Efficient, large-scale synthesis and preclinical studies of MRS5698, a highly selective A₃ adenosine receptor agonist that protects against chronic neuropathic pain

Dilip K. Tosh¹ · Janak Padia² · Daniela Salvemini³ · Kenneth A. Jacobson¹

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Abstract We reported that 2-(3,4-difluorophenylethynyl)-N⁶-3-chlorobenzyl (N)-methanocarba adenosine derivative **1** (MRS5698) binds selectively to human and mouse A₃ adenosine receptors (A₃ARs, K_i 3 nM). It is becoming an important pharmacological tool for defining A₃AR effects and is orally active in a chronic neuropathic pain model. Here, we introduce a new synthetic route for MRS5698 from D-ribose, suitable for a scale-up on a multi-gram scale, and we measure in vitro and in vivo ADME-Tox parameters. MRS5698 was very stable in vitro, failed to inhibit CYPs at <10 μM, and was largely bound to plasma proteins. It was well tolerated in the rat at doses of ≤200 mg/kg i.p. A 1 mg/kg i.p. dose in the mouse displayed *t*_{1/2} of 1.09 h and plasma C_{max} of 204 nM at 1 h with an AUC of 213 ng×h/mL. CACO-2 bidirectional transport studies suggested intestinal efflux of MRS5698 (efflux ratio 86). Although the oral %F is only 5 %, the beneficial effect to reverse pain lasted for at least 2 h in the CCI model in rats, using the same vehicle for oral administration of a high dose. The stability, low toxicity, lack of CYP interaction,

pharmacokinetic half-life, and in vivo efficacy suggest that MRS5698 is a preferred compound for further consideration as a treatment for neuropathic pain.

Keywords Nucleoside · Purines · G protein-coupled receptor · ADME-toxicity · Preclinical development

Abbreviations

ADME	Absorption, distribution, metabolism, and excretion
AR	Adenosine receptor
CCI	Chronic constriction injury
CHO	Chinese hamster ovary
CIPN	Chemotherapy-induced peripheral neuropathy
Cl-IB-MECA	1-deoxy-1-[2-chloro-6-[[[3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide
CNS	Central nervous system
CYP	Cytochrome P450
DMF	N,N-dimethylformamide
DCC	Dicyclohexylcarbodiimide
DMSO	Dimethylsulfoxide
EDC	N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide
EL	Extracellular loop
GMP	Good manufacturing practice
GPCR	G protein-coupled receptor
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HOBt	1-Hydroxybenzyltriazole
HP-β-CD	2-Hydroxypropyl-β-cyclodextrin
HRMS	High resolution mass spectroscopy

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✉ Kenneth A. Jacobson
kajacobs@helix.nih.gov

¹ Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

² PrimeTime Life Sciences, Germantown, MD, USA

³ Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, St. Louis, MO 63104, USA

IB-MECA	1-Deoxy-1-[6-[[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide
IND	Investigational New Drug
MRS5698	(1 <i>S</i> ,2 <i>R</i> ,3 <i>S</i> ,4 <i>R</i> ,5 <i>S</i>)-4-(6-((3-Chlorobenzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9H-purin-9-yl)-2,3-dihydroxy- <i>N</i> -methylbicyclo[3.1.0]hexane-1-carboxamide
MTD	Maximum tolerated dose
MW	Molecular weight
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
PDC	Pyridinium dichromate
PEG	Polyethylene glycol
PK	Pharmacokinetic
PWT	Paw withdrawal threshold
SAR	Structure-affinity relationship
SGF	Stimulated gastric fluid
SGF	Stimulated intestinal fluid
trp	Tryptophan
TBAF	Tetra- <i>n</i> -butylammonium fluoride
TBDPS	<i>tert</i> -Butyldiphenylsilyl
TEER	Trans epithelial electrical resistance
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
XTT	2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Introduction

Chronic severe pain is a significant global health problem [1]. In the USA alone, one third of Americans suffer from some form of chronic pain, and over 30 % of these individuals report pain that is resistant to analgesic therapy [1]. The economic impact of pain is similarly large at approximately \$100 billion annually [1]. While selective cyclooxygenase-2 (COX-2) inhibitors are effective for several forms of chronic pain, their occasional side effects including an increased risk of heart attack and stroke [2] prompted the precipitous withdrawal of some (i.e., Vioxx) from the market in 2004. Opiate/narcotic analgesics are the most effective treatments for acute and chronic severe pain. However, their clinical utility is often hampered by the development of analgesic tolerance as well as de novo painful hypersensitivity to innocuous and noxious stimuli. Such phenomena have been observed in both animal and human studies [3–5]. The development of tolerance necessitates escalating doses to achieve equivalent pain relief, even as the onset of opiate-induced hypersensitivity subverts its therapeutic impact [3–5]. This complex pathophysiology contributes significantly to a decreased quality of life in the growing population of subjects with chronic pain due to oversaturation, reduced

physical activity, respiratory depression, constipation, potential for addiction, and other side effects. Accordingly, there is growing interest in new approaches that would maintain opiate efficacy during repetitive dosing without engendering tolerance or unacceptable side effects.

