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Targeted drug delivery crossing cytoplasmic membranes of intended cells via ligand-grafted sterically stabilized liposomes

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Abstract

In this study, we tested whether sterically stabilized liposomes (SSL) with surface ligands specific for the mu opioid receptor (MOR) can actively target MOR-expressing cells. Dermorphin, a selective MOR agonist, was conjugated to DSPE-PEG$_{3400}$ to obtain DSPE-PEG$_{3400}$-dermorphin. Dermorphin-grafted SSL (dermorphin-SSL) was prepared by thin-film rehydration–extrusion and post-insertion method. DSPE-PEG$_{3400}$-dermorphin and dermorphin-SSL retained the affinity to MOR as determined by receptor binding assay using [^{3}H]DAMGO, whereas plain SSL without surface ligands showed no binding to the receptor. Cellular uptake of cholesteryl BODIPY encapsulated dermorphin-SSL was studied by microplate spectrofluorometry as well as fluorescent and confocal microscopy. Significant fluorescence signal was observed inside CHO-hMOR cells after the treatment with dermorphin-SSL, indicative of MOR-mediated endocytosis. In contrast, no uptake of dermorphin-SSL was found in naive CHO cells or CHO-hDOR cells that lack MOR. Taken together, these results demonstrate that dermorphin-SSL delivery system is capable of targeting intracellular components of MOR-expressing cells. Such a system may be applied to carry pharmaceutical agents to achieve region-specific delivery of analgesics and/or to attenuate side effects associated with opioids.
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1. Introduction

Pain especially that associated with cancer and neuropathy is extremely difficult to treat. Opioids, such as morphine, remain to be most efficacious analgesics. Repeated and prolonged use of these drugs is problematic as tolerance and drug dependence begin to occur, which drastically limits the effectiveness and application of these drugs. Extensive research has been carried out over the past several decades to understand the mechanisms underlying opioid tolerance and dependence. Whereas the exact mechanisms are yet to be completely defined, a number of novel targets, many intracellular, have been identified to attenuate opioid tolerance and dependence (e.g., PKC, CaMKII, CREB, etc) [1–7]. Despite these advances, it would be unlikely that these intracellular sites can be reached by conventional delivery methods. Moreover, region-specific intervention is advantageous to achieve specificity and reduce side effects. Developing therapeutically useful agents targeting these novel sites will require target-delivery methods.

Great progress has been made in targeted drug delivery area that it is possible to deliver agents (including peptides or nucleotides) to selected cells to modulate the intended intracellular targets. One strategy is to apply surface ligands to conventional [8–12] or sterically stabilized liposomes (SSL) [13–19] to achieve cell-specific targeting. Coupling of targeting peptides to liposomes has been widely used for targeting drug delivery [8,9,11,15,16,19]. Liposomal delivery systems provide high capacity of drug payload and drug stability, which may become especially beneficial for delivering peptides and nucleotides. Compared with conventional liposomes, SSL has advantages of longer circulating half-life and non-immunogenicity, which can improve the therapeutic efficacy of the

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encapsulated drugs. Addition of liposome surface ligands provides liposomes with the ability of targeting a specific cell population by recognizing receptors expressed on the targeted cells with high selectivity. Such a method, when in conjunction with receptor-mediated endocytosis, can be particularly powerful to deliver drugs across cytoplasmic membranes.

The mu opioid receptor (MOR), a member of G-protein-coupled receptors (GPCR), is primarily responsible for the analgesic and rewarding effects of opioids [20,21]. Targeting the receptor provides a unique opportunity for improving pain treatment by modulating the analgesic and addictive potential of opioid drugs. Activation of MOR by its agonists such as DAMGO and dermorphin is known to cause internalization of the receptor–ligand complex [22–24]. In fact, saporin, a cell impermeable cytotoxic agent, has been directly conjugated to dermorphin. Unlike saporin, dermorphin–saporin is capable of entering and killing MOR-expressing cells in rats [25]. However, it is advisory to simply eliminate MOR-expressing cells for most clinical therapies. In addition, dermorphin–saporin (1 : 1) conjugate provides relatively low drug payload.

In this study, we tested a dermorphin-grafted SSL system (dermorphin-SSL) to specifically target cells expressing MOR. We hypothesize that dermorphin-SSL can recognize cell-surface MOR and be internalized via MOR-mediated endocytosis, thus it is capable of delivering encapsulated pharmaceutical agents to reach intracellular targets.

2. Materials and methods

2.1. Materials

Egg-phosphatidylcholine (PC), cholesterol (CH), polyethylenimine (PEI) and bovine serum albumin were purchased from Sigma (St. Louis, MO). Dipalmitoyl phosphatidyl-glycerol (DPPG) was from Sygena (Switzerland). 1,2-distearoylglycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000] (DSPE-PEG$_{2000}$) was obtained from Shearwater Polymers Inc. (Huntsville, AL). DSPE-PEG$_{3400}$-maleimide (DSPE-PEG$_{3400}$-MAL) was purchased from Avanti Polar Lipids (Alabaster, AL). Cholesteryl BODIPY (fluorescent probe) was from Molecular Probes Inc. (Portland, OR). Dermorphin, DAMGO ([D-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin) and [^{3}H]DAMGO were provided by Multiple Peptide Systems (San Diego, CA). Dulbecco's modified eagle medium, Ham's F-12, penicillin and streptomycin, G418 and hygromycin B were from Cellgro (Herndon, VA). Newborn calf serum was from BioWhittaker Inc. (Walkersville, MD).

2.2. Cell culture

Chinese hamster ovary cells (CHO, ATCC, Manassas, VA) stably transfected with human mu opioid receptors (CHO-hMOR) [26,27], and CHO cells stably transfected with human delta opioid receptors (CHO-hDOR) [28,29] were cultured in 1 : 1 Dulbecco's modified eagle medium (DMEM) and Ham's F-12 supplemented with 10% newborn calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. To maintain stable selection, 200 μg/ml G418 or hygromycin B was added to CHO-hMOR or CHO-hDOR cells, respectively. Cells were cultured in incubators maintained at 37 °C with 5% CO_2 in humidified air.

