

Pharmacological validation of a novel nonhuman primate measure of thermal responsivity with utility for predicting analgesic effects

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Introduction: The development of novel analgesics to treat acute or chronic pain has been a challenge due to a lack of translatable measurements. Preclinical end points with improved translatability are necessary to more accurately inform clinical testing paradigms, which may help guide selection of viable drug candidates.

Methods: In this study, a nonhuman primate biomarker which is sensitive to standard analgesics at clinically relevant plasma concentrations, can differentiate analgesia from sedation and utilizes a protocol very similar to that which can be employed in human clinical studies is described. Specifically, acute heat stimuli were delivered to the volar forearm using a contact heat thermode in the same manner as the clinical setting.

Results: Clinically efficacious exposures of morphine, fentanyl, and tramadol produced robust analgesic effects, whereas doses of diazepam that produce sedation had no effect.

Conclusion: We propose that this assay has predictive utility that can help improve the probability of success for developing novel analgesics.

Keywords: pain, opioid, translatable, monkey, thermode, noxious heat

Introduction

There is an unmet need for pain relief medicines with improved efficacy and reduced side effects relative to the current standards of care. Recent efforts aimed at developing novel therapeutics that have produced very limited clinical success despite promising effects in preclinical models.¹⁻⁵ One potential reason for the inability to translate preclinical findings to clinical success is a lack of translatable biomarkers. Indeed, the ability to clearly measure target modulation clinically greatly increases the probability of success in the clinic.^{6,7} The challenge is then to identify pharmacodynamic end points that can be directly translated from preclinical species to humans despite a large species gap, particularly since the vast majority of the preclinical measures employed to assess in vivo analgesic potential utilize rodents.⁸⁻¹² Utilizing rodents presents at least four major challenges that might reduce translatability to the clinic: 1) inferring the subjective perception of pain by measuring behavior, 2) utilizing equipment and procedures that are not used in the clinical setting, 3) potential species differences in pain biology relative to human, and 4) potential differences in analgesic compound potency and/or affinity for rodent receptor vs human receptor.

In the studies reported here, we have validated a preclinical pharmacodynamic biomarker to better address the above challenges. Without the ability for a subject to report pain, it is not possible to fully address the first of three aforementioned gaps;

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we have therefore addressed gaps two through four in an effort to identify a biomarker with improved translatability. First, we used nonhuman primates (NHPs) instead of rodents. This is important not only because of potential differences in pain biology between rodents and NHPs but also because of species-specific differences in drug potency or affinity. For example, there are instances when a molecule binds with much greater affinity to the human receptor vs rodent receptor, prohibiting the compound from being characterized in rodents,^{13,14} whereas this issue is seldom if ever noted for NHPs. Second, we used a clinically validated pain assessment tool, the Medoc thermal stimulator, which is an identical instrument to that used clinically. Clinically effective doses of opioids and alpha-2 adrenergic agonists demonstrate clear analgesic efficacy in healthy human subjects receiving acute noxious stimuli delivered by this thermode device.^{15–19} We hypothesized that acute thermal stimulation in healthy NHPs might represent an end point sensitive to drug targets with therapeutic potential and at the very least would translate to healthy human assessment of target modulation in Phase I testing.

Methods

Subjects

Eight single- or pair-housed male and female rhesus macaques weighing 4–12 kg were used in the experiments (three males and five females). Personality profiles were not created. Subjects were maintained on a 12-h light cycle (06:30–18:30 h) with room temperatures maintained at $22 \pm 2^\circ\text{C}$. Testing was performed within a separate colony room, to which each animal was habituated for 15–30 min before testing, which took place between 10:00 and 14:30 h. Subjects were fed their full daily regimen of food (Purina High Protein Monkey Diet no. 5045) at 08:00 h, and water was available ad libitum. All monkeys were given various fresh fruits and vegetables daily in addition to the standard food regimen. Principles from the *Guide for the Care and Use of Laboratory Animals*, the National Institutes of Health, and the United States Department of Agriculture were followed, and all protocols were approved by the Merck & Co., Inc. (West Point, PA, USA) and the Institutional Animal Care and Use Committee.

