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Dr. Rainer Glaser, Professor of Chemistry

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1-(2,4-Dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4H-indazol-4-one: A Pain Reliever That Causes Less Gastrointestinal Dysfunction than Morphinek

By: Adit Shah and Stephanie Singh

Dear Dr. Glaser,

We would like to submit this paper, along with its supporting information, to *JOC*. In this article, we present a study done on an opioid painkiller that has less severe side effects than morphine and/or other opioids. Although opioids are well-known and popular painkillers, they ultimately result in addiction and severe side effects if used improperly and therefore, it is important to seek alternatives for pain relief. As a possible reviewer, we would like to suggest Kaidi Yang (Mizzou, kyn69@mail.missouri.edu).

Sincerely,

Adit and Stephanie

Abstract

Although opioids are well-regarded for their analgesic activity, they have the potential of becoming addictive and deadly if misused or used for long periods of time. Opioids bind to all three opioid receptors but the mu-receptor is very important for the analgesic effects of opioids. However, high affinity to the mu-receptor has been linked to respiratory depression and many other unpleasant side effects. In this article, we seek to explore using a new compound, 1-(2,4-Dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4H-indazol-4-one, as an alternative painkiller. Although it has high affinity for the mu-opioid receptor, it has shown potential as an alternative painkiller that less unpleasant side effects, such as gastrointestinal blockage. It also shows a promising route for the discovery of less dangerous painkillers.

Introduction

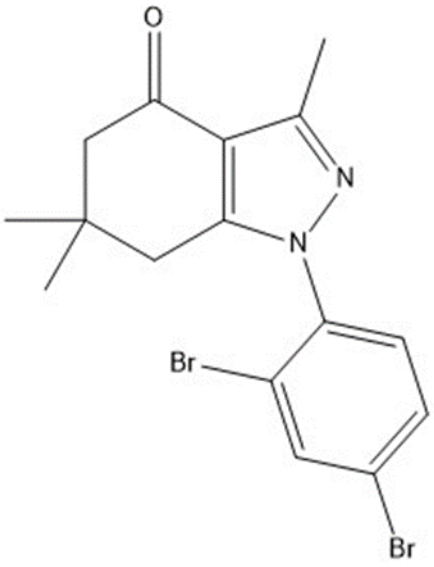
Materials and Methods

Animal

Wild-type male C57BL/6 mice and mu-opioid receptor knockout mice were kept in a temperature-controlled animal room in a 12 hour light and 12 hour dark cycle. Animal experiments complied with the Policies on the Use of Animals in Neuroscience Research and the ethical guidelines of investigating pain in conscious animals established by the International Association for the Study of Pain.

1-(2,4-Dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4H-indazol-4-one

Scheme 1: Chemical Structure of 1 as the free base.



1-(2,4-Dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4H-indazol-4-one

1 (Scheme 1) was synthesized by the regioselective condensation of 2,4-dibromophenylhydrazine with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione in the presence of catalytic acid at room temperature in methanol. The phenylhydrazine was prepared by the diazotization of 2,4-dibromoaniline with sodium nitrite followed by reduction with stannous chloride as reported in the literature. All reagents used were purchased commercially and used without further purification.[1]

Purification and Characterization

1 was purified using a Hitachi 2000 series HPLC system with a detection wavelength of 254nm and a C-18 column(ZORBAX Eclipse XDB-C18). The gradient was 10%-90% Acetonitrile and 10mM aqueous NH4OAc and 0.1% formic acid. NMR spectra were recorded using a Mercury-400 spectrometer. Chemical shifts are reported

with respect to the residual solvent signal. High resolution mass spectra were obtained using a VARIAN 901-MS by electrospray ionization.

