5** in which a phenol group is attached to the linker is shown in Scheme 2. Commercially available naltrexone **9** was loaded on a 2-hydroxyethylamino 2-chlorotrityl resin (0.17 mmol/g)[6] by the Mitsunobu reaction. The extent of loading was estimated by cleavage of resin **10** with 10% TFA/CH_2Cl_2 and found to be 65 μmol/g. Treatment of ketone **10** with phenylhydrazine gave the solid-supported hydrazone **11**, followed by cleavage by treatment with 10% TFA/CH_2Cl_2 to afford the NTI derivative **12**[7] in 20% yield from resin **10**. The amine **12** was converted to the acid **5** by acylation with succinic anhydride.



Scheme 2. Reagents and conditions: (a) 2-hydroxyethylamino 2-chlorotrityl resin, DEAD, PPh_3, THF, rt, 15 h; (b) phenyl hydrazine, 1% AcOH/DMF, rt, 3 h; (c) 10% TFA/CH_2Cl_2, rt, 1 h, 20% from **10**; (d) 40 mM succinic anhydride, 10 % triethylamine/dioxane, rt, 30 min. **12**: R = H, **5**: R = $CO(CH_2)_2COOH$.

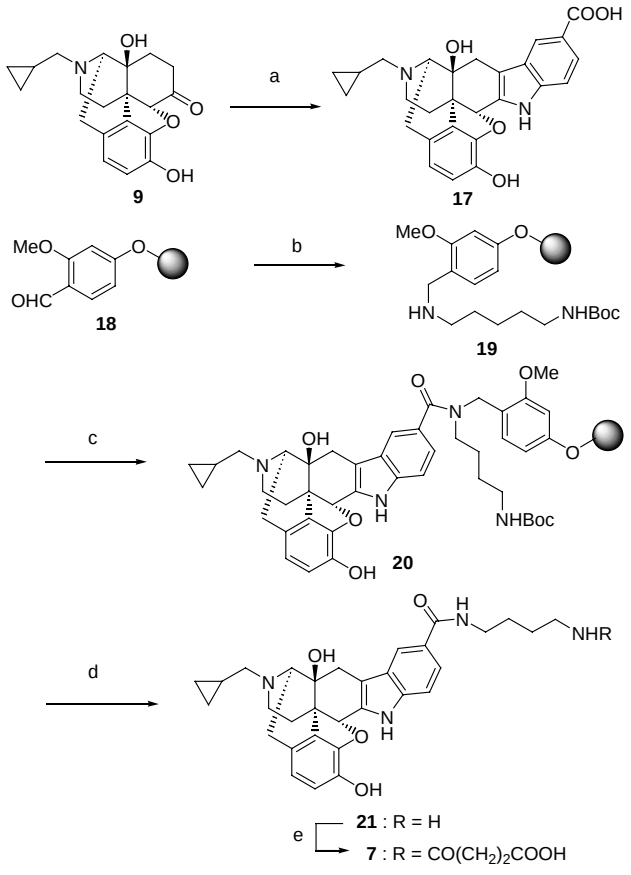
NTI derivative **6** was prepared by employing a solid-phase synthesis using one-pot release and cyclization methodology[8] as shown in Scheme 3. The loading of



Scheme 3. Reagents and conditions: (a) succinic anhydride, DMAP, CH_2Cl_2, 60 °C, 5 h; (b) 3-aminopropionaldehyde diethylacetal, HOBt H_2O, diisopropylethylamine, DMF, rt, 40 h; (c) 1 M HCl aq/THF (1/5), rt, 2 h; (d) $NaBH_3CN$, 1% AcOH/DMF, rt, 16 h; (e) phenylhydrazine, AcOH/CH_2Cl_2 (1/1), rt, 6 h; (f) 5% TFA/CH_2Cl_2, rt, 3 h.

succinic anhydride onto the Wang resin (1.22 mmol/g) was achieved using DMAP in CH_2Cl_2 at 60 °C to provide the solid-supported carboxylic acid **13**. The loading was confirmed by the appearance of the IR absorption of the ester group (1717 cm^{-1}). 3-Aminopropionaldehyde diethylacetal was coupled with solid-supported carboxylic acid **13** using HOBt and DIEA in DMF to afford the diethylacetal resin **14**. Deprotection of the solid-supported acetal **14** by treatment with 1 M HCl/THF, followed by reductive amination with commercially available noroxycodone (**15**) using $NaBH_3CN$ in 1% AcOH–DMF, provided the solid-supported ketone **16**. Hydrazone formation of the solid-supported ketone **16** by treatment with phenylhydrazine in AcOH–CH_2Cl_2 (1:1), and subsequent exposure of the solid-supported phenylhydrazone to 5% TFA in CH_2Cl_2, gave the NTI derivative **6**[9] in 2% yield from the Wang resin.

