An analgesic peptide H-20 attenuates chronic pain via the PD-1 pathway with few adverse effects

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1. Methods

Capsaicin-induced acute pain. Capsaicin-induced acute pain test was performed as described previously[1]. Mice were habituated to the experimental environment for 15 min and then treated with saline/H-20. Capsaicin (1.6 μg/paw) was intraplantarly administrated after 5 min. Then, the cumulative time of nociceptive behaviors were recorded for 5 min.

Hot plate. Hot plate test was performed using female mice as described previously[2,3]. Briefly, mouse was placed on the hot plate at 53 °C. The timing was stopped when the mouse showed signs of pain-related behavior (jumping or paw licking). The stimulus cutoff was set at 40 s to avoid scald.

Tail flick test. The tail-flick test was performed as detailed previously[3]. Briefly, mouse was gently restrained by hand. The tail (3 cm from the tip of the tail) was immersed into the water (50 °C). The tail-flick latency (tail withdrawal from the water) was measured at 30, 45, 60, 120, 180 and 300 min after i.t. injection of H-20. The stimulus cutoff was set at 10 s to avoid scald. The percent maximum possible effect: %MPE = 100 × (test latency–control latency) / (10 – control latency).

Assessment acute itch behaviors. The itch behaviors that caused by i.t. injection of saline/H-20/morphine were assessed as described previously[4]. Mice were acclimatized to the test environment for 15 min. After i.t. injection of drug, the scratch behaviors to the injected region of mice were recorded and counted during 30 min.

Open field test. The locomotor activity and anxiety-like behaviors were evaluated using open field test as described previously[5]. 30 min after i.t. injection of drug, mice were placed into the open field arena (40 × 40 × 40 cm) and video recorded for 10 min. The data analysis was perform using Noldus software.

2. REFERENCES

1 Kohro, Y. *et al.* Spinal astrocytes in superficial laminae gate brainstem descending control of mechanosensory hypersensitivity. *Nature neuroscience* **23**, 1376-1387, doi:10.1038/s41593-020-00713-4 (2020).

2 Chen, G. *et al.* PD-L1 inhibits acute and chronic pain by suppressing nociceptive neuron activity via PD-1. *Nature neuroscience* **20**, 917-926, doi:10.1038/nn.4571 (2017).

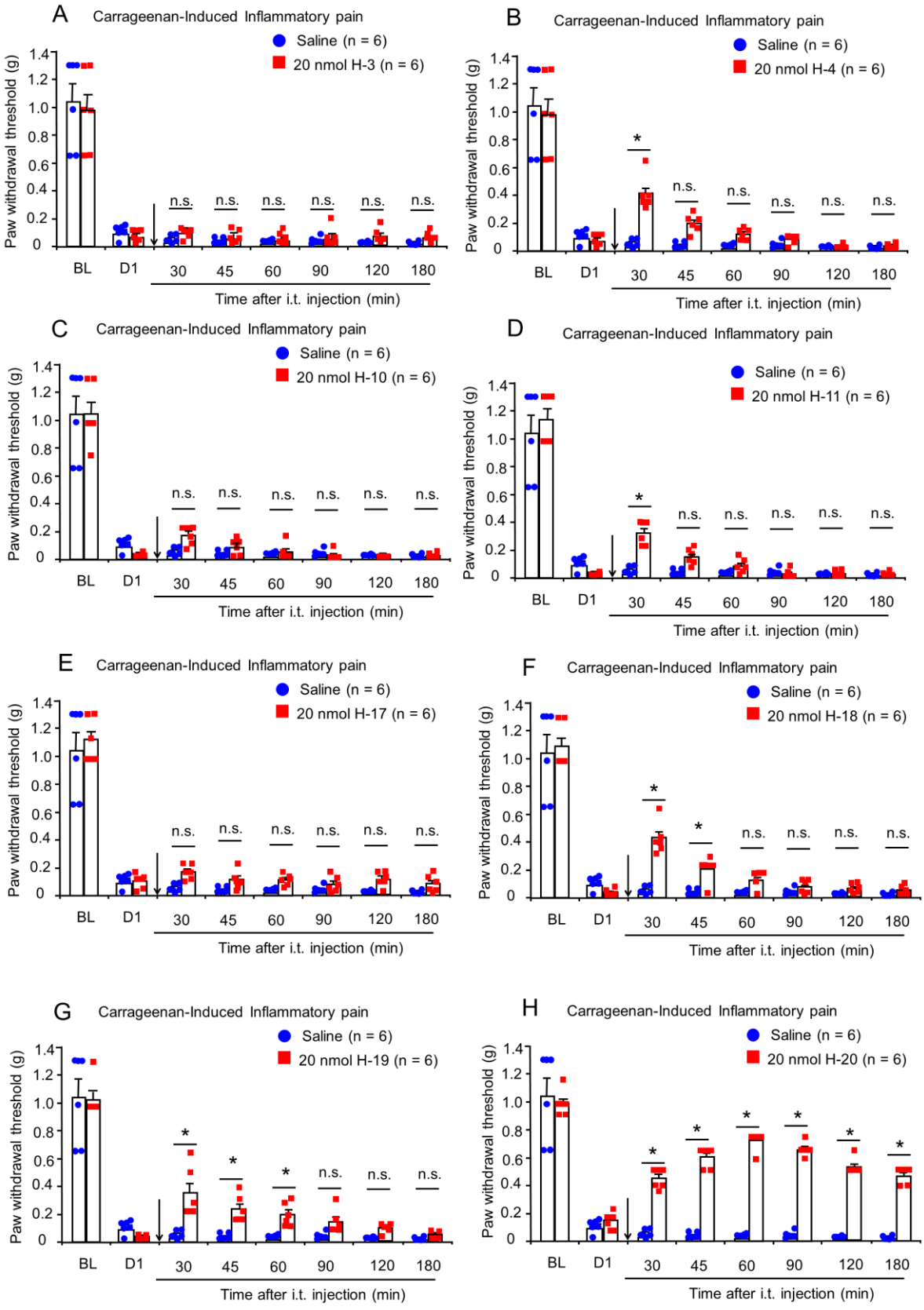
3 Wang, Z. *et al.* Anti-PD-1 treatment impairs opioid antinociception in rodents and nonhuman primates. *Science translational medicine* **12**, doi:10.1126/scitranslmed.aaw6471 (2020).

