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The design and synthesis of novel, phosphonate-containing transient receptor potential melastatin 8 (TRPM8) antagonists

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ABSTRACT

A series of benzothiophene-based phosphonates was synthesized and many analogs within the series were shown to be potent antagonists of the TRPM8 channel. The compounds were obtained as a racemic mixture in 5 synthetic steps, and were tested for TRPM8 antagonist activity in a recombinant, canine TRPM8-expressing cell line using a fluorometric imaging plate reader (FLIPR) assay. Structure–activity relationships were developed initially by modification of the core structure and subsequently by variation of the aromatic substituents and the phosphonate ester. Compound **9l** was administered intraperitoneally to rats and demonstrated engagement of the TRPM8 target in both prevention and reversal-modes in an icilin-induced 'wet-dog' shake model.

Transient receptor potential (TRP) channels are non-selective cation channels that are activated by a variety of stimuli. Numerous members of this ion channel family have been identified to date, a number of which are thermosensitive. Of particular interest to us is the melastatin family, type 8 channel (TRPM8), also called the cold-menthol receptor 1 (CMR1).[1] TRPM8 is stimulated by cool to cold temperatures as well as by the chemical agonists menthol and icilin,[2,3] which likely accounts for the cooling sensation that these agents provoke. TRPM8 is located on nociceptive A-delta and C-fiber neurons and is also modulated by inflammation-mediated second messenger signals,[4,5] thereby potentially providing a molecular basis for abnormal cold sensitivity in pathologic conditions wherein pain, often of a burning nature, is a hallmark.[6–8]

Hypersensitivity to cold stimuli is a well-documented symptom of inflammatory and neuropathic pain. Recent studies using TRPM8 knock-out mice demonstrate a critical role for TRPM8 in cold sensation.[9–11] TRPM8 expression on nociceptive neurons is up-regulated under pathological conditions, resulting in an enhanced sensitivity to innocuous cold (i.e., cold allodynia),[12,13] whereas cold allodynia seen following nerve ligation[14] is attenuated in TRPM8 null mice.[9] The effective treatment of chronic pain, especially following nerve injury, remains an elusive goal and still represents a large unmet clinical need. Because cold intolerance and paradoxical burning sensations induced by chemical or thermal cooling closely parallel symptoms seen in a wide range of clinical disorders, there is a strong rationale for the development of TRPM8 modulators as novel therapeutics.[15]

Recently, potent, small molecule TRPM8 antagonists have been reported.[16,17] This communication describes the discovery and optimization of a series of potent and selective TRPM8 antagonists that are based on a benzothiophene-phosphonate template. This structural motif afforded compounds that antagonize TRPM8-mediated behaviors in rats and offer the potential for treatment of cold allodynia and other aberrations in cold sensing.

In the present study, high-throughput screening in a TRPM8 canine FLIPR[18] assay at a concentration of 4 μM led to the identification and confirmation of a diaryl phosphonate lead, **1**, which demonstrated modest potency (IC_{50} = 3.6 μM). The low molecular weight and multiple points for chemical diversification were

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attractive features of this phosphonate hit. As shown in Figure 1, initial modification of **1** via insertion of a methylene group afforded homolog **2**, which demonstrated a four-fold improvement in potency (IC_{50} = 0.95 μM).

Investigation of alternate scaffolds related to compound **2**, using chemistry outlined in Scheme 1, resulted in the synthesis of compounds **4**,[19,20] as shown in Table 1. Though several of the compounds had similar potency to compound **2**, analogs 2-naphthyl **4c** (IC_{50} = 0.12 μM), 2-benzothienyl **4e** (IC_{50} = 0.25 μM), and 3-methyl-2-benzothienyl **4f** (IC_{50} = 0.184 μM) demonstrated a 4- to 8-fold improvement in potency. Additionally, compound **4f** represented a novel scaffold for further studies of structure–activity relationships.

Analogs of compound **4f** were prepared utilizing the synthetic approach outlined in Scheme 2. 3-Substituted benzothiophenes **5** were treated with *n*-butyllithium at reduced temperatures, followed by treatment of the resulting anion with DMF to give 2-formylbenzothiophenes **6**. Reduction of the aldehyde with sodium borohydride, followed by conversion of the resulting alcohol to the bromide with phosphorous tribromide afforded bromomethylbenzothiophenes **7**. Arbuzov reaction with a trialkylphosphite gave phosphonate intermediates **8**, which were deprotonated with *n*-butyl lithium and alkylated with an appropriately substituted benzyl bromide to afford compounds **9**.

