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Pharmacodynamic Relationships between Duration of Action of JDtic-like Kappa-Opioid Receptor Antagonists and Their Brain and Plasma Pharmacokinetics in Rats

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Abstract

JDtic is a potent and selective κ -opioid receptor (KOR) antagonist that reverses U50,488-induced diuresis in rats. It partitions into brain with a duration of action lasting for weeks. In a search for KOR antagonists that do not accumulate in the brain, we compared single doses of five methylated JDtic analogs (RTI-97, -194, -212, -240, and -241) for reversal of U50,488 diuresis and pharmacokinetic (PK) properties. All six compounds showed potent and selective KOR antagonism in a [³⁵S]GTP γ S binding assay. Plasma half-lives ranged from 24 to 41 h and brain half-lives from 24 to 76 h. JDtic and RTI-194 showed increasing brain to plasma ratios over time, indicating increasing partitioning into brain and a longer duration of action for reversal of diuresis than did RTI-97. RTI-240 did not show significant brain accumulation. RTI-212 showed no substantive difference between brain and plasma levels and was inactive against diuresis. RTI-241, with a lower brain to plasma ratio than JDtic and RTI-194, formed JDtic as a metabolite, which still reduced diuresis after 9 weeks. The fact that the duration of action was correlated with the brain to blood plasma ratios and area under the concentration-time curves suggests that PK properties could help to predict safety and acceptable duration of action for KOR antagonists.

Graphical Abstract

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Author Contributions

G.T.P., J.L.H., T.R.F., and R.W.S. performed the pharmacological and PK studies. S.M.O., G.T.P., J.L.H., T.R.F., and F.I.C. designed the studies, performed analysis of the data, and wrote the manuscript.

ASSOCIATED CONTENT

Supporting Information

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Group means and standard errors for continued weekly testing of dose groups for the analogs until there was no significant effect upon U50,488-induced diuresis and all antidiuresis data for JDtic sc and ip (PDF)

The authors declare no competing financial interest.

Keywords

brain; diuresis; JD1c; κ -opioid receptor antagonist; pharmacokinetics; prodrug; rat

Over the last three decades, potent and selective κ -opioid receptor (KOR) antagonists have been discovered. For example, norbinaltorphine (nor-BNI), 5'-guanidinonaltrindole (GNTI), and 5'-acetamidinoethylnaltrindole (ANTI) were developed with naltrexone as the lead structure.¹⁻³ Carroll and co-workers developed JD1c using N-substituted 3,4-dimethyl-4-(3-hydroxyphenyl)piperidine as the lead structure.^{4,5} In addition to being very potent as KOR antagonists in the [³⁵S]GTP γ S binding assay, nor-BNI, GNTI, and JD1c are extraordinarily long-lasting as *in vivo* KOR antagonists (2 or more weeks).^{4,6,7} Though the reasons for the long duration of action are not clear, mechanistic studies show nor-BNI and JD1c can induce long-lasting changes in c-Jun N-terminal kinase function 1 (JNK1).⁸ Another possible contributing factor is that these compounds are deposited and persist in brain cellular structures long after initial pharmacological effects are over.

We found that JD1c ((3*R*)-7-hydroxy-*N*-((1*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl)-2-methylpropyl)-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide⁹ and methylated analogs RTI-97, (3*S*)-7-hydroxy-*N*-((1*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl)-2-methylpropyl)-2-methyl-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide;⁹ RTI-194, (3*R*)-7-hydroxy-*N*-[(1*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylbutyl)-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide; RTI-212, (3*R*)-*N*-[(1*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]methyl]-2-methylpropyl]-7-methoxy-1,2,3,4-tetrahydroisoquinoline-3-carboxamide; RTI-240, (3*R*)-7-hydroxy-*N*-[(1*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]methyl]-(2-methylpropyl)-3-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxamide;¹⁰ and RTI-241, (3*R*)-7-hydroxy-*N*-[(1*S*)-1-[(3*R*,4*R*)-4-(3-methoxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-(2-methylpropyl)-1,2,3,4-tetrahydroisoquinoline-3-carboxamide¹⁰ (Figure 1; for clarity, the acronyms for the five JD1c analogs were abbreviated from RTI-5989-97, RTI-5989-194, RTI-5989-212, RTI-5989-240, and RTI-5989-241 to use of the last three numbers (e.g., RTI-5989-97 becomes RTI-97)) are potent KOR antagonists in the [³⁵S]GTP γ S binding assay.^{10,11} When tested as an antagonist of KOR agonist U50,488H-induced diuresis, RTI-194 has a potency and duration of activity similar to JD1c and nor-BNI, but RTI-212 is inactive.¹² However, little is known about how pharmacokinetic and metabolic mechanisms, including the possibility of pharmacologically active metabolites, affect efficacy and duration of action.

JDTic and its methylated analogs have similar structures, K_e values for inhibition of KOR agonist-induced binding, and selectivity relative to the μ and δ receptors. Therefore, this unique set of structurally related KOR antagonists offers the potential to correlate the duration of action with brain and plasma PK properties and to determine whether there are pharmacologically active metabolites.