Radioligand Binding Assay

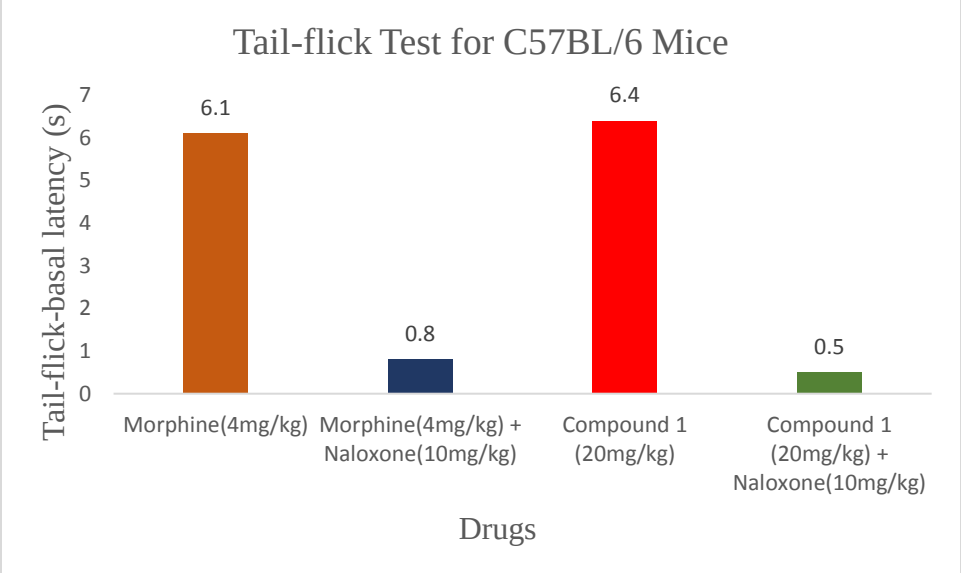
Human embryonic kidney 293 cells expressing human mu-opioid receptor, human delta-opioid receptor and human kappa-opioid receptor were harvested and homogenized in membrane preparation buffer that contained fresh protease inhibitor cocktail and centrifuged for 30 minutes. The pellets were resuspended, alliquoted and stored at -80˚C. For [3*H*]diprenorphine saturation binding assay, membranes were incubated with different concentrations of [3*H*]diprenorphine in binding buffer at 25˚C for 1 hour. For competitive binding experiments, [3*H*]diprenorphine was incubated with membranes in the absence or presence of various concentrations of compounds at 25˚C for 1 hour. Samples were filtered onto glass-fiber filters and washed three times with ice-cold phosphate-buffered saline. The results of the binding assay are shown in Table 1.

Table 1. **Opioid Receptor-binding Affinity of Morphine and 1-(2,4-Dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one(Compound 1) on mu-opioid, delta-opioid and kappa-opioid receptors**

		[^{3}*H*]diprenorphine binding, K_i	
Receptor	Mu-opioid Receptor	Delta-Opioid Receptor	Kappa-opioid Receptor
Morphine, nM	6.8±0.8	55±8	25±3
Ketamine[2]	4.38±.04	4.55±.04	3.57±.02
Naloxone[3]	8±1	4±1	2±1
6β-Naltrexol[3]	29±14	2±1	2±2
Compound 1, nM	15±2	82±7	76±9

Tail-flick Test

Mice with a basal latency of 2.5 and 3s were collected and randomly divided. The basal latency was recorded before treatment and tail-flick latencies were recorded 30, 60, 90, 120 and 180 minutes after iv, intraperitoneal, or intrathecal administration of the drugs. Morphine and naloxone were dissolved in saline. The iv solution of 1-(2,4-Dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4H-indazol-4-one (Compound 1) was prepared in 5% dimethyl sulfoxide, 5% cremophor and 90% saline. Cyprodime, naltrindole and nor-binaltorphimine were prepared in 5% dimethylsulfoxide and 95% saline. The analgesic effect was measured as the difference between the tail-flick latency and the basal latency. The results are shown in Figure 1.



