Title page

DSR-71167, a novel mineralocorticoid receptor antagonist with carbonic anhydrase inhibitory activity, separates urinary sodium excretion and serum potassium elevation in rats


Drug Research Division, Sumitomo Dainippon Pharma., Co., Ltd., Osaka, Japan
DSR-71167 is a novel mineralocorticoid receptor antagonist

Katsuya Fujita, PhD
Drug Research Division, Sumitomo Dainippon Pharma., Co., Ltd.,
33-94, Enoki-cho, Suita, Osaka, 564-0053, Japan
Phone: +81-6-6337-6201
Fax: +81-6-6337-0917
E-mail: katsuya-fujita@ds-pharma.co.jp

Text pages: 39 pages
Tables: 3
Figures: 6
References: 36
Words in the Abstract: 212 (<250)
Words in the Introduction: 443 (<750)
List of nonstandard abbreviations

ACZ: Acetazolamide
AR: Androgen receptor
CA: Carbonic anhydrase
C_{max}: Maximum concentration
DOCA: Deoxycorticosterone acetate
DSR-30192:
2-(hydroxymethyl)-N-(4-(methylsulfonyl)phenyl)-2′-(trifluoromethyl)biphenyl-4-carboxamide
DSR-71167:
2-[[2,2-difluoroethyl]amino]methyl]-2′-fluoro-N-(3-methoxy-4-sulfamoylphenyl)biphenyl-4-carboxamide hydrochloride
EPL: Eplerenone
GR: Glucocorticoid receptor
MR: Mineralocorticoid receptor
PR: Progesterone receptor
SBP: Systolic blood pressure
SM-368229:
N-4,4-dimethyl-2-thioxo-1,4-dihydro-2H-3,1-benzoxazin-6-yl-thiophene-2-sulfonamide
SPI: Spironolactone

**Recommended section**

Cardiovascular
Abstract

Mineralocorticoid receptor (MR) antagonists such as spironolactone (SPI) and eplerenone (EPL) are useful for treating hypertension and heart failure. However, these two agents have the serious side effect of hyperkalemia. We hypothesized that adding the ability to inhibit carbonic anhydrase (CA) would reduce the risk of hyperkalemia associated with MR antagonists. We investigated the profiles of DSR-71167 (MR antagonist with weak CA inhibitory activity) in regard to anti-mineralocorticoid actions by examining relationships between urinary excretion of sodium (index of anti-mineralocorticoid action) in deoxycorticosterone acetate (DOCA)-treated rats, and elevation of serum levels of potassium in potassium-loaded rats, compared with a DSR-71167 derivative without CA inhibition (DSR-30192), SPI and EPL. DSR-71167 dose-dependently increased urinary excretion of sodium in DOCA-treated rats without elevating serum levels of potassium in potassium-loaded rats. DSR-30192, SPI, and EPL elevated serum levels of potassium significantly in potassium-loaded rats at doses that increased MR inhibitory activity. We confirmed that DSR-71167 significantly increases urinary bicarbonate and decreases blood bicarbonate, as pharmacodynamic markers of CA inhibition, in intact rats. Chronic DSR-71167 administration showed anti-hypertensive effects in high salt-loaded Dahl hypertensive rats. These results demonstrate that DSR-71167 is a novel type of MR antagonist with CA inhibitory activity that is expected to become a safer MR antagonist with a low potential risk for hyperkalemia.
Introduction

Aldosterone contributes to hypertension and heart failure by promoting sodium retention through the mineralocorticoid receptor (MR) (Williams and Williams, 2003). However, experimental studies have shown that MR activation could also contribute to target-organ damage (Young and Funder, 1996; Rocha et al., 1998; Schiffrin, 2006; Nagase and Fujita, 2008; Nishiyama et al., 2009).

The role of aldosterone in the pathogenesis of cardiovascular diseases has been established by three main clinical trials: Randomized Aldactone Evaluation Study (RALES) (Pitt et al., 1999), Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) (Pitt et al., 2003), and Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure (EMPHASIS-HF) (Zannad et al., 2011). After publication of RALES, the prevalence of prescriptions for spironolactone (SPI) increased abruptly, and hyperkalemia-related morbidity and mortality also increased (Poggio et al., 2010).

Hyperkalemia is a mechanism-based and serious adverse effect of the use of MR antagonists (Chapagain and Ashman, 2012). Hyperkalemia increases the risk of death in hospitalized patients by approximately tenfold (Goyal et al., 2012). Risk of hyperkalemia after MR antagonist use is considered a major drawback for hypertension and heart failure therapy, and therapy using SPI and/or eplerenone (EPL) has been underused in the United States (Albert et al., 2009; Samuel and Delcayre, 2010).
Our research team has focused on a strategy for MR antagonists that carry a low risk of hyperkalemia. We reported that a MR antagonist with partial agonistic activity for MR showed reduced risk profiles for hyperkalemia in rats (Nariai et al., 2012b). In our search for another type of MR antagonist, we hypothesized that MR antagonists with diuretic targets could reduce the risk of hyperkalemia. Several types of diuretics, including carbonic anhydrase (CA) inhibitors, loop diuretics, and thiazide-type diuretics, increase urinary excretion of potassium (Brechue et al., 1990; Nagashima and Karasawa, 1996), which leads to hypokalemia. Moreover, CA and MR are localized to the kidney cytosolic fraction. Hence, a drug that has MR antagonism with CA inhibition can potentially modulate urinary excretion of potassium, and thereafter may reduce the elevation of potassium levels in serum caused by MR antagonists.

In an effort to discover novel MR antagonists with or without CA inhibition, we identified DSR-71167 (2-[[2,2-difluoroethyl]amino]methyl]-2’-fluoro-N-(3-methoxy-4-sulfamoylphenyl) biphenyl-4-carboxamide hydrochloride) and DSR-30192 (2-(hydroxymethyl)-N-(4-(methylsulfonyl)phenyl)-2’-(trifluoromethyl)biphenyl-4-carboxamide).