Chronic neuropathic pain is a major unresolved health-care issue with widespread human and socioeconomic impact (5–10 % occurrence in Europe and the USA [6]). Its general incidence is augmented by pain from chemotherapy-induced peripheral neuropathy (CIPN, 7). To this end, patients with malignancies of various etiologies who receive taxane, platinum complex, vinca alkaloids, and/or proteasome-inhibitors risk developing a chronic, distal, and symmetrical sensory peripheral neuropathy often accompanied by a neuropathic pain syndrome [7]. This condition may resolve soon after drug termination or could last for years [7, 8]. Thus, CIPN is one of the most common causes of discontinuation of what is otherwise a life-saving therapy [7, 8].

We aim to develop a small molecule that acts as an A₃ adenosine receptor (AR) agonist for the treatment of chronic neuropathic pain, as indicated in animal models [9–11]. There is a need to develop new drugs for this widespread condition, especially one acting by a novel mechanism, because current treatment is not very effective or is associated with severe or disturbing side effects. The current treatment is with tricyclic antidepressants, anticonvulsants, tramadol, or drugs with local anesthetic properties. Thus, neuropathic pain remains a challenge for the clinician and a burden for the patient.

We have performed extensive probing of the structure-affinity relationship (SAR) through the synthesis and pharmacological characterization of nucleoside analogs, leading to the identification of (1*S*,2*R*,3*S*,4*R*,5*S*)-4-(6-((3-chlorobenzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9H-purin-9-yl)-2,3-dihydroxy-*N*-methylbicyclo[3.1.0]hexane-1-carboxamide (MRS5698 **17**, Fig. 1) as a potent and very selective agonist of the A₃AR [12]. MRS5698 includes multiple structural features that were optimized for potency and selectivity at the target A₃AR, such as a [3.1.0]bicyclohexane ring system in place of ribose and a rigid extension at the C2 position. Several earlier A₃ agonists (ribosides IB-MECA (CF101), only 50- to 100-fold selective in rat, and Cl-IB-MECA (CF102)) from our lab are currently in clinical trials for other indications (inflammatory diseases and cancer) [13]. MRS5698 displays higher affinity and selectivity (>3000-fold) vs. other ARs in both human and mouse, and both factors are consistent across species. Thus, MRS5698 is favored due to less interaction with other ARs, which potentially will avoid side effects. We also have reported the A₃AR selectivity of MRS5698 in comparison to >50 other cell surface receptors and ion channels [14].

The unambiguous pharmacological characterization of chronic pain protection by A₃AR agonists in vivo at spinal and supraspinal sites is based on the use of selective agonists and antagonists, as well as A₃AR knock-out mice and the inhibition of adenosine kinase [15]. MRS5698 demonstrated

Fig. 1 Synthesis of MRS5698, with yields indicated (except for **2** and **3**, which were isolated in high recovery as crude products). Reagents and conditions: (i) acetone, H_2SO_4 , rt, 3 h; (ii) TBDPS-Cl, imidazole, CH_2Cl_2 , rt, 3 h; (iii) $\text{Ph}_3\text{PCH}_3\text{Br}$, potassium *t*-butoxide, THF, rt, 4 h; (iv) DCC, DMSO, TFA, pyridine, rt, overnight; (v) vinyl magnesium bromide, THF, -78°C , 2 h; (vi) Zhan catalyst-1B, toluene, 60°C ; (vii)