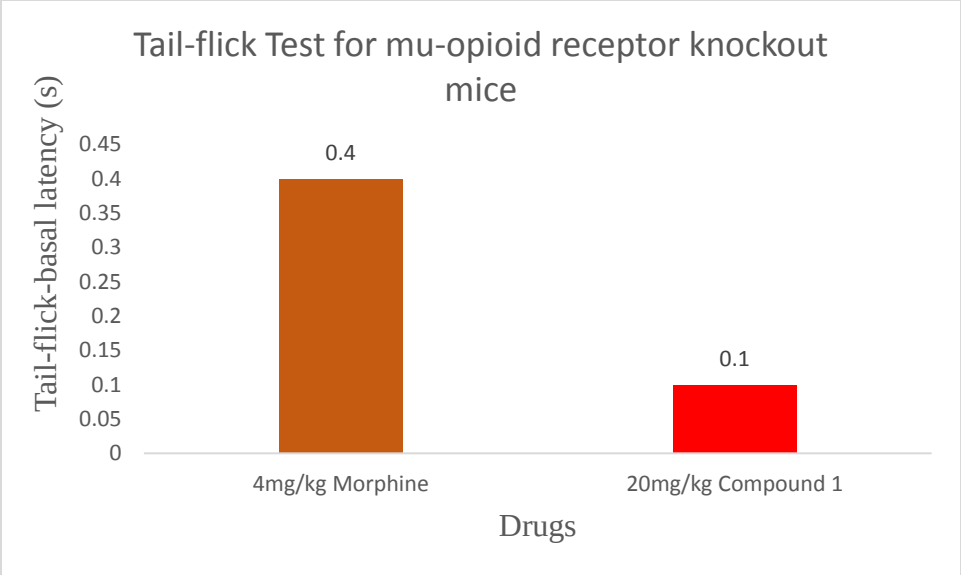


Figure 1:The results of the tail-flick test for both wild-type and mu-opioid receptor knockout mice.

Cancer-induced Pain and Mechanical Allodynia Test

Mouse B16-F1 cells were cultured in DMEM that contained 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin in T-175 tissue culture flasks and harvested with trypsin-EDTA solution. To introduce cancer pain, each mouse was injected with either 20µl phosphate-buffered saline or isoflurane anesthesia on postinoculation day 0. On postinoculation day 19, mice were placed on a mesh floor and allowed to adapt for 1 hour. Melanoma cell-injected mice were injected with vehicle, morphine or 1, 1-(2,4-dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4H-indazol-4-one and a 50% withdrawal threshold was evaluated with von Frey filaments. Testing began with a 0.5g force. If a withdrawal response occurred, the next weaker von Frey filament was applied but if no withdrawal response occurred, the next stronger filament was applied.

Mechanical allodynia refers to changes in pressure required to induce withdrawal. The results are shown in Figure 2.