We hypothesized that PK and efficacy studies could be used to establish pharmacodynamic relationships for the discovery of selective KOR antagonists without long duration of action. To test this hypothesis, we used male rats to determine (1) PK plasma and brain concentration-time profiles after ip administration, (2) whether active metabolites are formed by metabolism, and (3) the relative potency and duration of action of JDTic and five analogs (sc) in their ability to antagonize U50,488-induced diuresis. For the third objective, we collected new diuresis data for sc administered JDTic, RTI-194, RTI-240, and RTI-241 and used previously published data for RTI-97 and RTI-212.¹² Importantly, RTI-241 was determined to be a prodrug that formed JDTic as a metabolite and produced very long lasting effects. We found the analogs with the largest values for brain to plasma partitioning (JDTic, RTI-194, and JDTic formed from RTI-241) also had the longest duration of action for the reversal of U50,488-induced diuresis.

RESULTS AND DISCUSSION

[³⁵S]GTP γ S Binding.

As shown by Cueva et al.,¹¹ JDTic and the five analogs were potent KOR antagonists in an agonist-stimulated [³⁵S]GTP γ S binding assay (Table 1). The K_e values ranged from 0.02 nM for JDTic to 0.16 mM for RTI-97. The two most potent analogs were RTI-194 and RTI-240, both with K_e values of 0.03 nM. RTI-241 and RTI-212 had K_e values of 0.037 and 0.06 nM, respectively. JDTic and all five analogs had greater than 100-fold selectivity for KOR relative to μ (MOR) and δ (DOR) opioid receptors.

Pharmacokinetics.

Figure 2 shows the concentration vs time and brain to plasma ratio plots for JDTic and the five analogs after a 5 mg/kg ip dose in male rats. Table 2 shows the PK parameters calculated from these data by a noncompartmental analysis. In all cases, the data points used to calculate individual rat $t_{1/2}$ values were sufficient to include 100% of the AUC_{total} in plasma (i.e., a period of time equivalent to at over four half-lives) and 88–100% of the AUC_{total} in brain.

Figure 3 shows the concentration vs time and brain to plasma ratio plots for JDTic at 3, 5 (from Figure 2), and 10 mg/kg for direct comparison. After considering the data from the 5 mg/kg dose, we chose to collect an additional blood and brain sample at 336 h from the rats administered doses of 3 and 10 mg/kg. This was used to help determine whether JDTic was completely eliminated after 14 days. Table 3 shows parameters calculated by noncompartmental analysis. Since an iv dose of each compound was not administered for comparison to the ip data and there was a limited number of concentration-time data points,

we could not fully determine the PK profile for bioavailability, absorption, distribution, and elimination phases.

Figure 4 (lower panel) shows results of analysis of the RTI-241 brain and plasma samples for the presence of JD_{Tic} as a metabolite. The high concentrations of JD_{Tic} found in these samples showed that RTI-241 is a prodrug, with JD_{Tic} as the pharmacologically active metabolite. This metabolic conversion likely results from liver first pass effects.

U50,488H-Induced Diuresis.

Figure 5 shows effects of JD_{Tic} and its five analogs at the common dose levels of 0.3, 1, 3, and 10 mg/kg sc at weeks 0 and 1, the data sets to be compared to the PK results in Figure 2 and Table 2. Values are expressed as mean percent of water + U50,488, which was set at 100%. Thus, the lower the percentage, the greater the reversal of diuresis toward the water + water (Veh) level, and the greater the implied KOR antagonism. By inspection, the compounds can be divided into three categories: substantial functional KOR antagonism effect at week 0 but not week 1 (RTI-97 and RTI-240), substantial effect at week 0 and greater effect at week 1 (JD_{Tic}, RTI-194, and RTI-241), and no effect (RTI-212). RTI-241 was so potent that the time-related differences are small at 0.3 and 1 mg/kg and nonexistent at 3 and 10 mg/kg because of a ceiling effect.

Figure 6 shows effects of JD_{Tic} at 1, 3, and 10 mg/kg ip at weeks 0 and 1, with effects at the same doses sc from Figure 5 given for comparison, the data set to be matched to the PK results in Figure 3. There was no significant effect of route at week 0 ($F_{1,17} = 1.17$) or week 1 ($F_{1,17} = 3.11$).

Although the primary objective was to investigate the role of PK factors in the duration of *in vivo* KOR antagonism, with a common dose range and time range specified to allow direct comparison, there was in some cases an opportunity to extend the dose and time ranges. The entire data set is placed in the record as Table S1 in Supporting Information for the six compounds sc and Table S2 in Supporting Information for JD_{Tic} ip. These tables show mean, standard error, an indication of statistically significant difference from water + U50,488H (boldface), and percentage difference from water + U50,488H (italics) for all groups on all test days, including the data from Runyon et al.¹³ and Beardsley et al.¹² The values for water + water vs water + U50,488H range from -77% to -95%, so a reduction greater than about 80% is considered complete antagonism. Following is a detailed presentation, with results of analysis of variance and post hoc testing.

