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Elucidation on the In Vivo Activity of the Bivalent Opioid Peptide MACE2 against Several Types of Chronic Pain

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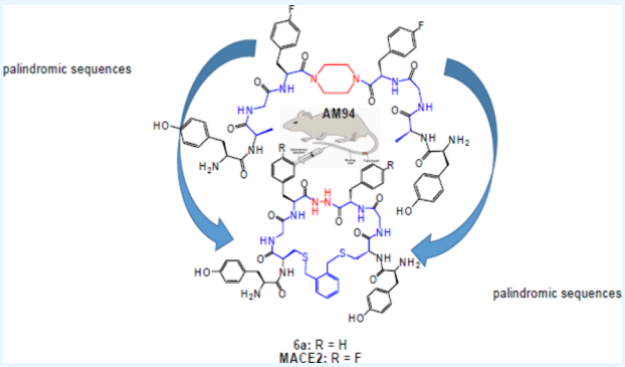
ABSTRACT: Biphalin is a bivalent μ/δ opioid receptor agonist showing a promising therapeutic profile with reduced side effects, but as a peptide is limited by poor metabolic stability and blood-brain barrier penetration. To improve these features, we developed the ligand **MACE2** and showed initial in vivo efficacy. To further explore the druggability of this ligand, in this report, we tested **MACE2** metabolic stability in human plasma, receptor engagement by 3 different routes of administration using the tail-flick test, and **MACE2** efficacy in 2 different pathological and chronic pain models. We found that **MACE2** had high stability in plasma and could produce target engagement and a tail flick response. We also showed that **MACE2** had high analgesic efficacy in CIPN but no efficacy in paw incision. Together, these findings suggest that **MACE2** has improved metabolic stability and brain penetration in vivo, prompting further development in clinical testing.



INTRODUCTION

Chronic pain is pain that lasts more than three months, as defined by the International Association for the Study of Pain (IASP). Currently available therapies comprise nonopioid analgesics such as nonsteroidal anti-inflammatory drugs (NSAIDs), acetaminophen, and aspirin, along with other drug classes like tricyclic antidepressants and gabapentinoids, which represent first-line therapies.[1,2] Second-line options include opioids, which are recommended for patients with moderate to severe pain or otherwise when pain has a negative impact on the patient's quality of life.[3,4] Unfortunately, opioid side effects are frequent and well documented in the literature, including hyperalgesia, dependence, addiction, and tolerance.[5]

Furthermore, escalating doses to combat severe pain or overcome tolerance can induce a dose-dependent risk of overdose.[6,7]

For this reason, the search for methods to improve opioid therapy, including the development of new opioid drugs, is of paramount importance. Co-administration of δ- (DOR) and μ-opioid receptor (MOR) agonists produces synergistic antinociception which could be advantageous over selective μ-agonists in therapy by improving the therapeutic index.[8−11] Bivalent MOR/DOR ligands are viable analgesics with improved therapeutic profiles versus selective MOR agonists; in this context, several small molecules and peptidomimetics have been described in the literature; however, none of them have been approved for human use.[12−18]

RV-JIM-C3 and biphalin represent two linear peptidomimetic-based molecules described as bivalent opioid ligands.[17,19,20] The first is a bivalent hybrid of fentanyl and the endogenous peptide enkephalin, which is able to induce dose-dependent antinociception in an in vivo model of pain.[17] Biphalin is a palindromic synthetic peptide endowed with high efficacy in animal models after intracerebroventricular administration but shows poor blood-brain barrier (BBB) penetration and low metabolic stability. However, unlike morphine, it produces less tolerance when administered in the spinal cord.[19,20]

These findings suggest that MOR/DOR bivalent peptides could be promising therapeutic options if their pharmacokinetic limitations can be overcome. To this end, we and others have explored various modifications to improve the metabolic stability and BBB penetration of these peptides and peptidomimetic ligands.[21−24] One promising option is cyclic peptides containing an *o*-bis(methyl)benzene bridge derived from the combination of local structural modifications and the global constriction of the parent linear peptide biphalin; they