2.3. Synthesis of DSPE-PEG$_{3400}$-dermorphin

First a modified dermorphin peptide (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-Cys) was synthesized by the Fmos solid-state peptide synthesis method using a Symphony peptide synthesizer (Protein Technologies, Tucson, AZ). The crude peptide was purified on a reversed-phase Vydac 218TP1010 C18 column (Hesperia, CA) using a HP1100 HPLC system (Agilent Technologies, Wilmington, DE). A flow rate of 5 ml/min using solvent A (0.1% TFA in Milli-Q water) and solvent B (0.1% TFA in acetonitrile) was used. The column was equilibrated with 5% solvent B. After sample injection, the column was eluted with a linear gradient from 5% solvent B to 100% solvent B in 60 min. The pure peptide fraction was identified by a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. Cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix for mass spectrometric analysis of the peptide product. Samples were mixed 1 : 1 with the matrix solution (10 mg CHCA in 1 ml aqueous solution of 50% acetonitrile containing 0.1% TFA). Aliquots (1.3 μl) were spotted onto a MALDI-TOF target plate and analyzed by a Voyager-DE PRO Mass Spectrometer (Applied Biosystems, Foster City, CA) equipped with a 337 nm pulsed nitrogen laser. The verified peptide was conjugated at room temperature overnight to the activated DSPE-PEG (DSPE-PEG$_{3400}$-MAL), at a molar ratio of 1 : 10 in PBS containing 5 mM EDTA. The conjugate was purified by HPLC and structurally verified by mass spectrometry using the above protocol. The masses of the peptide and DSPE-PEG$_{3400}$-dermorphin conjugate were measured using a positive-ion linear mode over the m/z 1000–6500. External mass calibration was performed using peaks of a mixture of bradykinin fragments 1–7 at 757 Da, angiotensin II (human) at 1046 Da, P14R (synthetic peptide) at 1533 Da, adrenocorticotropic hormone fragment 18–39 (human) at 2465 Da, insulin oxidized B (bovine) at 3494 Da, and insulin (bovine) at 5735 Da.

2.4. Preparation of dermorphin-SSL

Dermorphin-SSL was prepared by thin-film rehydration–extrusion and post-insertion method described previously [16,30,31]. Briefly, egg PC, DPPG, DSPE-PEG$_{2000}$, and cholesterol were dissolved in the mixture of chloroform and methanol (9 : 1 v/v) at the molar ratios of 0.50 : 0.10 : 0.03 : 0.35 and dried to a thin film in a round bottom flask using a rotary evaporator at 45 °C, 90 rpm, 600 mm Hg pressure under Argon. For fluorescent dermorphin-SSL preparation, a non-exchangeable fluorescent probe, cholesteryl BODIPY, was incorporated in the lipid mixture at 1 : 1500 molar ratio (probe : lipid). Complete dryness was achieved by desiccation under vacuum overnight. The dry lipid film was hydrated in 0.01 M isotonic HEPES buffer (pH 7.4) and then vortexed and

sonicated. The formed SSL (plain SSL) was extruded through a 100 nm pore size polycarbonate filter using a Liposofast extruder (Avestin, Canada). DSPE-PEG_{3400}-dermorphin was dissolved in pH 6.6 HEPES buffer and then inserted into preformed SSL by incubation for 3 h at 37 °C to obtain dermorphin-SSL. Free DSPE-PEG_{3400}-dermorphin was removed by passing SSL through an EconPac 10DG desalting column (Bio-Rad, Hercules, CA).

2.5. Characterization of dermorphin-SSL

The size of dermorphin-SSL was determined by quasi-elastic light scattering (QELS) method using a NICOMP Particle Sizer Model 370 (Particle Sizing Systems, Santa Barbara, CA). The phospholipid content of dermorphin-SSL was measured by the modified Bartlett phosphate assay [32]. The concentration of DSPE-PEG_{3400}-dermorphin in liposomes was determined by receptor binding assay using [^{3}H]DAMGO. The number of dermorphin molecules per liposome was then determined as DSPE-PEG_{3400}-dermorphin concentration divided by liposome concentration that was estimated according to the relationship between the known phospholipid concentration and liposome size [33].

2.6. In vitro receptor binding assay

Receptor binding assay was performed based on the method previously described [22,35]. Briefly, membranes were prepared from CHO-hMOR cells by Polytron homogenization at setting 6 for 2 min on ice, followed by centrifugation at 20,000 *g* for 30 min at 4 °C. Protein content was determined by the Coomassie protein assay method (Pierce Biotechnology, Rockford, IL) using bovine serum albumin as the standard. MOR receptor binding was conducted in triplicate with 1 nM [^{3}H]DAMGO in 50 mM Tris HCl buffer (pH 7.4) at 30 °C for 1 h (50 μg protein/reaction). Nonspecific binding was determined in the presence of 20 μM unlabelled DAMGO. Reactions were terminated by rapid vacuum filtration through GF/B filters presoaked with 0.2% polyethylenimine. Filter-bound radioactivity was determined by liquid scintillation counting (Beckman Coulter Inc., Fullerton, CA). Binding data representing the mean ± SD were analyzed using Prism program (GraphPad Software, San Diego, CA).

2.7. Cellular uptake of dermorphin-SSL

CHO-hMOR cells were plated into 24-well cell culture dishes at an initial density of 200,000 cells/well and grown for 24 h under the condition described above. Before experiments, cell monolayers were rinsed with serum-free growth medium for 3 times. Cells were then incubated in serum-free medium containing fluorescent dermorphin-SSL or plain SSL (final phospholipid concentration: 65 μM) for 0, 0.5, 1, 2, 4 and 8 h at 37 or 4 °C. Two temperatures were used in order to differentiate receptor binding and internalization. At 4 °C, it was expected that no receptor internalization occurred, whereas both receptor binding and internalization took place at 37 °C [22,34]. At the end of incubation, the cells were washed three times with ice-cold PBS before the cells were scraped off and lysed in the lysis buffer (1% triton X-100 in PBS). The lysate was vortexed and then centrifuged at 20,000 *g* for 15 min at 4 °C. The supernatant was collected and measured for fluorescence intensity using a SpectraMAX Gemini XS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA). To convert the fluorescence intensity to the number of liposomes, a standard curve was constructed by measuring the fluorescence intensity of cell-lysis buffer containing known numbers of liposomes.

2.8. Fluorescent imaging

CHO, CHO-hMOR and CHO-hDOR cells were seeded onto the 12-mm diameter circle coverslips precoated with 0.2% gelatin at a density of 50,000 cells/well and cultured 24 h before the experiment. To study receptor-mediated dermorphin-SSL uptake, cells were treated with fluorescent dermorphin-SSL or plain SSL (65 μM phospholipids) in serum-free growth medium for 4 h. Then cells were rinsed three times with phosphate-buffered saline and fixed with 3.7% paraformaldehyde for 10 min. Thereafter, coverslips containing cells were washed three times with phosphate-buffered saline and once more with deionized water. Coverslips were mounted onto slides using fluorescence mounting medium (Vector Laboratories, Inc., Burlingame, CA) and examined for fluorescent signals using Olympus IX70 inverted fluorescence microscope (Olympus Corp., Lake Success, NY) and Carl Zeiss Laser Scanning System 510 (Carl Zeiss MicroImaging, Thornwood, NY) [35].