In cases where the requisite 3-substituted benzothiophenes were not commercially available, Scheme 3 was utilized to synthesize the starting materials. For example, the 2-fluoro-aryl ketones **10** were treated with ethyl thioglycolate, in the presence of base and heat, to afford benzothiophene carboxylate esters **11**. Subsequent reduction of the ethyl ester gave alcohols **12**, which, using the steps outlined in Scheme 2(b–d), were converted to target compounds **13**.

The 3-isopropyl benzothiophene analogs were synthesized according to Scheme 4. Thiophenol **14** was treated with 1-bromo-3-methyl-butan-2-none[21] and sodium hydride, to afford 3-methyl-1-phenylsulfanyl-butan-2-one **16**, which was cyclized using polyphosphoric acid (PPA) in the presence of heat to give benzothiophene **17**. Compound **17** was converted to compound **18** using analogous chemistry to that depicted in Scheme 2 (steps a–d).

Compound **9l** was monodealkylated using DMF and sodium azide to afford the acid analog **19**, as shown in Scheme 5.

In addition, phosphine oxide analogs were prepared as shown in Scheme 6 to study the importance of the phosphonate ester moiety for TRPM8 antagonist activity. Diethyl phosphate **20** was treated with an *n*-propyl Grignard reagent to afford intermediate **21**,[22] which was subsequently treated with base and compound **7** to afford dialkyl-phosphine oxide **22**. Treatment of compound **22** with strong base and 3,4-difluorobenzyl bromide gave compound **23**.

The functional activity of compounds **4**, **9, 13**, **18, 19** and **23–26** was determined in HEK293 cells stably expressing canine TRPM8 by measuring changes in intracellular calcium concentration with a Ca^{2+}-sensitive fluorescent dye upon activation with icilin.[18,23,24] All analogs were similarly evaluated for potential cross-reactivity with the genetically related TRPV1 channel, another thermoTRP

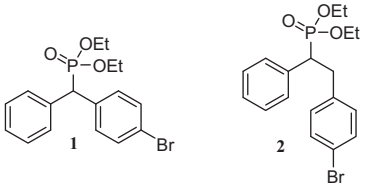
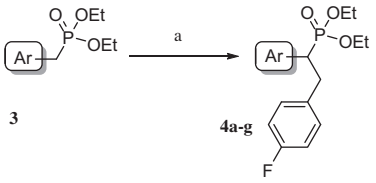
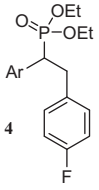


Figure 1. Structure of HTS lead **1** and homolog **2**.



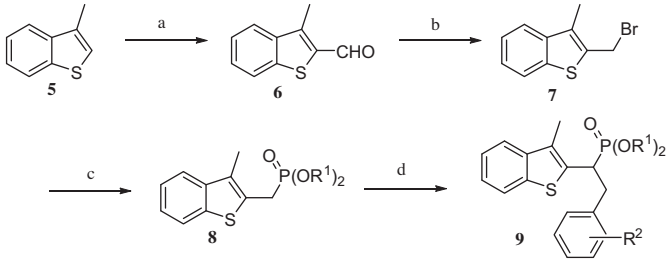
Scheme 1. Reagents and conditions: (a) THF, *n*-BuLi; 4-fluorobenzyl bromide, −70 °C (35–60%).

Table 1
In vitro TRPM8 IC_{50} data for analogs, **4a–g**

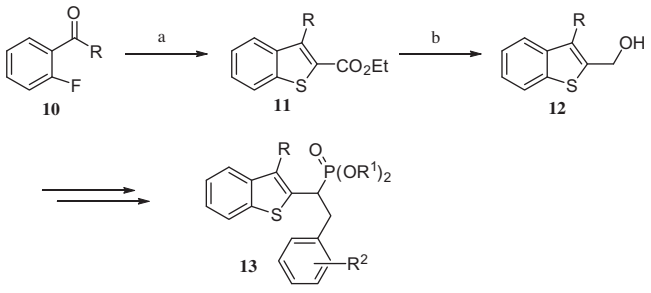


Compd[a]	Ar	IC_{50} (μM)
4a	5-Chloro-1-phenyl-3-indolyl	1.40
4b	5-Fluoro-3-benzothienyl	0.70
4c	2-Naphthyl	0.12
4d	1-Naphthyl	0.50
4e	2-Benzothienyl	0.25
4f	3-Methyl-2-benzothienyl	0.18
4g	2-Benzofuranyl	0.50

