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The Development of a Macromolecular Analgesic for Arthritic Pain

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Abstract

The addictive potential of clinically-used opioids due to their direct action on the dopaminergic reward system in the brain has limited their application. In an attempt to reduce negative side effects as well as to improve the overall effectiveness of these analgesics, we have designed, synthesized, and evaluated an *N*-(2-hydroxypropyl)methacrylamide (HPMA)-based macromolecular prodrug of hydromorphone (HMP), a commonly used opioid. To this end, P-HMP was synthesized via RAFT polymerization and a subsequent polymer analogous reaction. Its interaction with inflammatory cells in arthritic joints was evaluated *in vitro* using a RAW264.7 cell culture, and subsequent confocal microscopy analysis confirmed that P-HMP could be internalized by the cells via endocytosis. *In vivo* imaging studies indicated that the prodrug can passively target to the arthritic joint after systemic administration in a rodent model of monoarticular adjuvant-induced arthritis (MAA). The inflammatory pain-alleviating properties of the prodrug were assessed in MAA rats using the incapacitance test, and were observed to be similar to dose-equivalent HMP. Analgesia through mechanisms at the spinal cord level was further measured using the tail flick test and it was determined that the prodrug significantly reduced spinal cord analgesia versus free HMP, further validating the peripheral restriction of the macromolecular prodrug. Immunohistochemical analysis of cellular uptake of the P-HMP within the MAA knee joint proved the internalization of the prodrug by phagocytic synoviocytes, co-localized with HMP's target receptor as well as with pain-modulating ion channels. Therefore, it can be concluded that the novel inflammation-targeting polymeric prodrug of HMP (P-HMP) has the potential to be developed as an effective and safe analgesic agent for musculoskeletal pain.

Graphical Abstract

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Keywords

Rheumatoid arthritis; opioid; analgesic; ELVIS mechanism; HPMA copolymer

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Introduction

Pain poses significant clinical, economic, and social burden on humanity.¹ Causes of pain include postoperative tissue damage, malignancies, or conditions in which pain distinguishes the disease itself, such as neuropathic pains or headaches.² Short-lived acute pain, which arises as a normal trigger to alert an individual to possible injury, can develop into a persistent condition, causing further complications and long-term disability. This type of pain, defined by its persistence of longer than twelve weeks, is classified as chronic. The annual costs involved with control of chronic pain (\$560-\$635 billion) far outweigh those of other major diseases, namely, heart (\$309 billion), cancer (\$243 billion), and diabetes (\$188 billion).³ Chronic pain itself afflicts 20% of the adult population in developed countries alone,⁴ resulting in loss of productivity and overall decline in everyday quality of life. Clinically, when physical therapy is not sufficient or is not an option, the pain must be managed by analgesic agents. This is often the case with physically immobilizing illnesses, including fibromyalgia,⁵ osteoarthritis, rheumatoid arthritis, and lumbago.⁶ These ailments frequently require first and foremost alleviation of the accompanying pain.

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease affecting 0.5 to 1 percent of adults population worldwide, with its development two to three times more likely in women.⁷ Severe joint inflammation, structural damage and pain are hallmarks of this disease.⁸ There is no cure for RA at present. The current treatment options of RA includes nonsteroidal anti-inflammatory medications (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs) and glucocorticoids (GCs).⁹ While DMARDs treatments may help to address joint inflammation and articular damage, they often have slow onset, which necessitates the concurrent use of NSAIDs and opioids as analgesics to control the pain associated with RA.^{10–12} The use of opioids for the treatment of both acute and chronic pain has been practiced for thousands of years.¹³ Opioids modulate nociception, the sensory nervous system's response to potentially harmful stimuli, primarily through agonistic action on opioid receptors, which are ubiquitous within the central nervous system (CNS) and reside at the peripheral terminals of afferent nerves.^{14,15} These ligands act to inhibit neurotransmitter release in primary afferent terminals in the spinal cord and activate descending inhibitory controls, leading to their analgesic effect. Opioids are relatively safe when used correctly and with caution. However, long-term opioid use has been proven controversial.^{12,16–18} Mechanistically, these compounds act directly on the brain's natural reward system to beget dopamine,¹⁹ which produces an euphoric effect. Drug tolerance characterized by neuroadaptations and physical dependence can thus transition into addiction if not well-controlled. Therefore, the safe clinical use of opioid necessitates the mitigation of these undesired CNS effects.

Synthetic water-soluble polymers such as poly(N-(2-hydroxypropyl)methacrylamide) (PHPMA) cannot permeate the blood brain barrier (BBB).²⁰ On the other hand,

macromolecular prodrugs based upon HPMA copolymers have been found to passively target inflammation in several animal models of human diseases.^{21–24} This novel inflammation-targeting mechanism, termed ELVIS (Extravasation through Leaky Vasculature and Inflammatory cell-mediated Sequestration), can be utilized to selectively localize and retain therapeutic agents at the inflammatory sites. Given the ELVIS mechanism, which would facilitate the passive targeting of HPMA copolymers to the arthritic joints, and the impermeability of the BBB to water-soluble polymers, we hypothesized that upon systemic administration of an opioid-conjugated HPMA copolymer prodrug system in a rat model of monoarticular adjuvant-induced arthritis (MAA), a standard model often used in pain and inflammation research, the prodrug can preferentially extravasate through vascular leakage present in the arthritic joints and be sequestered by residential and infiltrated inflammatory cells that have been activated by inflammatory cytokines and possess high rates of endocytosis. The internalized prodrug will be processed in the acidic lysosomal compartments, releasing the opioid and promoting local analgesic effects. Spinal cord analgesia will be very limited due to the polymeric prodrug's restricted access to CNS.