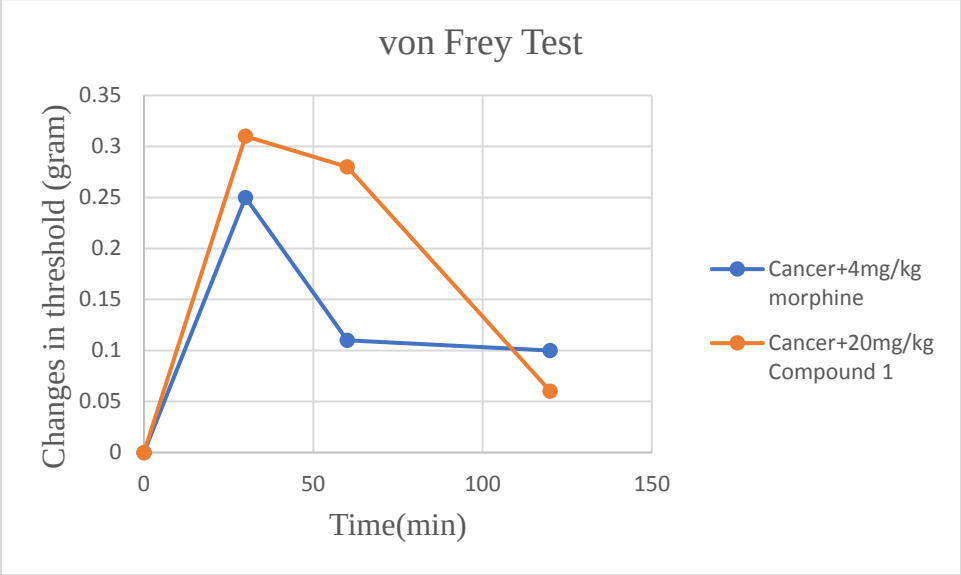
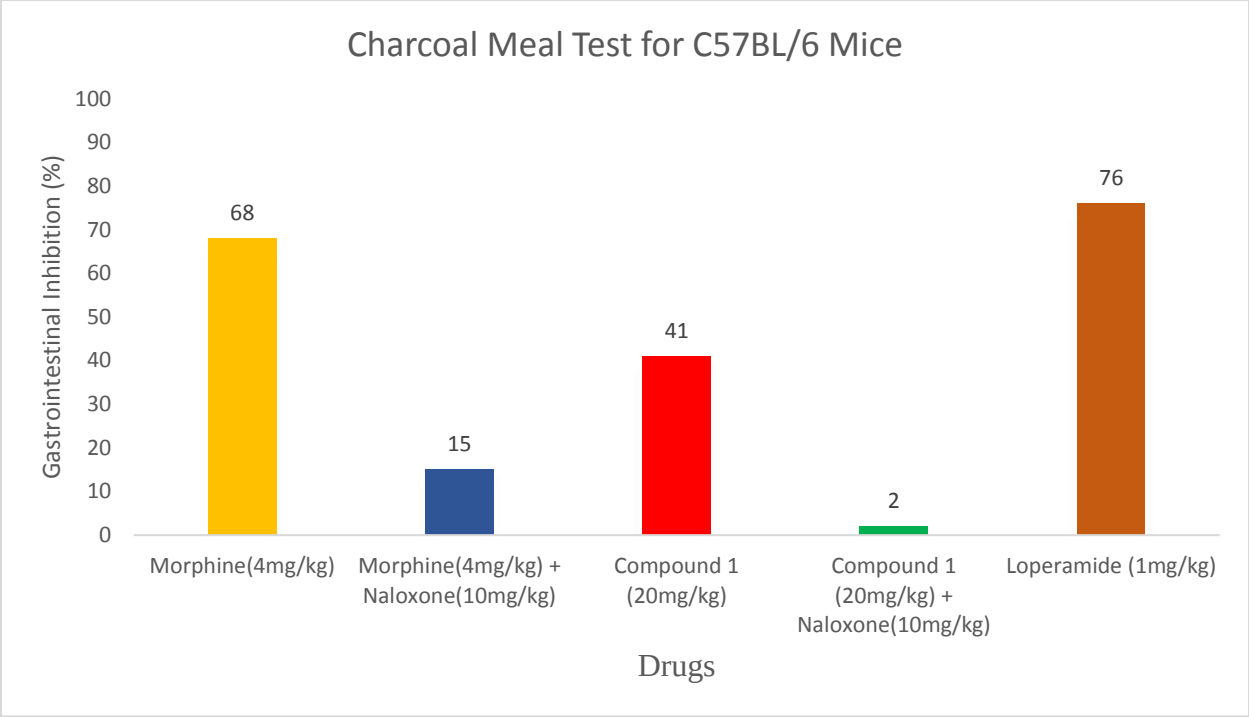


Figure 2. This graph displays the results of the von Frey Test.

Charcoal Meal Test

B6 mice were fasted for 16 hours before experiments with free access to water. Different doses of drugs were given to the mice 15 minutes before administering an aqueous activated charcoal suspension. After 30 minutes, the mice were euthanized with a ketamine-xylazine cocktail, followed by cervical dislocation. The length of migration of the charcoal meal from the pylorus to the ileocecal junction of the small intestine was measured. Gastrointestinal propulsion was calculated as the percentage of the distance traveled by the charcoal meal relative to the total length of the small intestine. If the propulsion rate was less than 55%, that indicated a clear inhibition of bowel propulsion. The results are shown in Figure 3.