In the present study, we investigated the pharmacological profiles of DSR-71167 compared with those of DSR-30192, SPI, and EPL. First, we evaluated the balance of risk (elevation of potassium levels in serum) compared with the benefit (anti-mineralocorticoid action) of DSR-71167 and that of other reference compounds. Next, we investigated if DSR-71167 does indeed exert CA inhibitory activity \textit{in vivo}. Finally, we examined the anti-hypertensive effects of
chronic administration of DSR-71167 on hypertensive rats.
Materials and Methods

Test compounds

Chemical structures of the test compounds used in the present study are shown in Figure 1. DSR-71167 and DSR-30192 were synthesized in our Drug Research division (Osaka, Japan). SPI was purchased from Sigma-Aldrich (St. Louis, MO, USA), and EPL was obtained from Inspra tablets (Pfizer, New York, NY, USA) by pulverization, extraction, and chromatographic purification. Test compounds were dissolved in dimethyl sulfoxide for in vitro experiments and were suspended in 0.5% methyl cellulose for in vivo experiments.

Evaluation of the effects of DSR-71167 and DSR-30192 on the transcriptional activity of MR and other steroid receptors in COS-7 cells

Effects of test compounds on the transcriptional activity of MR and other steroid receptors were evaluated using the method reported by Nariai et al. (Nariai et al., 2011). COS-7 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium supplemented with Minimum Essential Medium with non-Essential Amino Acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% fetal bovine serum, and maintained at 37°C in a humidified atmosphere containing 5% CO₂. In the assay, COS-7 cells were transferred into 96-well plates at 7.0×10⁴ cells/mL in Dulbecco’s modified Eagle’s medium containing 5% charcoal/dextran-treated fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA, USA).
Cells were transiently transfected using TransIT-L1 (Mirus Bio LLC, Madison, WI, USA) with human MR, androgen receptor (AR), progesterone receptor (PR), or glucocorticoid receptor (GR) expression vectors, pGL3-MMTV, and phRL-TK (Promega, Madison, WI, USA). The phRL-TK plasmid was used as the internal control. After incubation for 6 h, test compounds with or without ligands were added, and the mixture was incubated for 18 h. The final ligand concentration for each steroid receptor was: MR, 1 nM aldosterone; AR, 0.1 nM methyltrienolone; PR, 1 nM progesterone; GR, 1 nM dexamethasone. The following day, cells were collected and lysed, and luciferase activity was measured using a luminometer (LB96V; Berthold Technologies, Bad Wildbad, Germany). Half-maximal inhibitory concentration (IC$_{50}$) values were calculated as the concentrations of test compounds that inhibited luciferase activity by 50% compared with ligand-treated cells.

**Evaluation of the effects of test compounds on CA inhibitory activity in vitro**

Effects of test compounds on CA activity were evaluated using the modified CO$_2$ hydration assay reported by Puscas et al. (Puscas et al., 1999). CAII is the major CA cytosolic isoform in the kidney (Swenson, 2014), so we used recombinant human CAII in this assay. Test compounds were pre-incubated with 0.15 μg/mL of human CA II (Sigma-Aldrich) in 20 mM Tris, pH8.0 and 0.005% phenol red solution for 30 min at room temperature. The reaction was initiated by adding carbonated water from bubbling CO$_2$ in distilled water. Absorbance was measured sequentially by
a spectrophotometer at 555 nm (U-3310; Hitachi High-Tech Fielding Corp., Tokyo, Japan). IC₅₀ values were calculated as the concentrations of test compounds that inhibited enzyme activity by 50% compared with solvent-treated samples.

**Evaluation of the binding affinity of DSR-71167 for various receptors, channels, and transporters and on enzyme activity for various target molecules**

Selectivity of DSR-71167 for various molecular targets was examined by Ricerca Biosciences LLC (Painesville, OH, USA) using standard *in vitro* radioligand binding assays (34 targets) and enzyme assays (26 targets). DSR-71167 was tested at 10 μM (n = 2) (Supplemental Tables 1 and 2).

**Animals**

Male Sprague–Dawley (SD) rats were obtained from Japan SLC Co., Ltd. (Shizuoka, Japan) or Charles River Laboratories International, Inc. (Yokohama, Japan). Male Dahl salt-sensitive rats were obtained from Japan SLC Co., Ltd. Rats were quarantined and acclimatized for 1 week before use. Rats were housed in groups of 3–5 per cage in a controlled environment (23 ± 2°C, 55 ± 10% humidity) with a 12-h light–dark cycle (light on at 8:00 AM), and allowed free access to food (CE-2, Clea Japan, Inc., Tokyo, Japan) and filtered water. The study protocol was approved by the Animal Care and Use Committee of the Drug Research Division of Sumitomo Dainippon
Pharma Co., Ltd. (Osaka, Japan) and undertaken in accordance with the regulations for animal experiments in that division.

Evaluation of DSR-71167 and DSR-30192 on pharmacokinetic parameters

Pharmacokinetic parameters of test compounds were evaluated by modifying the method of Nariai et al. (Nariai et al., 2011). In brief, non-fasted SD rats aged 6–8 weeks were administered DSR-71167 (10 mg/kg, p.o.) or DSR-30192 (10 mg/kg, p.o.). Blood samples were collected from the jugular vein at 0.25, 0.5, 1, 2, 4, 6, and 24 h after drug administration. At the high dose of DSR-71167 (100 mg/kg), blood samples were collected at 1, 3, 6, and 24 h after drug administration. Drug concentration in each plasma sample was measured using a liquid chromatography/tandem mass spectrometry system (LC/MS/MS) (Alliance 2690, Waters Corp., Milford, MA, USA; and API3000, Applied Biosystems Inc., Foster City, CA, USA). Data are the mean ± S.D. (n = 3).