For control, each compound (JD_{Tic} and five analogs sc plus JD_{Tic} ip) had its own water + water and water + U50,488 groups. Water + water groups emitted a small volume of urine (range of means 1.7 to 2.3 g) initially, and the amount tended to rise slightly in subsequent weeks as they gained weight. Water + U50,488H group means varied (range 12.0 to 18.2 g) initially, and the amount also tended to rise as they gained weight. In all cases, water + U50,488 was significantly higher than water + water.

Initially, JD_{Tic} sc (Figure 5A and Table S1 in Supporting Information, data taken from Runyon et al.¹³) showed dose-related antagonism, with all doses significantly lower than

water + U50,488 control by Newman-Keuls, maximum –65% at 10 mg/kg. Antagonism increased systematically 1 week later, maximum –81% at 10 mg/kg. There were main effects of treatment ($F_{4,15} = 82.65$) and week ($F_{1,15} = 4.87$) and a treatment $\times$ week interaction ($F_{4,15} = 8.60$).

Initially, RTI-97 (Figure 5B and Table S1 in Supporting Information) showed dose-related antagonism, with 10 and 30 mg/kg lower than water + U50,488 by Bonferroni, maximum –56% at 30 mg/kg. Antagonism declined 1 week later, with only 30 mg/kg significantly lower (–25%) and was no more than a nonsignificant trend 2 weeks later. There were main effects of treatment ($F_{6,63} = 39.24$) and week ($F_{3,63} = 170.10$) and a treatment $\times$ week interaction ($F_{18,63} = 6.81$). Water + U50,488H was greater than water + water at all four times by Bonferroni. On a mg/kg basis, the compound was less potent than JDtic and had a shorter duration of action.

Initially, RTI-194 (Figure 5C and Table S1 in Supporting Information; data taken from Beardsley et al.¹²) showed dose-related antagonism, with 1, 3, and 10 mg/kg lower than water + U50,488 by Bonferroni, maximum –55% at 30 mg/kg. The reductions were greater 1 week later, with all four doses lower, maximum –80% at 10 mg/kg. There were main effects of treatment ($F_{5,18} = 82.77$) and week ($F_{1,18} = 16.54$) and a treatment $\times$ week interaction ($F_{5,18} = 13.60$). The compound was about as potent as JDtic initially and at 1 week.

RTI-212 up to 10 mg/kg (Figure 5D and Table S1 in Supporting Information; data taken from Beardsley et al.¹²) did not show antagonism initially or 1 week later.

Initially, RTI-240 (Figure 5E and Table S1 in Supporting Information) showed dose-related antagonism, with 3, 10, and 30 mg/kg lower than water + U50,488 by Bonferroni, maximum –57% at 30 mg/kg. Antagonism declined 1 week later, with only 30 mg/kg significantly lower (–24%) and no trend 2 weeks later. There were main effects of treatment ($F_{6,42} = 64.22$) and week ($F_{2,42} = 155.00$) and a treatment $\times$ week interaction ($F_{12,42} = 9.23$). The 30 mg/kg dose + water did differ from water + water: there were main effects of treatment ($F_{1,12} = 6.37$) and week ($F_{2,12} = 8.17$) and a treatment $\times$ week interaction ($F_{2,12} = 11.27$), with 30 mg/kg + water greater than water + water in the initial test only. We have no explanation for this reduction to 62%, other than random variation. This is the only instance in which water + high dose differed significantly from water + water. The compound was about as potent as JDtic initially but had a shorter duration of action.

Initially, RTI-241 (Figure 5F and Table S1 in Supporting Information) showed dose-related antagonism, with all doses lower than water + U50,488 by Bonferroni, maximum –95% at 30 mg/kg. Antagonism was about the same 1 week later, maximum –98% at 30 mg/kg, and nearly as great 2 weeks later, maximum –92% at 30 mg/kg. Thereafter, the effect declined gradually. There were main effects of treatment ($F_{6,84} = 83.99$) and week ($F_{4,84} = 65.94$) and a treatment $\times$ week interaction ($F_{24,84} = 7.29$) for weeks 0–4. A separate analysis of variance was run on all 11 determinations for 10 and 30 mg/kg, the lower dose groups having been removed after the week 4 determination, at which they showed no antagonism. There were main effects of treatment ($F_{3,120} = 49.89$) and week ($F_{10,120} = 46.71$) and a treatment $\times$

week interaction ($F_{30,120} = 9.50$). The 10 mg/kg dose significantly antagonized U50,488H up to 7 weeks later, and 30 mg/kg dose did so up to 9 weeks later. The compound was much more potent than JDtic and had an extraordinarily long duration of action.