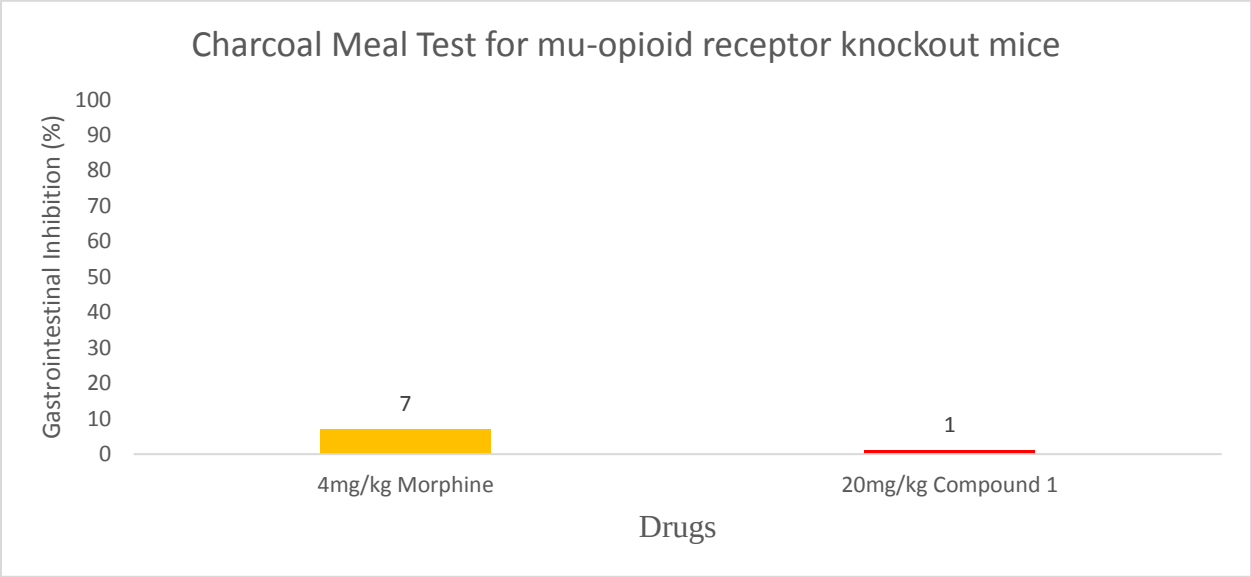


Figure 3: The results of the Charcoal Meal Test for gastrointestinal transit for both wild-type and mu-opioid receptor knockout mice.[4]

Results and Discussion

Conclusion

In this study, we synthesized and attempted to characterize 1-(2,4-Dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4H-indazol-4-one by running a series of assays and tests. These include the radioligand binding assay, tail-flick test, cancer-induced pain and mechanical allodynia test, the tail-clip test and the charcoal meal test. The radioligand binding assay proves that this compound has a high binding affinity for the mu-receptor but it binds less tightly than morphine. Furthermore, it proved to be an effective analgesic because the mu-opioid receptor knockout mice still managed to experience pain. More importantly, it showed potential as an alternative to morphine and other addictive opioids because it proved to have less unpleasant side effects, such as gastrointestinal blockage.

From the results of these tests, it is possible to conclude that 1-(2,4-Dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4H-indazol-4-one acts as a mu-opioid receptor agonist to relieve pain. Even though it still has addictive potential, it has proven to be a viable alternative to morphine because it causes less gastrointestinal blockage. Further modifications to this compound may be done in order to find new compounds that cause less adverse side effects.

Supporting Information Available

The appendix contains a detailed synthesis as well as the characterization of the compound.

