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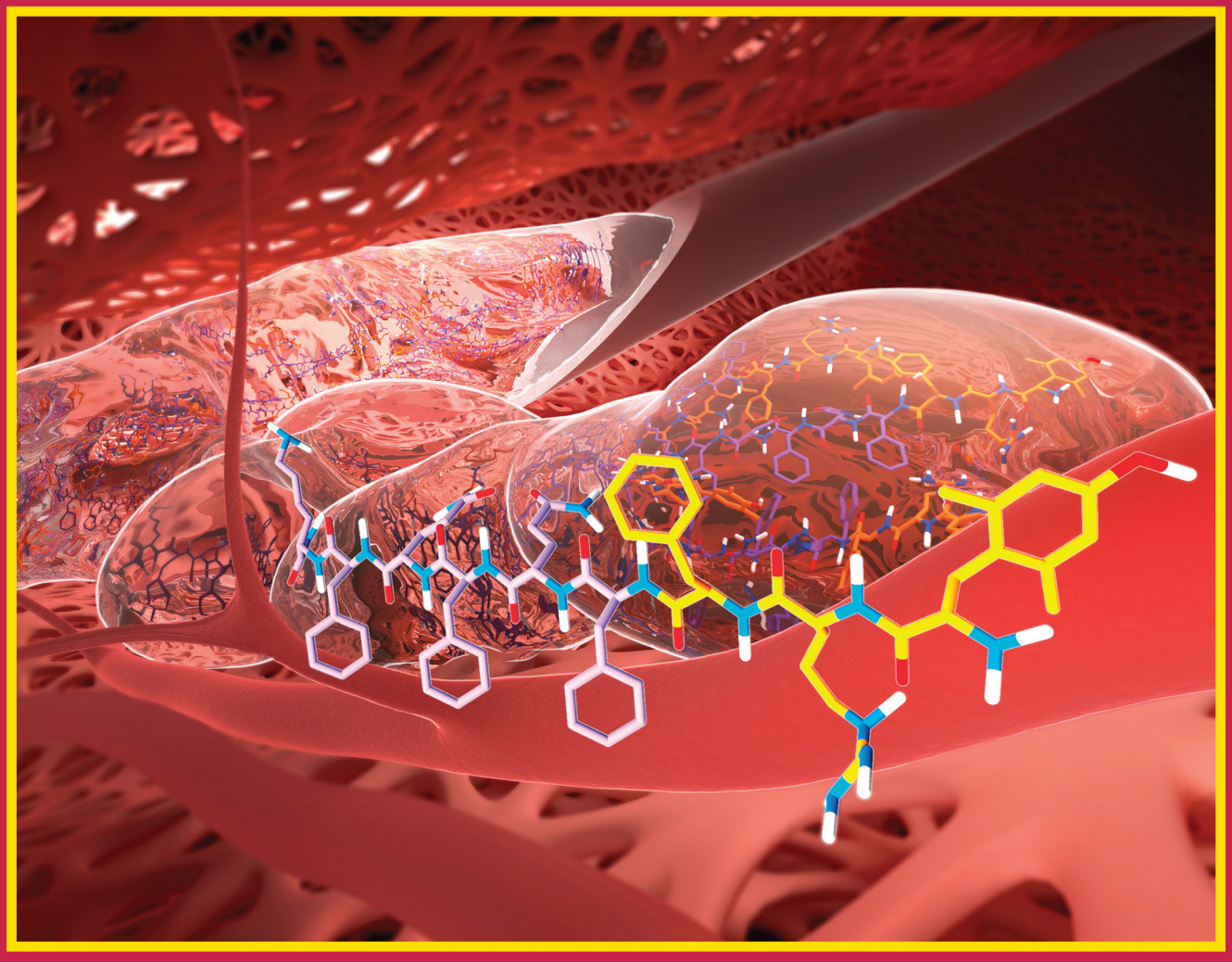

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Brief Article

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Biodegradable Amphipathic Peptide Hydrogels as Extended-Release System for Opioid Peptides