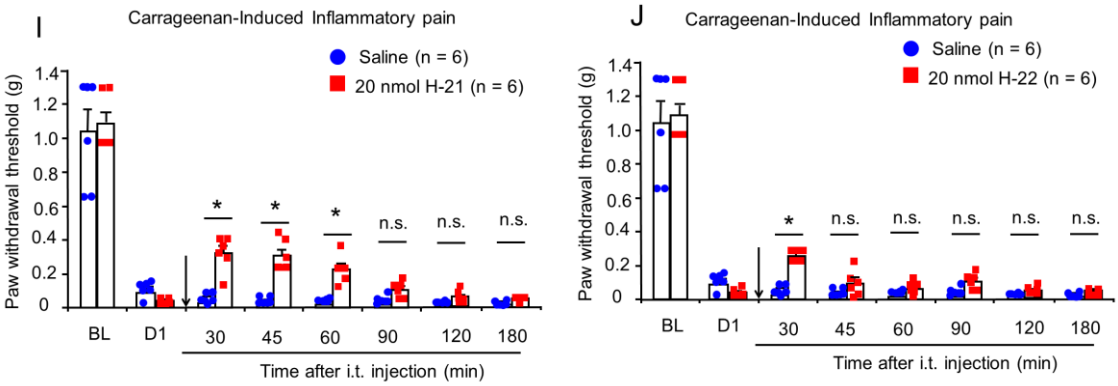
4 Wang, Z. *et al.* Central opioid receptors mediate morphine-induced itch and chronic itch via disinhibition. *Brain : a journal of neurology* **144**, 665-681, doi:10.1093/brain/awaa430 (2021).

5 Liu, Y. *et al.* Short-term resistance exercise inhibits neuroinflammation and attenuates neuropathological changes in 3xTg Alzheimer's disease mice. *Journal of neuroinflammation* **17**, 4, doi:10.1186/s12974-019-1653-7 (2020).

Supplemental Material Table 1. The analytical data of peptides targeting PD-1

NO.	Sequence	MW (g/mol)	Amber score	Purity (%)
H-1	VVEYGSNMT	999.09	-70.3	97.2
H-2	EYGSNMTIE	1043.1	-75.5	96.5
H-3	VEKQLDLAA	982.12	-85.8	98.9
H-4	VYWEMEDKN	1213.3	-91.8	97.6
H-5	EDLKVQHSS	1042.1	-76.7	99.1
H-6	VQHSSYRQR	1160.2	-68.9	98.1
H-7	QRARLLKDQ	1127.3	-63.6	96.6
H-8	ARLLKDQLS	1043.2	-72.5	97.6
H-9	LKDQLSLGN	987.11	-73.7	98.5
H-10	KDQLSLGNA	945.03	-84.9	97.4
H-11	DQLSLGNAA	887.93	-82.3	96.1
H-12	QLSLGNAAL	886.01	-71.3	97.8
H-13	SLGNAALQI	886.01	-77.5	98.5
H-14	ALQITDVKL	1000.2	-71.1	97.8
H-15	LQITDVKLQ	1057.2	-68.6	97.5
H-16	LQDAGVYRC	1024.2	-78.8	98.7
H-17	VYRCMISYG	1091.3	-89.3	97.6
H-18	YRCMISYGG	1049.2	-87.3	96.2
H-19	MISYGGADY	976.06	-84.2	96.8
H-20	ISYGGADYK	973.04	-81.0	98.0
H-21	SYGGADYKR	1016.1	-83.4	99.2
H-22	YGGADYKRI	1042.2	-80.1	98.6