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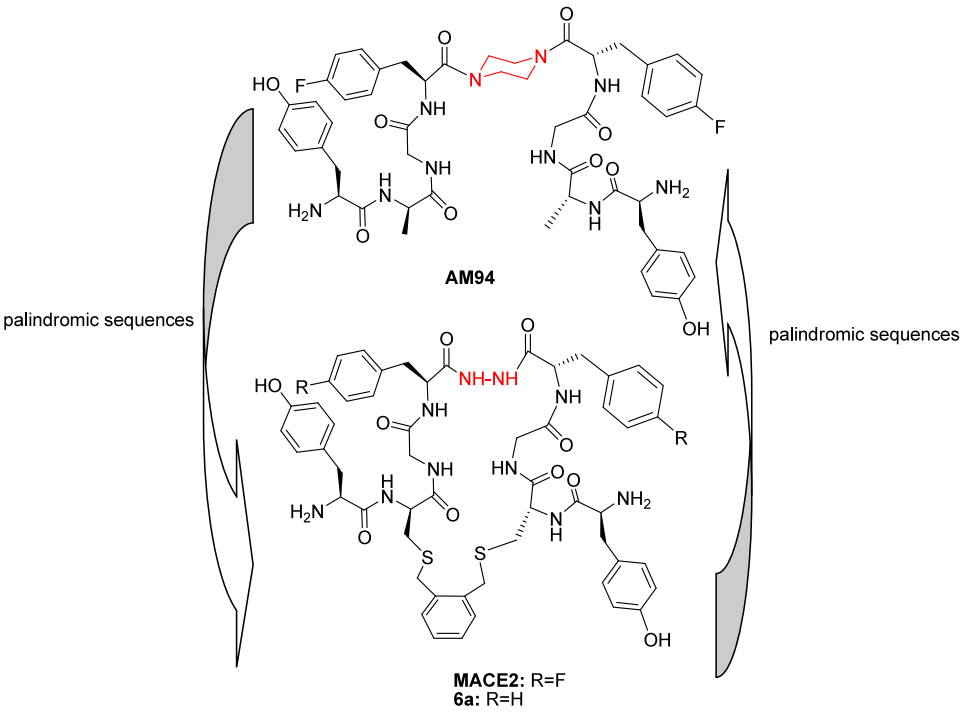

Figure 1. Rational design of cyclic peptide **MACE2** and its parent compound **6a**.

exhibit good stability in human plasma and higher antinociceptive effects in mice.[22] This renders them optimal lead compounds for further improvements.

One such cyclic peptide recently described by us, namely, **MACE2**, possesses an ideal pharmacological profile: it shows a strong affinity for MOR and DOR with a DOR/MOR ratio of 0.3 against selectivity ratios of 18 and 59 for KOR/MOR and KOR/DOR, respectively. Its high efficacy at MOR is associated with a strong analgesic activity in the hot-plate test after iv and in the formalin test after sc routes; **MACE2** showed higher activity in both than biphalin. Also, **MACE2** was shown to have a good resistance to metabolic degradation in human plasma after 240 min and the best permeability value in a PAMPA model among other compounds.[22] Since these are desirable features to develop an efficient and druggable opioid peptide analogue, we planned to further investigate the pharmacokinetic and pharmacodynamic properties of this ligand in order to complete its overall pharmacological profile as a safe and effective MOR/DOR opioid agonist for the management of chronic pain with reduced side effects.

RESULTS AND DISCUSSION

Design and Synthesis. The cyclic peptide **MACE2** is a hybrid peptidomimetic of biphalin composed of two palindromic primary sequences common to the parent compound **6a**,[25,26] joined together with an *o*-bis(methyl)-benzene bridge. In **MACE2**, the Phe$^{4,4'}$ residues present in compound **6a** have been substituted by a pF-Phe$^{4,4'}$, thus it could be considered as a cyclic analogue of the linear peptide **AM94** (Figure 1). Peptide **AM94** includes this type of local modification with the insertion of a piperazine central linker; this is the most potent and long lasting in vivo compound among the linear analogues.[26] For this work, the title peptide **MACE2**, compound **6a**, biphalin, and its analogue **AM94** were prepared according to our previously published procedures,[25,26] and then the human plasma stability was performed to test the resistance to metabolic degradation in 1 h.