Charlotte Martin,[†] Maria Dumitrascuta,[‡] Morgane Mannes,[†] Aquilino Lantero,[‡] Dominik Bucher,[‡] Katja Walker,[‡] Yannick Van Wanseele,[§] Edith Oyen,[†] Sophie Hernot,[∥] Ann Van Eeckhaut,[§] Annemieke Madder,[*,⊥] Richard Hoogenboom,[*,#] Mariana Spetea,[*,‡] and Steven Ballet[*,†]

[†]Research Group of Organic Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, Brussels B-1050, Belgium

[‡]Department of Pharmaceutical Chemistry, Institute of Pharmacy and Center for Molecular Biosciences (CMBI), University of Innsbruck, Innrain 80-82, 6020 Innsbruck, Austria

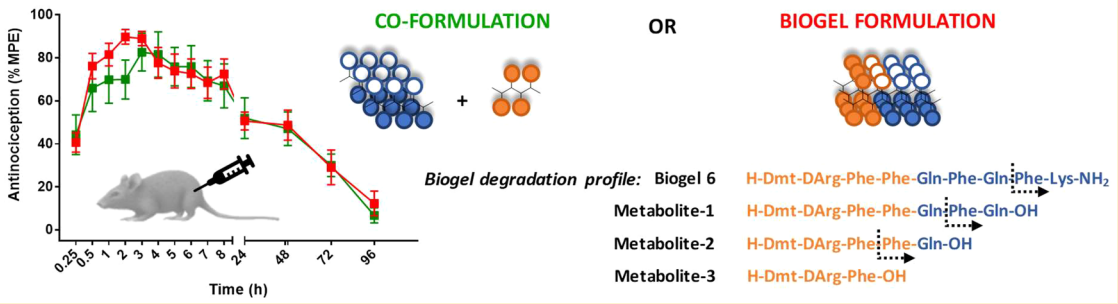
[§]Department of Pharmaceutical Chemistry, Drug Analysis and Drug Information, Center for Neurosciences (C4N), Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium

[∥]In Vivo Cellular and Molecular Imaging, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium

[⊥]Organic and Biomimetic Chemistry Research Group, Ghent University, Krijgslaan 281, 9000 Ghent, Belgium

[#]Supramolecular Chemistry Group, Centre of Macromolecular Chemistry (CMaC), Ghent University, Krijgslaan 281, 9000 Ghent, Belgium

Supporting Information



ABSTRACT: Chronic pain is currently treated with opioids that offer unsatisfactory long-term analgesia and produce serious side effects. There is a clear need for alternative therapies. Herein, peptide-based hydrogels are used as extended-release drug delivery carriers. Two different formulations were developed: the drug is coformulated within the hydrogel; the drug is an integral part of the hydrogelator. Both strategies afford a prolonged and significant antinociception up to 72 h after subcutaneous administration in mice.

INTRODUCTION

Chronic pain remains one of the main challenges in human medicine at the beginning of the third millennium, with about 20−30% of people worldwide suffering from chronic pain.[1] Opioid analgesics are the most effective and widely prescribed drugs to treat moderate to severe pain,[2] but their clinical use is often limited by serious side effects and complications, such as respiratory depression, constipation, nausea, sedation, development of analgesic tolerance, and abuse liability.[3] Furthermore, in light of the current opioid epidemic, there is a central need to find innovative, effective, and safe analgesics.[4] To treat chronic pain, opioids can be administered as short-acting opioid (SAO) or long-acting opioid (LAO) formulations. The SAOs require repeated administration, with a duration of action of 2−3 h, resulting in a poor and inconsistent pain relief with higher occurrence of adverse effects, while LAOs show a more gradual drug release in the bloodstream leading to prolonged effects of 8 up to 72 h.[5] Such LAOs use controlled drug delivery systems that provide a constant drug concentration in the systemic circulation, thus reducing the frequency of administration which in turn improves patient compliance.[6] Although various extended-release (ER) formulations/technologies have been developed over the years, consistent pain relief is still poorly managed, stressing the need for innovative and superior treatments. Notably, only conventional opioids, including morphine, oxycodone, buprenorphine, and fentanyl, all known to induce major side effects, are used to date in existing ER formulations.[6]