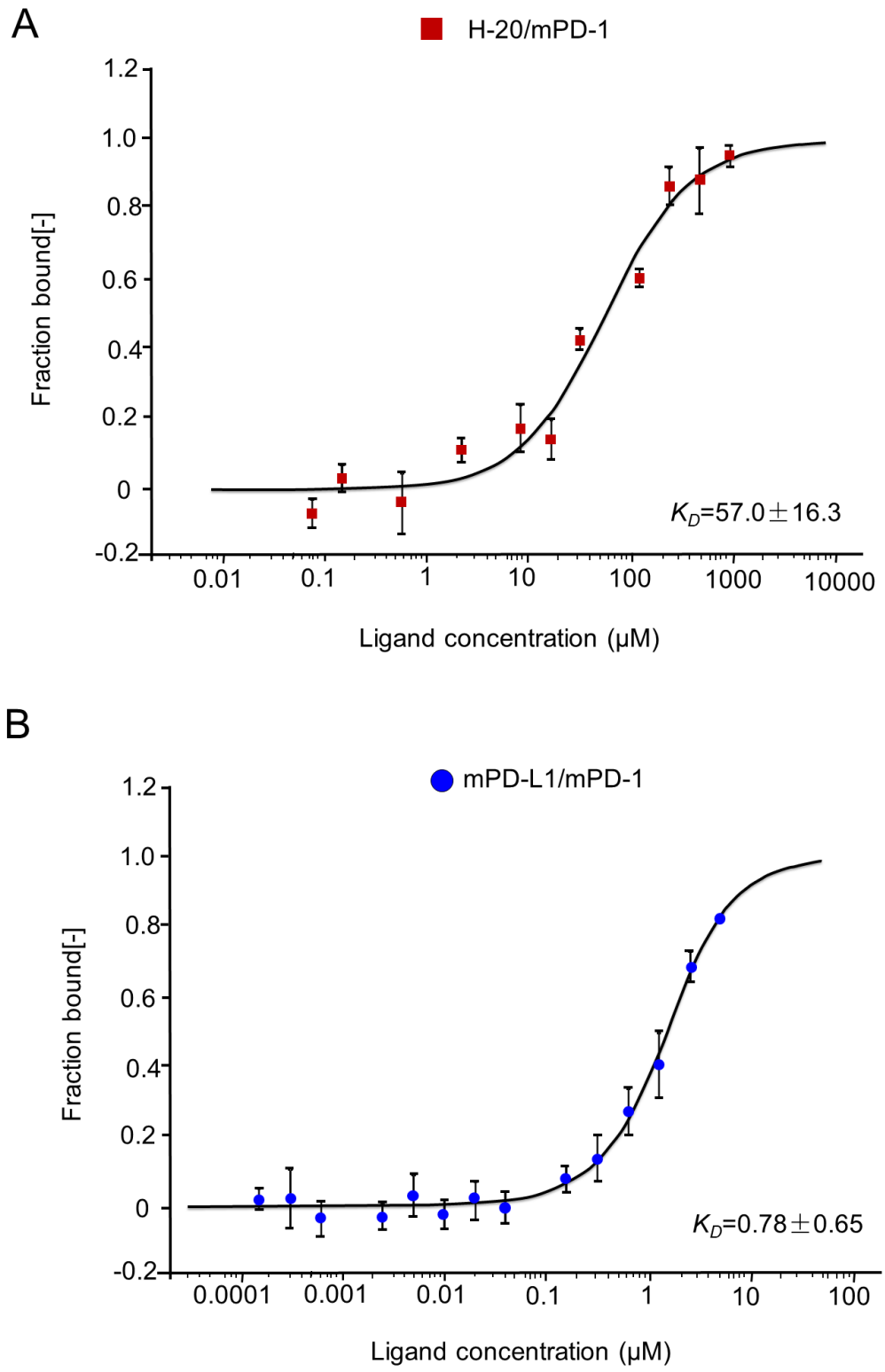

A
Carrageenan-Induced Inflammatory pain
Saline (n = 6)
20 nmol H-3 (n = 6)
B
Carrageenan-Induced Inflammatory pain
Saline (n = 6)
20 nmol H-4 (n = 6)
C
Carrageenan-Induced Inflammatory pain
Saline (n = 6)
20 nmol H-10 (n = 6)
D
Carrageenan-Induced Inflammatory pain
Saline (n = 6)
20 nmol H-11 (n = 6)
E
Carrageenan-Induced Inflammatory pain
Saline (n = 6)
20 nmol H-17 (n = 6)
F
Carrageenan-Induced Inflammatory pain
Saline (n = 6)
20 nmol H-18 (n = 6)
G
Carrageenan-Induced Inflammatory pain
Saline (n = 6)
20 nmol H-19 (n = 6)
H
Carrageenan-Induced Inflammatory pain
Saline (n = 6)
20 nmol H-20 (n = 6)
Paw withdrawal threshold (g)
BL
D1
30
45
60
90
120
180
Time after i.t. injection (min)
n.s.
*