In this manuscript, we report the design and synthesis of an HPMA copolymer conjugate of hydromorphone (HMP), a semi-synthetic opioid receptor agonist, which is estimated to be five to ten times more potent than morphine.²⁵ Marketed under the brand name Dilaudid, this narcotic analgesic has been in clinical use since the 1920s to alleviate a variety of painful conditions.^{26–29} Hydromorphone can produce neuroexcitatory metabolites in addition to its potentially addictive qualities.³⁰ In an effort to address these issues, HMP has been chemically conjugated to the main chain HPMA copolymer via an acid-labile hydrazone bond. We here demonstrate the preferential inflammation targeting ability of this macromolecular prodrug of HMP. The prodrug has significantly less analgesia at the spinal cord level, further suggestive of its restriction within the inflamed joint. Thus, the macromolecular prodrug strategy could be especially beneficial by way of limiting the BBB penetration of these narcotic analgesics.

Materials and Methods

Materials

N-(3-Aminopropyl) methacrylamide (APMA) hydrochloride was purchased from Polysciences, Inc. (Warrington, PA). *N*-(2-Hydroxypropyl) methacrylamide (HPMA), *S,S'*-bis(α,α' -dimethyl- α'' -acetic acid)-trithiocarbonate (CTA, purity >97%),³¹ *N*-methacryloylglycylglycyl hydrazide (MA-Gly-Gly-NHNH₂),³² and poly (HPMA-*co*-APMA)³³ were prepared as reported previously. Hydromorphone (HMP) hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). IRDye® 800CW carboxylate was purchased from LI-COR, Inc. (Lincoln, NE). Alexa Fluor® 488 NHS ester was purchased from Life Technologies, Inc. (Eugene, OR). All other reagents and solvents were purchased from either Sigma-Aldrich (St. Louis, MO) or Acros Organics (Morris Plains, NJ). All compounds were reagent grade and used without further purification.

Instruments

^1H and ^{13}C NMR spectra were recorded on a 500 MHz NMR spectrometer (Varian Medical Systems, Palo Alto, CA). The weight average molecular weight (M_w) and number average molecular weight (M_n) of copolymers were determined by size exclusion chromatography (SEC) using the ÄKTA FPLC system (GE Healthcare, Chicago, IL) equipped with UV and RI (Knauer, Berlin, Germany) detectors. SEC measurements were performed on a Superdex 200 column (HR 10/30) with phosphate-buffered saline (PBS, pH 7.4) as the eluent. HPMA homopolymer samples with narrow PDI values were used as calibration standards. HPLC analyses were performed on an Agilent 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA) with a reverse phase C_{18} column (Agilent, 4.6×250 mm, $5 \mu\text{m}$). Tail flick latencies were measured on an Ugo Basile Talk Flick Unit (Ugo Basile SRL, Varese, Italy) and static hind paw weight bearings were evaluated on an Incap incapacitance meter (Columbus Instruments, Columbus, OH). Semiquantitative biodistribution studies were carried out using an IVIS® Spectrum *in vivo* imaging system (PerkinElmer, Inc. Waltham, MA). A Faxitron® MX-20 Cabinet X-ray System (Faxitron Bioptics, LLC, Tucson, AZ) was used for detection of the decalcification progress. A Leica RM2255 microtome (Leica Biosystems, Buffalo Grove, IL) was used for paraffin-embedded tissue sectioning. For fluorescent microscopic analysis, both cultured cells and tissue sections were evaluated using a Zeiss LSM 800 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY). For microscopic analysis of H&E slides, an Olympus BX 51 (Olympus Corporation of the Americas, Center Valley, PA) was used. Spectrophotometric absorbance measurements were performed using a SpectraMax M2e UV/Vis spectrophotometer (Molecular Devices, Sunnyvale, CA).

Synthesis of poly(HPMA-co-HMP) (P-HMP)

HPMA (400 mg, 2.79 mmol) and MA-Gly-Gly-NHNH₂ (41.9 mg, 0.196 mmol) were dissolved in methanol (3 mL), and to this solution was added the initiator 2,2'-azobisisobutyronitrile (AIBN, 2.37 mg, 0.0145 mmol) and the RAFT agent *S,S'*-bis(α , α' -dimethyl- α'' -acetic acid)-trithiocarbonate (CTA, 2.27 mg, 0.008 mmol). The solution was purged with argon and polymerized at 50 °C for 48 hr. The resulting polymer was first purified on a LH-20 column (GE Healthcare, Waukesha, WI) to remove the unreacted low molecular weight compounds and obtain the desired product. The resulting copolymer (330 mg) and hydromorphone hydrochloride (33.5 mg, 0.11 mmol) were then dissolved in methanol (3 mL). Acetic acid (0.3 mL) was added to the reaction solution as a catalyst. The solution was again purged with argon and stirred at room temperature for 72 hr. After evaporation of the reaction solvent, the P-HMP product was purified by LH-20 column.