As an alternative to conventional opioid drugs displaying detrimental adverse effects, several endogenous and exogenous opioid peptides have been evaluated as potential analgesics

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over the years. One such peptide, dermorphin, is approximately 1000 times more potent in humans than morphine while inducing fewer side effects paralleled by longer analgesia.[7] Inspired by these promising findings, dermorphin was used as a platform for the development of various analgesic peptides,[8] and hence we envisaged them as useful leads for the design of peptidomimetic LAO formulations with better antinociceptive effects, as compared to morphine, and with a reduced propensity for side effects. In the domain of pain therapy, several controlled-delivery systems are available for hydrophobic analgesics,[6] but versatile systems for hydrophilic drugs, such as painkilling peptides, are still lacking.

This work describes new peptide-based hydrogels for chronic pain management by using them as biomaterial formulations for the extended release of opioid peptides. Among various types of hydrogels, peptide-based hydrogels are highly attractive due to their biocompatibility, biodegradability, and cytocompatibility, as they are made from natural building blocks, and their ease of synthesis and purification.[9] Hence, they found widespread use in biomedical applications, such as tissue engineering and drug delivery.[10] Recently, we designed a new family of short amphipathic peptide-based hydrogels, which form thixotropic injectable hydrogels upon dissolution in aqueous solutions.[11] The efficacy of the hydrogel networks as controlled drug delivery platform was demonstrated for morphine, showing extended antinociceptive effect up to 72 h after subcutaneous (sc) administration in mice.[12] In this study, we established the use of peptide hydrogels for the ER of opioid peptides, which is to the best of our knowledge unprecedented proof that amphipathic peptide hydrogels are compatible with peptide cargoes.

RESULTS AND DISCUSSION

In this study, two strategies were targeted: (i) the analgesic drug is encapsulated within the hydrogel network (i.e., a "coformulation"), and (ii) the analgesic pharmacophore is covalently linked to the hydrogelator, resulting in an analgesic hydrogel conjugate (hereafter called "biogel" formulation) (Figure 1). While the conjugation of drugs to macromolecule carriers (such as PEG) has been used to improve pharmacokinetic profiles, to our knowledge, the design of prodrugs solely based on biodegradable self-assembling peptides is unprecedented.

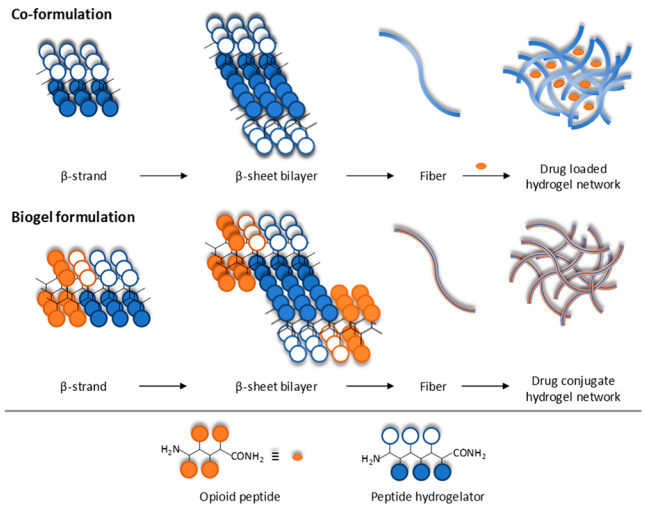

Figure 1. Illustration of self-assembling of supramolecular hydrogels based on amphipathic peptide sequences. The "coformulation" and "biogel" strategies are depicted.