3. Results

3.1. Synthesis of DSPE-PEG_{3400}-dermorphin

DSPE-PEG_{3400}-dermorphin was synthesized by a single step reaction of DSPE-PEG_{3400}-MAL with modified dermorphin peptide (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-Cys) as shown in Fig. 1. The reaction was carried out at room temperature overnight in phosphate-buffered saline containing 5 mM EDTA. DSPE-PEG_{3400}-dermorphin was purified by HPLC and structurally confirmed by mass spectrometry. The peak at 5200 mass–charge ratio at a charge of 1 verified that the mean molecular weight of DSPE-PEG_{3400}-dermorphin was 5200 Da (Fig. 2), which was in agreement with the calculated molecular weight of the conjugate. The sequences of the modified dermorphin and DSPE-PEG_{3400}-dermorphin were also confirmed by amino acid analysis. The final yield for the coupling reaction between DSPE-PEG_{3400}-MAL and the modified dermorphin was over 90%.

3.2. Affinity of DSPE-PEG_{3400}-dermorphin to MOR

The affinities of dermorphin and DSPE-PEG_{3400}-dermorphin were determined in CHO-hMOR cells by radioligand receptor binding assay. Dermorphin and DSPE-PEG_{3400}-

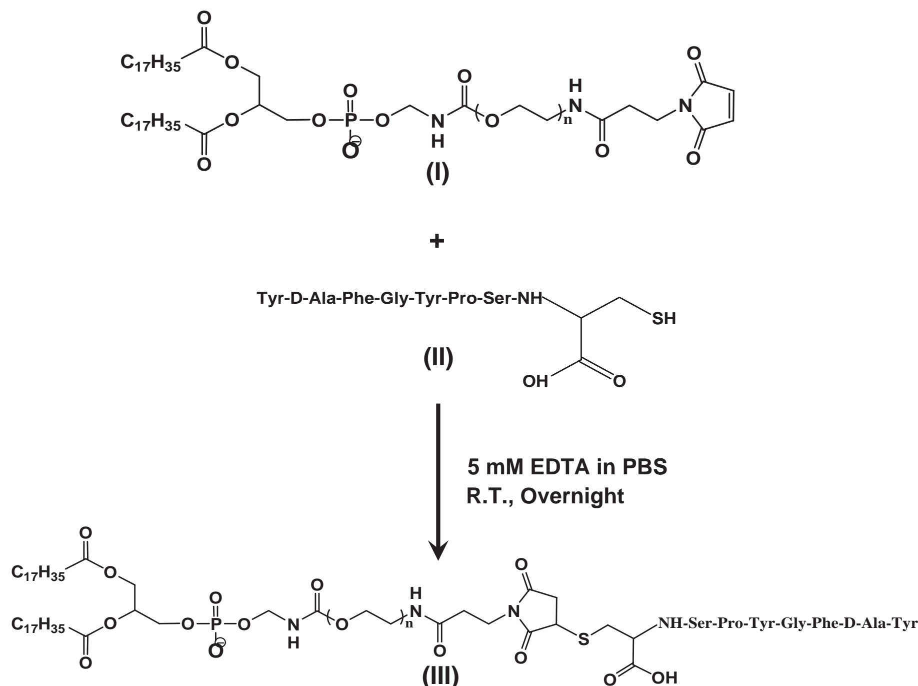

Fig. 1. The synthesis scheme of DSPE-PEG$_{3400}$-dermorphin. The conjugate was synthesized by coupling of the thiol group of modified dermorphin and the maleimide of DSPE-PEG$_{3400}$-MAL. (I) DSPE-PEG$_{3400}$-MAL; (II) modified dermorphin with cysteine residue at C-terminus; (III) DSPE-PEG$_{3400}$-dermorphin.

dermorphin showed identical binding property and their displacement curves were nearly superimposed. Both dermorphin and DSPE-PEG$_{3400}$-dermorphin completely displaced the binding of [^{3}H]DAMGO, a selective MOR agonist, to hMOR (Fig. 3). The IC50 values were determined to be 1.9 ± 0.6 nM and 1.6 ± 0.3 nM (S.D.) for DSPE-PEG$_{3400}$-dermorphin and dermorphin, respectively (not statistically different). These data indicated that DSPE-PEG modification of dermorphin did not alter its affinity to hMOR.

3.3. Characterization of dermorphin-SSL

After determining the retained affinity of DSPE-PEG$_{3400}$-dermorphin to MOR, DSPE-PEG$_{3400}$-dermorphin was micellized and incubated with preformed SSL to prepare dermorphin-grafted SSL as described in the method. The mean diameter of dermorphin-SSL was determined to be 150 ± 30 nm (S.D.) by the analysis of volume- or intensity-weighted distribution using NICOMP. The size of dermorphin-SSL exhibited the Gaussian distribution. Following gel filtration to remove DSPE-PEG$_{3400}$-dermorphin unincorporated into the phospholipid bilayer of SSL, the phospholipid content of dermorphin-SSL was determined to be 4.44 mM by the modified Bartlett phosphate assay and the number of dermorphin molecules per liposome was calculated to be around 250.

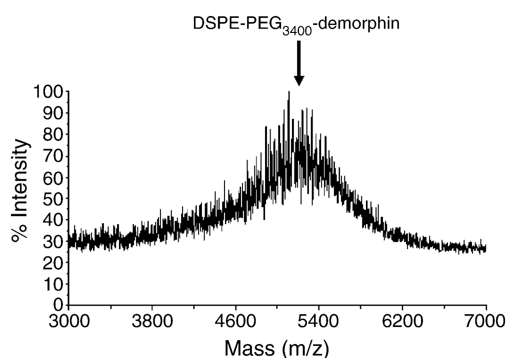

Fig. 2. Mass spectrum of DSPE-PEG$_{3400}$-dermorphin. The mean molecular weight of DSPE-PEG$_{3400}$-dermorphin was determined to be around 5200 Da as indicated by the arrow.