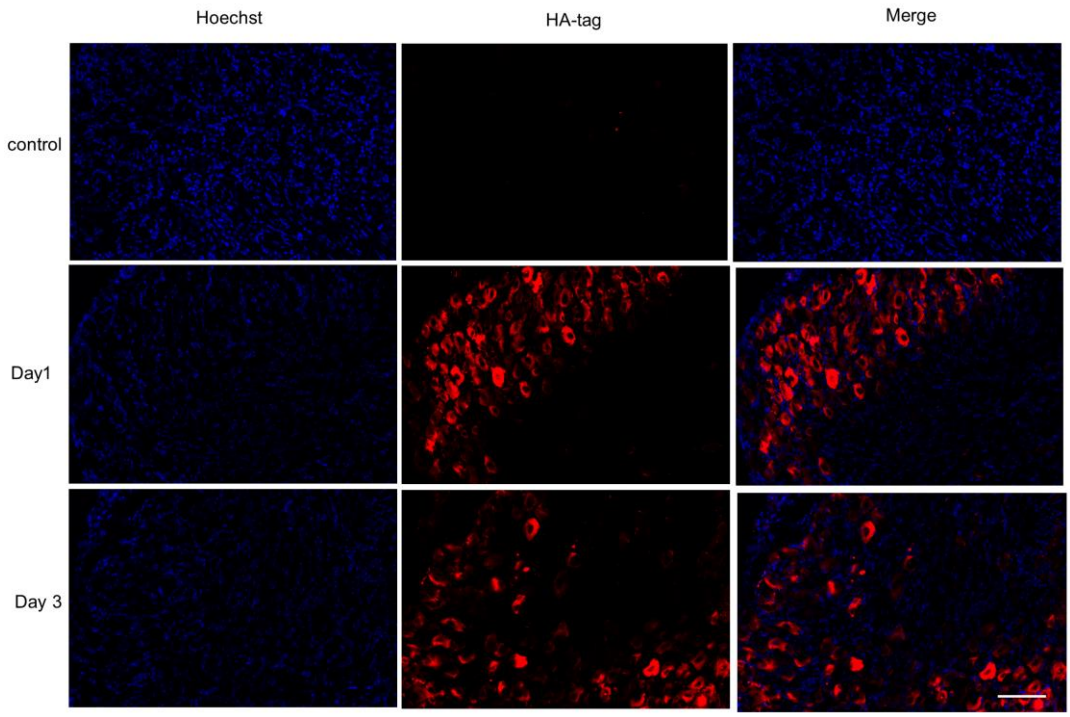
Supplemental Material Figure 1. Antinociceptive effects of spinal PD-1 targeted peptides in carrageenan-induced acute inflammatory pain model. Mean ± SEM, 6 mice per group. *p < 0.05, versus Saline group, n.s., no significance. Two-way RM ANOVA.

Supplemental Material Table 2. The analytical data of H-20

No.	TOF MS $[M+H]^+$		Purity	Docking score	MMGBSA dG Bind	Half-lives ($t_{1/2}$, min)
	Calcd	Found	(%)	(kcal/mol)	(kcal/mol)	Mouse Brain
H-20	973.04	973.6	98	-8.767	42.61	100



Supplemental Material Figure 2. MST-binding curve for K_D determination of the (A) H-20-mPD-1 and (B) mPD-L1-mPD-1 interaction in triplicates.