While in the coformulation the drug loading and release depend on diffusion and complexation/desorption processes, guided by the interaction of the drug with the hydrogel's fibers, the biogel concept presents advantages such as a lowered drug release rate via implementation of a covalent linkage between the opioid pharmacophore and the peptide hydrogelator, thus limiting the risk of burst release and protecting the peptide drug from rapid clearance.[13]

Herein, the opioid pharmacophores **1**−**5** (Table 1) were designed based on dermorphin (H-Tyr-DAla-Phe-Gly-Tyr-Pro-Ser-NH_2) and endomorphin-2 (H-Tyr-Pro-Phe-Phe-NH_2), two endogenous opioid peptides displaying high potency and selectivity for the μ-opioid receptor (MOR), the primary target for effective analgesia.[14] Since it was demonstrated that the N-terminal tetrapeptide of opioid peptides is commonly accepted to be the minimum sequence required for high opioid activity, N-terminal [Dmt^1,D-Xaa^2]-tetrapeptide analogues were designed. While **3** is an optimized and balanced MOR/δ-opioid receptor (DOR) agonist,[15] opioid peptides **4** and **5** correspond to the [Dmt^1]-endomorphin-2 and [Dmt^1]endomorphin-1 sequences, respectively. All opioid peptides, **1**−**5**, showed very high affinity to the MOR, with **1** and **5** demonstrating picomolar affinity (Table S2).

Generally, all opioid peptides displayed decreased affinities to the DOR and κ-opioid (KOR) receptors, thus providing MOR selectivity. Also, the opioid receptor binding affinity of **3** established in this study is in line with the profile recently described.[16] On the basis of functional activity at the human MOR as determined in the [^{35}S]GTPγS binding assay, **1**−**5** were very potent agonists (EC_{50} ranging from 0.10 to 0.86 nM) and showed high efficacy acting as full agonists except for **4**, which was a potent MOR partial agonist (Table S3).

Accumulated evidence indicates that MOR-mediated antinociception results from G-protein-mediated signaling, while β-arrestin-2 signaling pathways promote the unwanted effects of opioids.[17] The concept of biased agonism at the MOR has gained significance to drug discovery, where the development of G-protein-biased MOR agonists may deliver the desired analgesia while avoiding the side effects. To verify MOR biased agonism of the opioid peptides (**1**−**5**) toward activation of G protein- over β-arrestin-2-mediated signaling, we compared their functional activity, i.e., potency and efficacy, across two functional assays that measure G protein coupling (the [^{35}S]GTPγS binding assay) and β-arrestin-2 translocation (the DiscoveRx PathHunter β-arrestin-2 recruitment assay) at the human MOR (Table S3). We established that opioid peptides **1**−**5** activate G protein with high potency as full MOR agonists while displaying much lower potencies in inducing β-arrestin-2 recruitment, thus stimulating the MOR in a manner that is preferentially biased toward G protein signaling.

In a next stage, these opioid peptides **1**−**5** (coded as OP1−5) were coformulated or covalently linked to recently described hexapeptide hydrogelators **14**−**16** (coded as GEL1−4)[11,12] giving way to a set of biogel sequences (Table 1).

The biogel conjugates were constructed by linking the opioid part to the peptide hydrogelator via an amide bond. The resulting biogel sequences can be considered as prodrugs

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Table 1. Opioid Pharmacophores (OP), Biogel Sequences, and Selected Hydrogelator Sequences (GEL)