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Supporting Information

1-(2,4-Dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one: A Novel Opioid Receptor Agonist with Less Accompanying Gastrointestinal Dysfunction than Morphine

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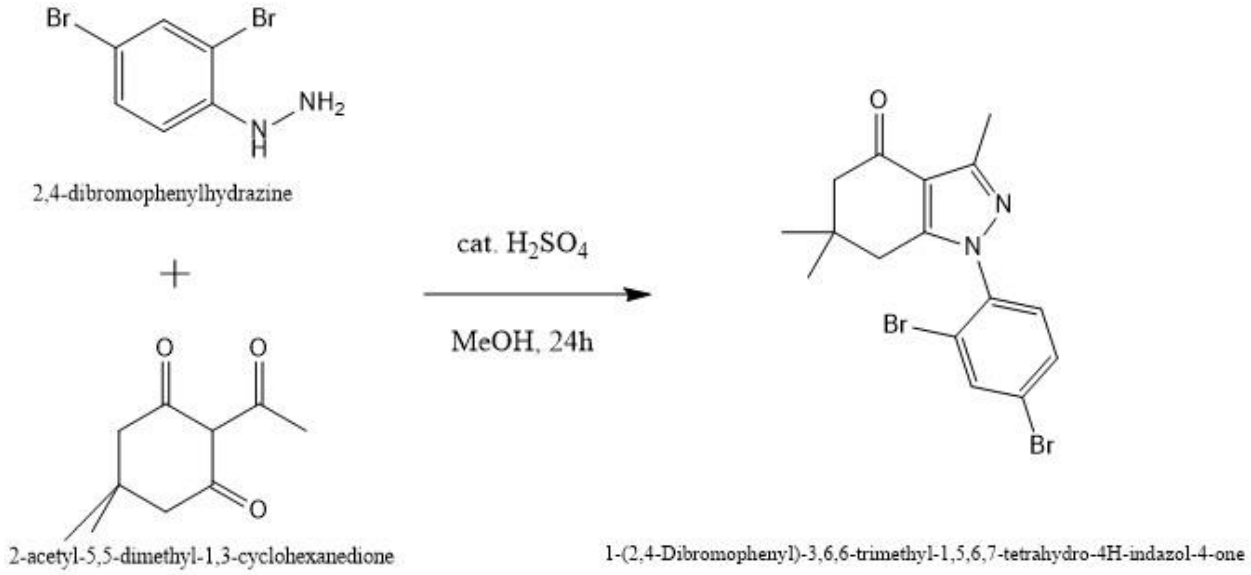
Synthesis of 1-(2,4-Dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one

To a mixture of 1-(2,4-dibromophenyl)hydrazine hydrochloride (121.0 mg .40 mmol) and 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (72.9 mg, .40 mmol) in ethanol (1.2 mL) was added a few drops of concentrated sulfuric acid. The reaction mixture was stirred at room temeprature for 24 hours. The mixture was diluted with ethyl acetate (8.0 mL) and washed with water (4.0 mL), saturated sodium bicarbonate (4.0 mL) and brine (4.0 mL). The organic was dried through anhydrous magnesium sulfate and concentrated by vacuum. The residue was purified by silica gel column chromatography (0-70% EtOAC/hexane) to give **1** as a powder (100.2 mg, 61% yield). (Scheme S1**)**

Scheme S1: Synthesis of Compound 1



^{1}H NMR

1-(2,4-Dibromophenyl)-3,6,6-trimethyl-1,5,6,7-tetrahydro-4*H*-indazol-4-one:

(CD_3OD, 400 MHz) δ 8.07 (d, J = 2.0 Hz, 1H), 7.76 (dd, J = 2.0, 8.4 Hz, 1H), 7.44 (d, J = 8.4 Hz, 1H), 2.54 (s, 2H), 2.46 (s, 3H), 2.40 (s, 2H), 1.10 (s, 6H)

1-(2,4-Dibromophenyl)hydrazine hydrochloride: (DMSO, 400 MHz) δ 10.11 (br, 2H), 8.01 (br, 1H), 7.79 (d, J = 2.0 Hz, 1H), 7.88 (dd, J = 2.0, 8.8 Hz, 1H), 6.97 (d, J = 8.8 Hz, 1H)

Mass Spectrometry

Calculated for $C_{16}H_{19}Br_2N_2O$ (M+H): 412.9864

Experimental: 412.9864

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