Plasma Stability. To check the resistance of the freshly prepared peptides **MACE2** and **AM94** to metabolic degradation, we determined the percentage of the remaining compound in human plasma after 1 h of incubation at 37 °C. Both ligands were stable in human plasma, with 95.44 and 98.75% remaining for **MACE2** and **AM94,** respectively, after 1 h (Figure 2). Both of them show stability in human plasma higher than that of biphalin.[27]

Nociceptive Pain Model. In order to complete the panel of responses to thermal stimuli previously explored for **MACE2,**[22] the tail-flick test was used to evaluate the antinociceptive efficacy after intracerebroventricular (i.c.v.), intrathecal (i.t.), and intravenous (i.v.) administration in mice and in comparison with the parent compounds biphalin and **6a** (Figure 3). When administered i.c.v. (Figure 3A), biphalin, **6a,** and **MACE2** induced an efficacious antinociceptive effect in comparison with vehicle-treated animals. Both **6a** and **MACE2** induced a greater effect than biphalin, although statistical analysis revealed a significant difference only for **6a** compared to biphalin (Figure 3A). After i.t. administration, biphalin, **6a,** and **MACE2** were able to exert an efficacious antinociceptive effect in comparison with animals treated with vehicle, but **6a** and **MACE2** induced a greater antinociceptive effect than biphalin-treated animals (Figure 3B). A similar behavior was observed after iv administration (Figure 3C), in which biphalin exerted a modest antinociceptive effect, while **6a** and **MACE2** produced a maximal degree of antinociception, similar to that observed after i.c.v. or i.t. administration. Together, these results suggest that our modifications to the biphalin parent structure have improved the metabolic stability and enabled **6a** and **MACE2** to act also after i.v. administration. These results also expand the behavioral repertoire first reported for **MACE2** in our previous work.[22]

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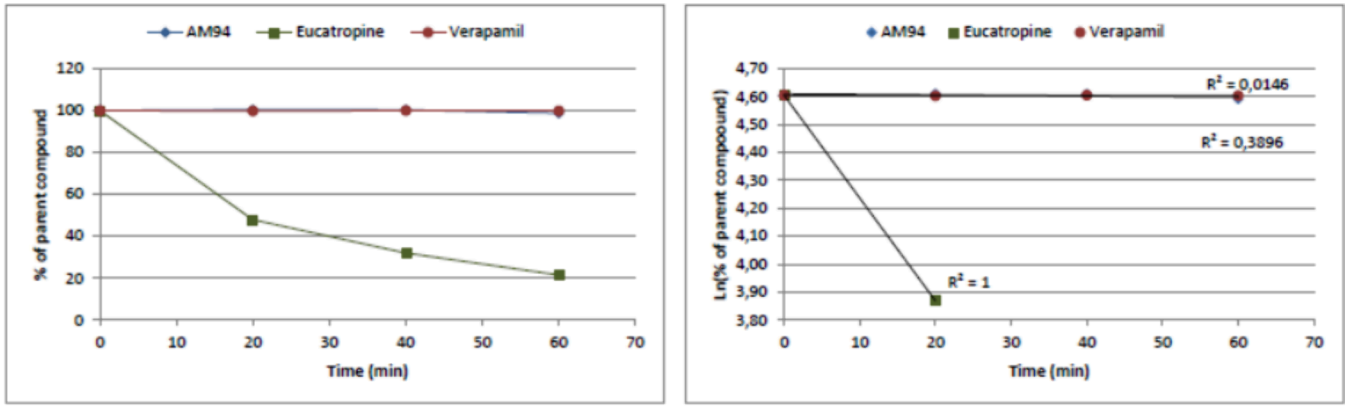
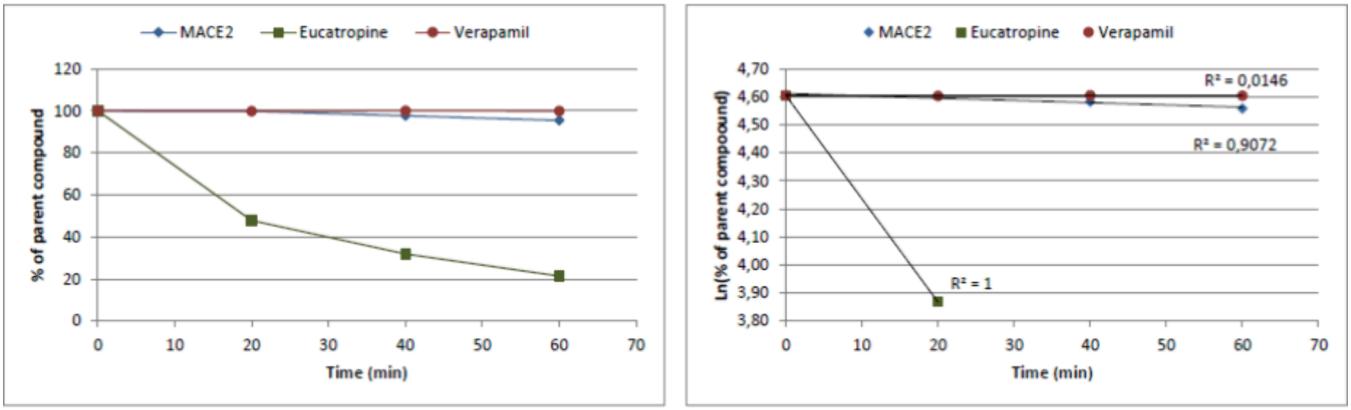