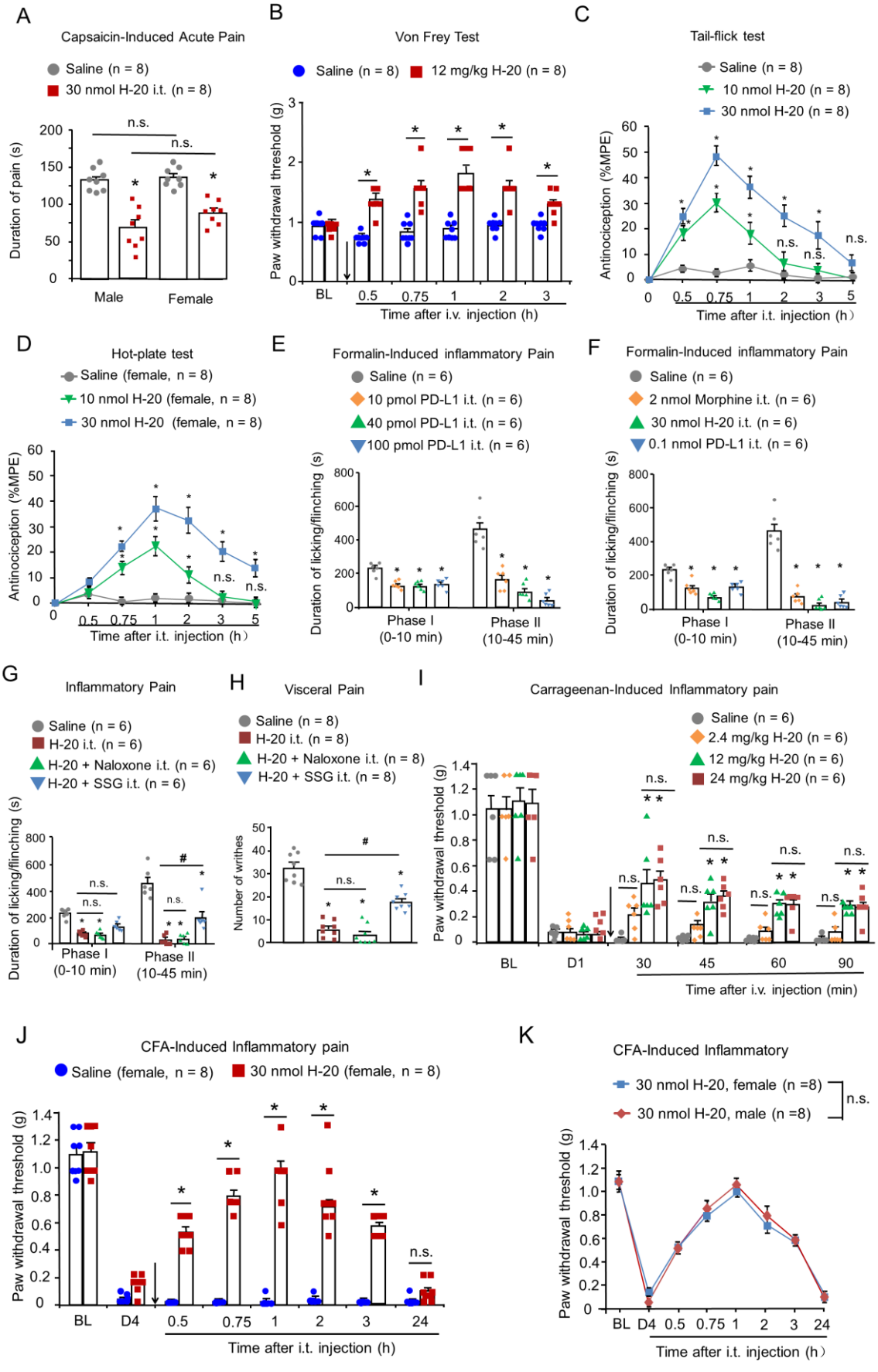


Supplemental Material Figure 3. After i.t. injection of HA-H-20 (30 nmol, Day 1/3), the combination of HA-H-20 with PD-1 on DRGs. Scale, 50 μm. 4 mice per group.

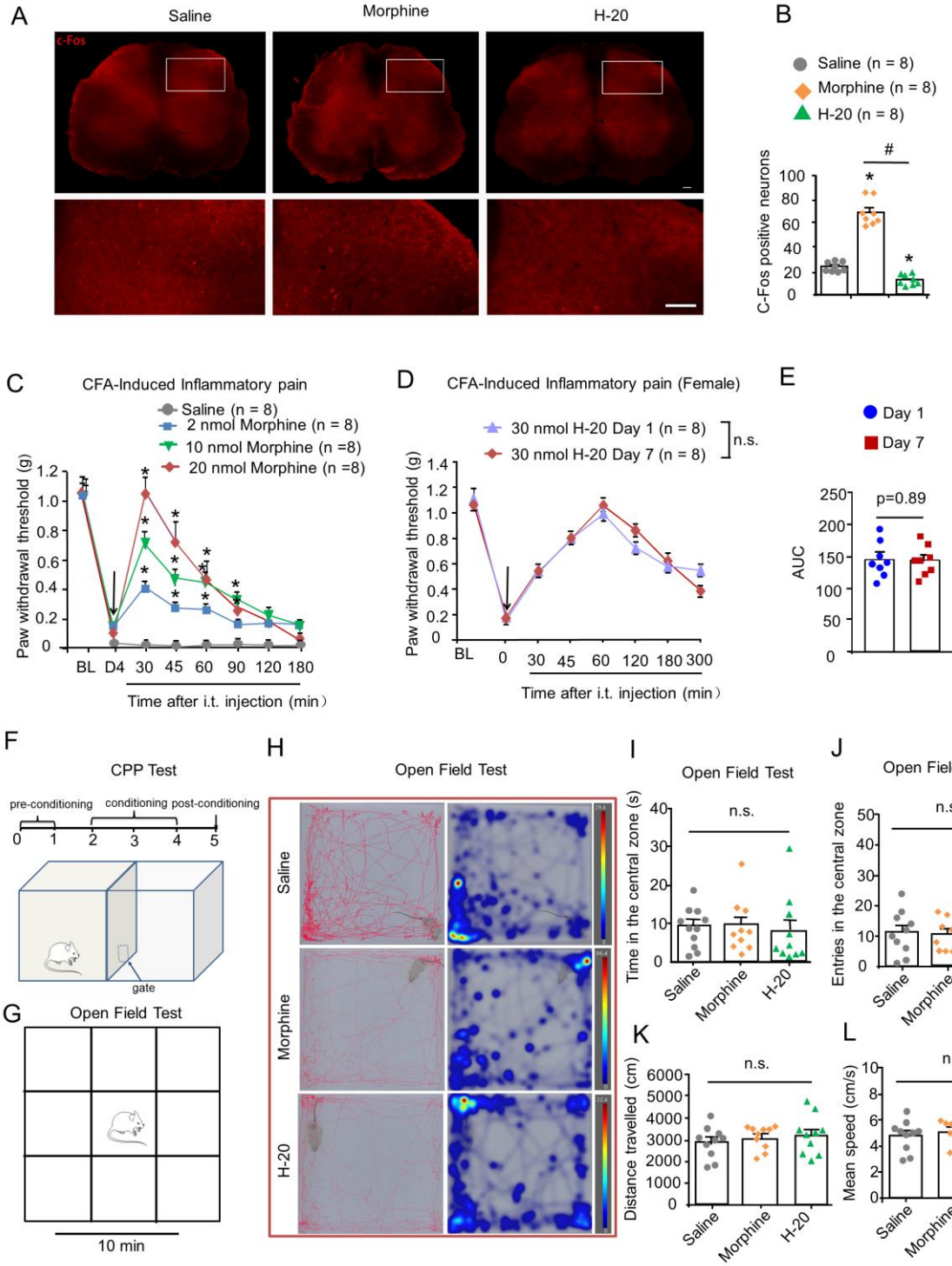
Supplemental Material Table 3. Bioactivity comparison of mPD-L1 and H-20