Evaluation of the effects of test compounds on urinary excretion of sodium in DOCA-treated rats

The following assay method was used by modifying the methods of Nariai et al. and Brandish et al. (Brandish et al., 2008; Nariai et al., 2012b). Male SD rats aged 6–8 weeks were used for experiments. Animals were divided into five groups (n = 5). On the day of the experiment, rats were administered test compounds (5 mL/kg, p.o.). Thirty minutes later, they were given 0.9%
NaCl (20 mL/kg, p.o.) and 0.3 mg/mL DOCA (1 mL/kg, s.c.). After drug administration, they were maintained in metabolic cages (Natsume Seisakusho Co., Ltd., Tokyo, Japan) for 8 h without food or water. Urinary sodium concentration was measured by a flame spectrophotometry system (JCA-BM 1650, Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan). Median effective dose (ED$_{50}$) values were calculated as the doses of test compounds that reversed urinary sodium excretion by 50% compared with DOCA-treated rats.

**Evaluation of the effects of test compounds on serum levels of potassium-loaded rats**

The potassium loading test was modified from the method reported by Nariai et al. (Nariai et al., 2012b). In brief, male SD rats (n = 4–5) were administered test compounds (5 mL/kg, p.o.). Sixty minutes later, 15% KCl solution (10 mL/kg, p.o.) was administered. Serum levels of potassium were measured 8 h after administration. ED$_{0.5}$ values were calculated as the doses of test compounds that increased serum levels of potassium by 0.5 mM compared with the vehicle group.

**Evaluation of the effects of DSR-71167 on the pharmacodynamic markers for CA in normal rats**

Non-fasted male SD rats (n = 4–5) were used for experiments. Animals were administered test compounds (5 mL/kg, p.o.). Sixty minutes later, they were given distilled water (10 mL/kg, p.o.) and maintained in metabolic cages for 3 h without food or water. Whole-blood and urinary
samples were collected 4 h after drug administration. Blood/urinary bicarbonate concentration and pH were measured using a portable blood gas analyzer (i-STAT; Fuso Pharmaceutical Industries, Ltd., Osaka, Japan). Blood/urinary bicarbonate concentration was calculated using the following formula:

$$\log (\text{HCO}_3^-) = \text{pH} + \log (\text{pCO}_2) - 7.608$$.

**Evaluation of the effects of DSR-71167 on systolic blood pressure (SBP) in high salt (HS)-treated Dahl hypertensive rats**

A HS-treated Dahl hypertensive rat model was created, as described previously (Zhou et al., 2011). Briefly, rats aged 6 weeks were divided randomly into five groups (n = 5–8) and given a HS diet containing 4% NaCl and administered DSR-71167 (10, 30, 100 mg/kg, p.o.) or EPL (100 mg/kg, p.o.) for 3 weeks. Control rats were given a normal-salt diet containing 0.3% NaCl. Trough SBP was measured in conscious rats in the morning by the tail-cuff method (BP-98A, Softron Co., Ltd., Tokyo, Japan).

**Statistical analyses**

Data are the mean ± S.D. Significance was determined using a one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test or Student’s t-test. P<0.05 was considered significant. Statistical calculations were done using Stat Preclinica (Takumi...
Information Technology, Inc., Tokyo, Japan).
Results

Effects of DSR-71167 and DSR-30192 on the transcriptional activity of MR and other steroid receptors in COS-7 cells

Maintaining selectivity for MR compared with other hormone receptors in the drug development of MR antagonists is important because SPI therapy has been limited by the disturbances it causes to the endocrine system (mainly gynecomastia) (Jeunemaitre et al., 1987). DSR-71167 inhibited MR-initiated transcription in the reporter-gene assay with selectivity for the MR that was 42-fold higher than for the other steroid receptors tested (Table 1). DSR-30192 also inhibited the MR with selectivity for the MR that was 19-fold higher than that for the other steroid receptors tested.

Effects of test compounds on CA inhibitory activity in vitro

ACZ, the most popular and classical CA inhibitor, was developed 60 years ago. We used ACZ as a positive control for a CA inhibitor. The IC\textsubscript{50} value of ACZ was 4.8 ± 2.4 (nM) (data not shown). DSR-71167 showed weak CA inhibitory activity from 3 μM. The IC\textsubscript{50} value of DSR-71167 was 19 μM. DSR-30192 (100 μM), SPI (500 μM), and EPL (500 μM) did not inhibit CA activity at all (Table 1).

Pharmacokinetic parameters of DSR-71167 and DSR-30192
Before the *in vivo* experiment, we measured drug concentrations of DSR-71167 and DSR-30192 in rats. DSR-71167 (10, 100 mg/kg, p.o.) and DSR-30192 (10 mg/kg, p.o.) showed sustained systemic exposure in rats. Moreover, the maximum concentration ($C_{\text{max}}$) and the area under the curve for DSR-71167 (10 mg/kg) were approximately tenfold higher than those for DSR-71167 (10 mg/kg, p.o.) (Table 2).

**Effects of test compounds on urinary excretion of sodium in DOCA-treated rats**

We used DOCA-treated rats to evaluate anti-mineralocorticoid action *in vivo* (Table 3). Compared with the vehicle group, urinary excretion of sodium in DOCA-treated rats decreased significantly over the 8-h experimental period. DSR-71167 significantly increased DOCA-suppressed urinary excretion of sodium at 30 and 100 mg/kg (Figure 2A). DSR-30192 showed mild natriuresis, but could not completely prevent the effects of DOCA even at the highest dose (300 mg/kg) (Figure 2B). SPI and EPL dose-dependently increased DOCA-suppressed urinary excretion of sodium (Figure 2C and D). ED$_{50}$ values for each compound are presented in Table 3.

**Effects of test compounds on serum levels of potassium in potassium-loaded rats**

To evaluate the risk of hyperkalemia in rats, we used a potassium-loaded rat model (Table 3). DSR-71167 showed only slight effects on serum levels of potassium (Figure 3A). DSR-30192 (100, 300 mg/kg), SPI (100 mg/kg), and EPL (30, 100 mg/kg) significantly increased serum levels.
Effects of DSR-71167 on pharmacodynamic markers for CA in rats

We measured urinary/blood bicarbonate and pH as pharmacodynamic markers of CA inhibition (Figure 4). The CA inhibitor ACZ (1 mg/kg) significantly changed urinary bicarbonate, urinary pH, and blood bicarbonate, but not blood pH. DSR-71167 showed dose-dependent increases in urinary bicarbonate and urinary pH, but only urinary bicarbonate was increased significantly. Moreover, DSR-71167 (30, 100 mg/kg) decreased blood bicarbonate significantly. However, EPL (100 mg/kg) did not change any pharmacodynamic markers for CA inhibition.

