Nanoconjugated NAP as a Potent and Periphery Selective Mu Opioid Receptor Modulator to Treat Opioid Induced Constipation

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Materials

Copper (I) bromide (CuBr), 2,2'-bipyridyl-4-dimethylaminopyridine (DMAP), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), triethylamine (Et_3N), magnesium sulfate ($MgSO_4$), deuterated solvents, and routine organic solvents were purchased from Acros (Morris Plains, NJ). A short heterobifunctional PEG derivative, acid-PEG-azide (MW=335 Da, n=5) was purchased from Biomatrik Inc. (Jiaxing, Zhejiang, China). Dialysis tubing and snakeskin MWCO 3500 were obtained from Thermo Fisher Scientific (Pittsburg, PA).

Instrumentation

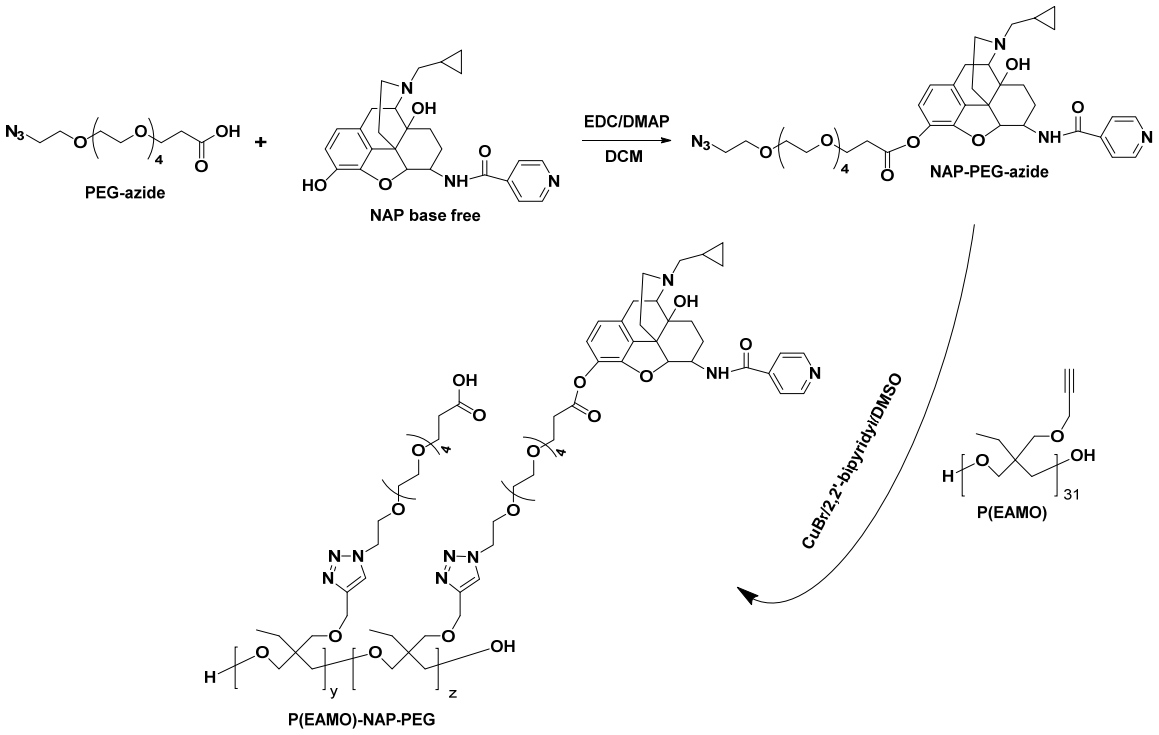
^1H NMR spectra were recorded on a Bruker AVANCEIII 600 MHz spectrometer. FTIR spectra were obtained on a Megna-IR 760 spectrometer using KBr pellets. Fluorescence emission spectra were recorded on a fluorescence spectrometer QM4 (Photon Technology International, Birmingham, NJ). Particle size and zeta potential were measured on Malvern Zetasizer Nano ZS90 apparatus (Malvern Instruments, Worcestershire, U.K.)