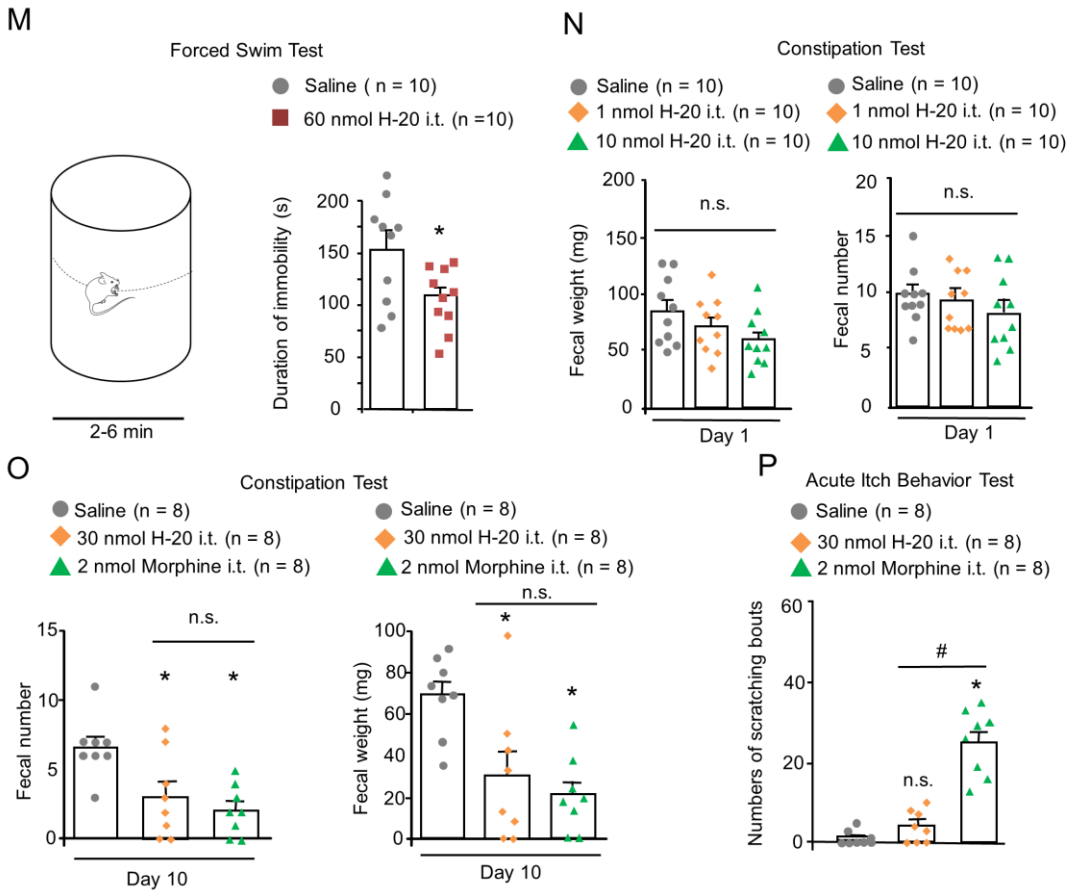
Name	MW(g/mol)	Length	ED_{50} values [a] (nmol)
mPD-L1	3,3275	290	0.004 (0.002-0.011)
H-20	973.04	9	0.63 (0.35-1.16)

[a]The ED_{50} was calculated in the ^{2}nd phase formalin-induced pain model, 6 mice per group.


A
Capsaicin-Induced Acute Pain
Saline (n = 8)
30 nmol H-20 i.t. (n = 8)
Duration of pain (s)
n.s.
n.s.
Male
Female
B
Von Frey Test
Saline (n = 8)
12 mg/kg H-20 (n = 8)
Paw withdrawal threshold (g)
BL
Time after i.v. injection (h)
C
Tail-flick test
Saline (n = 8)
10 nmol H-20 (n = 8)
30 nmol H-20 (n = 8)
Antinociception (%MPE)
Time after i.t. injection (h)
D
Hot-plate test
Saline (female, n = 8)
10 nmol H-20 (female, n = 8)
30 nmol H-20 (female, n = 8)
Antinociception (%MPE)
Time after i.t. injection (h)
E
Formalin-Induced inflammatory Pain
Saline (n = 6)
10 pmol PD-L1 i.t. (n = 6)
40 pmol PD-L1 i.t. (n = 6)
100 pmol PD-L1 i.t. (n = 6)
Duration of licking/flinching (s)
Phase I
(0-10 min)
Phase II
(10-45 min)
F
Formalin-Induced inflammatory Pain
Saline (n = 6)
2 nmol Morphine i.t. (n = 6)
30 nmol H-20 i.t. (n = 6)
0.1 nmol PD-L1 i.t. (n = 6)
Duration of licking/flinching (s)
Phase I
(0-10 min)
Phase II
(10-45 min)
G
Inflammatory Pain
Saline (n = 6)
H-20 i.t. (n = 6)
H-20 + Naloxone i.t. (n = 6)
H-20 + SSG i.t. (n = 6)
Duration of licking/flinching (s)
Phase I
(0-10 min)
Phase II
(10-45 min)
H
Visceral Pain
Saline (n = 8)
H-20 i.t. (n = 8)
H-20 + Naloxone i.t. (n = 8)
H-20 + SSG i.t. (n = 8)
Number of writhes
I
Carrageenan-Induced Inflammatory pain
Saline (n = 6)
2.4 mg/kg H-20 (n = 6)
12 mg/kg H-20 (n = 6)
24 mg/kg H-20 (n = 6)
Paw withdrawal threshold (g)
BL
D1
Time after i.v. injection (min)
J
CFA-Induced Inflammatory pain
Saline (female, n = 8)
30 nmol H-20 (female, n = 8)
Paw withdrawal threshold (g)
BL
D4
Time after i.t. injection (h)
K
CFA-Induced Inflammatory
30 nmol H-20, female (n =8)
30 nmol H-20, male (n =8)
n.s.
Paw withdrawal threshold (g)
BL D4
Time after i.t. injection (h)