Effects of DSR-71167 on SBP in HS-treated Dahl hypertensive rats

Compared with normal salt-treated rats, SBP in HS-treated Dahl rats increased (128.4 vs. 157.1 mmHg, respectively). DSR-71167 (10, 30, 100 mg/kg) dose-dependently and significantly suppressed the increase in SBP (138.5, 133.0, and 127.6 mmHg, respectively). EPL (100 mg/kg) also significantly suppressed the increase in SBP (129.6 mmHg) (Figure 5). Compared with the HS-treated group, serum levels of potassium were not changed significantly by administration of DSR-71167 or EPL (data not shown).
Discussion

The risk of hyperkalemia has been considered to be the main drawback of therapy with MR antagonists, and use of SPI and EPL has been limited (Albert et al., 2009; Samuel and Delcayre, 2010). To test our hypothesis that a MR antagonist with CA inhibition can reduce the risk of hyperkalemia, we evaluated the pharmacological profiles of a novel MR antagonist, DSR-71167. DSR-71167 showed potent MR and weak CA inhibitory activity with IC50 values of 0.26 and 19 μM, respectively. Moreover, DSR-71167 strongly increased DOCA-suppressed natriuresis and exerted anti-hypertensive effects in HS-treated Dahl hypertensive rats. However, DSR-71167, in contrast to SPI and EPL, did not cause elevation of serum levels of potassium in potassium-loaded rats.

The relationship between anti-mineralocorticoid action (urinary excretion of sodium in DOCA-treated rats) and risk of hyperkalemia (elevation of serum levels of potassium in potassium-loaded rats) is shown in Figure 6. DSR-71167 has wide separation between anti-mineralocorticoid actions and elevation of serum levels of potassium. DSR-30192, SPI, and EPL increase urinary excretion of sodium in parallel with elevation of serum levels of potassium. We calculated the safety margin for hyperkalemia risk as the ratio between ED0.5 and ED50 (Table 3). DSR-71167 showed a wide margin (>18) as compared with SPI, EPL, and DSR-30192 (0.62, 1.3, and 0.27, respectively). Taken together, it can be assumed that a MR antagonist with CA inhibition shows minimal effects on elevation of serum levels of potassium while maintaining its anti-mineralocorticoid action.
In the present study, we used urinary excretion of sodium as an index of anti-mineralocorticoid action even though we used sodium balance as an index of anti-mineralocorticoid action in a previous study (Nariai et al., 2012b). Pharmacokinetic profiles of DSR-71167 are different from those of SM-368229. DSR-71167 showed a shorter time for the maximum effect compared with that of SM-368229. Hence, we changed the index of the anti-mineralocorticoid action from sodium balance to sodium excretion, and the evaluation time from 23 h to 8 h. Nonetheless, DSR-71167 showed significant anti-hypertensive effects in HS-treated Dahl hypertensive rats at a dose close to the ED$_{50}$ value in the DOCA administration test.

The limitation of this study include that we did not evaluate the anti-hypertensive effects of DSR-71167 in animal models with fully developed hypertension. It will be necessary to evaluate the effects of DSR-71167 on blood pressure in hypertensive animals to capture the “true” therapeutic potential of DSR-71167.

We focused on adding CA inhibitory activity to MR antagonists because many CA inhibitors have been reported, and it is known that unsubstituted sulfonamide moieties are important for inhibiting of CA activity (Supuran, 2008; Swenson, 2014). We then added the sulfonamide structure to scaffolds of MR antagonists and, as a result, obtained DSR-71167. In contrast, DSR-30192 was synthesized without a sulfonamide moiety and, as we expected, did not show any CA inhibitory activity. Determining the balance between CA inhibition and MR antagonism is important, but could not be investigated because we could not obtain various MR antagonists with
CA inhibition for in vivo experiments. However, we conclude that the weak CA inhibitory activity of DSR-71167 is appropriate (though not optimal). Excess CA inhibition can cause mechanism-based serious side effects such as metabolic acidosis and urolithiasis (Kass et al., 1981; Heller et al., 1985).

CA inhibition of DSR-71167 in vitro was relatively weak, so we wondered if CA inhibition actually contributes to the low risk profiles of hyperkalemia of DSR-71167. We approached this question in three ways. First, as shown by pharmacokinetic analyses, the $C_{\text{max}}$ of DSR-71167 at 100 mg/kg (66 μM) exceeded the IC50 value for CA inhibition (19 μM). Second, DSR-71167 dose-dependently increased urinary bicarbonate (pharmacodynamic marker for CA inhibition) in vivo. Third, MR antagonists without CA inhibition (SPI, EPL, DSR-30192) elevated serum levels of potassium in potassium-loaded rats. Taken together, these data suggest that the CA inhibitory activity of DSR-71167 has an important role in minimizing elevation of serum levels of potassium.

CA inhibitors are used to cause maximal dilation in the cerebral vasculature (Grossmann and Koeberle, 2000; Swenson, 2014). There may be long-term positive or negative effects of increased cerebral blood flow in hypertensive patients. Hence, DSR-71167 also may have other potential effects, including cerebral vasodilation. We did not examine the other potential effects of DSR-71167 because the CA inhibitory activity of DSR-71167 was very weak compared with that of the major CA inhibitor ACZ.

As mentioned above, measuring CA inhibitory activity in vivo is important. We have explored
the highly sensitive pharmacodynamic markers of CA using urine, blood, and renal tissue in intact rats treated with ACZ. In our preliminary experiments, ACZ (0.1 mg/kg) increased urinary bicarbonate excretion and urinary pH in intact rats (data not shown). Other markers of CA inhibition were changed by administering ACZ at 1 or 10 mg/kg (data not shown). We suggest that urinary bicarbonate could be one of the most sensitive pharmacodynamic markers of CA inhibition. Urinary bicarbonate can be measured in humans, so confirming that DSR-71167 increases urinary bicarbonate excretion in clinical trials is important.