Synthesis of poly(HPMA-co-HMP-co-APMA)

As the precursor to the dye-containing copolymers, poly(HPMA-co-HMP-co-APMA) was first synthesized. Briefly, HPMA (500 mg, 3.49 mmol), APMA (6.8 mg, 0.038 mmol), MA-Gly-Gly-NHNH₂ (53 mg, 0.247 mmol), AIBN (2.98 mg, 0.018 mmol) and CTA (2.38 mg, 0.01 mmol) were dissolved in methanol (6 mL) in a glass ampoule. After purging with argon for 5 min, the ampoule was flame-sealed and heated at 50 °C for 48 hr in the absence of light. The mixture was then purified by LH-20 column to remove the unreacted low

molecular weight compounds, dialyzed (molecular weight cutoff = 25 kDa) for 24 hr in ddH₂O and then lyophilized. The resulting copolymer (300 mg) and hydromorphone hydrochloride (32.3 mg, 0.1 mmol) were dissolved in methanol (3 mL). Acetic acid (0.3 mL) was added to the reaction solution as a catalyst. The solution was purged with argon and stirred for 72 hr at room temperature. Upon evaporation of the reaction solvent, the poly(HPMA-co-HMP-co-APMA) product was purified by LH-20 column.

Synthesis of poly(HPMA-co-HMP-co-IRDye 800CW) (P-HMP-IRDye)

Poly(HPMA-co-APMA-co-HMP) (150 mg, containing 0.0084 mmol amine) and IRDye 800CW carboxylate (1 mg, 0.0009 mmol) were dissolved in dimethylformamide (DMF, 300 μ L) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) activating agent (13 mg, 0.084 mmol), hydroxybenzotriazole (HOBt, 11.3 mg, 0.084 mmol) and *N,N*-diisopropylethylamine (DIPEA, 10.8 mg, 0.084 mmol). The mixture was stirred overnight in darkness at room temperature. Subsequently, the mixture was directly purified via LH-20 column and lyophilized.

Synthesis of poly(HPMA-co-HMP-co-Alexa Fluor 488) (P-HMP-Alexa)

Poly(HPMA-co-APMA-co-HMP) (150 mg, containing 0.0084 mmol amine), Alexa Fluor 488 NHS ester (1 mg, 0.0016 mmol) was dissolved in DMF (1 mL) and to the solution was added DIPEA (10.8 mg, 0.084 mmol). The mixture was stirred overnight in darkness at room temperature, immediately after which it was directly purified via LH-20 column and lyophilized.

***In vitro* HMP release from P-HMP**

The P-HMP conjugate (2 mg/mL) was dissolved in acetate buffers (0.01 M with 0.15 M NaCl, pH 5.0 and pH 6.5, respectively) or phosphate buffer (0.01 M with 0.15 M NaCl, pH 7.4) and incubated at 37 °C. At selected time intervals, aliquots (0.3 mL) were withdrawn and neutralized, if appropriate, for HPLC analysis (mobile phase acetonitrile-water 35:65 [v/v], containing sodium dodecyl sulfate [0.5%, w/v] as ion pairing reagent, and acetic acid [0.4% v/v]; injection volume = 10 μ L; flowrate = 1 mL/min; UV detection = 230 nm).

Cell culture study

RAW 264.7 cells (American Type Culture Collection, TIB-71) were grown in a 75 cm² flask to confluence in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 U/mL and 100 μ g/mL, respectively) under incubation (37 °C, 5% CO₂). Cells were scraped and their density counted using a hemacytometer visualized under a light microscope, and the suspension was thereafter diluted (2×10^5 cells/mL) in fresh supplemented media. To a 24-well plate was added 15-mm diameter round coverslips and suspension media (1 mL). The macrophages were allowed to adhere onto the coverslips under incubation (24 hr, 37 °C, 5% CO₂). The following day, coverslips were washed and aspirated (2 $\times$, PBS) and to each well was added LPS (10 μ g/mL) in fresh, unsupplemented media, and cells were again incubated (24 hr, 37°C, 5% CO₂). The P-HMP-Alexa was then directly added to each well (final concentration 200 μ g/mL) and placed under incubation (24 hr, 37 °C, 5% CO₂). The

following day, cells were aspirated and washed (2×, PBS), and to the wells was added media with LysoTracker^{TM} Red DND-99 (75 nM, Thermo Fisher Scientific, L7528). After incubation (3 hr, 37 °C, 5% CO₂), coverslips were washed and aspirated (2×, PBS), fixed, and mounted onto microscope slides using the ProLong^{TM} Gold Antifade mountant containing DAPI nuclear stain (Thermo Fisher Scientific, P36931). Upon drying, the slides were immediately visualized using confocal microscopy.