Drugs

Morphine, fentanyl, and tramadol were selected as clinically active positive controls and were purchased from Sigma-Aldrich Co., St Louis, MO, USA, dissolved in saline and administered subcutaneously (SC) at 0.2 mL/kg 30 min

prior to testing. Doses were calculated as a function of base to account for salt factors. The benzodiazepine diazepam (DZP) was selected as a negative control to assess whether a sedative compound without clinical analgesic properties would inhibit responding to thermode stimuli. The dose and route of DZP that produced modest sedation in rhesus macaque were selected,^{20–22} and it was obtained in solution form from Hospira (San Clemente, CA, USA) and administered SC at 0.4 mL/kg. Doses for positive controls were selected to produce exposures matching those reported as active in postoperative pain.^{23–25} Specifically, NHP exposures were intended to match the clinical minimum efficacious concentration (MEC) defined as the trough plasma level measured just prior to patient-controlled administration of another dose of analgesics.

Thermode behavioral testing

Animals were chaired with their arms restrained against the front horizontal panel using an umbilical tape. Veterinary wrap was applied to each wrist to prevent abrasive contact with the tape, which was then tied around the wrists and affixed to a lower front portion of the chair with sufficient slack to avoid distress and allow for visible withdrawal motions. Each animal's hair on the underside of its forearms was shaved, and the thermode stimulator was attached to the shaved area of the left or right forearm using veterinary wrap. Thermal stimulation was delivered via Medoc Thermode software and triggered with an external handheld trigger. Four heat stimuli (44°C , 46°C , 48°C , or 50°C) were presented pseudorandomly in six blocks (Table 1). These temperatures were selected based on reported human pain thresholds with the same thermode device.^{15,19,26,27} Each stimulus was presented for 5 s, and stimuli were presented under a variable interval of 22.5 s (range = 15–30 s). Each response was assessed using a 3-point scale, where 0 = no response, 1 = response consisting of a single clear arm movement, and 2 = multiple arm or body movements. Occasionally, a score of 0.5 was used to indicate a very small or questionable response. All experiments were performed within subjects; thus, each animal

Table 1 Stimulus intensity ($^\circ\text{C}$) in each block of four stimuli, in sequential order from top to bottom

Block					
1	2	3	4	5	6
46	50	44	48	48	46
50	48	46	50	44	50
44	46	48	44	50	44
48	44	50	46	46	48

received all treatments in pseudorandom order. Testing was performed twice per week, and the experimenter was blind to drug treatments. At least half of the animals initially selected for testing did not exhibit adequate behavioral responses to thermode stimulation and were therefore not selected for testing. Only animals exhibiting a minimum mean score of 0.5 at 48°C and of 1.0 at 50°C under repeated baseline conditions were selected for study. A sample of subjects from the first behavioral screening is shown in Figure 1, though it does not represent all animals ultimately included in drug studies.

Pharmacokinetics

Concentrations were quantified using a Transcend LX2 Multiplexed UPLC system coupled with a SCIEX (API4500 for morphine and API6500 for fentanyl and tramadol) triple quadruple mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The study samples and triplicate standard curves spiked in control matrix (30 µL of 2% formic acid per 100 µL of monkey plasma) were prepared for analysis using a protein precipitation extraction method. The

chromatographic separation was performed using an Acquity UPLC HSS T3 (50 × 2.1 × 1.8 µm) column. The flow rate was 0.750 mL/min, and the liquid chromatography (LC) gradient method was started with 80% water with 0.1% formic acid and ramped to 98% acetonitrile with 0.1% formic acid for morphine, 100% water with 0.1% formic acid and ramped to 98% acetonitrile with 0.1% formic acid for fentanyl, and 95% water with 0.1% formic acid and ramped to 98% acetonitrile with 0.1% formic acid for tramadol. The concentration of L-005346293-001D005 in the samples was determined using MultiQuant 3.0.1 based on triplicate standard curves ranging from 5 to 10,000 nM for morphine, from 0.1 to 1,000 nM for fentanyl, and from 2 to 10,000 nM for tramadol.