EPL is contraindicated in patients with severe renal impairment, but the renoprotective effects of MR antagonists in experimental research (Shibata et al., 2007; Nagase and Fujita, 2008; Nariai et al., 2012a) and clinical research (Mavrakanas et al., 2014) are well-known. However, patients with impaired renal function are at a high risk of hyperkalemia (Einhorn et al., 2009; Roscioni et al., 2012). Khosla et al. reported that patients with an estimated baseline glomerular filtration rate of ≤45 mL/min/1.73 m² in whom the serum level of potassium is >4.5 mM have a nine-fold higher odds ratio of hyperkalemia when being treated with MR antagonists (Khosla et al., 2009). MR antagonists that can be used readily in patients with kidney disease are desired. Hence, it will be necessary to evaluate the effects of DSR-71167 on urinary albumin excretion and serum levels of potassium in a model of impaired renal function, such as 5/6 nephrectomized rats.

Having low-risk profiles for hyperkalemia is important for the discovery of third- or fourth-generation MR antagonists. From this viewpoint, the novel mechanism of action of
DSR-71167 is promising. Three other MR antagonists have been reported to have low risk profiles for hyperkalemia in rats. First, Pfizer has discovered PF-3882845 (potent and selective MR antagonist with a pyrazoline scaffold). PF-3882845 shows attenuation in blood pressure and reduction in urinary albumin that is significantly greater than those with EPL in HS-treated Dahl hypertensive rats, with potentially fewer effects on elevation of serum levels of potassium. (Meyers et al., 2010). However, the mechanisms underlying these findings have yet to be determined. Further information regarding hyperkalemia risk is expected from clinical trials (Clinical Trials.gov, NCT01488877). Second, we have discovered SM-368229 (potent and selective MR antagonist with a benzoxazin scaffold). SM-368229 prevents increases in SBP without elevation of serum levels of potassium in spontaneously hypertensive rats, but treatment with SPI prevents SBP increase with elevation of serum levels of potassium (Nariai et al., 2012b). The mechanism for this phenomenon is that the partial agonistic activity of SM-368229 for the MR promotes a neutral effect on serum levels of potassium. Further research is needed to characterize the effects of SM-368299 in humans. Third, Bayer has discovered finerenone (BAY 94-8862; potent and selective MR antagonist with a dihydronaphthyridine scaffold). Finerenone shows natriuretic effects greater than those of EPL in conscious rats, with less effect on kaliuresis. Finerenone possesses tissue selectivity towards more pronounced cardiac and/or vascular activity than renal activity, which can offer end-organ protection with a reduced risk of electrolyte disturbances (Kolkhof et al., 2014). Finerenone has been tested in a Phase II trial in patients with
heart failure in which it reduced the left ventricular ejection fraction associated with mild or moderate chronic kidney disease (Pitt et al., 2013). Low doses of finerenone tend to reduce levels of brain natriuretic peptide and amino-terminal pro brain natriuretic peptide similar to that seen with SPI, though finerenone increases serum levels of potassium less than that observed with SPI. Further large scale clinical evaluations are underway to demonstrate the clinical efficacy of finerenone.

Our data showed a low hyperkalemia risk profile for DSR-71167 in potassium-loaded rats. However, we have confirmed that chronic DSR-85955 administration reduces SBP in spontaneous hypertensive rats without elevating serum levels of potassium (Supplemental Figure 1). The free-base form of DSR-85955 is the same as that of DSR-71167. Hence, DSR-71167 is expected to mitigate elevation in serum levels of potassium in models other than the potassium-loaded rat.

This study revealed that the effect of DSR-71167 on serum levels of potassium is greatly different than that of SPI or EPL. The weak CA inhibitory activity of DSR-71167 has a crucial role in suppressing elevation of serum levels of potassium in rats. DSR-71167 can be a safer MR antagonist with a low risk of hyperkalemia, and so could aid treatment of hypertension and heart failure.
Acknowledgments

We thank Dr. Kazuto Yamada and Setsuko Yamamoto for their valuable comments, support, and encouragement. We also thank Shoji Ogawa, Yuka Yamane, Kayo Watanabe, Masakazu Shintome, Satoshi Suetsugu, Toshihide Takagi, and Jun Tadano for their excellent technical assistance.
Authorship Contributions

Participated in research design: Nariai, Fujita, Kawane, and Kato

Conducted experiments: Nariai, Fujita, Kawane, Nakayama, and Matsuda

Contributed new reagents or analytical tool: Katayama, Fukuda, Hori, Hasegawa, Iwata, and Suzuki

Undertook data analyses: Nariai, Fujita, and Mori

Wrote or contributed to writing of the manuscript: Nariai, Fujita, Mori, and Kato
References


(3S,3aR)-2-(3-chloro-4-cyanophenyl)-3-cyclopentyl-3,3a,4,5-tetrahydro-2H-benzo[g]indazole-7-carboxylic acid (PF-3882845), an orally efficacious mineralocorticoid receptor (MR) antagonist for hypertension and nephropathy. *J Med Chem* **53**:5979-6002.


Footnotes

(a) None

(b) None

(c) Tetsuro Nariai, PhD

Drug Research Division, Sumitomo Dainippon Pharma., Co., Ltd., Osaka, Japan

3-1-98, Kasugade-naka, Konohana-ku, Osaka, 554-0022, Japan

E-mail: tetsuro-nariai@ds-pharma.co.jp

(d) None
Figure Legends

Figure 1

Chemical structures of DSR-71167, DSR-30192, spironolactone, and eplerenone

Figure 2

Effects of test compounds on urinary excretion of sodium in deoxycorticosterone acetate (DOCA)-treated rats

Sprague–Dawley rats were orally administered (A) DSR-71167 (10, 30, 100 mg/kg), (B) DSR-30192 (30, 100, 300 mg/kg), (C) spironolactone (SPI) (3, 10, 30 mg/kg), or (D) eplerenone (EPL) (3, 10, 30 mg/kg). Thirty minutes later, they were given 0.9% NaCl solution (20 mL/kg, p.o.) and DOCA (0.3 mg/kg, s.c.). After drug administration, they were maintained in metabolic cages for 8 h and urine samples were collected. Data are the mean ± S.D. (n = 4–5). ## P < 0.01 vs. vehicle by Student’s t-test, *P < 0.05, **P < 0.01 vs. DOCA by ANOVA followed by Dunnett’s multiple comparison test.