[a] Target compounds were purified by reverse-phase semi-prep HPLC and purities were judged by reverse-phase HPLC/MS with UV detection at 215 and 254 nm (YMC J'Sphere C-18 column, 0.4 × 5 cm; mobile phase: MeCN–H_2O (0.1% TFA)). All new compounds were characterized by ESI-MS and 400-MHz ^1H NMR. Compounds were isolated as racemic mixtures.



Scheme 2. Reagents and conditions: (a) (1) THF, *n*-BuLi; (2) DMF; (3) 1 N HCl, −70 °C (99%); (b) (1) THF, MeOH, $NaBH_4$ (84%); (2) CH_2Cl_2, PBr_3 (98%); (c) Toluene, $P(OR_1)_3$ (85–98%); (d) THF, *n*-BuLi, R_2 benzylbromide (23–66%).



Scheme 3. Reagents and conditions: (a) DMF, NaH, ethyl thioglycolate, 90 °C (39–81%); (b) (1) THF, $LiAlH_4$ (53–100%).

channel that is activated by, inter alia, noxious heat, protons and capsaicin.[25]

Initially, the focus was on exploring the substituents on the benzyl moiety using chemistry depicted in Scheme 1. As illustrated in Table 2, replacement of the 4-fluoro group with hydrogen gave an analog (**9a**) that was slightly less potent than compound **4f** in the functional assay. However, analogs with small, lipophilic groups in the 3-position of the phenyl ring (*cf.* **9b**, **9d**, and **9f**), were more potent than lead compound **4f**. Disubstitution of the 3,4-and 3,5-positions with fluoro groups was also well tolerated (*cf.* **9k** and **9l**). All analogs were inactive against TRPV1 at 10 μM.

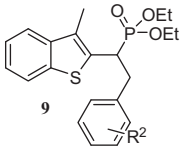
As it was assumed that the 3,4-difluorobenzyl substitution of compound **9l** would prevent extensive metabolism of the benzyl ring, we next altered the 3-position of the benzothiophene ring (Table 3) and the R^1 phosphonate ester moiety (Table 4), while holding the benzyl moiety constant.

Potency in the functional assay was somewhat sensitive to the steric bulk of the 3-position substituent, as cyclopropyl-(**13b**), cyclobutyl-(**13c**) and cyclohexyl-(**13e**) analogs exhibited higher IC_{50} values than compound **9l**. Nevertheless, potency could be maintained or further improved through the judicious choice of a substituent with the appropriate steric and electronic properties. For instance the cyclopentyl analog **13d**, with an IC_{50} value of 0.026 μM, demonstrated similar potency to **9l**. Isopropyl analog **18** demonstrated a marked improvement in functional activity (IC_{50} = 0.012 μM) when compared to compound **9l**.

The effect of the ester functionality of compound **9l** on TRPM8 antagonist potency was investigated by either altering the size of the ester moiety or by removal of one of the ester groups. As illustrated in Table 4, conversion to the mono-acid/mono-ester **19** as per the chemistry in Scheme 5, resulted in complete loss of TRPM8 functional activity, demonstrating the vital importance of a hydrophobic moiety in this portion of the molecule. We next explored the size requirement of the ester, and showed that optimal potency was realized when the ethyl esters were replaced by isopropyl esters (e.g., **24**: IC_{50} = 0.024 μM). However, replacement of the alkoxy moieties of the phosphonate ester with the alkyl chains of a phosphine oxide (e.g., **23** and **25**) using the chemistry shown in Scheme 6 resulted in considerably less potent analogs, with IC_{50} values of 0.165 and 0.123 μM, respectively.