New dose response data for JDtic ip (Figure 6 and Table S2 in Supporting Information) were generated in Sprague-Dawley rats to allow comparison to the earlier data for JDtic sc in Sprague-Dawley rats (Figure 5 and Table S1 in Supporting Information). These data helped to determine whether efficacy differed between the two routes. Initially, the compound showed dose-related antagonism, with 10 and 30 mg/kg doses lower than control by Bonferroni, maximum -51% at 30 mg/kg, and nonsignificant trends at 1 and 3 mg/kg. Antagonism was greater 1 week later, with 3, 10, and 30 mg/kg lower, maximum -85% at 30 mg/kg; and the effect was slightly less 2 weeks later, though the maximum was still -74% at 30 mg/kg. There were main effects of treatment ($F_{4,45} = 18.75$) and week ($F_{2,45} = 3.90$) but no treatment $\times$ week interaction. The reductions for 1, 3, and 10 mg/kg were as follows: initially, -21%, -30%, and -44% ip vs -23%, -36%, and -64% sc; 1 week later, -30%, -43%, and -75% ip vs -45%, -66%, and -81% sc. That is, the degree of antagonism did not differ greatly as a function of route.

To compare the two routes, the values for the common doses 1, 3, and 10 mg/kg were converted to percent of their respective water + U50,488 means and submitted to analysis of variance. At week 0, there was an outlier in the 10 mg/kg ip group, 101%, vs 34%, 41%, and 50% for the other three rats. We concluded that this was the result of a missed ip injection and so removed the value before further analysis. There was no significant effect of route ($p = 0.29$ at week 0 and 0.10 at week 1) and no interaction ($p = 0.94$ at week 0 and 0.26 at week 1).

Some of the compounds showed complex pharmacokinetics profiles, long half-life values in plasma and brain (Figure 2 and Table 2), and extremely long duration of action. Absorption and distribution after ip administration were not complete until at least 24 h for most compounds, and elimination from brain was much slower than from plasma for three of the six (JDtic, RTI-194, and RTI-212). Since the estimates of half-life values were based on only two or three concentration-time points and the brain concentrations often appeared to be increasing relative to plasma concentrations at the end of the time period for sample collections, we did not assume that the $t_{1/2}$ values were always an accurate reflection of the terminal elimination half-life. An exception was RTI-212, which showed significantly faster absorption and distribution and apparently more complete elimination than the other five compounds. Importantly, it also did not reverse U50,488-induced diuresis.

The time-dependent values for the brain to plasma ratios up to the last measured concentrations at 168 h (Figure 2) also suggested that elimination from brain based on $t_{1/2}$ values was significantly slower than that from plasma for some compounds (JDtic, RTI-194, and RTI-212, Table 2) or that they were still redistributing and accumulating in brain tissue or both. Data for two additional doses of JDtic, 3 and 10 mg/kg ip, showed that the brain to plasma ratios were starting to decrease by 336 h (i.e., 14 days; Figure 3 and Table 3).

To assess the possibility of PK dose dependency, we conducted PK studies of JD_{Tic} using doses of 3, 5, and 10 mg/kg (Figure 3 and Table 3). Comparison of the doses was performed by dividing the AUC_{last} values by the dose of JD_{Tic} administered. The mean, standard deviation, and percent coefficient of variation for the plasma and brain AUC_{last} values (Table 3) were 322 ± 103 h·ng/mL, 32%, and 2184 ± 329 h·ng/g, 9%, respectively. Since the AUC_{last} value for the 10 mg/kg dose was about 1.7 times greater than the values for the other two doses, this suggested possible PK dose-dependent changes with higher doses of JD_{Tic}. The *t*_{1/2} values for JD_{Tic} in brain tissue were 1.8 to 2.9 times longer than plasma values.

RTI-241 appeared to be a prodrug that was metabolized to form significant concentrations of JD_{Tic} through metabolic O-demethylation (see Figure 1 for chemical structures). The absorption of RTI-241 along with the formation rate and an apparently slow disposition process for JD_{Tic} led to sustained plasma and brain concentrations of JD_{Tic} over time (Figure 4). These impactful PK processes produced the most prolonged duration of action of all of the compounds in the antidiuresis test, the highest value for a brain to plasma ratio (113 to 1), and the highest brain to plasma AUC_{last} ratio (17.4).

JD_{Tic} (as a parent compound or metabolite from RTI-241) and RTI-194 had by far the highest brain to plasma ratios at the 168 h time point (Figure 2). They also had the largest AUC_{last} values and two of the three longest *t*_{1/2} values. RTI-212 had the longest brain *t*_{1/2}, but the brain AUC_{last} was substantially lower, and the compound was inactive in the antidiuresis test.

In a 2011 study, the effects of naloxone, nor-BNI, JD_{Tic}, RTI-97, RTI-194, RTI-212, RTI-240, RTI-241, LY2456302, PF-4455242, FP3FBZ, and JSPA071B stimulation of phospho-JNK-immunoreactivity in spinal cords of injected mice and in κ receptor-transfected HEK293 cells *in vitro* were found to be strongly correlated with *in vivo* duration of antagonism of U50,488-induced analgesia in mice.¹⁴ Thus, the very long duration of antagonist action of nor-BNI and JD_{Tic} *in vivo* was attributed to processes related to c-Jun N-terminal kinase (JNK1) activation. However, the persistent *in vivo* effects could also be due to slow PK clearance from the brain (Figures 2, 3, and 4 and Tables 2 and 3). With the exception of RTI-212, which did not reverse diuresis, JD_{Tic} and the other four RTI compounds had a duration of antagonism of U50,488-induced analgesia in mice¹⁴ similar to the duration of antagonism of U50,488-induced diuresis in rats.