3.4. Affinity of dermorphin-SSL to MOR

In order to determine whether dermorphin-SSL could still bind to the mu opioid receptor, a series of different concentrations of dermorphin-SSL were used to compete the binding of

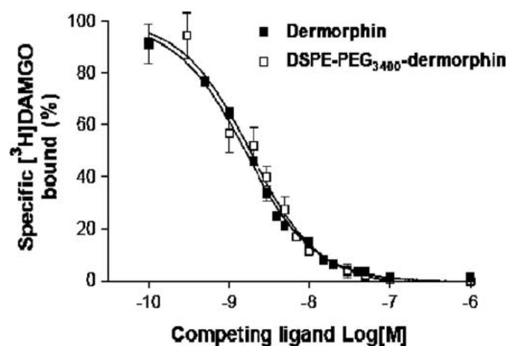

Fig. 3. Displacement binding of [^{3}H]DAMGO (1 nM) by dermorphin (■) or DSPE-PEG$_{3400}$-dermorphin (□) to human mu opioid receptors expressed in CHO cells. The specific radioactivity of [^{3}H]DAMGO bound to hMOR in the absence of competing ligands was set to 100%. There was no significant difference between the two groups ($P > 0.05$, two-tailed Student's t-test). Each point represents the mean ± S.D. of three experiments (each performed in triplicate).

[^{3}H]DAMGO to CHO-hMOR (Fig. 4). At high concentrations, dermorphin-SSL completely displaced the [^{3}H]DAMGO binding, indicating that dermorphin-SSL retained its receptor affinity. In contrast, plain SSL without surface ligands was not able to compete the binding of [^{3}H]DAMGO to CHO-hMOR (Fig. 4). Therefore, only dermorphin-grafted liposomes were able to recognize and bind to MOR.

3.5. Cellular uptake of dermorphin-SSL

To study the kinetics of liposome uptake by MOR-expressing cells, CHO-hMOR cells were incubated with fluorescent dermorphin-SSL or plain SSL containing 65 μM phospholipids for various time periods. To distinguish cell membrane-bound from internalized liposomes, CHO-hMOR cells were incubated at either 4 or 37 °C. At 37 °C, the apparent uptake of dermorphin-SSL (including membrane-bound and internalized) increased rapidly during the first hour of incubation in a linear manner (Fig. 5). The uptake increased only slightly over the next 7 h, suggesting that the system was saturated. In contrast, very little uptake of dermorphin-SSL occurred at 4 °C. Since receptor internalization was not expected to occur at 4 °C, cell-associated liposomes at this temperature were due to the binding of dermorphin-SSL to the cell-surface receptors. Therefore, the different amount of cell-associated liposomes at either temperature could be used to estimate the number of internalized liposomes. For example, 2.5×10^4 liposomes were estimated to be internalized into each cell after 4 h incubation, representing approximately 81% of total cell-associated liposomes. These data suggested that a majority of cell-associated liposomes were taken up by the internalization mechanism. However, there was no cellular uptake of plain SSL at either temperature (Fig. 5). Therefore, only dermorphin-SSL, not plain SSL, could be taken up by CHO-hMOR cells.

3.6. Fluorescent imaging

Cellular uptake of dermorphin-SSL was further studied using fluorescent microscopy. CHO, CHO-hMOR and CHO-hDOR cells were incubated with dermorphin-SSL or plain

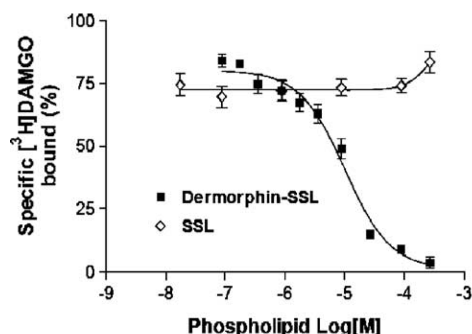

Fig. 4. Displacement binding of [^{3}H]DAMGO (1 nM) by dermorphin-SSL to CHO-hMOR. Receptor bound [^{3}H]DAMGO was displaced by dermorphin-SSL (■), but not by plain SSL (◊). The specific radioactivity of [^{3}H]DAMGO bound to hMOR in the absence of SSL or dermorphin-SSL was set to 100%. Each point represents the mean ± S.D. of three experiments (each performed in triplicate).

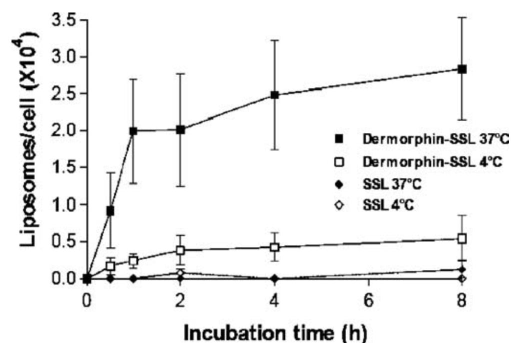

Fig. 5. Time course of dermorphin-SSL and SSL uptake by CHO-hMOR cells. CHO-hMOR cells were incubated with dermorphin-SSL or plain SSL (65 μM phospholipids) at 37 or 4 °C for various lengths of time in serum-free growth medium. (■), cells treated with dermorphin-SSL at 37 °C; (□), cells treated with dermorphin-SSL at 4 °C; (♦), cells treated with SSL at 37 °C; (◊), cells treated with SSL at 4 °C. Each point represents the mean ± S.D. of two experiments (each performed in triplicate).

SSL for 4 h at 37 °C. In these experiments, cholesteryl BODIPY was encapsulated in liposomes for two purposes: 1) to simulate encapsulation of a chemical compound in our system; 2) to monitor the whereabout of liposomes by fluorescent signals. To avoid non-specific adhesion, cells were washed extensively with phosphate-buffered saline. Significant fluorescent signals were observed in CHO-hMOR cells after the treatment with dermorphin-SSL for 4 h (Fig. 6). In contrast, no fluorescence signal was observed in either CHO-hDOR or CHO cells after the treatment with dermorphin-SSL (Fig. 6). Moreover, no fluorescence was detected after the treatment of CHO, CHO-hMOR and CHO-hDOR cells with plain SSL (data not shown). After 4 h incubation with liposomes, no significant cell morphology changes were observed for all three types of CHO cells, suggesting little apparent cell toxicity of dermorphin-SSL.

To exclude the possibility that dermorphin-SSL is simply associated with the outer cell surface, but does not get internalized by cells, we further examined the dermorphin-SSL treated CHO-hMOR cells using confocal laser microscopy. Cells were examined by scanning confocal plain every 400 nm. Indeed, intense fluorescence signal was detected inside cells, indicating cellular uptake of dermorphin-SSL (Fig. 7). These results suggested that dermorphin-SSL was capable of recognizing MOR and being internalized into CHO-hMOR cells with the encapsulated fluorescent probe. Therefore, dermorphin-SSL system has the potential to carry drugs to intracellular components of intended cells.