Statistics

Two-factor (temperature [temp] and group) repeated measures analyses of variance (ANOVAs) were performed to test for the main effects of temperature and group, as well as interactions between these factors. At temperatures where significant treatment effects were observed,

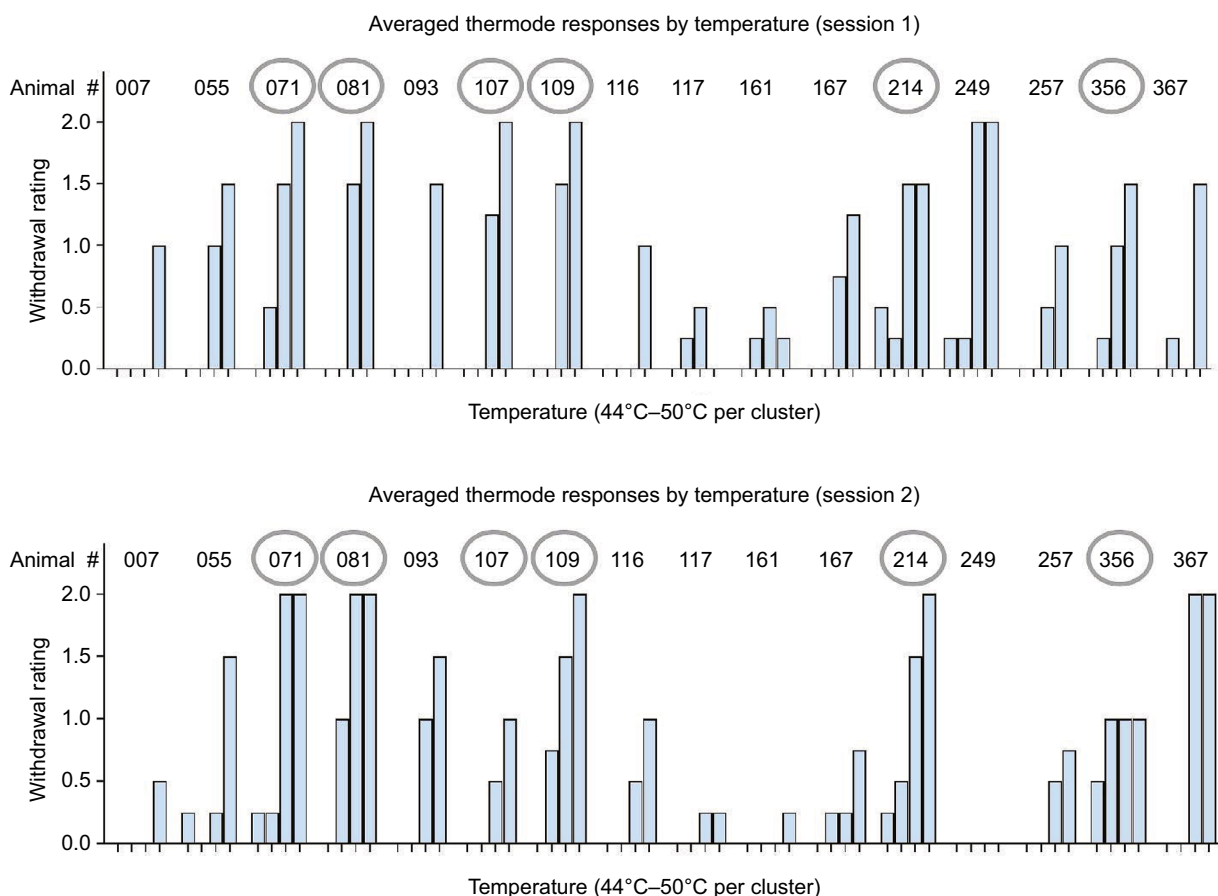


Figure 1 Baseline response of subject candidates over two sessions.

Note: Only six animals met criteria from the initial cohort of 16 tested (circled).

paired samples *t*-tests were used to test for the effects of individual treatment conditions. To control multiplicity, individual treatments were compared to control only at temperatures where significant treatment effects (*F*-test) were observed. This comparison consisted of paired *t*-tests of each active treatment vs the zero (vehicle) dose. To further control for multiplicity, a “step-down” approach was then used for *t*-tests, whereby the highest dose was tested first (vs vehicle), and the next dose was only tested if the highest dose was significant (and so on until the *t*-test was nonsignificant).