A tool compound was required to establish proof of pharmacodynamic activity in vivo for a TRPM8 antagonist. Racemates **9l** and **24** were identified as phosphonate analogs of interest in light of their potency in the calcium mobilization assay (TRPM8 IC_{50} = 64 and 24 nM, respectively). Both compounds were assessed for their metabolic stability in rat and human liver microsomes and for their absorption potential in Caco-2 cells. Compounds **9l** and **24** exhibited similar microsomal stability, with 53% and 63% remaining, respectively, after incubation in rat liver microsomes for 10 min, and 60% and 39% remaining, respectively, after incubation in human liver microsomes for 10 min.[26] However, compound **9l** demonstrated a much higher absorption potential in Caco-2 cells, with a P_{app} A to B of 3.99×10^{-6} cm/s, compared to compound **24**, with a P_{app} A to B of 0.075×10^{-6} cm/s. Despite the modest

Table 2
In vitro TRPM8 IC_{50} data for compounds **9a**–**9o**



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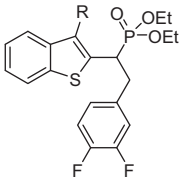
Compd[a]	R^2	IC_{50} (μM)
9a	H	0.360
9b	3-Fluoro	0.031
9c	2-Fluoro	0.114
9d	3-Chloro	0.022
9e	3-Bromo	0.145
9f	3-Methyl	0.065
9g	3-Trifluoromethyl	0.138
9h	3-Methoxy	0.121
9i	4-Trifluoromethyl	0.365
9j	4-Trifluoromethoxy	0.240
9k	3,5-Difluoro	0.058
9l	3,4-Difluoro	0.064
9m	3,4-Dichloro	0.300
9n	4-Chlroro-3-trifluoromethyl	0.525
9o	4-Fluoro-3-trifluoromethyl	0.120

[a] Target compounds were purified by reverse-phase semi-prep HPLC and purities were judged by reverse-phase HPLC/MS with UV detection at 215 and 254 nm (YMC J'Sphere C-18 column, 0.4 × 5 cm; mobile phase: MeCN–H_2O (0.1% TFA)). All new compounds were characterized by ESI-MS and 400-MHz ^{1}H NMR. Compounds were isolated as racemic mixtures. All analogs were inactive when counter-screened against the human TRPV1 cell line at 10 μM.

Table 3
In vitro TRPM8 IC_{50} data for compounds **13a**–**13f** and **18**

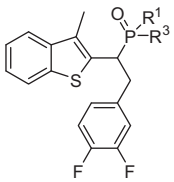


Compd[a]	R	IC_{50}[b] (μM)
13a	H	0.390
13b	-*c*-C_3H_5	0.116
13c	-*c*-C_4H_7	0.146
13d	-*c*-C_5H_9	0.026
13e	-*c*-C_6H_{11}	0.350
13f	$-CH_2C(CH_3)_3$	0.160
18	$-CH(CH_3)_2$	0.012

[a] Target compounds were purified by reverse-phase semi-prep HPLC and purities were judged by reverse-phase HPLC/MS with UV detection at 215 and 254 nm (YMC J'Sphere C-18 column, 0.4 × 5 cm; mobile phase: MeCN–H_2O (0.1% TFA). All new compounds were characterized by ESI-MS and 400-MHz ^{1}H NMR. Compounds were isolated as racemic mixtures.

[b] Inhibition of canine TRPM8 is reported as mean IC_{50} values. All analogs were inactive when counter-screened against the human TRPV1 cell line at 10 μM.

Table 4
In vitro TRPM8 IC_{50} data for compounds **19**, **23**–**26**



Compd[a]	R^1	R^3	IC_{50}[b] (μM)
19	–OEt	–OH	NA
23	$-C_3H_7$	$-C_3H_7$	0.165
24	$-O-CH(CH_3)_2$	$-O-CH(CH_3)_2$	0.024
25	$-CH_2CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	0.123
26	-O-*c*-C_6H_{13}	-O-*c*-C_6H_{13}	0.750

[a] Target compounds were purified by reverse-phase semi-prep HPLC and purities were judged by reverse-phase HPLC/MS with UV detection at 215 and 254 nm (YMC J'Sphere C-18 column, 0.4 × 5 cm; mobile phase: MeCN–H_2O (0.1% TFA). New compounds were characterized by ESI-MS and 400-MHz ^{1}H NMR. Compounds were isolated as racemic mixtures.

[b] Inhibition of canine TRPM8 is reported as mean IC_{50} values; NA, <20% inhibition at 5 μM. All analogs were inactive when counter-screened against the human TRPV1 cell line at 10 μM.