Establishment of the monoarticular adjuvant-induced arthritis (MAA) model

Male Lewis rats (175–200 g) were obtained from Charles River Laboratories and allowed to acclimate for at least one week under standard housing conditions. Freund's complete adjuvant was freshly prepared by mixing methylated bovine serum albumin (mBSA, 2 mg/mL) in distilled water with *Mycobacterium tuberculosis* strain H37RA (2 mg/mL, heat-killed) in paraffin oil to form an emulsion. Rats were injected with adjuvant (0.5 mL) subcutaneously (s.c.), twice at two different sites on the back during a one-week interval for immunization. After an additional two weeks, animals were anesthetized (1–1.5% isoflurane and 1 L/min O₂), and given an intraarticular injection of mBSA (500 µg in 50 µL distilled water) to the left knee joint cavity to induce monoarticular adjuvant-induced arthritis (MAA). Successful establishment of arthritis was confirmed by the presence of edema in the left hindlimbs and of the rats' compromised gaits. All animal experiments were carried out in accordance by guidelines evaluated and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center.

Near-infrared biodistribution study of P-HMP in MAA rats

The day after arthritis induction, rats (n = 4 per imaging day) were administered a mixture of P-HMP-IRDye (IRDye equivalent 3.5×10^{-7} mol/kg) and P-HMP (altogether 6 mg/kg HMP equivalent) via tail vein injection. Rats were imaged using the IVIS Spectrum imaging system on days 1 and 3 to evaluate distribution and retention of the IRDye-labeled prodrug. Prior to acquisition, rats were anesthetized (2% isoflurane and 1 L/min O₂) and hair was removed from the hindquarters to avoid attenuation of fluorescence signal. Briefly, hair removal cream (Veet Gel Hair Remover Cream, Sensitive Formula) was applied directly to the hindquarters imaging area and rinsed off with warm water until complete elimination of the hair. Upon acquisition, the captured images were then analyzed using the Living Image 4.5 software (PerkinElmer, Inc.).

Immunohistochemical and histological analysis of the knee joints

To MAA rats was intravenously injected P-HMP-Alexa (HMP equiv. 6 mg/kg, n = 12) one day post-induction. On day 1 post-dosing, rats were perfused intracardially with saline, then fixed with 4% (w/v) paraformaldehyde in saline. Both the diseased (left) and healthy control (right) hind limbs were collected then decalcified using 20× specimen volume of freshly-prepared, neutralized 10% (w/v) EDTA solution. The decalcifying solution was changed daily in the first week, then three times a week, until complete removal of calcium was confirmed via X-ray. Each tissue sample was then cut to size directly around the joint and synovial areas and then paraffin-embedded. Slices (20 µm) were cut using a microtome and placed onto tissue-adherent slides. After antigen retrieval using sodium citrate buffer and blocking using 10% normal goat serum, the slides were immunohistochemically stained

separately with mouse anti-rat CD68 (Bio-Rad, MCA341R, dilution 1:100) and rabbit anti-rat P4HB (ProSci Inc., 8213, dilution 1:100). Dually-stained slides were treated with antibodies for rabbit anti-rat TRPV1 (Abcam, ab31895, dilution 1:100) and guinea pig anti-rat mu opioid receptor (μ OR, Novus Biologicals, NB100–1618, dilution 1:100). Slides were then treated with goat anti-rabbit Alexa Fluor 568 (dilution 1:1000), goat anti-mouse Alexa Fluor 647 (dilution 1:1000), or goat anti-guinea pig Alexa Fluor 647 (dilution 1:1000) at room temperature in the dark (1 hr). The stained slides were imaged using a ZEISS LSM 800 confocal microscope after mounting in either ProLong® Gold antifade mountant with DAPI (Thermo Fisher Scientific, P36931) or Fluoromount-G® (Southern Biotechnology, 00–4958-02). Additional paraffin slides (10 μ m) were then hematoxylin and eosin (H&E) stained and evaluated under microscope (Olympus BX51) to capture the histological differences between the arthritic and healthy knee joints.

Analgesic efficacy study of P-HMP on MAA rats

Hydromorphone hydrochloride (6 mg/kg, n = 10) or P-HMP (HMP equiv. 6 mg/kg, n = 10) were administered to MAA rats by tail vein injection (i.v., 1 mL/kg). Saline was injected intravenously as a control (n = 10). The weight distribution between the animal's hind limbs was measured using the incapitance tester. This apparatus consists of two force transducers capable of measuring the body weight that the animal places on each hind limb. Animals were placed on the incapitance tester with their hind paws centered on the two force transducers, and the average body weight distribution in grams was calculated over a period of three seconds. The weight bearing score is expressed as a ratio of the weight placed through the limb ipsilateral to the inflammation versus the sum of the weights placed through both the contralateral and ipsilateral limbs, with a ratio of 50% resulting from equal weight distribution across both hind limbs. Weight distribution was measured before induction, before dosing, and at several time intervals following drug administration, up to seven days.