Synthesis of P(EAMO)-NAP-PEG Nanoconjugates

The synthesis involved three steps. Step 1) Synthesis of NAP-PEG-azide conjugates. NAP was synthesized following our previous report [1] and then converted to NAP free base. Step 2) Coupling of NAP-PEG-azide to P(EAMO). Briefly, to a suspension of NAP (50 mg) in 5 ml of dichloromethane (DCM) at 0 ºC was added dropwise 30 µl of Et_3N. The solution was stirred for 30 minutes and subjected to rotary evaporation to remove DCM. The obtained crude solid NAP free base (77 mg, 0.17 mmol), PEG-azide (75 mg, 0.22 mmol),

and DMAP (20 mg, 0.16 mmol) were dissolved in 5 ml of DCM. Following addition of EDC (75 mg, 0.4 mmol), the reaction mixture was stirred overnight at room temperature and then poured into 10 ml of cold water. Following extraction with DCM (20 ml × 3), the combined DCM solution was dried over $MgSO_4$. Crude NAP-PEG-azide product was then obtained by removing DCM under reduced pressure and used without further purification.

Scheme S1. Synthesis of P(EAMO)-PEG-NAP nanoconjugates.



Step 3) Coupling of NAP-PEG-azide to P(EAMO). A mixture of $P(EAMO)^2$ (23 mg, 4.75 μmol, possessing 31 repeat units, i.e., n=31), crude NAP-PEG-azide (100 mg), and 2, 2'-bipyridyl (24 mg, 0.16 mmol) was dissolved in 1.5 ml of dry dimethyl sulfoxide (DMSO) and degassed using the freeze-pump-thaw method. The flask was filled with nitrogen and a DMSO solution of CuBr (11 mg, 77 μmol) was added. After an overnight reaction at room temperature, the reaction solution was poured into 20 ml of cold water

and stirred for 1 h. The resulting solid was filtered, washed with water, and dried. The crude P(EAMO)-NAP-PEG conjugates were dissolved in DCM and centrifuged at 15.6 kg force for 1 h. Following removal of DCM under reduced pressure, the obtained residue was suspended in 10 ml of ether, stirred overnight, filtered, washed with ether three times (4 ml × 3), and then vacuum dried. For further purification, the conjugates were washed with ethylenediaminetetraacetic acid (EDTA)/DCM, filtered, and vacuum dried. P(EAMO)-NAP-PEG was characterized by ^{1}HNMR and IR (Figure S1). ^{1}HNMR: (DMSO-d_6, 600MHz): δ (ppm) 8.87 (br, s, 2H), 7.93 (br, s, 3H), 6.76 (d, 2H), 4.76 (s, 1H), 4.43 (br, s, 4H), 4.10-2.00 (m, 37H), 1.92-0.9 (m, 5H), 1.20 (br, s, 2H), 0.67 (s, 3H), 0.85-0.2 (m, 6H). IR ν_{max} (neat) cm^{-1}: 2922, 2853, 1653, 1539, 1444, 1229, 1091.

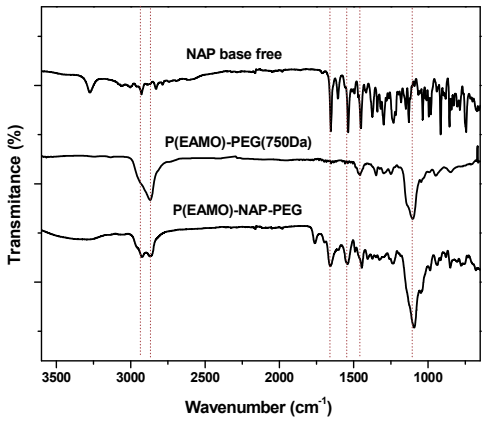


Figure S1. FTIR spectra of NAP free base, P(EAMO)- PEG(750Da)*, and P(EAMO)-NAP-PEG. *adapted from our prior work for comparison.

Critical Micelle Concentration (CMC) Determination

The pyrene I1/I3 ratio method[3] was applied to determine critical micelle concentration of nanoconjugated NAP. A series of NAP nanoparticle solutions from 1 mg/L up to 5 g/L containing 0.6 μM of pyrene were prepared by dissolving appropriate

amounts of nanoconjugated NAP in the pyrene water solution followed by heating at 65 °C for 3 h and equilibration at room temperature for 16 h. Fluorescent emission spectra of the pyrene-containing solutions were then recorded using an excitation wavelength of 335 nm. The intensities of I1 at the wavelength of 370 nm and I3 at the wavelength of 390 nm were measured and the ratio of I1/I3 against NAP nanoconjugate concentration was plotted.

Particle Size and Zeta Potential Measurements

PBS was filtered through a 20 nm filter. The size and zeta potential of nanoconjugated NAP at concentration above CMC were measured at room temperature using a Malvern Zetasizer Nano ZS90 apparatus (Malvern Instruments, Worcestershire, U.K.).