Parameter	unit	AM94	Eucatropine	Verapamil
h	1/min	0,0002	0,0369	0,000004
T1/2	min	>60	18,80	>60
% remaining	%	98,75	21,43	99,90



Parameter	unit	MACE2	Eucatropine	Verapamil
h	1/min	0,0008	0,0369	0,000004
T1/2	min	>60	18,80	>60
% remaining	%	95,44	21,43	99,90

Figure 2. Human plasma metabolic stability of **MACE2** and **AM94**. Plots represent the total amount of remaining parent compound versus time. Least-squares linear regression analysis was used to calculate the degradation half-life ($t_{1/2}$) of **MACE2** and **AM94** in human plasma. Verapamil and Eucatropine were used as internal reference compounds.

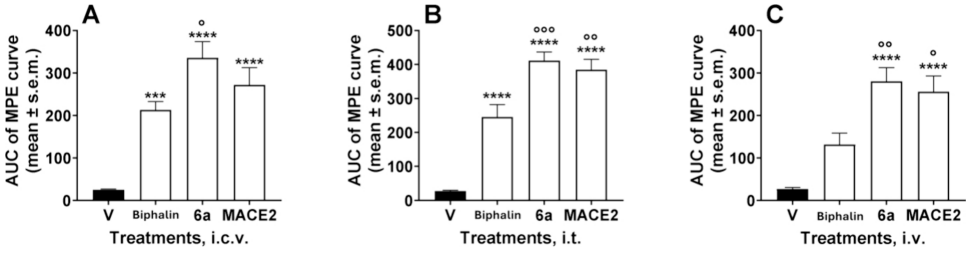


Figure 3. Effects induced by vehicle (V), biphalin, **6a,** and **MACE2** in the tail flick test. Data are reported as area under the curve of the maximum possible effect (%MPE) = (postdrug latency − baseline latency)/(cutoff time − baseline latency) × 100. In panel A, the effects induced by peptides administered i.c.v. at the dose of 0.6 nmol/mouse are reported. The effects obtained after i.t peptide administration at the dose of 0.6 nmol/mouse are reported in panel B. In C are reported the effects obtained after i.v. peptide administration at the dose of 1.5 μmol/kg. **** is for $P < 0.0001$ and *** is for $P < 0.001$ vs V; ° is for $P < 0.05$, °° is for $P < 0.01$ and °°° is for $P < 0.001$ vs Biphalin. $N = 8–10$ mice/group.

Pathological and Chronic Pain Models. Nociceptive pain assays like the tail-flick test can provide evidence of receptor engagement; however, they are not highly translationally relevant to pain patients. We thus sought to test **MACE2** in the postsurgical paw incision and chemotherapy-induced peripheral neuropathy (CIPN) pain models. These models are highly