Results

Thermode stimulation resulted in a temperature-dependent increase in the withdrawal response. At 44°C no appreciable response was observed, whereas at 46°C–50°C, temperature-dependent responses were produced. In the morphine dose–response (Figure 2), a two-factor (temp × group) repeated measures ANOVA indicated significant main effects of temp ($F_{3,90} = 91.1, p < 0.001$) and group ($F_{3,90} = 48.4, p < 0.001$) and a significant interaction of temp and group ($F_{9,90} = 8.1, p < 0.001$). Repeated measures ANOVA at each temperature revealed a significant effect of group at 46°C ($F_{3,18} = 4.6, p < 0.05$), 48°C ($F_{3,18} = 26.3, p < 0.001$), and 50°C ($F_{3,18} = 24.9, p < 0.001$), but not 44°C ($F_{3,18} = 3.0, p = 0.056$). At temperatures where significant group effects were observed, morphine produced a significant reduction in responding at both 1 mg/kg ($p < 0.05, p < 0.01, \text{ and } p < 0.01$ at 46–50°C, respectively) and 3 mg/kg ($p < 0.01$ at all temps; paired samples *t*-test, $N = 7$). In contrast, the GABA_A receptor-positive allosteric modulator DZP (2 mg/kg) had

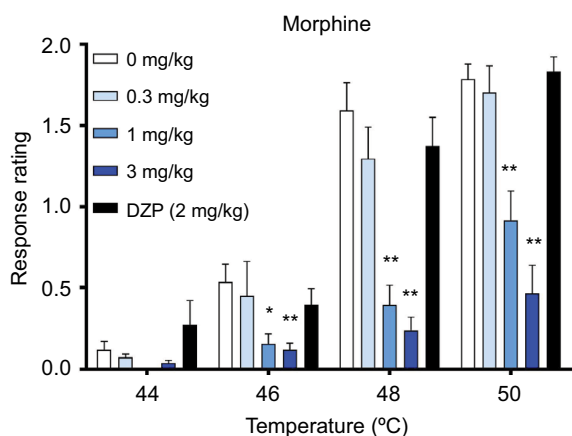


Figure 2 Baseline response to heat stimulation (0 mg/kg, white bars) and effect of morphine and DZP on heat-induced responses.

Note: * $p < 0.05$, ** $p < 0.01$, paired samples *t*-test ($N = 7$, mean \pm SEM).
Abbreviations: DZP, diazepam; SEM, standard error of the mean.

no effect on responding despite producing visible sedation. Total plasma exposures resulting from the doses of morphine tested are shown in Table 2.

A two-factor (temp × group) repeated measures ANOVA also indicated significant main effects of temp ($F_{3,77} = 210.4, p < 0.001$) and group ($F_{2,77} = 44.6, p < 0.001$) and a significant interaction of temp and group ($F_{6,77} = 6.4, p < 0.001$) in the fentanyl dose–response (Figure 3, total plasma exposures in Table 3). Repeated measures ANOVA at each temperature revealed a significant effect of group at 46°C ($F_{2,14} = 13.3, p < 0.001$), 48°C ($F_{2,14} = 24.8, p < 0.001$) and 50°C ($F_{2,14} = 12.3, p < 0.001$), but not 44°C ($F_{2,14} = 1.9, p = 0.18$). At temperatures where significant group effects were observed, fentanyl produced a significant reduction in responding at both 0.005 mg/kg ($p < 0.05$ at 46°C and

Table 2 Plasma concentration of morphine 0.5 h after administration^a

Dose (mg/kg)	Total plasma (μM)
0.3	0.51 \pm 0.12
1.0	1.41 \pm 0.59
3.0	4.22 \pm 0.72

Note: ^aMean \pm SEM, $N = 7$ /group.

Abbreviation: SEM, standard error of the mean.

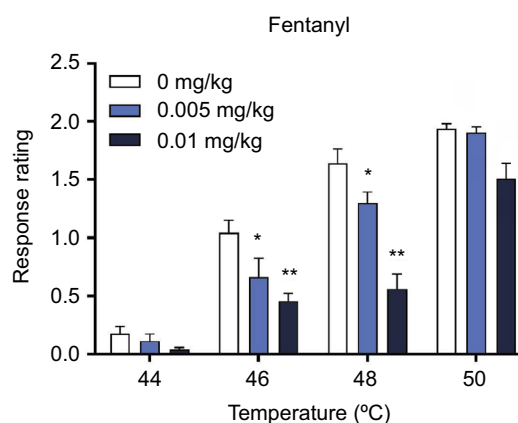


Figure 3 Effect of fentanyl on heat-induced responses.

Note: * $p < 0.05$, ** $p < 0.001$, paired samples *t*-test ($N = 8$, mean \pm SEM).