The tail flick test was performed as a supplemental method to determine whether the analgesic properties of our prodrug are restricted from off-target effects within the spinal cord. For this test, rats' tails were exposed to a focused beam of radiant heat at a point 3 cm from the tip using the tail flick unit. Tail flick latencies were defined as the interval between the onset of the thermal stimulus and the reflexive response of the tail. Animals not responding within 15 sec were removed from the tail flick unit and assigned a withdrawal latency of 15 sec. Tail flick latencies were measured at pre-induction, pre-dosing, and at several other time points following drug administration, up to seven days.

Statistics

Data are shown as mean $\pm$ SD. Statistical analysis was performed using Prism version 7.03 (GraphPad Software, Inc.). The results of the incapitance and tail flick assays were analyzed using two-way ANOVA, and individual groups were compared using Tukey's HSD test for multiple comparisons.

Results

Synthesis and characterization parameters of HPMA-co-HMP conjugates

After 48 hours of copolymerization of HPMA, APMA and MA-Gly-GlyNHN=Dex, the resulting polymer was purified by LH-20 column and dialyzed against ddH₂O to remove the unreacted low molecular weight compounds. Subsequently, HPLC analysis of the polymer indicated that there was no free Dex in the copolymer. In addition, P-Dex-APMA was labeled with Alexa Fluor 488 and IRDye 800CW via conjugation with their corresponding NHS esters. After overnight reaction, the solutions were purified by LH-20 column to remove small molecule reactants. The M_w , PDI, and fluorescent tag contents of the copolymers are detailed in Table 1.

In vitro release of HMP from P-HMP conjugate

Three different buffer conditions were used to mimic the environments to which the HMP copolymer conjugate is exposed *in vivo*. The buffer at pH 7.4 reflects the initial condition of the intravenously-injected prodrug into the bloodstream. Gradual release at this pH is observed over the time course measured. Once the P-HMP is extravasated to the site of inflammation, a pH of 6.5 often dominates due to increased glycolysis under the hypoxic conditions present in chronic inflammation. In simulating conditions, the drug demonstrates a similar release pattern to that in the neutral condition, with more rapid release kinetics. Furthermore, uptake of the prodrug into phagocytic vesicles exposes them to a pH of approximately 5, and it is apparent that this can result in rapid HMP release from the polymer backbone; ~90% of the drug content is released within 8 hours in corresponding *in vitro* conditions.

Phagocytosis of P-HMP by murine macrophages

As evident in Figure 2, the LPS-activated macrophage cells are able to internalize the P-HMP-Alexa copolymer after 24 hr incubation. The drug signal also coincides with the red fluorescence of the LysoTracker, indicating that the copolymer does indeed reside within the intracellular lysosomal compartments.

Near-infrared imaging-based biodistribution study of P-HMP in MAA rats

There is strong fluorescent signal at the left knee joint at days 1 and 3 post-injection of the P-HMP-IRDye, as apparent in Figure 3. This sustained near-infrared signal which accumulates primarily at the site of inflammation is indicative of P-HMP's ability to preferentially target areas of joint inflammation.

Immunohistochemical and histological analysis of knee joints

The image panels shown in Figure 4A indicate the co-localization of P-HMP (green) with CD68 (macrophage marker, magenta) and P4HB (fibroblast marker, red) within the arthritic knee joints of MAA rats. DAPI (blue) indicates nuclei. There is visible co-existence of the prodrug with its target μ OR (magenta) and as well as with the TRPV1 ion channel (red). The contralateral healthy control knee joints (Figure 4B) display very little to no prodrug fluorescence and no infiltrated inflammatory cells within the joint synovium. The TRPV1

ion channels and the μ ORs appear to be downregulated in contrast to the inflammatory MAA knee joints. In conjunction with these images, the acquisition areas of the IHC panels as captured by confocal microscopy are indicated by the asterisk in the H&E images of Figure 5. There is clear evidence of cellular infiltration (CI) within the synovium and tissue (cartilage/bone) destruction (TD) within the arthritic joint. No signs of arthritis, such as inflammatory cell infiltration, bone erosion, cartilage damage and synovial hyperplasia, is visible in the contralateral healthy control knee joints (Figure 5).

Analgesic efficacy of P-HMP on MAA rats

The mean weight distribution ratio (percent weight bearing score, %WBS) were calculated for each group using the following formula:

$$\%WBS = \left[\frac{\text{weight on injured leg}}{\text{weight on uninjured leg} + \text{weight on injured leg}} \right] * 100$$

While there is a marked difference in WBS values for the P-HMP versus saline within the first 8 hours after dosing, there is no significant variation amongst the P-HMP and free HMP groups at the time points measured (Figure 6). This indicates that the P-HMP exhibits rapid therapeutic onset and duration similar to the free HMP injection, but the effect does not last beyond the first day. Statistical significance is observed within the first 8 hours, and in particular at the onset of dosing, between the saline control and HMP groups, indicative of the free drug's immediate analgesic effect. However, there is a delay in analgesic response observed within the P-HMP group.