4. Discussion

Although the exact mechanisms behind opioid tolerance and dependence are not fully understood, a number of intracellular targets have been unveiled to play important roles such as PKC, CREB and CaMKII [1–7]. Therapeutic interventions at these intracellular proteins present unique opportunities to improve opioid analgesia and reduce tolerance and addiction. However, many of these molecules have diverse physiological functions. For instance, PKC is also involved in memory

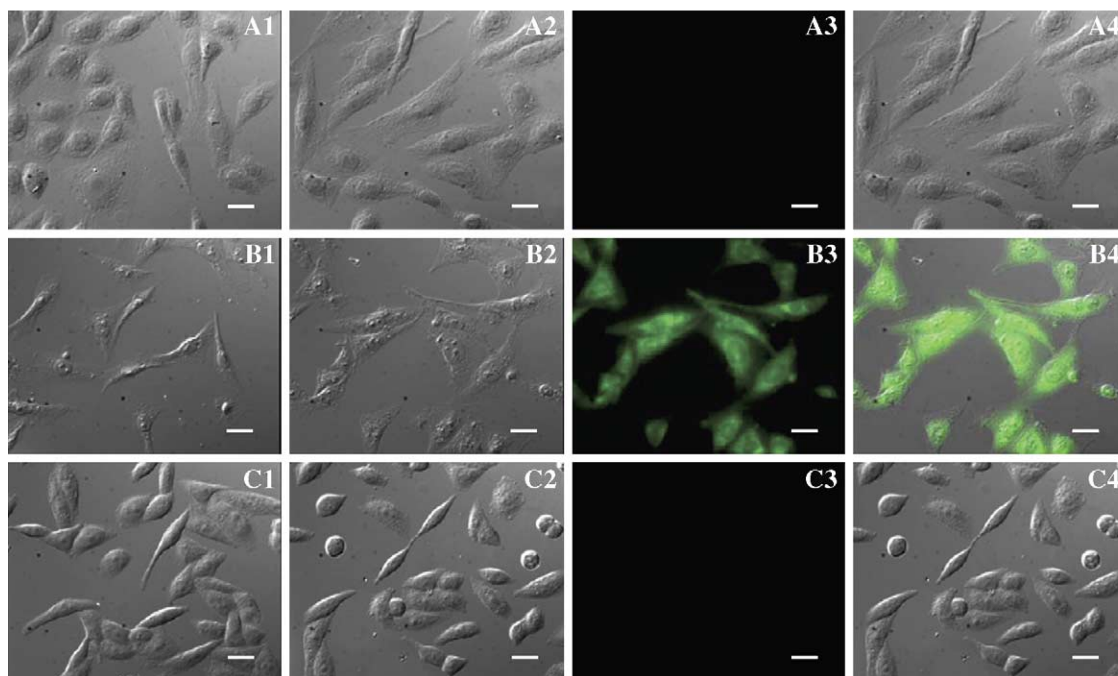

Fig. 6. Internalization of cholesteryl BODIPY encapsulated dermorphin-SSL by CHO-hMOR cells, but not by CHO and CHO-hDOR cells, examined by fluorescent microscopy. CHO (A), CHO-hMOR (B) and CHO-hDOR (C) cells were incubated with dermorphin-SSL (65 μM phospholipids) for 4 h at 37 °C in serum-free growth medium. Cells were fixed with 3.7% paraformaldehyde and viewed by either differential interference contrast (DIC) (A1, B1, C1: before dermorphin-SSL treatment; A2, B2, C2: after dermorphin-SSL treatment) or fluorescent microscopy (A3, B3, C3: after dermorphin-SSL treatment). Images of A4, B4 and C4 are the overlay of DIC (A2, B2, C2) and fluorescence (A3, B3, C3), respectively. The absence of fluorescence in CHO and CHO-hDOR cells in contrast to the intense cell-associated fluorescence in CHO-hMOR cells indicated the cell-specific association of dermorphin-SSL. Scale bars represent 20 μm.

function [36,37], hence non-selective delivery of PKC inhibitors may also affect learning and memory. Therefore, a cell-specific delivery system is more desirable in designing therapeutic agents for improving opioid analgesia and minimizing side effects. The mu opioid receptor (MOR) is primarily responsible for the analgesic and rewarding effects of opioids. In this study, a dermorphin-grafted sterically stabilized liposome (dermorphin-SSL) has been developed to selectively deliver drugs targeting intracellular components of MOR-expressing cells.

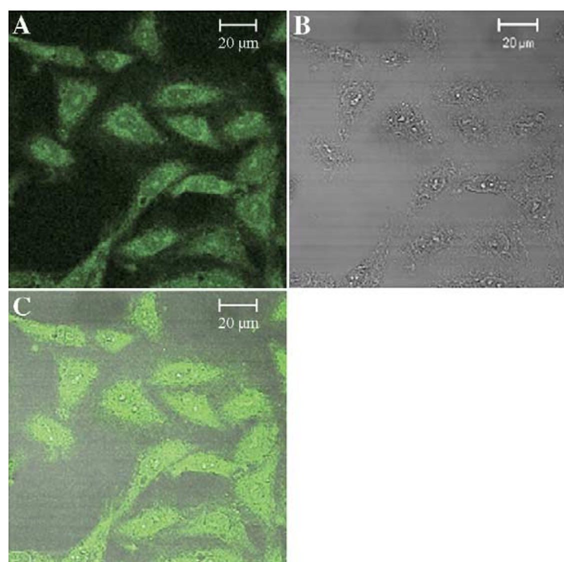

Fig. 7. Confocal image of CHO-hMOR cells after 4 h of incubation with cholesteryl BODIPY encapsulated dermorphin-SSL. (A) Fluorescent image; (B) DIC image; (C) Overlay image of (A) and (B). Scale bars represent 20 μm.

Theoretically any molecule can be directly conjugated to a ligand to achieve targeting delivery to the cells expressing specific receptors; however, ligand-grafted SSL with therapeutic agents encapsulated is preferred for a number of reasons. PEGylated liposomes are sterically stabilized, providing longer circulating half-life and little immunogenicity [13–15]. Each liposome can carry over 10^4 drug molecules depending on the size of liposomes. The payload is several orders of magnitude greater than that of ligand-drug conjugate [38]. In addition, ligand-grafted liposomes provide a general approach to deliver a number of potential drugs without the need for chemical linkers that must be specifically designed for particular drugs on a case-by-case basis [14,38]. Although PEG chains can prolong the circulating half-life of liposomes, some liposomes will still be taken up by the reticuloendothelial system (RES) and the encapsulated drugs will be released into the RES, not the intended location. Liposomes with surface targeting-ligands are expected to be enriched in targeted cell areas, which will decrease RES uptake [39,40].