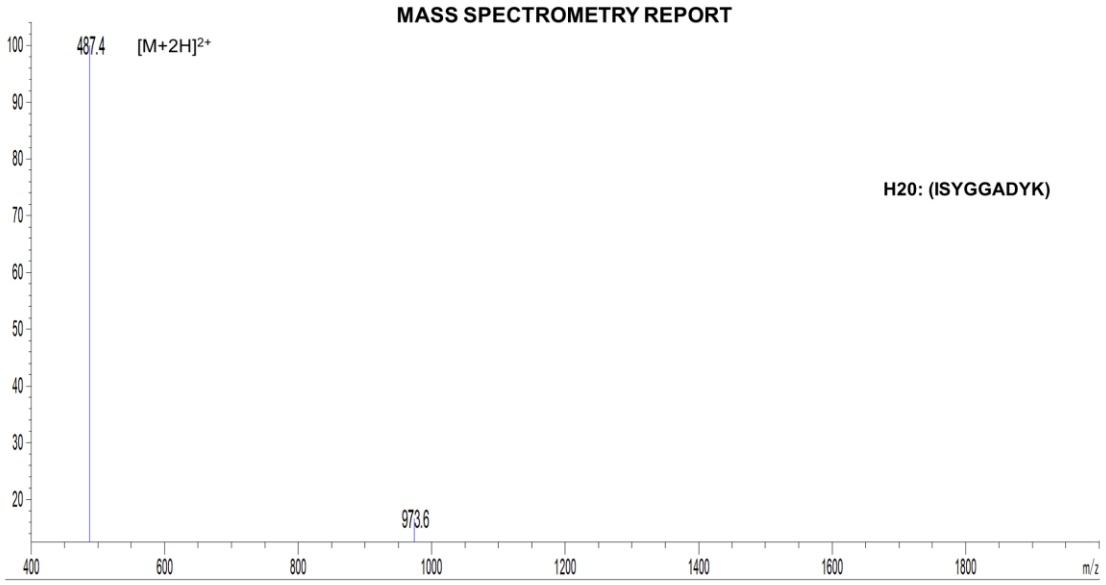
Supplemental Material Figure 4. (A) I.t. H-20 attenuates capsaicin-induced nociceptive behaviors. Mean ± SEM. *p < 0.05, versus Saline group. N.s., no significance. 8 mice per group. Two-way ANOVA. (B) I.v. H-20 (12 mg/kg) modulates basal mechanical pain thresholds in male mice. Mean ± SEM. *p < 0.05, versus Saline group. 8 mice per group. Two-way RM ANOVA. (C, D) Basal thermal pain thresholds are evaluated after i.t. injection of H-20 via tail-flick test (C) and hot plate test (D). Mean ± SEM. *p < 0.05, versus Saline group. N.s., no significance. 8 mice per group. Two-way RM ANOVA. (E) Analgesic effects of i.t. PD-L1 (10, 40, and 100 pmol) in formalin model. Mean ± SEM. 6 mice per group. *p < 0.05, versus Saline group. Two-way RM ANOVA. (F) Analgesic effects of i.t. morphine (2 nmol)/H-20 (30 nmol)/PD-L1 (0.1 nmol) in formalin model. Mean ± SEM. 6 mice per group. *p < 0.05, versus Saline group. Two-way RM ANOVA. (G) The effects of SHP-1 inhibitor sodium stibogluconate (SSG, co-injection, 10 nmol) or naloxone (co-injection, 5 nmol) on H-20 (30 nmol) induced-analgesia in formalin test. Mean ± SEM. 6 mice per group. *p < 0.05, versus Saline group, #p<0.05, versus H-20 group. N.s., no significance. Two-way RM ANOVA. (H). The effects of SSG (10 nmol) or naloxone (5 nmol) on H-20 (30 nmol) induced-analgesia in visceral pain test. Mean ± SEM. 8 mice per group. *p < 0.05, versus Saline group, #p<0.05, versus H-20 group. N.s., no significance. One-way ANOVA. (I) Analgesic effects of i.v. H-20 (2.4, 12, 24 mg/kg) in carrageenan-induced inflammatory pain model. Mean ± SEM. 6 mice per group. *p < 0.05, versus Saline group. N.s., no significance. Two-way RM ANOVA. (J) Effects of spinal H-20 on CFA-induced inflammatory pain model in female mice. Mean ± SEM. 8 mice per group. *p < 0.05, versus Saline group. N.s., no significance. Two-way RM ANOVA. (K) Comparison of male and female mice. N.s., no significance. Mean ± SEM. 8 mice per group. Two-way RM ANOVA.