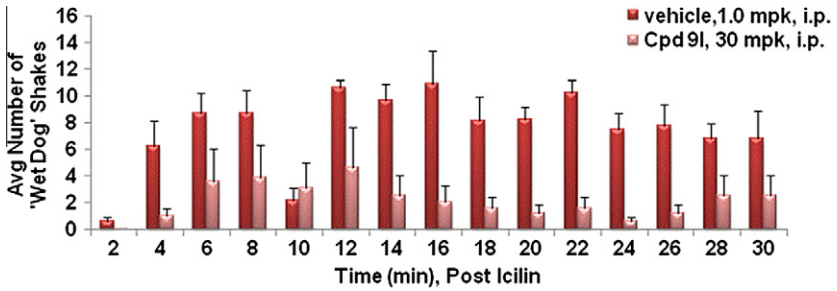


Figure 2. Time course of icilin-induced 'wet-dog' shakes in rats after ip administration of vehicle or Cpd **9l**.

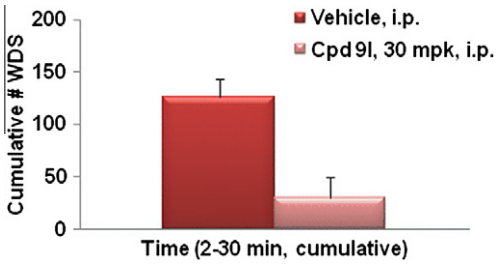


Figure 3. Cumulative reduction of subsequent icilin-induced 'wet-dog' shakes behavior in rats after ip administration of vehicle or Compd **9l**.

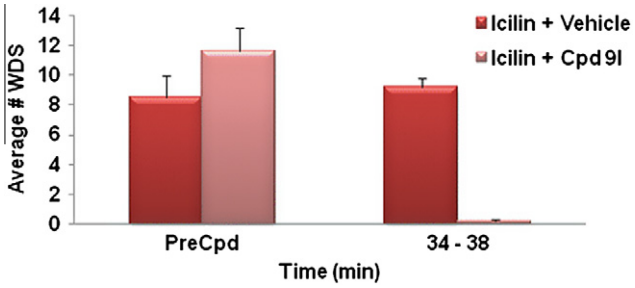
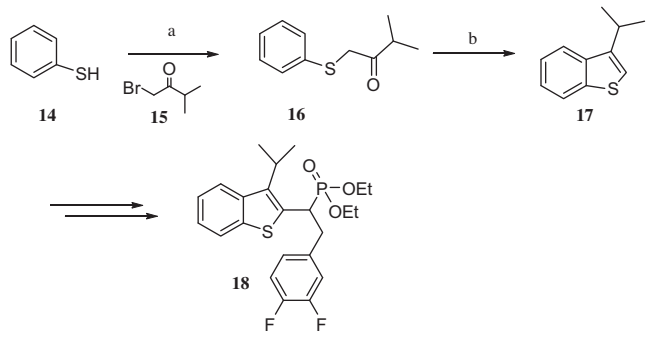
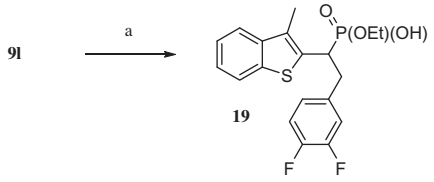


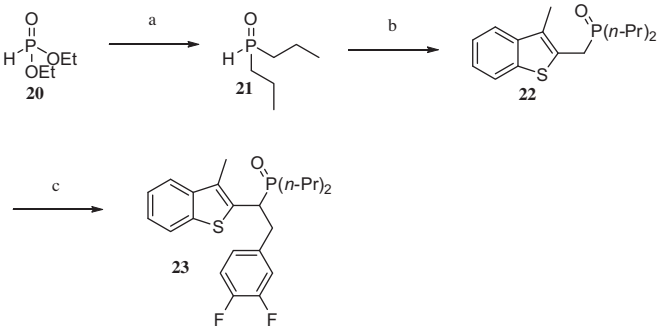
Figure 4. Reversal of icilin-induced 'wet-dog' shakes in rats after oral administration of vehicle or Compd **9l**.



Scheme 4. Reagents and conditions: (a) DMF, NaH, **15**,[21] 90 °C (69%); (b) PPA, 140 °C (82%).



Scheme 5. Reagents and conditions: (a) DMF, NaN_3 (82%).