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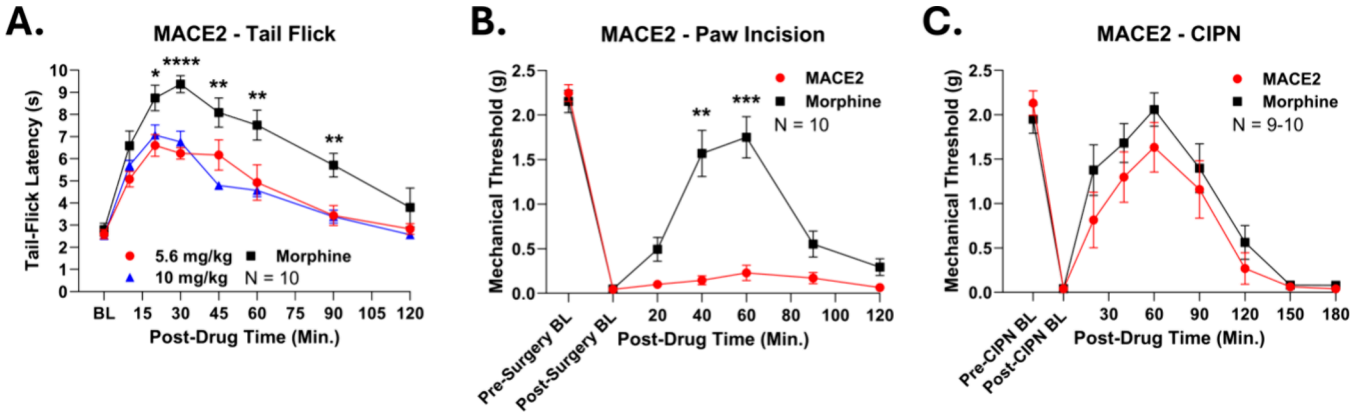

Figure 4. MACE2 is efficacious against chemotherapy-induced peripheral neuropathy (CIPN) but not postsurgical pain. Male and female CD-1 mice in each experiment, data as the mean ± SEM. Each experiment was completed in 2 technical replicates. (A) Dose ranging performed by administering 5.6−10 mg/kg **MACE2** or 10 mg/kg morphine by the s.c. route in the tail flick assay. Both 5.6 and 10 mg/kg **MACE2** produced efficacious antinociception albeit significantly less than morphine. BL = baseline. (B) Paw incision with pre- and postsurgical mechanical thresholds measured using von Frey filaments. Twenty-four h after surgery, 10 mg/kg **MACE2** or 10 mg/kg morphine administered s.c. and a time course measured. **MACE2** produced no measurable antinociception in this assay. (C) CIPN induced using paclitaxel as described in the Methods. On day 8, 10 mg/kg **MACE2** or 10 mg/kg morphine delivered s.c., and a time course was performed. **MACE2** produced efficacious antinociception that was statistically the same as that of morphine ($p > 0.05$). *, **, ***, **** = $p < 0.05$, 0.01, 0.001, 0.0001 vs same time point **MACE2** group by 2 Way ANOVA with Dunnett's (tail flick) or Sidak's (paw incision, CIPN) post hoc test. We have previously tested the drug vehicle used in these experiments, which had no measurable effect on these pain types.[30,31]

translationally relevant and include pathological elements such as neuroinflammation, central sensitization, and more.[28,29]

We also sought to improve our translational relevance by delivering **MACE2** via the s.c. route. However, we had not previously delivered **MACE2** by this route, so we first did dose-ranging using the tail flick assay. Both 5.6 and 10 mg/kg **MACE2** s.c. are able to induce efficacious tail flick antinociception, albeit less than a comparison dose of 10 mg/kg morphine (Figure 4A). Thus, as established, we used a 10 mg/kg dose of **MACE2** in both pathological pain assays. Interestingly, we found that **MACE2** produced no measurable antinociception in the paw incision assay (Figure 4B). However, **MACE2** produced efficacious antinociception that was statistically indistinguishable from 10 mg/kg morphine in CIPN (Figure 4C). Our results together suggest that **MACE2** is metabolically stable and can penetrate to sites of action in the brain or spinal cord; however, this drug may have specific pharmacodynamics that make it more effective in some pain assays (tail flick, CIPN) and ineffective in others (paw incision). Alternatively, it is possible that the drug could engage peripheral vs central receptors to evoke these specific effects on pain; the use of naloxone methiodide or similar peripherally restricted antagonists could be used to answer this question. Note, however, that it is at least possible for **MACE2** to engage central receptors based on the i.t. and i.c.v. observations from Figure 3. Lastly, these results do suggest target engagement and efficacy in acute and chronic pain; however, we have not yet assayed the side effect profile of **MACE2**, which will be critical for potential future translation.