***In Vitro* Competitive Radioligand Binding and Functional Assays**

The radioligand binding and the [^{35}S]GTPγS binding assays were conducted using monocloned opioid receptor-expressed Chinese hamster ovarian (CHO) cell lines as described previously.[1] Briefly, for the competition binding assay, [^{3}H]naloxone, [^{3}H]diprenorphine, and [^{3}H]naltrindole were used to label MOR, KOR, and DOR, respectively. Aliquots of a membrane protein (30 μg) were incubated with the corresponding radioligand in the presence of seven different concentrations of the ligand under investigation in TME buffer (50 mM Tris, 3 mM $MgCl_2$, 0.2 mM EGTA, pH 7.7) at 30 °C for 1.5 h. The bound radioactive ligand was separated from the free radioligand by filtration using the Brandel harvester (Biomedical Research & Development Laboratories, MD).

Specific (i.e., opioid receptor-related) binding was determined as the difference in binding obtained in the absence and presence of 5 μM naltrexone, 5 μM U50,488, and 5 μM SNC80 for MOR, KOR, and DOR, respectively. The potency of a drug in displacing the specific binding of the radioligand was determined by linear regression analysis of Hill plots. IC_{50} values were then determined and converted to K_i values using the Cheng–Prusoff equation. MOR [^{35}S]GTPγS functional assays were conducted in the same cell membranes used in the receptor binding assays. Membrane proteins (10 μg) were incubated with varying concentrations of compounds, GDP (10 μM) and 0.1 nM [^{35}S]GTPγS in assay buffer (50 mM Tris, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM EGTA, pH 7.7) for 1.5 h at 30 °C. Nonspecific binding was determined with 20 μM unlabeled GTPγS. DAMGO (3 μM) was included in the assay for a maximal effect of a full agonist for MOR.

In Vivo Activity Studies

P(EAMO)-NAP-PEG was dissolved in DMSO and then diluted to the desired concentration such that the DMSO concentration did not rise above ten percent.

Tail Immersion Test. The warm water tail-immersion test was performed according to previously described methods using a water bath with the temperature maintained at 56 ± 0.1°C.[4] Briefly, before giving the mice injections, a baseline (control) latency was determined. Only mice with a control reaction time of 2 to 4 s were used. The test latency after drug treatment was assessed at the appropriate time, and a 10 s maximal cutoff time was imposed to prevent tissue damage. Antinociception was quantified according to established procedures as the percentage of maximum possible effect (%MPE), which was calculated as follows: %MPE = [(test latency - control latency)/(10 - control latency)] × 100.[5] Percentage MPE was calculated for each mouse, using at least six mice per group.

Charcoal Meal Test for Gastrointestinal Transit Analysis. Forty-eight hours before testing, mice were placed in cages with raised mesh wire to suspend them above their bedding and prevent ingestion of feces or bedding. The animals were habituated for 24 h in the presence of food and water and then fasted for 24 h with free access to water as previously reported. [6] This time frame was chosen to deplete the intestine and colon of any feces. To maintain caloric intake and to avoid hypoglycemia, mice had access to a sugar water solution consisting of a final concentration of 5% dextrose for the first 8 h of the fasting period. In control experiments mice were treated with either saline (10 μl/g s.c.) or morphine (2 mg/kg s.c.), and 20 min later they were given an oral gavage consisting of 5% aqueous suspension of charcoal in a 10% gum Arabic solution. At 30 min after the administration of the charcoal meal, the mice were euthanized by cervical dislocation, and the small intestine from the jejunum to the cecum was dissected and placed in cold saline to stop peristalsis. The distance traveled by the leading edge of the charcoal meal was measured relative to the total length of the small intestine, and the percentage of intestinal transit for each animal was calculated as percentage transit (charcoal distance)/(small intestinal length) × 100. This was referred to as intestinal transit in the text. In drug treated mice, mice were treated with P(EAMO)-NAP-PEG either by subcutaneous injection or through oral gavage 15min prior to the administration of either saline (10 μl/g s.c.) or morphine (2 mg/kg s.c.). 20 min following this, mice were then given an oral gavage consisting of 5% aqueous suspension of charcoal in a 10% gum Arabic solution. At 30 min past administration of the charcoal meal, the mice were euthanized by cervical dislocation, and the small intestine from the jejunum to the cecum was dissected and placed in cold

saline to stop peristalsis. The percentage of intestinal transit for each animal was calculated as described above.

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