Figure 3

Effects of test compounds on serum levels of potassium in potassium-loaded rats

Sprague–Dawley rats were orally administered (A) DSR-71167 (30, 100, 300 mg/kg), (B) DSR-30192 (100, 300 mg/kg), (C) spironolactone (SPI) (10, 30, 100 mg/kg), or (D) eplerenone
(EPL) (10, 30, 100 mg/kg). Serum levels of potassium were measured 8 h after KCl (1.5 g/kg, p.o.) administration. Data are the mean ± S.D. (n = 4–5). *P < 0.05, **P < 0.01 vs. vehicle by ANOVA followed by Dunnett's multiple comparison test.

Figure 4

Effects of DSR-71167 on pharmacodynamic markers for carbonic anhydrase in rats

Sprague–Dawley rats were orally administered acetazolamide (ACZ) (1 mg/kg), DSR-71167 (10, 30, 100 mg/kg), or eplerenone (EPL) (100 mg/kg). Sixty minutes later, they were given distilled water (10 mL/kg, p.o.) and maintained in metabolic cages for 3 h. Urine and blood samples for urinary/blood bicarbonate and pH were collected after 4 h of drug administration. Data are the mean ± S.D. (n = 4–5). #P < 0.05, ##P < 0.01 vs. vehicle by Student's t-test, *P < 0.05, **P < 0.01 vs. vehicle by ANOVA followed by Dunnett's multiple comparison test.

Figure 5

Effects of DSR-71167 on systolic blood pressure (SBP) in high salt (HS)-treated Dahl hypertensive rats

Dahl salt-sensitive rats were given a HS diet containing 4% NaCl. DSR-71167 (10, 30, 100 mg/kg) or eplerenone (EPL) (100 mg/kg) was administered for 3 weeks at the same time as high salt loading. Control rats were given a normal-salt (NS) diet containing 0.3% NaCl. Data are the
mean ± S.D. (n = 5–8). ## P < 0.01 vs. NS by Student’s t-test, **P < 0.01 vs. HS by Dunnett’s multiple comparison test.

Figure 6

Anti-mineralocorticoid action and elevation of serum levels of potassium in response to DSR-71167 and reference compounds

Urinary excretion of sodium (%) was calculated as the efficacy of test compounds that reversed urinary excretion of sodium compared with deoxycorticosterone acetate-treated rats. Changes in serum levels of potassium were calculated as compared with the vehicle group in potassium-loaded rats.
**Tables**

Table 1

*In vitro* pharmacological profiles of DSR-71167 and reference compounds in steroid receptor reporter gene assays and in carbonic anhydrase inhibitory activity

<table>
<thead>
<tr>
<th></th>
<th>MR</th>
<th>AR</th>
<th>PR</th>
<th>GR</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSR-71167</td>
<td>0.26 ± 0.11</td>
<td>11 ± 6.9</td>
<td>11 ± 2.2</td>
<td>30 ± 8.7</td>
<td>19 ± 7.7</td>
</tr>
<tr>
<td>DSR-30192</td>
<td>0.39 ± 0.12</td>
<td>&gt;10</td>
<td>7.4 ± 0.3</td>
<td>&gt;10</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>0.012 ± 0.004</td>
<td>0.011 ± 0.008</td>
<td>0.35 ± 0.4</td>
<td>2.2 ± 0.6</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Eplerenone</td>
<td>0.42 ± 0.07</td>
<td>4.5 ± 1.5</td>
<td>24 ± 6.5</td>
<td>&gt;100</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

Data are the mean ± S.D. (n = 3–5). IC$_{50}$ values of spironolactone and eplerenone for nuclear steroid receptors are cited from Nariai et al., 2011.

MR: mineralocorticoid receptor, AR: androgen receptor, PR: progesterone receptor, GR: glucocorticoid receptor, CA: carbonic anhydrase
Table 2

Pharmacokinetic parameters of DSR-71167 and DSR-30192

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>$C_{\text{max}}$ (μg/mL)</th>
<th>$C_{\text{max}}$ (μM)</th>
<th>$T_{\text{max}}$ (hours)</th>
<th>$T_{1/2}$ (hours)</th>
<th>AUC (μg•hour/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSR-71167</td>
<td>3.0 ± 1.5</td>
<td>5.6 ± 2.8</td>
<td>2.0 ± 1.7</td>
<td>3.1 ± 0.4</td>
<td>21 ± 8.6</td>
</tr>
<tr>
<td>DSR-71167</td>
<td>35 ± 10</td>
<td>66 ± 19</td>
<td>1.7 ± 1.2</td>
<td>3.0 ± 0.3</td>
<td>247 ± 66</td>
</tr>
<tr>
<td>DSR-30192</td>
<td>7.4 ± 3.8</td>
<td>16 ± 8.5</td>
<td>4.0 ± 2.0</td>
<td>4.4 ± 0.5</td>
<td>77 ± 41</td>
</tr>
</tbody>
</table>

Data are the mean ± S.D. (n = 3). Rats were orally administered DSR-71167 (10, 100 mg/kg) or DSR-30192 (10 mg/kg) and drug concentration in plasma was measured.