Dermorphin is a biologically active opioid heptapeptide that is highly selective and active for MOR [41,42]. One report has used the peptide to target a conjugated toxin selectively to MOR-expressing cells in vivo [25]. The same study further identified dermorphin-induced internalization of the dermorphin–toxin/receptor complex as a mechanism for the entry of MOR-expressing cells by the toxin. In this

study, we took advantage of the affinity, selectivity, and internalization properties of dermorphin at MOR for cell-specific targeting. It has been reported that the N-terminal dermorphin tetrapeptide represents the minimal sequence that is required for biological activities [42,43]. To maintain its high affinity, only the C-terminus of dermorphin was modified by adding an additional cysteine residue, so the peptide can be readily conjugated to DSPE-PEG$_{3400}$-MAL to form DSPE-PEG$_{3400}$-dermorphin by coupling of thiol and maleimide groups. The conjugation reaction is highly specific and takes place under mild conditions. The formed thiol–ether bond is not readily hydrolyzed in vivo which confers the stability of DSPE-PEG$_{3400}$-dermorphin. DSPE-PEG$_{3400}$-dermorphin retained affinity and selectivity to MOR (IC50: 1.9 nM), which was not significantly different from that of dermorphin (IC50: 1.6 nM). Our data supported the previous finding that N-terminal dermorphin is essential for its affinity to MOR [42,43]. The mass distribution of DSPE-PEG$_{3400}$-dermorphin shown in mass spectrogram was due to the PEG polymer. The mean molecular weight, determined by MALDI-MS, was 5200 Da, which was in agreement with the calculated size of DSPEP-EG$_{3400}$-dermorphin.

Dermorphin-SSL was prepared by post-insertion method. As reported in previous studies using this method [30,31], it is advantageous that DSPE-PEG can be transferred into the membrane of the preformed liposomes by one-step incubation with very little drug release during the transfer and only small increases in liposome diameters. Moreover, liposomes can be constructed with defined number of targeting ligands that could be controlled by the alteration of incubation time, temperature and ligand concentration.

To study the receptor affinity and cell uptake of dermorphin-SSL, MOR-transfected CHO cells were established. CHO cells do not express endogenous opioid receptors, and they are highly transfectable. Opioid receptors transfected in CHO cells are common models widely employed to study receptor–ligand interaction, receptor activation, signal transduction, and adaptive changes of these receptors. Receptor activation, phosphorylation, internalization, downregulation, dimerization, and receptor tolerance have all been studied in these cells [44–49]. CHO cells expressing only a single opioid receptor subtype provide a unique system to study a receptor-selective mechanism. To study the cell-specific uptake of dermorphin-SSL, we also employed another cell line expressing the human delta opioid receptors (CHO-hDOR). Both hMOR and hDOR belong to the family of seven transmembrane G-protein-coupled receptors, sharing extensive sequence and structural homologies. Among all known receptors, hDOR is the closest to hMOR [50]; therefore, CHO-hDOR along with untransfected CHO cells were used as controls in our studies. Using these receptor-specific cell lines, we observed that dermorphin-SSL was only taken up by CHO-hMOR cells, not CHO or CHO-hDOR cells. It should, however, be noted that these are non-neuronal artificial cell lines that express high levels of receptors. Future studies will be carried out to confirm the results in neuronal cells with endogenous receptors.

We applied 4 h treatment based on the protocol that we had previously identified to induce significant receptor internalization by a variety of opioid agonists [22] as well as pilot studies. Liposome uptake by CHO-hMOR cells reached plateau at 4 h, as no significant increase of liposome internalization was observed thereafter. We identified cell uptake of dermorphin-SSL only by CHO-hMOR cells, but not by CHO or CHO-hDOR cells. Differential interference contrast (DIC) cell images before and after dermorphin-SSL treatment were taken to demonstrate intact cell morphology, which also indicated low apparent cell toxicity of dermorphin-SSL. Interestingly, fluorescence signal was observed inside the nuclei of CHO-hMOR cells after the treatment with dermorphin-SSL. This observation may suggest that targeted agents could enter nuclei after the degradation of liposomes, which will be essential to deliver gene (e.g. antisense)-based agents encapsulated in liposomes. For instance, various PKC isoforms are major mediators for the development of opioid tolerance, which is a common mechanism leading to inadequate pain control. Gene-based agents (e.g., antisense) offer the most selective inhibition of PKC isoforms. Such gene-based drugs can be incorporated into liposomes and tested for the treatment of opioid tolerance in future studies. However, further studies are required to investigate the intracellular trafficking of the liposome molecules. Liposome size is another important variable that may affect the outcome. Since receptor-mediated endocytosis involves clathrin-coated pits [34,51,52], which shows an upper size limit for internalization of approximately 200 nm [53,54], it is very likely that liposomes with a size larger than 200 nm may not be internalized by cells. It was reported that folate liposomes with a mean diameter of about 200 nm were not internalized by KB cells [14]. In our study, we prepared dermorphin-SSL with a mean diameter of 150 nm and found these liposomes could be internalized by CHO-hMOR cells.

Our results demonstrated that dermorphin-SSL was capable of recognizing specifically the cell-surface MOR, leading to MOR-mediated endocytosis of liposomes in vitro. For future in vivo studies, dermorphin-SSL will be given intrathecally to deliver drugs in animals, thus bypass the blood brain barrier. Using the dermorphin-grafted SSL delivery system, we will test in these future studies the effects of encapsulated therapeutic agents on the treatment of pain, opioid tolerance and drug addiction.

5. Conclusion

Dermorphin was successfully conjugated to DSPE-PEG$_{3400}$ without affecting the affinity to its receptor. The synthesized conjugate was inserted into the preformed sterically stabilized liposome to obtain dermorphin-grafted SSL. DSPE-PEG$_{3400}$-dermorphin and dermorphin-SSL retained their affinity and selectivity for MOR. Moreover, dermorphin-SSL and encapsulated probe were taken up by CHO-hMOR cells, but not by naive CHO cells or very closely related CHO-hDOR cells. These results suggest that dermorphin-SSL can be used to deliver drugs to the intracellular component of intended cells with high fidelity.

References

[1] K.O. Aley, J.D. Levine, Different mechanisms mediate development and expression of tolerance and dependence for peripheral mu-opioid antinociception in rat, J. Neurosci. 17 (1997) 8018–8023.

[2] F.L. Smith, R.R. Javed, M.J. Elzey, W.L. Dewey, The expression of a high level of morphine antinociceptive tolerance in mice involves both PKC and PKA, Brain Res. 985 (2003) 78–88.