Munro et al.¹⁵ measured physicochemical properties of nor-BNI, GNTI, and JD_{Tic} in mice to gain information about their long duration of antagonist action. The results did not support the hypothesis of slow absorption or that they dissolve and persist in cell membranes. The results are more consistent with a JNK1 desensitization-based mechanism.^{8,14,16} However, a 2013 PK study that evaluated the time-course of the brain content and plasma concentration of nor-BNI after sc administration in mice suggested that the long-lasting antagonist effects are due to residual nor-BNI detected in brain for 21 days.¹⁷ In another study, the concentration of nor-BNI in perfused brain homogenates was determined in mice up to 21 days after administration of nor-BNI at 30 nmol icv or 10 mg/kg ip.¹⁸ In subsequent experiments, Nor-BNI was administered at doses based on the amount of nor-

BNI detected in the brain homogenates of nor-BNI treated mice. The results showed significant antagonism of U50,488-induced antinociception, and the levels were consistent with the observed KOR antagonism. Overall, the physical presence of nor-BNI in the mouse brain paralleled its *in vivo* pharmacological profile over time, suggesting that physicochemical and PK properties of this KOR antagonist may contribute to its long duration of action. However, both studies^{17,18} indicated that the long duration of nor-BNI might also involve the activation of JNK.

JDTic's and RTI-194's PK properties and antidiuresis potencies were very similar to each other. However, no compound was as potent as RTI-241 in the antidiuresis test. While this difference in potency was attributed to JDTic as a metabolite of RTI-241 and subsequent prolonged PK properties of JDTic, the long-lasting activity could also be due to the induction of long-lasting pharmacological effects after most of the compound was removed from the brain. For example, at week 0, RTI-194 reduced diuresis at 1, 3, and 10 mg/kg, and the effects were even greater at week 1. In contrast, RTI-212 did not significantly reduce diuresis at week 0 or week 1 at any dose; this lack of potency occurred even though RTI-212 brain concentrations were high. However, the brain concentrations were virtually the same as plasma concentrations, which was unlike the PK profile of any of the other compounds.

In conclusion, the results provide valuable information about what mechanism(s) are driving the long duration of response for some KOR antagonists. The experiments addressed whether the long duration is predominantly receptor antagonism, PK-related mechanism(s), or JNK phosphorylation. The answer is best understood by considering the sequence of pharmacological events that leads to the long response. Even though all six compounds produce *in vitro* KOR antagonism in a [³⁵S]GTPγS binding assay, the degree to which they cross the blood-brain barrier is widely different, so PK-related processes substantially affect their *in vivo* potency (e.g., RTI-212). In addition, without the conversion of RTI-241 to JDTic by liver first pass metabolism, RTI-241 would not be expected to still reduce diuresis 9 weeks after administration. The fact that the duration of action is correlated with the brain to blood plasma ratios and area under the concentration-time curves suggests that PK properties are an important factor in pharmacological action. Without delivery of the compounds to their sites of action, the important mechanism of JNK phosphorylation could not occur. Thus, all of these factors are important, but the contribution of PK-related events has been underappreciated in the overall action and safety of KOR antagonists.

METHODS

Chemistry.

RTI-97, RTI-194, RTI-212, RTI 240, and RTI-241 were synthesized as previously reported.^{5,10,11}

Subjects.

For PK studies, adult male experimentally naive Sprague-Dawley rats (251–275 g) were received from Charles River Laboratories (Raleigh, NC) and group-housed in a temperature-controlled (22 °C) room on a regular 12-h light/dark cycle in the animal research facility of

Research Triangle Institute (RTI). They were provided water and pelleted certified Purina Rodent Chow (5002) ad libitum and quarantined for at least 1 week prior to commencement of testing. For diuresis studies, adult male experimentally naive Long-Evans rats at 251–275 g were received from Charles River Laboratories (Raleigh, NC), housed one or two per cage, and maintained in the RTI animal research facility as stated above. In addition, to generate diuresis data for JD_{Tic} ip to match the ip route used for PK studies, Sprague-Dawley rats as described above for PK were used. The use of the ip route was needed to generate PK data to determine the effects of liver first-pass metabolism. All rats received care according to the *Guide for the Care and Use of Laboratory Animals*, National Research Council, Revised, 2010. The research facility was certified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The study was approved by the RTI Institutional Animal Care and Use Committee.