CONCLUSIONS

The investigation of multiple opioid receptors to develop novel bivalent peptidomimetics represents a key approach in pain medicinal chemistry research. A key requirement for the creation of these bifunctional ligands is the identification of a linker inside the main pharmacophore. Some of them with a small molecule's structure, e.g., Eluxadoline and nalmefene have entered the clinical space for the management of diseases and pathological conditions such as irritable bowel syndrome, diarrhea, opioid overdose, and alcohol abuse. Others are in late preclinical testing or in clinical trials for pain, suggesting the hypothesis that such types of analgesics could overcome some drawbacks related to classical opioids used in therapy. The cyclic peptide **MACE2** is a bivalent MOR/DOR agonist that is viable as a lead compound for further structural modifications due to its good stability in human plasma and strong antinociceptive effect in vivo, including in CIPN chronic pain. In this work, we corroborate its efficacy in vivo through the tail flick test via three different administration routes, demonstrating target engagement by a translatable peripheral route of administration. We also tested its suitability for chronic pain management in two diverse chronic pain models compared with morphine, finding that **MACE2** was highly effective in relieving CIPN. Further work is needed to optimize the pharmacokinetics of this molecule and advance it to clinical trials.

METHODS

Plasma Stability. The assay of plasma stability is carried out by choosing an initial concentration of the compound (i.e., 5 μM) following and by incubating the substance in the human plasma at 37 °C. The amounts of the resulting mixture of plasma and compound incubated are taken at the beginning (T0) and after 1 h (T2). The samples collected were treated with an aliquot volume of acetonitrile (kept at 0 °C) in order to precipitate the proteins and stabilize the sample from degradation. Simultaneously, the experiments with **MACE2** and the reference compound **AM94** are carried out in the same manner in order to ensure the reliability of the stability test. The tests are performed in triplicate. The supernatant and the proteins were separated by centrifugation, and the supernatant was subjected to LC-MS analysis. The data are referenced to the T0 collected sample as 100% (no degradation), and then the percentage of the substance remaining after incubation at a given time point is calculated. $T_{1/2}$ (half-life) will be calculated from linear regression of time course data. Human plasma, the

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incubated solutions of the investigated compounds, and the control solutions (at working concentration) were kept at 37 °C.

Procedure. 99 μL of human plasma was distributed into a prelabeled 96-well plate (in triplicates per time point). Then 1.0 μL of tested substance and standard compounds were poured into wells prefilled by plasma. 200 μL of acetonitrile was added immediately into wells marked as "T0" time point to precipitate the proteins and avoid (refrigerated at 0 °C) sample degradation. Plates were incubated in a thermostatic shaker at 37 °C while shaken at 350 rpm. The reaction was quenched by the addition of 200 μL of cold acetonitrile to the respective wells at selected time points (20, 40, and 60 min). Then, the last experiment (at the last time point) was stopped, and the plate was subjected to centrifugation at 4000*g* for 20 min at 4 °C in order to separate the proteins and the liquid. 200 μL of supernatant was used for further analysis and moved to 96-well plates and covered with plate mats. LC-MS analysis was carried out.

LC-MS System Description. The LC-MS system is composed of a degaser, pump.autogaser, autosampler, RS column compartment with detector Photodiode Array (all Ultimate 3000 series by Orion Scientific, Veggiano, Pd, IT). Mass spectrometer Bruker Daltonics amaZon Speed (Bruker Daltonik GmbH, Bremen, Germany).

Animals (Facilities of the National Centre for Drug Research and Evaluation, Istituto Superiore di Sanità). In the designed experiments, CD-1 male mice (Charles River, Italy, 25−30 g) were used and maintained in colonies, housed in cages (7 units per cage) under standard light and dark cycles (from 7:00 a.m. to 7:00 p.m.), temperature regulated at 21 ± 1 °C, and relative humidity regulated at 60% ± 10% for at least 7 days. Food and water were available freely. The "Service for Biotechnology and Animal Welfare" of the "Istituto Superiore di Sanità and the Italian Ministry of Health" authorized the experimental protocol on animals, following the law number 26 of 2014 (authorization number 756/2018-PR).

Injection Procedure. Dimethylsulfoxide (DMSO) was purchased from Merck (Rome, Italy). All of the solutions were freshly prepared on each experimental day. The peptide compound solutions were prepared using saline containing 0.9% sodium chloride and DMSO in a ratio of 1:5 v/v. These solutions were injected at a volume of 10 μL/mouse for intracerebroventricular (i.c.v.) administrations, or 5 μL/mouse for intrathecal (i.t.) administrations, or in a volume of 150 μL/mouse for intravenous (i.v.) administrations. The administrations were carried out in animals anesthetized with isoflurane. For i.c.v. injections, an incision was practiced in the scalp, and the bregma was located. Injections were performed using a 10 μL Hamilton microsyringe equipped with a 26-G needle, 2 mm caudal, and 2 mm lateral from the bregma at a depth of 3 mm, as previously described.[32] The i.t. injections were made by direct lumbar puncture into the spinal subarachnoid space by a Hamilton microsyringe equipped with a 30-G needle.[31] The i.v. administrations were carried out in the lateral vein of the tail heated with the aim of a lamp and using a Hamilton syringe equipped with a 25 G needle.[33] The doses of biphalin, **6a,** and **MACE2** used were chosen based on the results obtained.[22]