compd	code	sequence
1	OP1	H-Dmt-DArg-Phe-Phe-NH_2
2	OP2	H-Dmt-DLys-Phe-Phe-NH_2
3	OP3	H-Dmt-DArg-Aba-βAla-NH_2
4	OP4	H-Dmt-Pro-Phe-Phe-NH_2
5	OP5	H-Dmt-Pro-Trp-Phe-NH_2
6	OP1-GEL2	H-Dmt-DArg-Phe-Phe-Gln-Phe-Gln-Phe-Lys-NH_2
7	OP1-GEL4	H-Dmt-DArg-Phe-Phe-Gln-β^3hPhe-Phe-Gln-Phe-Lys-NH_2
8	OP2-GEL1	H-Dmt-DLys-Phe-Phe-Glu-Phe-Gln-Phe-Lys-NH_2
9	OP2-GEL2	H-Dmt-DLys-Phe-Phe-Gln-Phe-Gln-Phe-Lys-NH_2
10	OP3-GEL2	H-Dmt-DArg-Aba-βAla-Phe-Gln-Phe-Gln-Phe-Lys-NH_2
11	OP4-GEL1	H-Dmt-Pro-Phe-Phe-Glu-Phe-Gln-Phe-Lys-NH_2
12	OP4-GEL2	H-Dmt-Pro-Phe-Phe-Gln-Phe-Gln-Phe-Lys-NH_2
13	OP5-GEL2	H-Dmt-Pro-Trp-Phe-Gln-Phe-Gln-Phe-Lys-NH_2
14	GEL1	H-Phe-Glu-Phe-Gln-Phe-Lys-NH_2
15	GEL2	H-Phe-Gln-Phe-Gln-Phe-Lys-NH_2
16	GEL4	H-Phe-Glu-β^3hPhe-Phe-Gln-Phe-Lys-NH_2

that release active pharmacophores after proteolytic degradation, wherein the hydrogelators were selected based on our recent findings related to thixotropic behavior, as well as extended in vivo release properties.[11,12]

Biogels **6**, **9**, **10**, **12**, and **13** are based on the hexapeptide hydrogelator H-Phe-Gln-Phe-Gln-Phe-Lys-NH_2 (**15**) and biogel **8** and **11** on H-Phe-Glu-Phe-Gln-Phe-Lys-NH_2 (**14**) with Glu in position 2 instead of Gln, while biogel **7** contains a mixed α/β sequence, H-Phe-Gln-β^3hPhe-Phe-Gln-Phe-Lys-NH_2 (Table 1). The latter hydrogel was designed to stabilize the peptide sequence against enzymatic degradation, in view of a potential extended release of the drug by slowing down its degradation.[12] In order to evaluate the in vivo stability of the peptide hydrogels, in vivo SPECT/CT imaging experiments in mice were performed (Figures S3.6A, S3.6B, S3.6C). The degradation of the hydrogel was followed by determination of the hydrogels' volume at the site of the injection. The selected hydrogelators **14** and **15** showed a slow in vivo degradation profile, with around 20% of the hydrogel still present 72 h after sc injection in mice (Supporting Information, Figure S3.6B).

The in vitro pharmacological profiles of the opioid pharmacophores **1**−**5** and biogels **6**−**13** (Table 1) were evaluated and compared in terms of binding affinities and functional activities at the human opioid receptors (Supporting Information, Tables S2 and S3). Covalent attachment of the opioid peptides **1** to **5** to recently reported peptide hydrogelators,[11,12] resulting in biogels **6**−**13** (Table 1) considerably decreased affinity, selectivity, and agonist potency at the MOR. In the series of biogel sequences, affinities in the nanomolar range were shown at the MOR (K_i ranging from 5 to 141 nM), with biogels **6**, **7**, and **9** being the most active and potent agonists in vitro (Table S3). Biogels **8**, **11**, **12**, and **13** displayed moderate MOR affinity and low agonist potency, some of them even showing partial agonism at the MOR. This lower activity of the biogels enhances their safety as prodrugs as they only become active after metabolic degradation (vide infra).

Central goals in chronic pain control are to provide analgesia of adequate efficacy and duration, to balance the patient's pain relief, to limit potential harmful consequences of opioids, and to improve the overall quality of life. In the current study, we assessed and compared antinociceptive properties of the opioid peptides in solution, coformulated with hydrogels, and as biogels (Figure 2a, Figures S3.4A, S3.4B, and S3.4C). Antinociception was evaluated in a model of acute thermal nociception, the tail-flick test, after systemic sc administration in mice. When administered in solution, compounds **1**−**3** produced a significant antinociceptive effect (up to 7 h for **1**, 8 h for **2**, and 4 h for **3**, Figure S3.4B). Opioid peptides **4** and **5** had a much shorter duration of the antinociceptive effect without reaching the desired 80% maximum possible effect (% MPE).