PDC, $4\text{ \AA}$ molecular sieves; (viii) NaBH_4 , $\text{CeCl}_3\cdot 7\text{H}_2\text{O}$, MeOH, 0°C , 60 min.; (ix) Et_2Zn , CH_2Cl_2 , rt, overnight; (x) 2-iodo-6-chloro-purine **20**, Ph_3P , DIAD, THF, rt; (xi) 3-chloro-benzylamine, Et_3N , MeOH; (xii) TBAF, THF, rt; (xiii) PDC, DMF; (xiv) MeNH_2 , EDC, HOBT, THF; (xv) 3,4-difluorophenylacetylene **23**, $\text{PdCl}_2(\text{Ph}_3\text{P})_2$, Et_3N , DMF; (xvi) 6 N HCl, MeOH, 50°C

pharmacological activity in multiple mouse and rat models of chronic neuropathic pain induced by chronic constriction injury (CCI) of the sciatic nerve [9, 16] or by the chemotherapeutic agent oxaliplatin [10]. The development of chemotherapy-induced neuropathic pain in mice in the CCI model was completely prevented by MRS5698 (1.0 mg/kg, i.p. given at the time peak pain is reached, i.e., 7 days). A_3AR agonists are inactive in models of acute pain, such as tail flick and hot plate assays [9]. Here, we delve further into the drug-like properties of MRS5698, which we prepare on a large scale by a more practical synthetic route from bulk starting materials (Figs. 1 and 2). In vivo pharmacokinetics and some in vitro characteristics of MRS5698 were determined, including inhibition of CYP 2C9, 2D6, and 3A4, cytotoxicity in HepG2 cells, stability in simulated body fluids, stability in liver microsomes, plasma protein binding and stability, aqueous solubility and stable in plasma and in simulated gastric and intestinal

fluids. We have evaluated pharmacokinetics (PK) and pharmacodynamics (PD), including oral bioavailability and half-life in plasma and developed the associated bioanalytical methods. High doses of MRS5698 were both efficacious in reversing neuropathic pain and well tolerated in vivo.

Materials and methods

Chemical synthesis

(3*aR*, 6*R*, 6*aR*)-6-(Hydroxymethyl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-*d*][1,3]dioxol-4-ol (**2**)

D-Ribose (**1**, 1.50 kg, 9.99 mol, 1.00 equiv.) and acetone (1.50 L) were stirred in a 20-L four-necked round-bottom

Fig. 2 Synthesis of intermediates **20** and **23**. Reagents and conditions: **a** (i) Ac_2O , DMF; (ii) iodine, isoamyl nitrite, THF. **b** (i) trimethylsilylacetylene, $\text{PdCl}_2(\text{Ph}_3\text{P})_2$, CuI , Et_3N , DMF; (ii) K_2CO_3 , EtOH

flask, and sulfuric acid (45 mL) was added dropwise at 0 °C. The resulting solution was stirred for 3 h at room temperature. The reaction was repeated 13 times. The pH value of the reaction mixture was adjusted to 7 by the addition of solid sodium bicarbonate and the resulting solids were removed by filtration. The filtrate was concentrated under vacuum to give compound **2** (29.4 kg, crude) as a yellow oil.

(3*aR*,6*R*,6*aR*)-6-[[*(tert*-Butyldiphenylsilyl)oxy]methyl]-2,2-dimethyl-tetrahydro-2*H*-furo[3,4-*d*][1,3]dioxol-4-ol (**3**)

Imidazole (650 g, 9.55 mol, 1.20 equiv.) was added to a solution of isopropylidene derivative **2** (1.90 kg, 8.00 mol, 1.00 equiv., 80 %) in dichloromethane (13 L) in a 20-L three-necked round-bottom flask under a nitrogen atmosphere, and the resulting solution was stirred for 0.5 h at room temperature. TBDPS-Cl (2.27 kg, 8.26 mol, 1.00 equiv.) was added dropwise into the reaction mixture at 10 °C, and after addition the mixture was stirred for 4 h at room temperature. This reaction was repeated 14 times. After completion of the reaction, the reaction mixture was washed with 4 L of water. The organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum, and the residue was purified on a silica gel column chromatography, eluting with ethyl acetate/petroleum ether (1:30–1:10) to yield compound **3** (23.8 kg, 40 %) as a yellow oil.

2-((*tert*-Butyldiphenylsilyl)oxy)-1-((4*R*,5*S*)-2,2-dimethyl-5-vinyl-1,3-dioxolan-4-yl)ethan-1-ol (**4**)

Potassium *t*-butoxide (790 g, 7.04 mol, 1.50 equiv.) was added portionwise to a solution of compound **3** (2.00 kg, 4.67 mol, 1.00 equiv.) in THF (7 L), and the mixture was stirred in a 20-L three-necked round-bottom flask under a nitrogen atmosphere at 0 °C. The resulting solution was stirred for 2 h at 25 °C. A solution of methyltriphenylphosphonium bromide (2500 g, 7.02 mol, 1.50 equiv.) in THF (8 L) was added dropwise to the reaction mixture at 0 °C, and the mixture was stirred for additional 4 h at room temperature. This reaction was repeated 10 times. The reaction was quenched by the addition of 3 L of water followed by NaCl (2 kg) at 0 °C. The organic layer was dried over anhydrous

sodium sulfate and concentrated under vacuum, and the residue was purified on a silica gel column chromatography, eluting with ethyl acetate/petroleum ether (1:20–1:10) to give compound **4** (14 kg, 70 %) as a yellow oil.