$C_{\text{max}}$: maximum concentration; $T_{\text{max}}$: time for the maximum effect; AUC: area under the curve.
Table 3

Safety margin for hyperkalemia risk for DSR-71167 and reference compounds

| Sodium excretion | Serum potassium | Ratio  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ED&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</td>
<td>ED&lt;sub&gt;0.5&lt;/sub&gt; (mg/kg)</td>
<td>ED&lt;sub&gt;0.5/ED&lt;sub&gt;50&lt;/sub&gt;&lt;/sub&gt;</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------</td>
<td>--------</td>
</tr>
<tr>
<td>DSR-71167</td>
<td>17</td>
<td>&gt;300</td>
</tr>
<tr>
<td>DSR-30192</td>
<td>131</td>
<td>36</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>10</td>
<td>6.0</td>
</tr>
<tr>
<td>Eplerenone</td>
<td>8.4</td>
<td>11</td>
</tr>
</tbody>
</table>

ED<sub>50</sub> values were calculated as doses of test compounds that reversed urinary excretion of sodium by 50% compared with deoxycorticosterone acetate-treated rats. ED<sub>0.5</sub> values were calculated as the doses of test compounds that increased serum levels of potassium by 0.5 mM compared with the vehicle group in potassium-loaded rats.
Figure 1

DSR-71167

DSR-30192

Spironolactone

Eplerenone
Figure 2

(A) Urinary sodium excretion (mmol) for Vehicle DOCA and DOCA+DSR-71167.

(B) Urinary sodium excretion (mmol) for Vehicle DOCA and DOCA+DSR-30192.

(C) Urinary sodium excretion (mmol) for Vehicle DOCA and DOCA+SPI.

(D) Urinary sodium excretion (mmol) for Vehicle DOCA and DOCA+EPL.
Figure 3

(A) Serum potassium (mmol/L) for Vehicle, DSR-71167 (30, 100, 300 mg/kg).

(B) Serum potassium (mmol/L) for Vehicle, DSR-30192 (100, 300 mg/kg).

(C) Serum potassium (mmol/L) for Vehicle, SPI (10, 30, 100 mg/kg).

(D) Serum potassium (mmol/L) for Vehicle, EPL (10, 30, 100 mg/kg).

This article has not been copyedited and formatted. The final version may differ from this version.

JPET Fast Forward. Published on April 28, 2015 as DOI: 10.1124/jpet.114.221341

at ASPET Journals on July 8, 2017 jpet.aspetjournals.org Downloaded from
Figure 4

(A) Urinary bicarbonate (mmol) vs. vehicle treatment and dose (mg/kg).
(B) Urinary pH vs. vehicle treatment and dose (mg/kg).
(C) Urinary bicarbonate (mmol) vs. vehicle treatment and dose (mg/kg).
(D) Urinary pH vs. vehicle treatment and dose (mg/kg).
(E) Blood bicarbonate (mmol/L) vs. vehicle treatment and dose (mg/kg).
(F) Blood pH vs. vehicle treatment and dose (mg/kg).

This article has not been copyedited and formatted. The final version may differ from this version.

JPET Fast Forward. Published on April 28, 2015 as DOI: 10.1124/jpet.114.221341
at ASPET Journals on July 8, 2017 jpet.aspetjournals.org Downloaded from
Figure 5

This article has not been copyedited and formatted. The final version may differ from this version.

JPET Fast Forward. Published on April 28, 2015 as DOI: 10.1124/jpet.114.221341

at ASPET Journals on July 8, 2017 jpet.aspetjournals.org Downloaded from
Figure 6

(A) Urinary sodium excretion (%) and Serum potassium (mmol/L) for DSR-71167 (mg/kg)

(B) Urinary sodium excretion (%) and Serum potassium (mmol/L) for DSR-30192 (mg/kg)

(C) Urinary sodium excretion (%) and Serum potassium (mmol/L) for SPI (mg/kg)

(D) Urinary sodium excretion (%) and Serum potassium (mmol/L) for EPL (mg/kg)
Supplemental data

DSR-71167, a novel mineralocorticoid receptor antagonist with carbonic anhydrase inhibitory activity, separates urinary sodium excretion and serum potassium elevation in rats