We then evaluated the coformulation and biogel strategies in terms of their extended release profile of the embedded/attached opioid peptides. Compound **1** coformulated with hydrogelator **15** or conjugated to hydrogelator **15** (to give biogel **6**) produced prolonged and significant antinociception up to 72 h when sc administered to mice (Figure 2a). Moreover, no significant differences were noted when comparing the effect of the two formulation strategies, supporting the previously proposed hypothesis that the controlled drug release is based on hydrogel fiber erosion rather than the proteolytic degradation of the hydrogelating sequences.[12] On the other hand, when **1** is conjugated to a stabilized hydrogelator sequence resulting in biogel **7**, a noticeable difference was found with **7** being significantly less efficacious in inducing an antinociceptive response with a much shorter duration action (up to 3 h) (Figure S3.4A, panel a). The reduced potency of **7** may be explained by its increased proteolytic stability due to the presence of a mixed α/β sequence obstructing its metabolic degradation into the active form; vide infra (Table 1). In contrast, a promising effect was shown for opioid peptide **2** coformulated with hydrogelator **15** or conjugated to hydrogelator **14** (biogel **8**) or to hydogelator **15** (biogel **9**), producing prolonged and significant antinociception up to 72 and 24 h, respectively (Figure S3.4A, panel b). When comparing **2** formulated in **8** or **9**, sc administered to mice, there was no significant difference in the antinociceptive effect. When coformulated with hydrogelator **15**, the more evolved peptidomimetic **3** was also highly effective, with a significant antinociceptive effect up to 72 h postadministration (Figure S3.4C). As **4** and **5** were established not to be very efficacious in causing an antinociceptive response and their corresponding biogels **11**, **12** and **13** exhibited fairly weak binding and agonist potencies at the MOR (Tables S2 and S3), eventually due to the rapid

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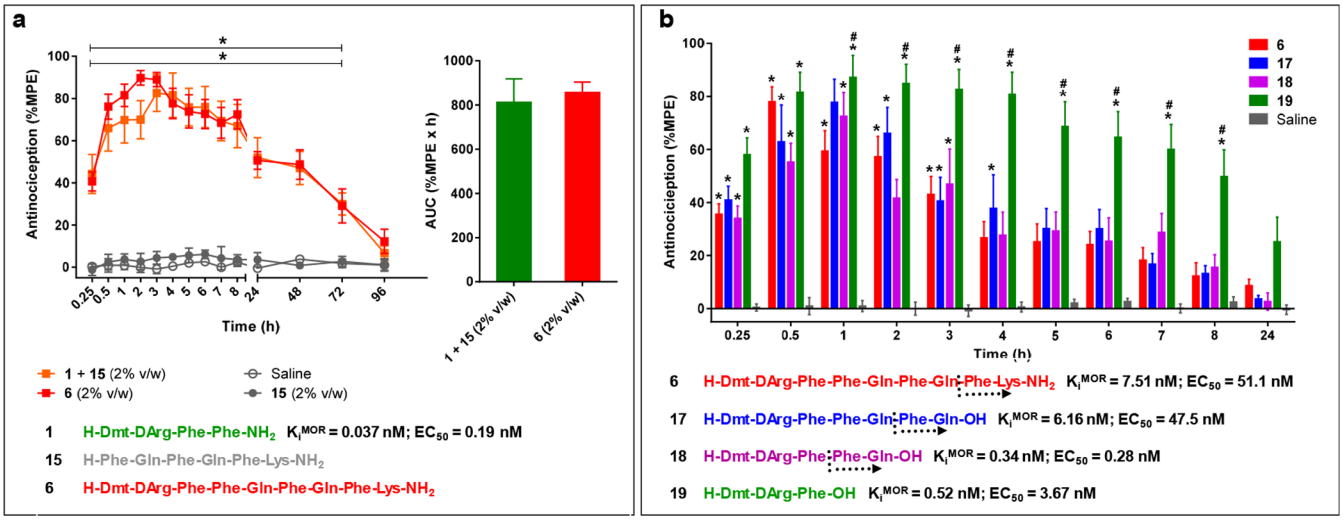