Previously published sc data for JD_{Tic}¹³ were included in the analysis. The subjects were experimentally naive adult male Sprague-Dawley rats at 165–200 g received from Charles River Laboratories (Raleigh, NC), housed four per cage, and maintained in the RTI animal research facility as stated above. Previously published sc data for RTI-194 and RTI-212¹² were also included in the analysis. The subjects were adult male Sprague-Dawley rats at 176–200 g received from Charles River Laboratories (Raleigh, NC), housed one to four per cage, and maintained in the RTI animal research facility as stated above.

In summary, all data were generated in adult male rats maintained under similar conditions (except number per cage, which was varied to accommodate increasing weight and to prevent fighting) in the animal research facility of RTI. The data were generated by the sc route (with additional data ip for JD_{Tic}) in either Sprague-Dawley or Long-Evans rats, $N = 3$ for PK and $N = 4$ for diuresis, with minor exceptions noted. All diuresis experiments were conducted by the same researchers.

Compound Preparation.

For PK studies, compounds were dissolved in saline; volume of injection was 5 mL/kg ip. For diuresis testing, compounds (as the hydrochloride salt) and U50,488H were dissolved in distilled water; lower dose concentrations were made by taking aliquots from the highest concentration and diluting with water; the volume of injection was 1 mL/kg sc (5 mL/kg for JD_{Tic} ip).

Pharmacokinetics.

For the main experiment, JD_{Tic} or each of the five methylated analogs was administered ip at 5 mg/kg. Three rats per time point were euthanized by CO₂ inhalation at 15 min and 4, 24, 72, and 168 h (exception, for RTI-212, $N = 2$ at 168 h and 1 at the other points). Blood was obtained by cardiac puncture, with heparin as an anticoagulant, and the tube was placed immediately in wet ice. Brain was removed and placed immediately in a vial, which was put into liquid nitrogen for about 1 min, then placed on dry ice, and later transferred to a –80 °C freezer. Within 1 h of collection, blood samples were conveyed to the PK lab for centrifugation. After centrifugation, the plasma fraction was collected and stored frozen until analysis.

In addition, JDTC was administered at 3 and 10 mg/kg, and blood and brains were collected at 4, 24, 72, 168, and 336 h to allow determination of whether PK values were independent of dose. The 336 h point was added because concentrations were still significant at 168 h after the 5 mg/kg dose. Samples were collected and stored as described above.

In preparation for analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS), whole brain samples were homogenized separately in an ethanol/water (50:50, v/v) solution (3 mL/g of brain). A 50- μ L aliquot of brain homogenate or plasma was transferred to a microcentrifuge tube followed by 10 μ L of a 1- μ g/mL solution of a buspirone internal standard in acetonitrile. Each sample was prepared in duplicate. Acetonitrile containing 0.1% formic acid (300 μ L) was added, and the samples were vortexed and then centrifuged at 1600g. A 100- μ L aliquot of the acetonitrile fraction was removed and diluted with 900 μ L of a solution of 0.5% formic acid in acetonitrile/water (85:15, v/v) containing 5 mM ammonium acetate.

LC-MS/MS calibration standards were prepared by combining 40 μ L of blank plasma or blank brain homogenate and 10 μ L of analyte spiking solution into microcentrifuge tubes, along with 10 μ L of internal standard solution. To generate standard curves for analyte quantitation, each ligand in plasma (in ng/mL) or brain (in ng/g) homogenate was prepared over a range of concentrations (e.g., for plasma 0.01–10 ng/mL, for brain 0.025–1000 ng/mL), which was based on the expected concentrations in each sample. All final reported plasma and brain concentrations were carefully monitored to ensure that they were within the range of the calibration curve for each analyte and sample.

Samples were analyzed with an Applied Biosystems API 4000 triple quadrupole mass spectrometer (Foster City, CA) interfaced to an Agilent 1100 binary pump and autosampler. A Valco valve and Agilent 1100 isocratic pump were used to divert the first minute of the run to waste while infusing 50:50 acetonitrile/water makeup solvent to the mass spectrometer. Chromatography was conducted with a Phenomenex Luna C8 column (150 mm $\times$ 4.6 mm i.d., 5- μ m particle size) fitted with a C8 guard cartridge. Injection volumes were 10 μ L. Two mobile phase solutions were used: solution A was 0.5% formic acid in water containing 5 mM ammonium acetate, and solution B was 0.5% formic acid in acetonitrile/water (85:15, % v/v) containing 5 mM of ammonium acetate. The elution program consisted of a linear gradient starting at 95% A 1 min following injection to 5% A over 5 min with a flow rate of 750 μ L/min.

The analytes and internal standard were monitored by LC-MS/MS in the positive ion mode, using multiple reaction monitoring of m/z 466 $\rightarrow$ 148 for JDTC, m/z 480 $\rightarrow$ 162 for RTI-97, m/z 480 $\rightarrow$ 162 for RTI-212, m/z 480 $\rightarrow$ 162 for RTI-240, and m/z 480 $\rightarrow$ 148 for RTI-241. For buspirone, the transition monitored was m/z 386 $\rightarrow$ 122.