The preparation of the NTI derivative **7** is shown in Scheme 4. NTI derivative **17**, containing a carboxylic acid at the 5′ position of the indole ring, was prepared from the commercially available naltrexone (**9**) by the Fischer indole synthesis using 4-carboxylphenylhydrazine in AcOH at reflux. *N*-(4-Aminobutyl)carbamic acid *tert*-butyl ester was coupled with 4-(4-formyl-3-methoxyphenoxy)butyryl aminomethyl polystyrene **18**[10] (0.94 mmol/g) by reductive amination with $NaBH(OAc)_3$ in 1% AcOH in DMF to afford the secondary amine resin **19**. Conversion of the carboxylic acid **17** with the solid-supported amine **19** to an amide was accomplished using standard conditions (DIC and HOBt) to give resin **20**. The cleavage of the product from the resin, followed by simultaneous deprotection of the Boc group, was achieved by treatment with 10% TFA/CH_2Cl_2 affording amine **21**[11] in 23% yield from the resin **18**. The amino group of **21** was converted to derivative **7** by treatment with succinic anhydride.



Scheme 4. Reagents and conditions: (a) 4-carboxylphenylhydrazine, AcOH, 130 °C, 5 h, 12%; (b) *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester, $NaBH_3CN$, 1% AcOH/DMF, rt, 60 h; (c) **17**, DIC, HOBt H_2O, DMF, rt, 40 h; (d) 10% TFA/CH_2Cl_2, rt, 0.5 h, 23% from **18**; (e) 40 mM succinic anhydride, 10% triethylamine/dioxane, rt, 30 min.

Latex nanoparticles **2**–**4** were prepared by coupling with the carboxylic acid derivatives **5**–**7** with an amino group on latex nanoparticles **8** in the presence of 30 mM each of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide with 2–10 mM concentrations of **5**–**7** in DMF (500 μl) for 5 h room temperature, followed by acetylation of the remaining amino groups with acetic anhydride. The capping of any remaining amino groups by acetylation was effective for reducing non-specific interactions of proteins with the particles. The acetyl groups were removed from the phenolic hydroxyl group and amino group in the indole skeleton by treatment with 0.1 M sodium hydroxide for 30 min. The immobilization of each derivative to the nanoparticles was confirmed by LC–MS analysis of the acid degradation product from **2** to **4**.

To demonstrate the feasibility of using latex particles, the affinity concentration of the δ-opioid membrane-associated protein was determined. Based on previously reported structure–activity relationships, it would be difficult to determine the most suitable particles for use in affinity concentration. The results of some affinity binding experiments of the nanoparticles carrying three NTI derivatives using cell membrane extracts of CHO cells carrying the recombinant δ-opioid receptor[12] are shown in Figure 1. Membrane-associated proteins were extracted using a surfactant using a previously reported method, with minor modifications.[13] Nanoparticles **2**–**4** and control nanoparticles, which contained no ligands, were mixed with the crude cell extracts. Each binding fraction was eluted by heat denaturation into a buffer containing sodium dodecyl sulfate (SDS) for the following SDS–PAGE analysis. SDS–PAGE and a subsequent Western blot analysis using an anti-δ-opioid receptor antibody revealed that the δ-opioid receptor was present in the

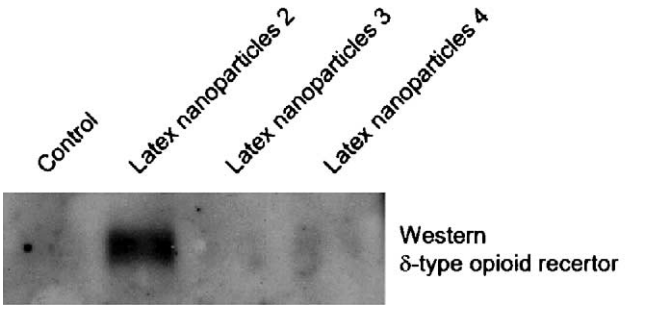


Figure 1. Elucidation of NTI derivative specific binding proteins in CHO cell carrying recombinant δ-opioid receptor (DOR). The 40–50 kDa molecular weight range was visualized by an anti-DOR antibody.

binding fraction of nanoparticles **2**. On the other hand, the control and the latex nanoparticles **3** and **4** showed no binding of δ-opioid receptor. These data suggest that the environment around the *tert*-amine in NTI is critical for direct binding with the δ-opioid receptor and/or that the site on NTI that binds to the δ-opioid receptor is limited to a narrow region including the *tert*-amine. In addition, these findings indicate that the phenol of NTI interacts with the δ-receptor near the entrance of the binding pocket instead of the bottom of the pocket. The results also show that a phenolic hydroxyl group is not an essential pharmacophore for binding the δ-opioid receptor.

In conclusion, the synthesis of latex nanoparticles carrying the three NTI derivatives, which have three different sites for coupling to nanoparticles, is described. Only the latex nanoparticles carrying the NTI derivative, which contains the linker on the phenolic part and have a *tert*-amine group facing the outside, was useful for isolating the δ-opioid receptor. Recently, several reports have proposed that unknown NTI receptors may exist.[14] Latex nanoparticles carrying NTI may be a useful tool, not only for the isolation of known δ-opioid receptors, but also for the elucidation of unique NTI receptors, which could be either a membrane-associated protein or a cytosolic protein. These three latex nanoparticles may be useful tools for a study of novel NTI receptors. Investigations in this regard are currently underway.