Figure 2. Coformulation and biogel strategies for the extended release profile of opioid peptide **1**. (a) Comparison of antinociceptive activity of **1** coformulated with hydrogelator 15 (GEL2) or conjugated to hydrogelator **15** resulting in biogel **6**, in the tail-flick test after sc administration in mice. Groups of mice received **1** (0.56 μmol/mouse) coformulated with **15** (2% v/w), **6** (2% v/w), **15** (2% v/w) alone (control), or saline (control) in a volume of 150 μL per mouse. (b) In vitro degradation profile of **6** (in solution) in human plasma. Antinociceptive activity of **6** and its metabolites **17**−**19** (Met-1 to -3, respectively) in the tail-flick test after sc administration in mice. Groups of mice received solutions of **6** (3.0 μmol/kg), **17** (3.9 μmol/kg), **18** (4.2 μmol/kg), **19** (6.8 μmol/kg), or saline (control) (right panel). Antinociceptive response, as %MPE, was measured over time, and area-under-the-curve (AUC) values for the respective time curves were calculated. Data are the mean ± SEM of 6−8 mice per group: (*) $P < 0.05$ vs respective control groups, (#) $P < 0.05$ vs 6 group, two-way ANOVA followed by Bonferroni post hoc test.

cleavage of the Dmt[1]-Pro[2], no formulations of these peptides were investigated in vivo for antinociceptive properties.

To study the drug release mechanism from the biogel conjugates, in vitro biostability experiments were performed in human plasma at physiological (37 °C) temperature. From this study, the half-life of each sequence was calculated and the major metabolites were identified at different time-points. Comparison of the half-lives highlighted that the presence of Dmt at the N-terminal end of the sequence (**6**, $t_{1/2}$ = 16 min) does not increase the stability of the sequence, compared to the Tyr analogue ($t_{1/2}$ = 18 min), while insertion of a β^3 homoresidue within the hydrogelator sequence (biogel **7**) gives rise to a highly stable peptide ($t_{1/2}$ = 123 min). To understand the metabolic degradation profile of these biogel sequences, the structures of the main metabolites were determined by LC−MS analysis of collected samples from the plasma stability experiments. The results showed that in human plasma, the biogels are degraded from the C- to N-terminus, with preferential amide cleavage at the Gln[5]-Phe[4] or Glu[5]-Phe[4] site. From this degradation mechanism, three main metabolites were identified corresponding to the hepta-, penta-, and tripeptides. For example, in the case of **6**, the main metabolites are **17**, **18**, and **19** (Figure 2b). Regarding biogels **7** and **10**, which contain unnatural amino acids, the degradation pattern seems to be similar. However, the presence of β^3hPhe and βAla residues, respectively, is suggested to prevent the release of an active pharmacophore, able to induce a biological effect in vivo.

Among the three metabolites of **6** (Figure 2b), **18** was identified as the most potent MOR agonist in vitro, being about 130 times more active than **6** (Table S3). Overall, the rank order of affinities/agonist potencies of the biogel **6** metabolites at the human MOR in vitro was **18** > **19** > **17** ~ **6**. In vivo, they all produced potent antinociceptive effect in the tail-flick test after sc administration in mice. While **17** and **18** displayed a similar time-course of the antinociceptive response to **6** when given at equianalgesic doses and a relatively short effect (up to 3−4 h), **19** showed an extremely long duration of action for such a short, nonformulated peptide (up to 8 h, $P < 0.05$ vs saline and **6** treated mice, two-way ANOVA with Bonferroni post hoc test) (Figure 2b).

CONCLUSIONS

Two peptide-based hydrogel strategies were deployed to achieve effective and prolonged antinociception. Herein, the released opioid compounds consisted of potent opioid tetrapeptides, when applying the coformulation method, or truncated peptide-derivatives resulting from the proteolytic degradation of the gelator segment in the biogel sequences. The degradation profile of one biogel sequence, **6**, unveiled the discovery of an ultrapotent and long-acting tripeptidic metabolite **19**. The biodegradability of the gelator sequence is extremely favorable, since conjugation to the described hydrogelators can be regarded as a prodrug approach applicable to many other biologically active sequences, which would gain therapeutic potential when existing in a controlled-release format.