2-[(*tert*-Butyldiphenylsilyl)oxy]-1-[(4*S*,5*S*)-5-ethenyl-2,2-dimethyl-1,3-dioxolan-4-yl]ethan-1-one (**5**)

DCC (1.26 kg, 6.11 mol, 2.00 equiv.), DMSO (595 g, 7.62 mol, 2.5 equiv.), and pyridine (241 g, 3.05 mol, 1.00 equiv.) were added to a solution of compound **4** (1300 g, 3.05 mol, 1.00 equiv.) in ethyl acetate (12 L) in a 20-L round-bottom flask. Subsequently, TFA (348 g, 3.05 mol, 1.00 equiv.) was added dropwise to the reaction mixture at 5 °C, and the reaction mixture was stirred overnight at room temperature. This reaction was repeated 10 times. After completion of the reaction, solids were removed by filtration and filtrate was washed with 3 × 2 L of water, dried over sodium sulfate, and evaporated under vacuum. The residue was purified by silica gel column chromatography, eluting with ethyl acetate/petroleum ether (1:30–1:10) to give the desired compound **5** (10 kg, 77 %) as a yellow oil.

1-((*tert*-Butyldiphenylsilyl)oxy)-2-((4*S*,5*S*)-2,2-dimethyl-5-vinyl-1,3-dioxolan-4-yl)but-3-en-2-ol (**6**)

Vinylmagnesium bromide solution (4.2 L of 1 M solution in THF, 1.78 equiv.) was added dropwise to a solution of compound **5** (1.0 kg, 2.36 mol, 1.00 equiv.) and THF (8 L) in a 20-L four-necked round-bottom flask at −78 °C, and the mixture was stirred for 2 h in the same conditions. The reaction was quenched by the addition of 3 L of saturated aqueous NH_4Cl solution and was extracted with 1 × 3.0 L of ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum, and the residue was purified on silica gel column chromatography, eluting with ethyl acetate/petroleum ether (1:30–1:10) to give compound **6** (700 g, 66 %) as a yellow oil.

(3*aS*,4*S*,6*aS*)-4-(((*tert*-Butyldiphenylsilyl)oxy)methyl)-2,2-dimethyl-3*a*,6*a*-dihydro-4*H*-cyclopenta[*d*][1,3]dioxol-4-ol (7)

Zhan catalyst-1B (3.3 g, 4.50 mmol, 0.4 %) was added to a solution of compound **6** (500 g, 1.1 mol, 1.00 equiv.) in toluene in a 5-L four-necked round-bottom flask under a nitrogen atmosphere at 10 °C. The resulting solution was heated at 60 °C for 2 h. This reaction was repeated six times. After completion of the reaction, the reaction mixture was evaporated under vacuum, and the residue was purified on silica gel column chromatography, eluting with ethyl acetate/petroleum ether (1:5–1:15) to give compound **7** (2.6 kg, 79 %) as a yellow oil.

(3*aR*,6*aR*)-6-(((*tert*-Butyldiphenylsilyl)oxy)methyl)-2,2-dimethyl-3*a*,6*a*-dihydro-4*H*-cyclopenta[*d*][1,3]dioxol-4-one (8)

Compound **7** (1.3 kg, 3.06 mol, 1.00 equiv.), 4 Å molecular sieves (2.6 kg, 2.00 equiv.) and DMF (6.5 L) were placed in a 20-L four-necked round-bottom flask. PDC (3.45 kg, 9.18 mol, 3.00 equiv.) was added portionwise to the reaction mixture at 10 °C for 2 h, and the reaction mixture was stirred overnight at room temperature. The reaction mixture was diluted with 20 volumes of ethyl acetate, and the mixture was stirred for 30 min. Double weight of silica gel (2.6 kg) was added to the reaction mixture, and the mixture was stirred for another 1 h. The solids were removed by filtration and the filter cake was washed with 10 volumes of ethyl acetate and the organic phase was washed with 4×5 volumes of brine. The resulting solution was dried, filtered, and evaporated under vacuum, and the residue was purified by silica gel column chromatography, eluting with ethyl acetate/petroleum ether (1:2–1:1) to provide compound **8** (0.802 kg, 62 %) as a yellow oil.