Because multiple animals were terminally sampled at each time point to obtain blood and brain, the method for pharmacokinetic analysis used was to model mean data from each time point, resulting in a single set of pharmacokinetic values for each dose group. As a consequence, statistical comparisons between analogs were not conducted.

For the model-independent (noncompartmental) analysis of average plasma or brain concentration-time data, the area under the compound plasma concentration-time curve (AUC) from the time of the first compound administration to the last observed concentration-time point (AUC_{last}) used for the analysis (e.g., 72 h for RTI-97 in plasma and brain, and 168 h for the other test compounds) was calculated using the linear trapezoid rule. A linear regression best-fit line to the terminal log-linear plasma concentration-time data was used to obtain an estimate of terminal disposition rate constant (λ_n) and half-life ($t_{1/2}$) by the equation: $t_{1/2} = 0.693/\lambda_n$. The remaining area to time infinity was determined from the predicted concentration on the best-fit line at the time point (t_n) of the last measured concentration (C_n) by C_n/λ_n . The sum of this area plus AUC_{last} was the AUC from time zero to infinity (AUC_{total}). For comparisons of different doses for studies of JDTC (3, 5, and 10 mg/kg), AUC_{total} data were normalized for dose. The brain concentration-time data were analyzed in a similar manner. The brain to plasma ratio (as a measure of time-dependent partitioning into the brain) for each test article was determined by dividing the brain AUC_{last} value by the plasma AUC_{last} value over the same time period.

Since these PK studies were conducted with ip doses, we did not know the exact dose of each compound that reached the systemic circulation, and therefore we could not accurately calculate the volume of distribution or the total body clearance.

The accuracy of these PK noncompartmental analysis calculations was checked by analysis of three representative concentration-time plasma data sets from JDTC, RTI-194, and RTI-241 by Phoenix WinNonlin V6.4 (Certara USA, Princeton, NJ). Values for $t_{1/2}$, AUC_{last} , and AUC_{total} by the two methods differed by <1.0% in all cases.

U50,488H-Induced Diuresis.

The 32 metabolism cages were standard units from Lab Products, Inc. (Seaford, DE). As noted above, both Sprague-Dawley and Long-Evans rats were used; strain is indicated for each test article under Results and Discussion and Supporting Information. The method was adapted from Leander¹⁹ and was similar to that used in previous evaluations of JDTC.^{4,12} On the first day, water (vehicle) or test article was injected sc. Immediately thereafter, water or U50,488H was injected sc. The rat was then placed in a metabolism cage, and urine output was measured from time 0 to 5 h by determining the weight of the urine in the collection tube. The process was repeated at weekly intervals except that only the second injection (water or U50,488H) was given; that is, JDTC and its five analogs were injected only once, at week 0.

The JDTC sc dose groups,¹³ the RTI-194 groups,¹² and the RTI-212 groups¹² were tested twice (i.e., on the day of test compound injection and 1 week later). The RTI-97 groups were tested four times, and the RTI-240 groups were tested three times. The three lower dose groups of RTI-241 were tested five times, until the rats no longer showed an effect; the two higher dose groups were tested 11 times. The JDTC ip groups were tested three times. After the last day of testing for each dose group, the rats were euthanized under CO₂. Data showing other longer periods of testing and a higher dose of 30 mg/kg are given in the Supporting Information. Thus, the data set for all six test articles allowed direct comparison

at the same four half-log dose levels (0.3, 1, 3, and 10 mg/kg) and two time points (week 0 and week 1). *This was the comparison of primary interest.*

The basic datum was the total weight of urine in grams produced over the 5 h test period. Values were submitted to two-way analysis of variance, with treatment as the between-subjects factor and week as the within-subjects factor. A post hoc test was applied to compare water + U50,488H to water + water and each dose of compound + U50,488H to water + U50,488H, that is, to same-day control (GraphPad Prism, GraphPad Software, Inc., La Jolla, CA). Other comparisons were made, as explained under Results and Discussion. An effect was considered significant if $p < 0.05$. The following data were lost: for RTI-97 at 2 weeks, two rats in the water + water group; for RTI-241 at 7 weeks, one rat in the water + water group. For analysis of variance, the missing value was replaced by the mean of the previous week and the following week. To derive a measure reflecting KOR antagonism, data were also expressed as percent of same-day U50,488H-induced output as follows. The mean for each dose + U50,488H was divided by the mean for water + U50,488H, the result was multiplied by 100, and that percentage was subtracted from 100. This was also done for water + water and highest dose of compound + water.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AUC	area under the serum or brain concentration-time curve
B/P	blood to plasma ratio at specific time points after test compound administration
PK	pharmacokinetic
$t_{1/2}$	apparent half-life in plasma or brain
[³⁵S]GTPγS	sulfur-35 guanosine-5'-O-(3-thio)triphosphate
KOR	κ -opioid receptor
MOR	μ -opioid receptor
DOR	δ -opioid receptor

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Compound	R ₁	R ₂	R ₃	R ₄	R ₅
	JDTic	H	H	H	H	H
	RTI-97	H	H	H	H	CH ₃
	RTI-194	H	H	CH ₃	H	H
	RTI-212	CH ₃	H	H	H	H
	RTI-240	H	H	H	CH ₃	H
	RTI-241	H	CH ₃	H	H	H

Figure 1.
Chemical structures of JD₂Tic and five methylated analogs.