The tail flick test revealed that the P-HMP lacks significant analgesic action upon the spinal cord. Data were expressed as tail flick latency (sec), and the percentage of the maximal possible effect (%MPE, 15 sec) was calculated as follows:

$$\%MPE = \left[\frac{\text{postdrug latency} - \text{predrug latency}}{15 \text{ sec} - \text{predrug latency}} \right] * 100$$

For the HMP (6 mg/kg) group, since rats lost consciousness from 0–3 hrs after drug administration, tail flick latency was kept at the maximal value (Figure 7). There are significant differences between the prodrug and the free HMP in their spinal cord analgesic effects. While the free drug positive control is responsible for significantly increased tail flick latencies within the first day, the latency values of the prodrug are significantly reduced and return to those of the saline control within the first five hours of administration.

Discussion

Oftentimes a primary symptom of its underlying disease, the pain associated with a chronic ailment usually requires potent analgesic therapy with immediate effect. Opioids such as hydromorphone and fentanyl have shown similar or superior pain modulating efficacy to over-the-counter NSAID analgesics and acetaminophen,¹⁰ highly depending on the nature of the pain³⁴ and drug tolerability in individual sufferers. In the particular case of inflammatory conditions like rheumatoid arthritis, long-term opioid therapy is a mainstay treatment.⁶

However, with the infamous opioid epidemic currently on the rise, a modified drug approach to improve the pharmacokinetic and biodistribution profiles of these medications is warranted. It has long since been established the ability of HPMA copolymers to extravasate into solid tumor tissue via the enhanced permeability and retention (EPR) effect, due to the polymer's uncharged and flexible nature which allows passage through endothelial tissue layers.³⁵ Analogous to tumor targeting, our previous research^{21–24} has proven the ability of HPMA copolymers to passively and selectively target inflamed tissue through what we have coined as the ELVIS mechanism; i.e. Extravasation through Leaky Vasculature and Inflammatory cell-mediated Sequestration. HPMA copolymers are also known to not permeate the blood brain barrier (BBB).²⁰ Therefore, by administering a polymeric conjugate of the model opioid hydromorphone (HMP) and a biocompatible HPMA copolymer carrier, we hypothesized that the increased vascular permeability present at the arthritic joints and restricted CNS access to water-soluble polymers would enable preferential targeting to areas of inflammatory lesion, potentially decreasing the spinal cord analgesia of opioids that lead to tolerance, dependence, and addiction.

The general design of P-HMP structure was partially based upon our previous PK/BD analysis of HPMA copolymer-dexamethasone conjugates (P-Dex) on an arthritis rat model.³⁶ A moderate M_w of 35 kDa was selected to provide a favorable joint relative exposure (AUC_{joint}/AUC_{blood}) and a reasonable absolute joint distribution value. A lower M_w would further increase the AUC_{joint}/AUC_{blood} value, but significantly reduce the P-Dex' absolute distribution to the arthritic joint due to reduced serum half-life. While further increase of the M_w would raise P-Dex' absolute joint distribution, it would reduce joint specificity. Such an increase must also be limited to within the 45 kDa glomerular filtration threshold³⁷ to allow the polymeric prodrug's renal clearance. A hydrazone bond was used to conjugate HMP to the HPMA copolymer carrier because of the convenient presence of a ketone in HMP. Due to the lack of local π conjugation, this hydrazone bond is more prone to acid-catalyzed hydrolysis (Figure 1) when compared to the P-Dex copolymers.²⁴

According to the results of the weight bearing scores after a single i.v. administration, the analgesic effect of P-HMP is almost identical to that of the free HMP. The slightly delayed therapeutic effect seen with P-HMP can be construed as a result of the time it takes the macromolecules to permeate into and extravasate from the network of vessels present at the inflammatory site and to be activated. As evidenced in the tail flick assay, the spinal cord analgesic effects of P-HMP vs. HMP is significantly reduced within the first 8 hrs of administration, and almost entirely eliminated after 5 hrs post-dosing. Spinal cord analgesia is a complex phenomenon, partially a result of descending mechanisms which modulate the activity of dorsal horn neurons. Opioids are known to reduce pain transmission at this level by inhibiting neurotransmitter release from the dorsal horn. In both the incapacitance (Figure 6) and tail flick (Figure 7) tests, the free HMP is demonstrated to have significant analgesic effects. Since the opioid alone does not have particular affinity for inflammatory pathologies, it is distributed to the area of arthritis as well as to the spinal cord region upon systemic administration, through penetration of the BBB. However, data from the tail flick test indicates that P-HMP does not cross the BBB or the dorsal horn to give rise to spinal cord analgesia. Because the tail does not possess inflammatory properties, the macromolecular P-HMP does not accumulate within this region, and we posit that the

existence of modest analgesia within the P-HMP group at earlier time points is most likely due to the diffusion of hydrolyzed HMP from the inflammatory depots back into circulation. The arthritic joint-targeting property of the prodrug is supported in the near-infrared imaging-based biodistribution data as well. Therefore, the two methods here used to measure analgesia together conclude that the P-HMP prodrug works primarily peripherally within the inflamed joint. Previous work has indicated that opioid tolerance is associated with the development of a compensatory response among spinal cord dorsal horn neurons, contributing to central sensitization that ultimately leads to tolerance, dependence, and addiction.³⁸ Given the results from these experiments, our macromolecular prodrug strategy may thus be able to allay the detrimental CNS effects commonly associated with opioid use.