Drug Research Division, Sumitomo Dainippon Pharma., Co., Ltd., Osaka, Japan

Corresponding author

Katsuya Fujita, PhD

Drug Research Division, Sumitomo Dainippon Pharma., Co., Ltd., Osaka, Japan

33-94, Enoki-cho, Suita, Osaka, 564-0053, Japan

Phone: +81- 6-6337-6201

Fax: +81-6-6337-0917

E-mail: katsuya-fujita@ds-pharma.co.jp
Supplemental Table 1

**Radioligand binding assays**

<table>
<thead>
<tr>
<th>Target molecules</th>
<th>Species</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine A\textsubscript{1}</td>
<td>Human</td>
<td>42</td>
</tr>
<tr>
<td>Adenosine A\textsubscript{2A}</td>
<td>Human</td>
<td>21</td>
</tr>
<tr>
<td>Adenosine A\textsubscript{3}</td>
<td>Human</td>
<td>-1</td>
</tr>
<tr>
<td>Adrenergic α\textsubscript{1B}</td>
<td>Rat</td>
<td>13</td>
</tr>
<tr>
<td>Adrenergic α\textsubscript{1D}</td>
<td>Human</td>
<td>20</td>
</tr>
<tr>
<td>Adrenergic α\textsubscript{2A}</td>
<td>Human</td>
<td>22</td>
</tr>
<tr>
<td>Adrenergic β\textsubscript{1}</td>
<td>Human</td>
<td>9</td>
</tr>
<tr>
<td>Adrenergic β\textsubscript{2}</td>
<td>Human</td>
<td>3</td>
</tr>
<tr>
<td>Angiotensin AT\textsubscript{1}</td>
<td>Human</td>
<td>-1</td>
</tr>
<tr>
<td>Angiotensin AT\textsubscript{2}</td>
<td>Human</td>
<td>1</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>Rat</td>
<td>77</td>
</tr>
<tr>
<td>Androgen</td>
<td>Rat</td>
<td>9</td>
</tr>
<tr>
<td>Bradykinin B\textsubscript{1}</td>
<td>Human</td>
<td>11</td>
</tr>
<tr>
<td>Bradykinin B\textsubscript{2}</td>
<td>Human</td>
<td>2</td>
</tr>
<tr>
<td>Calcium Channel L-Type, Benzothiazepine</td>
<td>Rat</td>
<td>-9</td>
</tr>
<tr>
<td>Calcium Channel L-Type, Dihydropyridine</td>
<td>Rat</td>
<td>6</td>
</tr>
<tr>
<td>Calcium Channel N-Type</td>
<td>Rat</td>
<td>9</td>
</tr>
<tr>
<td>Endothelin ET\textsubscript{A}</td>
<td>Human</td>
<td>6</td>
</tr>
<tr>
<td>Receptor/Channel/Transporter</td>
<td>Species</td>
<td>% Inhibition</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td>---------</td>
<td>--------------</td>
</tr>
<tr>
<td>Endothelin ET&lt;sub&gt;B&lt;/sub&gt;</td>
<td>Human</td>
<td>0</td>
</tr>
<tr>
<td>Epidermal Growth Factor</td>
<td>Human</td>
<td>15</td>
</tr>
<tr>
<td>Estrogen Er&lt;sub&gt;α&lt;/sub&gt;</td>
<td>Human</td>
<td>3</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>Human</td>
<td>2</td>
</tr>
<tr>
<td>Histamine H&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Human</td>
<td>22</td>
</tr>
<tr>
<td>Histamine H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Human</td>
<td>14</td>
</tr>
<tr>
<td>Histamine H&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Human</td>
<td>-3</td>
</tr>
<tr>
<td>Muscarinic M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Human</td>
<td>9</td>
</tr>
<tr>
<td>Muscarinic M&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Human</td>
<td>10</td>
</tr>
<tr>
<td>Muscarinic M&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Human</td>
<td>-2</td>
</tr>
<tr>
<td>Potassium Channel K&lt;sub&gt;ATP&lt;/sub&gt;</td>
<td>Human</td>
<td>15</td>
</tr>
<tr>
<td>Potassium Channel HERG</td>
<td>Human</td>
<td>-12</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Human</td>
<td>22</td>
</tr>
<tr>
<td>Retinoid X Receptor RXRα</td>
<td>Human</td>
<td>20</td>
</tr>
<tr>
<td>Sodium Channel, Site 2</td>
<td>Human</td>
<td>22</td>
</tr>
<tr>
<td>Thyroid Hormone</td>
<td>Rat</td>
<td>1</td>
</tr>
</tbody>
</table>

Binding affinity of DSR-71167 for various receptors, channels, and transporters are shown by % inhibition as compared to DMSO-treated samples. DSR-71167 was used at 10 μM (n = 2).
**Supplemental Table 2**

*Enzyme activity of DSR-71167 for various target molecules*

<table>
<thead>
<tr>
<th>Enzyme assays</th>
<th>Species</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin Converting Enzyme</td>
<td>Rabbit</td>
<td>-1</td>
</tr>
<tr>
<td>ATPase, Na⁺/K⁺</td>
<td>Pig</td>
<td>10</td>
</tr>
<tr>
<td>Carnitine Palmitoyltransferase-1</td>
<td>Rat</td>
<td>-5</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>Human</td>
<td>0</td>
</tr>
<tr>
<td>Ca²⁺/calmodulin-dependent protein kinase 2</td>
<td>Human</td>
<td>18</td>
</tr>
<tr>
<td>Ca²⁺/calmodulin-dependent protein kinase 4</td>
<td>Human</td>
<td>12</td>
</tr>
<tr>
<td>Cyclooxygenase COX-1</td>
<td>Human</td>
<td>-15</td>
</tr>
<tr>
<td>CYP450, 1A2</td>
<td>Human</td>
<td>-2</td>
</tr>
<tr>
<td>CYP450, 2C19</td>
<td>Human</td>
<td>34</td>
</tr>
<tr>
<td>CYP450, 2D6</td>
<td>Human</td>
<td>9</td>
</tr>
<tr>
<td>CYP450, 3A4</td>
<td>Human</td>
<td>37</td>
</tr>
<tr>
<td>Factor VIIa</td>
<td>Human</td>
<td>2</td>
</tr>
<tr>
<td>Factor Xa</td>
<td>Human</td>
<td>-18</td>
</tr>
<tr>
<td>Neutral Endopeptidase</td>
<td>Human</td>
<td>-7</td>
</tr>
<tr>
<td>Nitric Oxide Synthase, Endothelial</td>
<td>Bovine</td>
<td>1</td>
</tr>
<tr>
<td>Nitric Oxide Synthase, Neuronal</td>
<td>Rat</td>
<td>6</td>
</tr>
<tr>
<td>Pepsin</td>
<td>Pig</td>
<td>3</td>
</tr>
<tr>
<td>Phosphodiesterase PED3</td>
<td>Human</td>
<td>5</td>
</tr>
<tr>
<td>Enzyme Activity</td>
<td>Human</td>
<td>% Inhibition</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------</td>
<td>--------------</td>
</tr>
<tr>
<td>Phosphodiesterase PED6</td>
<td>Human</td>
<td>-8</td>
</tr>
<tr>
<td>Proteasome</td>
<td>Human</td>
<td>0</td>
</tr>
<tr>
<td>Renin</td>
<td>Human</td>
<td>4</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Human</td>
<td>7</td>
</tr>
<tr>
<td>Tissue Plasminogen Activator</td>
<td>Human</td>
<td>1</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Human</td>
<td>2</td>
</tr>
<tr>
<td>Tyrosine Kinase, Insulin Receptor</td>
<td>Human</td>
<td>-1</td>
</tr>
<tr>
<td>UDP Glucuronosyltransferase 1A1</td>
<td>Human</td>
<td>36</td>
</tr>
</tbody>
</table>

Enzyme activity of DSR-71167 for various target molecules is shown by % inhibition as compared to DMSO-treated samples. DSR-71167 was used at 10 μM (n = 2).
Supplemental Figure 1

Effects of DSR-85955 on systolic blood pressure and serum levels of potassium in spontaneous hypertensive rats

The male spontaneous hypertensive rats aged 11 weeks were orally administered DSR-85955 (10, 30, 100 mg/kg) for 2 weeks. (A) Systolic blood pressure and (B) serum levels of potassium were measured at the last day of experiment. The free base form of DSR-85955 is the same as that of DSR-71167. Data are the means ± S.D. (n = 8). * P<0.05, ** P<0.01 vs. vehicle by ANOVA followed by Dunnett's multiple comparison test.