Abbreviation: SEM, standard error of the mean.

Table 3 Plasma concentration of fentanyl 1.5 h after administration^a

Dose (mg/kg)	Total plasma (μM)
0.005	0.0014 \pm 0.000
0.010	0.0031 \pm 0.001

Note: ^aMean \pm SEM, $N = 8$ per group.

Abbreviation: SEM, standard error of the mean.

48°C) and 0.01 mg/kg ($p < 0.001$ at 46°C and 48°C; paired samples t -test, $N = 8$).

In the tramadol dose–response (Figure 4, total plasma exposures in Table 4), a two-factor (temp \times group) repeated measures ANOVA indicated significant main effects of temp ($F_{3,77} = 257.1, p < 0.001$) and group ($F_{2,77} = 20.8, p < 0.001$) and a significant interaction of temp and group ($F_{6,77} = 2.5, p < 0.05$). Repeated measures ANOVA at each temperature

revealed a significant effect of group at 46°C ($F_{2,14} = 23.6, p < 0.001$) and 48°C ($F_{2,14} = 8.4, p < 0.01$) but not 44°C ($F_{2,14} = 1.8, p = 0.20$) or 50°C ($F_{2,14} = 3.7, p = 0.05$). At temperatures where significant group effects were observed, tramadol produced a significant reduction in responding at both 2.5 mg/kg ($p < 0.001$ and $p < 0.01$ at 46°C and 48°C, respectively) and 5 mg/kg ($p < 0.01$ and at 46°C and 48°C; paired samples t -test, $N = 8$).

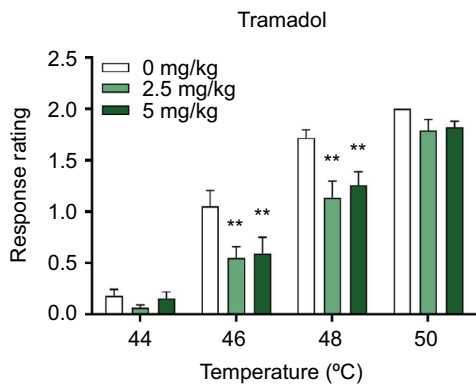


Figure 4 Effect of tramadol on heat-induced responses. **Note:** ** $p < 0.01$, paired samples t -test ($N = 8$, mean \pm SEM). **Abbreviation:** SEM, standard error of the mean.

Table 4 Plasma concentration of tramadol 1 h after administration^a

Dose (mg/kg)	Total plasma (μM)
2.5	2.11 \pm 0.94
5.0	4.16 \pm 1.29

Note: ^aMean \pm SEM, $N = 8$ per group. **Abbreviation:** SEM, standard error of the mean.

Discussion

We present the first report showing heat-induced arm-withdrawal behavior in NHP using a Medoc CHEPS protocol similar to that employed in clinical pain testing, and we have attempted to validate the paradigm with clinically relevant exposures of analgesics. In all cases, these analgesics were effective. In contrast, the GABA_A receptor-positive allosteric modulator DZP (2 mg/kg, selected as a negative control to test whether reductions in arm withdrawal were related to sedation) had no effect on the response despite producing observable sedation. Thus, despite using a behavioral end point in place of clinical subject reports, we believe this assay presents an improvement in translation relative to other preclinical assays available.

The doses of each positive control tested were selected based on their ability to produce clinically relevant plasma exposures (Tables 2–4). However, in order to more systematically determine whether the concentration–effect functions for the NHP thermode test are predictive of effects in the clinic, the MEC of each compound was compared clinically