Acknowledgments

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6. 2-Chlorotrityl resin was purchased from Novabiochem, Switzerland.
7. Compound **12**: ^{1}H NMR (300 MHz, CD_3OD): δ 0.24–0.32 (2H, m), 0.58–0.68 (2H, m), 0.99 (1H, m), 1.76–1.79 (1H, m), 2.41–2.54 (2H, m), 2.57–2.70 (2H, m), 2.61 (1H, d, J = 15.1 Hz), 2.83–2.90 (1H, m), 2.87 (1H, d, J = 15.9 Hz), 2.98 (1H, dd, J = 19.0, 6.3 Hz), 3.08–3.10 (2H, m), 3.25–3.30 (1H, m), 3.62 (1H, d, J = 6.3 Hz), 4.09–4.20 (2H, m), 5.69 (1H, s), 6.68 (1H, d, J = 8.1 Hz), 6.75 (1H, d, J = 8.3 Hz), 6.95 (1H, dd, J = 8.1, 7.0 Hz), 7.09 (1H, dd, J = 8.1, 7.1 Hz), 7.31 (1H, d, J = 8.3 Hz), 7.38 (1H, d, J = 8.1 Hz), 8.54 (0.6H, s); ^{13}C NMR (75.4 MHz, CD_3OD)(HCOOH salt): δ 4.1, 5.2, 9.5, 24.5, 30.0, 32.0, 41.0, 45.5, 49.5, 60.0, 63.4, 68.9, 74.1, 86.5, 110.9, 112.1, 118.7, 119.4, 119.8, 120.2, 123.3, 127.8, 128.1, 130.0, 132.4, 138.7, 142.6, 145.9, 170.0; HRMS (ESI) 458 $(M+H)^+$, calcd: 458.2444. Found: 458.2413.
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9. Compound **6**: ^{1}H NMR (300 MHz, CD_3OD): δ 1.78–2.04 (3H, m), 2.37–2.52 (3H, m), 2.56–2.63 (2H, m), 2.65 (1H, d, J = 15.9 Hz), 2.73–2.87 (2H, m), 2.97 (1H, d, J = 16.1 Hz), 3.12–3.27 (4H, m), 3.41–3.52 (2H, m), 3.75 (3H, s), 3.84 (1H, d, J = 6.3 Hz), 5.73 (1H, s), 6.74 (1H, d, J = 8.3 Hz), 6.78 (1H, d, J = 8.3 Hz), 6.94–6.98 (1H, m), 7.08–7.12 (1H, m), 7.32 (1H, d, J = 8.3 Hz), 7.37 (1H, d, J = 7.8 Hz), 8.51 (0.6H, s); ^{13}C NMR (75.4 MHz, CD_3OD)(HCOOH salt): δ 24.8, 26.1, 30.3, 30.4, 32.9, 33.1, 35.8, 47.5, 48.0, 49.5, 49.7, 50.9, 57.3, 63.2, 73.8, 85.6, 109.6, 112.2, 116.7, 119.2, 119.8, 120.2, 123.4, 124.6, 127.7, 130.1, 130.8, 138.8, 145.0, 145.9, 177.0; HRMS (ESI) 532 $(M+H)^+$, calcd: 532.2448. Found: 532.2430.
10. 4-(4-Formyl-3-methoxyphenoxy)butyryl AM resin was purchased from Novabiochem, Switzerland.
11. Compound **21**: ^{1}H NMR (300 MHz, CD_3OD): δ 0.52–0.58 (2H, m), 0.76–0.90 (2H, m), 1.16 (1H, m), 1.71–1.73 (4H, m), 1.94 (1H, m), 2.71–2.79 (1H, m), 2.73 (1H, d, J = 16.8 Hz), 2.90–3.03 (4H, m), 3.07 (1H, d, J = 16.1 Hz), 3.17–3.21 (1H, m), 3.32–3.50 (5H, m), 4.25 (1H, d, J = 6.3 Hz), 5.72 (1H, s), 6.66 (1H, d, J = 8.1 Hz), 6.69 (1H, d, J = 8.1 Hz), 7.40 (1H, d, J = 8.8 Hz), 7.64 (1H, dd, J = 8.8, 1.7 Hz), 8.02 (1H, d, J = 1.5 Hz); ^{13}C NMR (75.4 MHz, CD_3OD) δ 3.5, 6.4, 7.0, 25.1, 26.0, 27.7, 29.9, 30.3, 40.1, 40.5, 47.6, 48.1, 58.9, 63.7, 73.6, 84.8, 110.7, 112.1, 119.3, 119.8, 120.5, 122.5, 122.5, 126.0, 127.4, 130.0, 132.0, 140.5, 141.9, 144.6, 171.2; HRMS (ESI) 529 $(M+H)^+$, calcd: 529.2815. Found: 529.2808.
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