The *in vitro* cell culture (Figure 2) and IHC (Figure 4) data prove that the prodrug can indeed be internalized by resident and infiltrating phagocytic immune cells, as shown via the co-local fluorescence of the Alexa-labeled prodrug with the activated macrophage cell line *in vitro* and with the antibody markers for macrophage-like (CD68) and fibroblast-like (P4HB) synoviocytes in the *ex vivo* processed tissues. Moreover, hydromorphone is known to elicit its analgesic effects through selective binding to opioid receptors, and in particular the mu subtype (μ OR).³⁰ Primary afferent nerves in vertebrate animals exist as pseudounipolar neurons, wherein their cell body, or soma, resides in the dorsal root ganglion or in the trigeminal ganglion for cranial nerves. Mu opioid receptors are expressed on the two axons emanating from these neural cells, the central projection terminating in the dorsal horn of the spinal cord and the peripheral projection terminating in the skin or in the organs of the body. Though it is widely acknowledged that under normal conditions analgesia via μ OR agonism is most prominent in the spinal cord and other central nervous system regions,³⁹ in inflammatory conditions, there is ample evidence of increased functionality of opioid receptors on primary afferent neurons and even on immune cells.⁴⁰ Data from the IHC analysis supports this mechanism of pain attenuation that may be brought about by our peripherally-restricted macromolecular prodrug. In Figure 4A, the positive fluorescent IHC staining of the μ OR receptor co-existent with P-HMP in processed arthritic tissue confirms the presence of the target receptor within the joint synovial cavity and corroborates our prodrug's ability to interact with these receptors. In this same slide, the dual staining of the TRPV1 nociceptive ion channel identifies an important element in the transmission and modulation of pain. There is substantial co-localization of this channel with μ OR, as well as with P-HMP. As expected, P-HMP was not observed in the healthy joint (Figure 4B) due to the absence of ELVIS-enabling pathology (Figure 5). The TRPV1 ion channels and the μ ORs were also downregulated when compared to the arthritic joints (Figure 4B). Activation of peripheral opioid receptors on nerve endings is known to suppress TRPV1 currents via the Gi/o and the cAMP/protein kinase A pathway.⁴¹ In addition, though originally believed to be to be exclusively associated with sensory neurons, this ion channel has also been identified on immune cells, from which its manipulation can mediate immune functions such as inflammation.⁴² In particular, its suppression in macrophages has been shown to reduce the cellular production of reactive oxygen species, which play a crucial role in the perpetuation of pain and inflammation.⁴³ Therefore, through binding to its receptor on immune cells and possibly on peripheral nerve endings could the hydromorphone mitigate the activities of co-localized TRPV1 receptors to produce analgesia within the inflamed

joint. However, as the therapeutic data suggests, the 1-day post-dosing analgesic efficacy of P-HMP is minimal, since HMP may have been entirely cleaved from the prodrug and metabolized by this time.

To further improve the efficacy of the macromolecular HMP prodrug, new linker chemistry for P-HMP may be designed wherein the hydrolysis rate is adjusted for sustained release. This is possible because the fine-tuning of the microchemical environment (e.g. introduction of an aromatic structure) of the cleavable linkers, such as hydrazone, in HPMA copolymers are known to affect the pH-dependent cleavage rate.⁴⁴ The utility of an acetal linker can also be explored due to the availability of a phenol in the HMP structure. This may, however, involve more complex chemistry. The *in vivo* imaging data indicates that the polymer is present at the inflamed tissue site at least three days post-dosing. Therefore, if a new prodrug design can afford a sustained P-HMP activation for a prolonged period of time *in vivo*, a persistent analgesic effect beyond the dosing day may be achievable. The increase of P-HMP MW could also be a valuable strategy to improve its retention within the inflammatory regions and therefore would require less frequent dosing than with the free drug alone, which can reduce toxic metabolite accumulation and adverse effects on the CNS. Such an increase is known to be accompanied by increased HPMA copolymer prodrug distribution to the mononuclear phagocyte system (MPS),³⁶ which warrants close monitoring of the liver function panel for potential signs of hepatotoxicities. To ensure proper renal clearance, any necessary M_w increase beyond the 45 kDa glomerular filtration threshold may justify the use of biodegradable polymeric carriers (e.g. dextran, hyaluronic acid, etc.) or the use of hybrid carriers^{45–49} which may partially degrade and yield HPMA copolymers of much shorter length. Future therapeutic efficacy studies should also include repeated dosing regimens, the data from which could further demonstrate the superior efficacy and safety of the macromolecular prodrug design as a new class of analgesic.