[3] Z.J. Wang, L. Tang, L. Xin, Reversal of morphine antinociceptive tolerance by acute spinal inhibition of Ca(2+)/calmodulin-dependent protein kinase II, Eur. J. Pharmacol. 465 (2003) 199–200.

[4] Z. Wang, W. Sadee, Tolerance to morphine at the mu-opioid receptor differentially induced by cAMP-dependent protein kinase activation and morphine, Eur. J. Pharmacol. 389 (2000) 165–171.

[5] G.H. Fan, L.Z. Wang, H.C. Qiu, L. Ma, G. Pei, Inhibition of calcium/calmodulin-dependent protein kinase II in rat hippocampus attenuates morphine tolerance and dependence, Mol. Pharmacol. 56 (1999) 39–45.

[6] R. Maldonado, J.A. Blendy, E. Tzavara, P. Gass, B.P. Roques, J. Hanoune, G. Schutz, Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB, Science 273 (1996) 657–659.

[7] A.S. Leonard, J.W. Hell, Cyclic AMP-dependent protein kinase and protein kinase C phosphorylate *N*-methyl-D-aspartate receptors at different sites, J. Biol. Chem. 272 (1997) 12107–12115.

[8] R.M. Straubinger, N.G. Lopez, R.J. Debs, K. Hong, D. Papahadjopoulos, Liposome-based therapy of human ovarian cancer: parameters determining potency of negatively charged and antibody-targeted liposomes, Cancer Res. 48 (1988) 5237–5245.

[9] K.K. Matthay, A.M. Abai, S. Cobb, K. Hong, D. Papahadjopoulos, R.M. Straubinger, Role of ligand in antibody-directed endocytosis of liposomes by human T-leukemia cells, Cancer Res. 49 (1989) 4879–4886.

[10] V. Weissig, J. Lasch, A.L. Klibanov, V.P. Torchilin, A new hydrophobic anchor for the attachment of proteins to liposomal membranes, FEBS Lett. 202 (1986) 86–90.

[11] B.J. Hughes, S. Kennel, R. Lee, L. Huang, Monoclonal antibody targeting of liposomes to mouse lung in vivo, Cancer Res. 49 (1989) 6214–6220.

[12] J.P. Leonetti, P. Machy, G. Degols, B. Lebleu, L. Leserman, Antibody-targeted liposomes containing oligodeoxyribonucleotides complementary to viral RNA selectively inhibit viral replication, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 2448–2451.

[13] A. Mori, S.J. Kennel, L. Huang, Immunotargeting of liposomes containing lipophilic antitumor prodrugs, Pharm. Res. 10 (1993) 507–514.

[14] R.J. Lee, P.S. Low, Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis, J. Biol. Chem. 269 (1994) 3198–3204.

[15] J.W. Park, K. Hong, P. Carter, H. Asgari, L.Y. Guo, G.A. Keller, C. Wirth, R. Shalaby, C. Kotts, W.I. Wood, et al., Development of anti-p185HER2 immunoliposomes for cancer therapy, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 1327–1331.

[16] S. Dagar, M. Sekosan, B.S. Lee, I. Rubinstein, H. Onyuksel, VIP receptors as molecular targets of breast cancer: implications for targeted imaging and drug delivery, J. Control. Release 74 (2001) 129–134.

[17] R.E. Eliaz, F.C. Szoka Jr., Liposome-encapsulated doxorubicin targeted to CD44: a strategy to kill CD44-overexpressing tumor cells, Cancer Res. 61 (2001) 2592–2601.

[18] E. Mastrobattista, G.A. Koning, L. van Bloois, A.C. Filipe, W. Jiskoot, G. Storm, Functional characterization of an endosome-disruptive peptide and its application in cytosolic delivery of immunoliposome-entrapped proteins, J. Biol. Chem. 277 (2002) 27135–27143.

[19] C. Mamot, D.C. Drummond, U. Greiser, K. Hong, D.B. Kirpotin, J.D. Marks, J.W. Park, Epidermal growth factor receptor (EGFR-targeted immunoliposomes mediate specific and efficient drug delivery to EGFR- and EGFRvIII-overexpressing tumor cells, Cancer Res. 63 (2003) 3154–3161.

[20] H.W. Matthes, R. Maldonado, F. Simonin, O. Valverde, S. Slowe, I. Kitchen, K. Befort, A. Dierich, M. Le Meur, P. Dolle, E. Tzavara, J. Hanoune, B.P. Roques, B.L. Kieffer, Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene, Nature 383 (1996) 819–823.

[21] I. Sora, N. Takahashi, M. Funada, H. Ujike, R.S. Revay, D.M. Donovan, L.L. Miner, G.R. Uhl, Opiate receptor knockout mice define mu receptor roles in endogenous nociceptive responses and morphine-induced analgesia, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 1544–1549.

[22] J.R. Arden, V. Segredo, Z. Wang, J. Lameh, W. Sadee, Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged mu-opioid receptor expressed in HEK 293 cells, J. Neurochem. 65 (1995) 1636–1645.

[23] G. Gaudriault, D. Nouel, C. Dal Farra, A. Beaudet, J.P. Vincent, Receptor-induced internalization of selective peptidic mu and delta opioid ligands, J. Biol. Chem. 272 (1997) 2880–2888.

[24] V.A. Alvarez, S. Arttamangkul, V. Dang, A. Salem, J.L. Whistler, M. Von Zastrow, D.K. Grandy, J.T. Williams, mu-opioid receptors: ligand-dependent activation of potassium conductance, desensitization, and internalization, J. Neurosci. 22 (2002) 5769–5776.

[25] F. Porreca, S.E. Burgess, L.R. Gardell, T.W. Vanderah, T.P. Malan Jr., M.H. Ossipov, D.A. Lappi, J. Lai, Inhibition of neuropathic pain by selective ablation of brainstem medullary cells expressing the mu-opioid receptor, J. Neurosci. 21 (2001) 5281–5288.

[26] J.M. Macdougall, X.D. Zhang, W.E. Polgar, T.V. Khroyan, L. Toll, J.R. Cashman, Synthesis and biological evaluation of some 6-arylamidomorphines as analogues of morphine-6-glucuronide, Bioorg. Med. Chem. 12 (2004) 5983–5990.

[27] N. Zaveri, W.E. Polgar, C.M. Olsen, A.B. Kelson, P. Grundt, J.W. Lewis, L. Toll, Characterization of opiates, neuroleptics, and synthetic analogs at ORL1 and opioid receptors, Eur. J. Pharmacol. 428 (2001) 29–36.