EXPERIMENTAL SECTION

Peptide Synthesis. The syntheses were performed manually using standard Fmoc-based solid phase peptide synthesis (SPPS). Rink amide AM resin was used for the synthesis of all sequences (OP, biogels, and GELs) with the exception of the three metabolites (MET) that were prepared starting from commercially available preloaded Wang resin. Amino acids (3 equiv) were coupled using HBTU (3 equiv) and DIPEA (5 equiv) in DMF for 40 min. The coupling of Boc N-protected Dmt (1.5 equiv) required the use of DIC (1.5 equiv) and HOBt (1.5 equiv) in DMF as coupling mixture with a reaction time of 2 h. Fmoc removal was performed using a 20% 4-methylpiperidine in DMF solution 2 times (5 and 15 min, respectively). After completion of the sequences, the peptides were cleaved from the resin by treatment with a cleavage cocktail constituted of TFA/TIPS/H_2O (95:2.5:2.5) for 3 h at room

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temperature. After filtration, the solvent was evaporated in vacuo and the residue dissolved in water/acetonitrile (1:1) and lyophilized. Crude peptides were purified by preparative reverse phase HPLC to yield pure peptide as a white powder after lyophilization, with a purity >95%, as assessed by analytical HPLC. Details about peptide purification are provided in the Supporting Information.

Peptide Gelation. Peptide gelation was performed, as previously reported.[12] Details are included in the Supporting Information.

Pharmacology. In Vitro Opioid Receptor Activities. Binding assays were conducted on human opioid receptors stably transfected into CHO cells according to the published procedures.[18] Binding of [^{35}S]GTPγS to membranes from CHO cells stably expressing the human MOR (CHO-hMOR) was conducted according to the published procedure.[18] The measurement of MOR stimulated β-arrestin-2 recruitment was performed using the DiscoveRx PathHunter β-arrestin-2 assay (DiscoveRx, Birmingham, U.K.) according to the manufacturer's protocol and published procedures.[19] Details are reported in the Supporting Information.

Nociceptive Assessment. The radiant heat tail-flick test was used to assess antinociceptive effects of the test peptides after sc administration in mice using an UB 37360 Ugo Basile analgesiometer (Ugo Basile s.r.l., Varese, Italy), as described previously.[20] Details are described in the Supporting Information.

Stability Studies. In vitro proteolytic stability tests were performed using human plasma, and the in vivo stability of hydrogel networks composed of GEL1−4 was controlled through in vivo imaging as previously reported.[21] Details are included in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.8b01282.

Additional chemical and pharmacological information (PDF)

Molecular formula strings (CSV)

■ AUTHOR INFORMATION

Corresponding Authors

*A.M.: e-mail, annemieke.madder@ugent.be.
*R.H.: e-mail, richard.hoogenboom@ugent.be.
*M.S.: e-mail, mariana.spetea@uibk.ac.at.
*S.B.: e-mail, steven.ballet@vub.be.

ORCID

Annemieke Madder: 0000-0003-0179-7608
Richard Hoogenboom: 0000-0001-7398-2058
Mariana Spetea: 0000-0002-2379-5358
Steven Ballet: 0000-0003-4123-1641

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

%MPE, percentage of maximum possible effect; Aba, 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one; CHO, Chinese hamster ovary; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; Dmt, 2′,6′-dimethyltyrosine; DOR, δ-opioid receptor; ER, extended release; hMOR, human μ-opioid receptor; HBTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole hydrate; KOR, κ-opioid receptor; LAO, long-acting opioid; MOR, μ-opioid receptor; SAO, short-acting opioid; SEM, standard error of the mean; SPECT-CT, single-photon emission computed tomography−X-ray computed tomography

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