Tail Flick Test. The tail flick latency was obtained using an infrared radiant light source (100 W, 15 V bulb by Ugo Basile, Gemonio, VA, IT), focalized onto a photocell by an aluminum parabolic mirror. The mice were hand-restrained using leather gloves during the trials (gently). The tails were subjected to radiant heat (focused 3−4 cm from the tip), and the latency (s) of the tail withdrawal to the thermal stimulus was recorded. The cut-off time was set at 15 s. The baseline latency was calculated as the mean of three readings recorded before testing at intervals of 15 min, and the time points to record the data of latency have been determined at 15, 30, 45, 60, 90, and 120 min after treatment. The resulting data are reported as the area under the curve (AUC) of the maximum effect % (% MPE) = (postdrug latency − baseline latency)/(cutoff time − baseline latency) × 100.

Animals—University of Arizona (UofA). Male and female CD-1 mice were obtained from Charles River at 5−8 weeks of age. The mice were housed for at least 5 days after arrival for acclimation. They were housed no more than 5 per cage in the *UofA* vivarium on a 12 h light-dark cycle with food (standard lab chow) and water available freely. All experiments were approved by the *UofA* IACUC and were carried out following the NIH guidelines "Care and Use of Laboratory Animals". All mice were moved to the experimental laboratory at least 30 min prior to the start of the experiment for acclimation. All experimenters were blinded to the treatment group by the delivery of bar-coded compound vials. All experimental apparatuses were cleaned between uses.

Behavioral Experiments—University of Arizona. The tail flick assay was carried out as in our previous work.[29] Pre- and postinjection baselines were measured by dipping the animal's tail into a warmed water bath (52 °C); the latency to withdraw was measured, and the cutoff was fixed at 10 s. Paw incision surgery was also carried out as described in our previous work,[34,35] with the mechanical threshold measured using von Frey filaments. Pre- and postbaseline measurements were taken, and the animals recovered for 24 h after surgery prior to drug injection. CIPN was induced as in our previous work,[36] by the injection of paclitaxel (2 mg/kg i.p.) on days 1, 3, 5, and 7 with drug injections on day 8. Pre- and postbaselines were also measured using von Frey filaments. For each assay, the drug was injected s.c., and a 2−3 h time course was performed as noted in each figure legend. For all assays, the behavioral data are reported as raw, without normalization or further modification.

Data Analysis and Statistics. The data of the in vivo experiments are reported as mean ± s.e.m. Significant discrepancies among the groups for tail flick AUC were evaluated by one-way ANOVA analysis followed by Dunnett's multiple comparisons test. Time course behavioral data were subjected to a two-way ANOVA analysis with Dunnett's (for the tail flick test) or Sidak's (for the CIPN, paw incision) post hoc test. The suite program GraphPad Prism 10.0.0 was used. Statistical significance was set at $p < 0.05$. The data and statistical analysis match with the recommendations on experimental design and analysis in pharmacology.

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Author Contributions

The study was designed by A.S. and A.M.; L.M. prepared the tested synthetic compounds and performed the plasma stability assay; J.A.M., C.S., and J.M.S. performed the CIPN and paw incision tests; and S.P. performed the tail flick in vivo assays.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DOR, δ-opiod receptor; MOR, μ-opioid receptor; i.c.v., intracerebroventricular; i.v., intravenous; i.t., intrathecal; s.c., subcutaneous; CIPN, chemotherapy-induced peripheral neuropathy; CD-1, Hsd:ICR; IASP, International Association for the Study of Pain; NSAIDs, nonsteroidal anti-inflammatory drugs; BBB, blood-brain barrier penetration; SEM, standard error of mean; BL, baseline; LC-MS, liquid chromatography-mass spectrometry; DMSO, dimethyl sulfoxide; AUC, area under curve

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