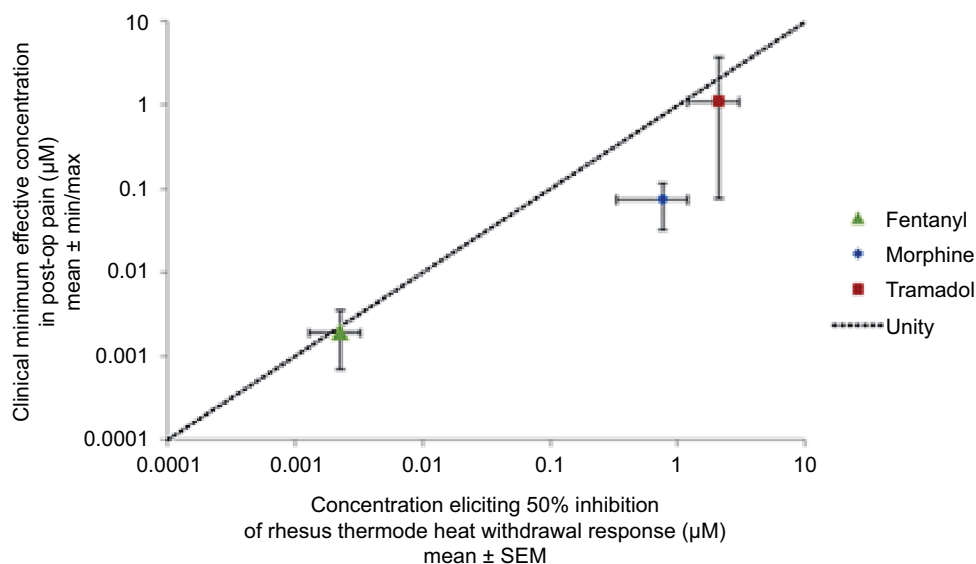


Figure 5 Human post–op MEC vs rhesus IC_{50} at 46°C. **Abbreviations:** max, maximum; MEC, minimum efficacious concentration; min, minimum; post–op, post–operative; SEM, standard error of the mean.

with its IC_{50} in the NHP test at 46°C (Figure 5). The clinically efficacious opioids morphine, fentanyl, and tramadol were active at doses matching their clinical MEC,^{23,24} and we therefore believe that their activity in the present thermode paradigm aligns well with clinical target modulation. Some of these doses are also known to be active in other NHP pain models with less translatable methodology.^{28–30} Interestingly, clinical populations afflicted with chronic pain report a hypersensitivity to thermode-evoked pain scores, ranging from allodynia to hyperalgesia.²⁷ It is therefore possible that inducing thermode hypersensitivity in NHPs could further increase the assay's similarity to these patient populations for future preclinical studies. Topical capsaicin application may represent one practical avenue for inducing hypersensitivity to thermode stimuli, as it is already used in clinical thermode paradigms.^{31,32}

Of note, more than half of the subjects in the initial screening cohort did not exhibit robust and/or reproducible responses to thermode stimulation and were not selected for drug testing. The reason for this observation cannot be objectively determined from the present data, but is likely the result of the limited temperatures employed. For human safety, the clinical device used in these experiments has a restricted temperature range at which stimuli can be maintained (if the stimuli are intended to last for a second or more), peaking around 50°C. As humans are shown to rate 50°C anywhere from 20% to 75% of “maximal pain”,^{15,19,26,27} it is expected that NHPs would express a range of responses between subjects and that many of these responses would not be overtly observable. Indeed, much of the reported NHP experiments employing noxious heat use higher, potentially tissue-damaging temperatures^{28,33} in order to evoke a robust response. However, the stimuli used herein are very typical of clinical experiments, and opioid analgesics are known to markedly affect human pain reporting at this range irrespective of whether individuals rate these temperatures as moderate or extreme.^{15,19} As a species, male NHPs have also likely evolved to be somewhat less expressive of pain behaviors than females, as they are known to compete for colony dominance and be potential targets for aggression.³⁴ The present study found less males meeting selection criteria than females, which may be consistent with this notion. Whatever the case, the goal of the present study was to generate a group of animals that showed a reproducible response against which analgesics could be tested, and therefore, the selection of animals that responding to these temperatures was necessary. Furthermore, the drug sensitivity in these animals appears to align well with human (at least for the compounds tested),

which helps mitigate concerns about whether the subjects employed here are unique and not predictive of effects in humans.

It is also worth noting that any mechanism intended for translation from NHP to human dose should be characterized with regard to its active metabolites, as the potential exists for species differences in metabolite clearance. In the present study, the active morphine exposures appear somewhat less similar to the clinical MEC relative to fentanyl and tramadol, and this could potentially be explained by species differences in metabolism of morphine-6-glucuronide, a metabolite known to be active in human.¹⁹ Indeed, this slight discrepancy between active human and rhesus exposure seems to be consistent with other morphine dose–responses observed in NHP.^{28–30} In any case, active doses of opioids in this assay – especially fentanyl and tramadol – align extremely well with the clinic, and the methods presented here may present an improvement in translational methodology.

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Disclosure

The authors report no conflicts of interest in this work.

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