[28] E. Malatynska, Y. Wang, R.J. Knapp, G. Santoro, X. Li, S. Waite, W.R. Roeske, H.I. Yamamura, Human delta opioid receptor: a stable cell line for functional studies of opioids, Neuroreport 6 (1995) 613–616.

[29] E. Malatynska, Y. Wang, R.J. Knapp, S. Waite, S. Calderon, K. Rice, V.J. Hruby, H.I. Yamamura, W.R. Roeske, Human delta opioid receptor: functional studies on stably transfected Chinese hamster ovary cells after acute and chronic treatment with the selective nonpeptidic agonist SNC-80, J. Pharmacol. Exp. Ther. 278 (1996) 1083–1089.

[30] S. Dagar, A. Krishnadas, I. Rubinstein, M.J. Blend, H. Onyuksel, VIP grafted sterically stabilized liposomes for targeted imaging of breast cancer: in vivo studies, J. Control. Release 91 (2003) 123–133.

[31] T. Ishida, D.L. Iden, T.M. Allen, A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs, FEBS Lett. 460 (1999) 129–133.

[32] M. Kates, Techniques in Lipidology, Elsevier, New York, 1972.

[33] H.G. Enoch, P. Strittmatter, Formation and properties of 1000-A-diameter, single-bilayer phospholipid vesicles, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 145–149.

[34] R.M. Straubinger, K. Hong, D.S. Friend, D. Papahadjopoulos, Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low pH compartment after internalization in coated vesicles, Cell 32 (1983) 1069–1079.

[35] Z. Wang, L.R. Gardell, M.H. Ossipov, T.W. Vanderah, M.B. Brennan, U. Hochgeschwender, V.J. Hruby, T.P. Malan Jr., J. Lai, F. Porreca, Pronociceptive actions of dynorphin maintain chronic neuropathic pain, J. Neurosci. 21 (2001) 1779–1786.

[36] D.J. Linden, Neuroscience. From molecules to memory in the cerebellum, Science 301 (2003) 1682–1685.

[37] M.A. Sutton, M.W. Bagnall, S.K. Sharma, J. Shobe, T.J. Carew, Intermediate-term memory for site-specific sensitization in aplysia is maintained by persistent activation of protein kinase C, J. Neurosci. 24 (2004) 3600–3609.

[38] J.W. Park, C.C. Benz, F.J. Martin, Future directions of liposome- and immunoliposome-based cancer therapeutics, Semin. Oncol. 31 (2004) 196–205.

[39] K. Maruyama, O. Ishida, T. Takizawa, K. Moribe, Possibility of active targeting to tumor tissues with liposomes, Adv. Drug Deliv. Rev. 40 (1999) 89–102.

[40] P. Sapra, T.M. Allen, Ligand-targeted liposomal anticancer drugs, Prog. Lipid Res. 42 (2003) 439–462.

[41] L.H. Lazarus, A. Guglietta, W.E. Wilson, B.J. Irons, R. de Castiglione, Dimeric dermorphin analogues as mu-receptor probes on rat brain membranes. Correlation between central mu-receptor potency and suppression of gastric acid secretion, J. Biol. Chem. 264 (1989) 354–362.

[42] M. Attila, S. Salvadori, G. Balboni, S.D. Bryant, L.H. Lazarus, Synthesis and receptor binding analysis of dermorphin hepta-, hexa- and pentapeptide analogues. Evidence for one- and two-side binding models for the mu-opioid receptor, Int. J. Pept. Protein Res. 42 (1993) 550–559.

[43] D. Cocchi, E.C. Degli Uberti, G. Trasforini, S. Salvadori, R. Tomatis, R. Torpia, R. Perelli-Cippo, Prolactin releasing and luteinizing hormone inhibiting activity of dermorphin shorter homologues in the rat, Life Sci. 36 (1985) 1707–1713.

[44] P.L. Prather, T.M. McGinn, L.J. Erickson, C.J. Evans, H.H. Loh, P.Y. Law, Ability of delta-opioid receptors to interact with multiple G-proteins is independent of receptor density, J. Biol. Chem. 269 (1994) 21293–21302.

[45] S. Chakrabarti, P.L. Prather, L. Yu, P.Y. Law, H.H. Loh, Expression of the mu-opioid receptor in CHO cells: ability of mu-opioid ligands to promote alpha-azidoanilido[32P]GTP labeling of multiple G protein alpha subunits, J. Neurochem. 64 (1995) 2534–2543.

[46] J.A. Becker, A. Wallace, A. Garzon, P. Ingallinella, E. Bianchi, R. Cortese, F. Simonin, B.L. Kieffer, A. Pessi, Ligands for kappa-opioid and ORL1 receptors identified from a conformationally constrained peptide combinatorial library, J. Biol. Chem. 274 (1999) 27513–27522.

[47] H.K. Kramer, E.J. Simon, mu and delta-opioid receptor agonists induce mitogen-activated protein kinase (MAPK) activation in the absence of receptor internalization, Neuropharmacology 39 (2000) 1707–1719.

[48] P.F. Zaratin, G. Petrone, M. Sbacchi, M. Garnier, C. Fossati, P. Petrillo, S. Ronzoni, G.A. Giardina, M.A. Scheideler, Modification of nociception and morphine tolerance by the selective opiate receptor-like orphan receptor antagonist (-)-*cis*-1-methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5H-benzocyclohepten-5-ol (SB-612111), J. Pharmacol. Exp. Ther. 308 (2004) 454–461.

[49] P.J. Emmerson, J.H. McKinzie, P.L. Surface, T.M. Suter, C.H. Mitch, M.A. Statnick, Na+modulation, inverse agonism, and anorectic potency of 4-phenylpiperidine opioid antagonists, Eur. J. Pharmacol. 494 (2004) 121–130.

[50] G.R. Uhl, I. Sora, Z. Wang, The mu opiate receptor as a candidate gene for pain: polymorphisms, variations in expression, nociception, and opiate responses, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 7752–7755.

[51] E. Smythe, G. Warren, The mechanism of receptor-mediated endocytosis, Eur. J. Biochem. 202 (1991) 689–699.

[52] J.L. Goldstein, R.G. Anderson, M.S. Brown, Coated pits, coated vesicles, and receptor-mediated endocytosis, Nature 279 (1979) 679–685.

[53] I.S. Zuhorn, R. Kalicharan, D. Hoekstra, Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis, J. Biol. Chem. 277 (2002) 18021–18028.

[54] J. Rejman, V. Oberle, I.S. Zuhorn, D. Hoekstra, Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis, Biochem. J. 377 (2004) 159–169.