From a more general prospective, polymers, either in the form of nanoparticles or water-soluble polymeric carriers, have been used extensively for the delivery of therapeutic and diagnostic agents to the CNS.⁵⁰ Active transport, opening of the tight-junction and the pathological breaching of the blood-brain barrier (BBB) and/or the blood-spinal cord barrier (BSCB) have been used to facilitate such delivery. The use of polymers for CNS delivery is mainly capitalizes on the platform nature of the carriers wherein different modalities can be easily installed. Intriguingly, neutral water-soluble polymers such as poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA) cannot permeate the BBB under normal physiological conditions.²⁰ Using a reverse thinking approach, we conjugated HMP to the HPMA copolymer. The resulting P-HMP was proven to elicit very little spinal cord analgesia, confirming the polymer prodrug's restricted access to the BBB/BSCB. Such conjugation also facilitates the passive targeting of HMP to the inflammatory arthritis pathology due to the ELVIS mechanism. As a further implication, we speculate that chemical conjugation to water soluble polymeric carriers may be used as a general strategy to reduce CNS side effects of therapeutic agents. The caveat of this strategy, obviously, is that the intrinsic inflammation tropism of these polymeric prodrugs must be simultaneously considered as favorable to promote targeted drug delivery.

Conclusion

A macromolecular prodrug of hydromorphone (a potent analgesic), has been successfully synthesized and characterized. We administered it as a single i.v. injection in a rodent model of monoarticular adjuvant-induced arthritis (MAA). In addition to an analgesic effect similar to the dose-equivalent HMP, the prodrug demonstrated significantly attenuated spinal cord analgesia. This may be attributed to the polymer prodrug's ability to preferentially target the site of inflammation, where it is internalized and activated via the phagocytic cells and readily accessible to local peripheral nerves, and to its restricted access to the CNS. Through this unique distribution pattern, the prodrug may mitigate opioid-induced tolerance, dependence, and addiction. With further structural optimization, we anticipate to create a P-HMP with potent and sustained analgesic effect and no off-target analgesia. In the future, these development schemes could be extrapolated to other analgesics for the more effective and safe management of chronic inflammatory pain.

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Figure 1.

In vitro release kinetics of P-HMP prodrug. Data are shown as mean $\pm$ standard deviation.

All analyses are performed in triplicate.

Figure 2.

Co-Localization of P-HMP-Alexa prodrug within mouse macrophage intracellular organelles. Representative confocal microscopy image of RAW 264.7 cells stained with LysoTracker Red DND 99 (red, lysosome marker) and P-HMP-Alexa conjugate (green). Nuclei are marked with DAPI (blue). The merged image of all three fluorescent signals is suggestive of co-localization of the P-HMP with intracellular lysosomes via the yellow color observed. Scale bars = 20 μm .

Figure 3.

In vivo visualization of prodrug targeting to arthritic joint. Representative 1 day and 3 days post-injection fluorescent images. Fluorescent signal intensity was normalized to the same scale for each image.

Figure 4. Confocal fluorescence microscopy analysis of arthritic (A) and healthy (B) joint synovium after systemic administration of P-HMP-Alexa. All scale bars = 20 μ m.

Figure 5. H&E stained histology images of (A) MAA knee joint and (B) healthy knee joint at 1-day post-dosing. CI = cellular infiltration; TD = tissue (bone/cartilage) destruction. Asterisks (*) indicate image acquisition area for IHC analysis. Scale bars = 1 mm.

Figure 6.

Percent weight bearing scores of MAA rats. For the free HMP group, incapacitance could not be measured from 0–3 hrs post-dosing, during which time the rats were comatose. **, $P < 0.01$ P-HMP vs. saline; ***, $P < 0.001$ P-HMP vs. saline; ****, $P < 0.0001$ P-HMP vs. saline; ##, $P < 0.01$ HMP vs. saline; ###, $P < 0.001$ HMP vs. saline; ####, $P < 0.0001$ HMP vs. saline.

Figure 7.

Tail flick latency values of MAA rats. For the free HMP group, tail flick latency was kept at the maximal value (15 sec) during the time rats were comatose. ††, $P < 0.01$ P-HMP vs. HMP; †††, $P < 0.001$ P-HMP vs. HMP; ††††, $P < 0.0001$ P-HMP vs. HMP; **, $P < 0.01$ vs. saline; ***, $P < 0.001$ vs. saline; ****, $P < 0.0001$ vs. saline.

Scheme 1.

Synthesis of HPMA copolymer-hydromorphone (HMP) conjugate (P-HMP). CTA, chain transfer agent; AIBN, 2,2'-azobisisobutyronitrile; MeOH, methanol.

Scheme 2.

The synthesis of P-HMP-IRDye (**A**) and the synthesis of P-HMP-Alexa (**B**).

Table 1. Characterization of HPMA Copolymer Conjugates^a

Polymer Conjugates	M_w ($\times 10^3$ g/mol)	PDI	[HMP] ($\mu\text{mol/g}$)	[Alexa 488] ($\mu\text{mol/g}$)	[IRDye 800CW]
P-HMP	32.5	1.12	578.34 ± 25.94	0	0
P-HMP-Alexa	33.4	1.16	452.16 ± 4.91	82.6 ± 1.1	0
P-HMP-IRDye	33.4	1.16	473.19 ± 15.07	0	76.4 ± 0.9

^a M_w : weight average molecular weight. PDI: polydispersity index.