A
Saline
Morphine
H-20
c-Fos
B
Saline (n = 8)
Morphine (n = 8)
H-20 (n = 8)
C-Fos positive neurons
C
CFA-Induced Inflammatory pain
Saline (n = 8)
2 nmol Morphine (n = 8)
10 nmol Morphine (n =8)
20 nmol Morphine (n =8)
Paw withdrawal threshold (g)
BL D4 30 45 60 90 120 180
Time after i.t. injection (min）
D
CFA-Induced Inflammatory pain (Female)
30 nmol H-20 Day 1 (n = 8)
30 nmol H-20 Day 7 (n = 8)
n.s.
BL 0 30 45 60 120 180 300
E
Day 1
Day 7
p=0.89
AUC
F
CPP Test
pre-conditioning
conditioning post-conditioning
gate
G
Open Field Test
10 min
H
Open Field Test
Saline
Morphine
H-20
I
Open Field Test
Time in the central zone (s)
J
Open Field Test
Entries in the central zone
K
Distance travelled (cm)
L
Mean speed (cm/s)



Supplemental Material Figure 5. (A) The expression of c-Fos in spinal cord after repeated i.t. H-20 (30 nmol) /morphine (2 nmol) for 7 continuously days. Scale, 50 μm. (B) Quantification of c-Fos positive neurons in spinal cord. Mean± SEM. *p < 0.05, versus Saline group. #p< 0.05, vs Morphine group. 4 mice per group, 8 images per group. One-way ANOVA. (C) Effects of spinal morphine (2, 10 and 20 nmol) on CFA-induced pain model. 8 mice per group. *p< 0.05, versus Saline group. Two-way RM ANOVA. (D) Antinociception of repeatedly i.t. injection of H-20 on CFA-induced inflammatory pain model in female mice. Mean ± SEM. 8 mice per group. N.s., no significance. Two-way RM ANOVA. (E) The area under the curve (AUC) values. Student's test. (F) Schematics of the conditioned place preference (CPP) test. (G-L) Open field test. Mean ± SEM. 10 mice per group. N.s., no significance. One-way ANOVA. (H) Locomotor traces and heatmap. (I) Time in the central zone. (J) The frequency of entering the central zone. (K) Distance travelled. (L)Mean speed. (M) Effect of i.t. H-20 (60 nmol) on the immobility time in the forced swim test. Mean ± SEM, 10 mice per group, *p < 0.05, versus Saline group. Student's test. (N) The

gastrointestinal function inhibition (GI) effect of intrathecal H-20 at single doses. Mean ± SEM, 10 mice per group. N.s., no significance. One -way ANOVA. (O) The GI effect of repeatedly i.t. injection of H-20/morphine for consecutive 10 days. Mean ± SEM, 8 mice per group. *$p < 0.05$, versus Saline group. N.s., no significance. One-way ANOVA. (P) Scratch behaviors were counted within 30 min after i.t. administration of H-20/morphine. Mean ± SEM, 8 mice per group. *$p < 0.05$, versus Saline group. #$p< 0.05$, vs Morphine group. N.s., no significance. One-way ANOVA.



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Sample:	H-20	CDL:	-20.0 v	T. Flow:	0.2 ml/min
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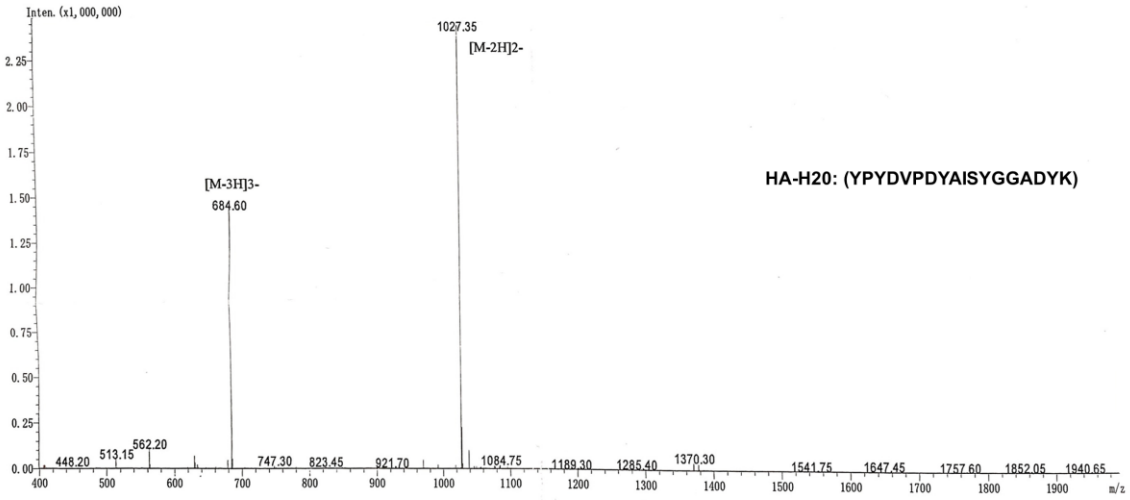

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CDL	:-20.0v	T.Flow	:0.2ml/min		
CDL Temp	:250℃	B.conc	:50%H2O/50%ACN		
Block Temp	:400℃				

Supplemental Material Figure 6. *Mass* spectrogram report of H-20 and HA-H-20.