Figure 2.

Concentration-time (+SD) and brain to plasma ratio (B/P) plots for JDITic and five analogs in Sprague-Dawley rats after a 5 mg/kg ip dose. The solid lines showing brain and plasma concentrations (left axis) are the best-fit lines to an apparent terminal disposition phase. The RTI-97 brain concentration appeared to increase at 168 h and so was excluded from the best-fit line for determining the apparent $t_{1/2}$. $N = 3$ rats per group for all data sets except for RTI 212, which was $N = 2$ at 168 h and $N = 1$ at all other time points.

Figure 3.

Plasma and brain concentration-time data (+SD) and brain to plasma ratio plots for JDtic at 3 and 10 mg/kg ip, including a time point of 336 h, in Sprague-Dawley rats ($N=3$). Data for the JDtic 5 mg/kg dose plots are from Figure 2. Concentrations at 336 h appeared not to differ from values at 168 h, but the blood to plasma ratio data were trending toward lower values.

Figure 4. Concentration-time and brain to plasma ratio for JDtic as a metabolite (lower panel) at 5 mg/kg ip and plots of RTI-241 (upper panel) from Figure 2 for comparison.

Figure 5.

Comparison of JDTic and five analogs sc for reversal of U50,488H-induced diuresis in rats. Compounds were administered only once, at week 0. At week 1, only water or U50,488H was administered. Data for JDTic were adapted from Runyon et al.¹³ Data for RTI-194 and RTI-212 were adapted from Beardsley et al.¹² RTI-97, RTI-240, and RTI-241 were tested in the current study. Rat strain was Sprague-Dawley for JDTic, RTI-194, and RTI-212 and Long-Evans for RTI-97, RTI-240, and RTI-241. $N = 4$. * indicates $p < 0.05$ vs concurrent water + U50,488H.

Figure 6.

Comparison of JDtic ip (left panel) to JDtic sc (right panel, from Figure 5A) for reversal of U50,488H-induced diuresis in Sprague-Dawley rats. $N = 4$. * indicates $p < 0.05$ vs concurrent water + U50,488H.

Table 1.

Inhibition of Agonist-Stimulated [35 S]GTP Binding in Cloned Human κ , μ , and δ Opioid Receptors by JDTic and Five Analogs^a

compound	κ U69,593 K_i , nM	μ DAMGO K_i , nM	δ DPDPE K_i , nM	ratio μ/κ	ratio δ/κ
JDTic	0.02 $\pm$ 0.01	251 $\pm$ 3.5	76.4 $\pm$ 2.7	1255	3830
RTI-97	0.16 $\pm$ 0.06	210 $\pm$ 60	491 $\pm$ 120	1313	3070
RTI-194	0.03 $\pm$ 0.02	3 $\pm$ 1	24 $\pm$ 4	100	800
RTI-212	0.06 $\pm$ 0.01	51 $\pm$ 15	118 $\pm$ 45	857	1970
RTI-240	0.03 $\pm$ 0.008	3.6 $\pm$ 1	854 $\pm$ 210	120	28500
RTI-241	0.037 $\pm$ 0.003	24 $\pm$ 8	21.2 $\pm$ 5	649	573

^aData from Cueva et al.¹¹

Table 2. Noncompartmental Pharmacokinetics for Plasma and Brain of JD7ic and Five Analogs in Sprague Dawley Rats after a 5 mg/kg ip Dose

parameter	JD7ic		RTI-97		RTI-194		RTI-212		RTI-240		RTI-241	
	plasma	brain	plasma	brain	plasma	brain	plasma	brain	plasma	brain	plasma	brain
$t_{1/2}$ (h)	28.4	51.8	23.7	24.3	33.5	51.2	35.2	76.2	29.3	30.7	41.4	29.5
AUC_{last} (h-ng/mL plasma; h-ng/g brain)	1316	9047	1096	325	799	7679	2128	1629	800	1367	815	1846
AUC_{total} (h-ng/mL plasma; h-ng/g brain)	1321	10114	1100	351	806	8624	2167	1846	802	1385	816	1852
ratio of brain/plasma AUC_{last} values	6.87		0.30		9.61		0.77		1.71		2.27	

Table 3.

Brain and Plasma Parameters of JDTC as a Function of Dose

parameter	3 mg/kg		5 mg/kg		10 mg/kg	
	plasma	brain	plasma	brain	plasma	brain
$t_{1/2}$ (h)	23.4	68.4	28.4	51.8	29.6	65.3
AUC_{last} (h·ng/mL plasma; h·ng/g brain)	784	6978	1316	9047	4410	24187
AUC_{total} (h·ng/mL plasma; h·ng/g brain)	787	7974	1321	10114	4425	27789
ratio of brain/plasma AUC_{last} values	8.90		6.87		5.48	