Scheme 6. Reagents and conditions: (a) *n*-PrMgCl, H_2O, K_2CO_3,[22] (b) Toluene, NaH (60%), **7** (42%); (c) THF, *n*-BuLi, 3,4-difluorobenzyl bromide (73%).

metabolic stability in the rat and human liver microsomes, compound **9l** was chosen for further in vivo evaluation. To overcome high metabolism and consequent low exposure (C_{max} = 41 ng/mL) when administered orally,[27] **9l** was administered intraperitoneally at 30 mg/kg in a rat pharmacodynamic 'wet-dog' shaking (WDS) assay. Icilin (AG 3–5) is a super cooling agent that activates the TRPM8 channel, and causes WDS behavior in rats after intraperitoneal administration at 1 mg/kg.[1,28–30] A study in TPRM8 knockout mice further demonstrated that icilin-induced WDS behavior was mediated by TRPM8.[9] To verify that the phosphonate series was acting at the TRPM8 channel, compound **9l** was initially tested in a preventive mode.[31] Specifically, **9l** was administered to rats (30 mg/kg, ip) 15 min prior to treatment with icilin (1 mg/kg, ip), the number of WDS behaviors was measured for 30 min in two-min intervals, and the results were compared to vehicle pretreatment. As shown in Figure 2, **9l** antagonized the effects of icilin throughout the 30-min time course of the experiment, cumulatively reducing WDS behaviors by 80% (Fig. 3).

To further characterize **9l** in vivo, the antagonism of the TRPM8 channel was also measured in the reversal mode of the rat icilin-induced WDS model. In this mode, WDS behaviors were measured between 10 and 14 min after administration of icilin (3 mg/kg, po), which served as baseline. Compound **9l** (30 mg/kg, ip) was administered at 14 min, the number of shakes was counted in 4-min bins for an additional 24 min, and the results were compared to vehicle control (Fig. 4, depicts final 4 min 4 bin). Compound **9l** is effective in both preventing—as seen in the previous experiment—and reversing existing icilin-induced behaviors.

In conclusion, members of a novel series of benzothiophene derived phosphonate esters have been shown to be potent

antagonists of the TRPM8 channel. Compound **9l** was identified as a highly selective and robust TRPM8 antagonist in vitro that inhibited icilin-induced behaviors in a rat WDS model. On the basis of these findings, the synthesis and identification of potent, selective, and orally bioavailable benzothiophene-derived phosphonate esters are being pursued. Ultimately, this work aspires to generate a safe and effective TRPM8 antagonist therapeutic that may be used to treat pain and other disorders in which patients suffer from a pathophysiologic hypersensitivity to cold.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2012.02.060.

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18. The functional activity of the analogs was determined by measuring changes in intracellular calcium concentration using a Ca^{2+}-sensitive fluorescent dye and FLIPRTM technology. Increases in intracellular Ca^{2+} concentration were readily detected upon activation with the TRPM8 agonist icilin. HEK293 cells stably expressing canine TRPM8 were grown on poly-D-lysine coated 384-well black-walled plates and loaded with Calcium 3 Dye (Molecular Devices) for 35 min at 37 °C, under 5% CO_2 and then for 25 min at room temperature and atmosphere. Cells were challenged with test compounds (at varying concentrations) and intracellular Ca^{2+} was measured for 5 min prior to the addition of icilin to all wells to achieve a final concentration of 60 nM which produces approximately an 80% maximal response. The IC_{50} value for each analog was determined from eight-point dose–response studies. Curves were generated using the average of quadruplicate wells for each data point.
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26. Studies performed in BD Gentest liver microsomes.
27. Compound **9l** was administered in a rat cassette PK study containing 4 compounds, with each compound administered intravenously at 1 mg/kg and orally at 5 mg/kg in a 15% solutol/D5W formulation. IV data: $T_{1/2}$ (77 min), V_z (3491 mL/kg), Cl (31.2 mL/min/kg); PO data: $T_{1/2}$ (106 min), T_{max} (105 min), C_{max} (41 ng/mL), $\%F$ (6%).
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31. Icilin was administered to male Sprague Dawley rats (275–500 g, Charles River Labs, $n = 4$–6/treatment) in PEG-400 at a concentration 1.0 mg/kg, ip Spontaneous WDS behaviors were counted in 2 min bins over 30 min post-icilin. Compound **9l** or vehicle was administered ip 15 min before icilin to measure its ability to inhibit this spontaneous WDS behavior.