(3*aS*,4*S*,6*aR*)-6-(((*tert*-Butyldiphenylsilyl)oxy)methyl)-2,2-dimethyl-3*a*,6*a*-dihydro-4*H*-cyclopenta[*d*][1,3]dioxol-4-ol (9)

NaBH₄ (87 g, 2.29 mol, 1.20 equiv.) was added portionwise to a solution of compound **8** (800 g, 1.89 mol, 1.00 equiv.), CeCl₃·7H₂O (600 g, 2.09 mol, 1.10 equiv.) in methanol (8 L) into 20 L four-necked round-bottom flask under a nitrogen atmosphere over 1.5 h at 0 °C. The reaction mixture was stirred for another 1 h at same condition and concentrated under vacuum. The reaction mixture was neutralized by the addition of 15 volumes of H₂O/AcOH (50:1), and the resulting solution was extracted with 2×15 volumes ethyl acetate. The combined organic layers were dried, filtered, and evaporated under vacuum, and the residue was purified by silica gel column chromatography, eluting with ethyl acetate/petroleum ether (1:2–1:8) to give compound **9** (546 g, 68 %) as a yellow oil.

(3*aR*,3*bR*,4*aS*,5*S*,5*aS*)-3*b*-(((*tert*-Butyldiphenylsilyl)oxy)methyl)-2,2-dimethylhexahydrocyclopropa[3,4]cyclopenta[1,2-*d*][1,3]dioxol-5-ol (10)

Et₂Zn solution (4.3 L of 1 M solution, 3.3 equiv.) was added dropwise to a solution of compound **9** (550 g, 1.30 mol, 1.00 equiv.) in dichloromethane (6.5 L) in a 20-L four-necked round-bottom flask under a nitrogen atmosphere at 0 °C over 1.5 h. After addition, the mixture was stirred for 30 min, and diiodomethane (1.39 kg, 5.19 mol, 4.00 equiv.) was added dropwise into the reaction mixture in the same conditions. Later, the reaction mixture was brought to room temperature, and the mixture was stirred overnight. The reaction was quenched by the addition of 10 volumes of aqueous NH₄Cl, and the mixture was stirred for another 30 min. The reaction mixture was extracted with two volumes of ethyl acetate and the combined organic layer was dried over sodium sulfate, filtered, and evaporated under vacuum. The residue was purified on column chromatography, eluting with ethyl acetate/petroleum ether (1:20–1:5) to give compound **10** (375 g, 66 %) as light yellow oil. MS: (ES, *m/z*): 461 [M+Na]⁺. H-NMR: (300 MHz, DMSO, ppm): δ 7.491–7.629 (m, 4H), 7.404–7.471 (m, 4H), 4.825 (d, *J*=18.9, 1H), 4.485 (d, *J*=7.5, 1H), 4.383–4.425 (t, 1H), 4.254–4.317 (m, 1H), 3.891 (d, *J*=7.5, 1H), 3.376 (d, *J*=10.8, 1H), 1.405–1.448 (m, 4H), 1.112–1.152 (t, 3H), 1.000 (s, 9H).

9-((3*aR*,3*bR*,4*aS*,5*R*,5*aS*)-3*b*-(((*tert*-Butyldiphenylsilyl)oxy)methyl)-2,2-dimethylhexahydrocyclopropa[3,4]cyclopenta[1,2-*d*][1,3]dioxol-5-yl)-6-chloro-2-iodo-9*H*-purine (11)

6-Chloro-2-iodo-purine **20** (527 g, 1.88 mol, 1.50 equiv.) and triphenylphosphine (658 g, 2.51 mol, 2.00 equiv.) and THF (5.5 L) were placed in a 20-L four-necked round-bottom flask under a nitrogen atmosphere. DIAD (500 g, 2.47 mol, 2.00 equiv.) was added dropwise into the reaction at 0 °C, and the mixture was stirred for 30 min at room temperature. Compound **10** (550 g, 1.25 mol) in THF (1.4 L) was added dropwise into the reaction mixture over 1 h at 10 °C, and the mixture was stirred overnight at room temperature. Solids were removed by filtration, and the filter cake was washed with three volumes of THF and the filtrate was evaporated under vacuum. The residue was purified on column chromatography, eluting with ethyl acetate/petroleum ether (1:15–1:3) to give compound **11** (339.4 g, 39 %) as an off white solid. MS: (ES, *m/z*): 701 [M+H]⁺. H-NMR (300 MHz, CDCl₃, ppm): δ 8.294 (s, 1H), 7.595–7.647 (m, 4H), 7.281–7.439 (m, 6H), 5.276 (d, *J*=6.6, 1H), 5.048 (s, 1H), 4.601 (d, *J*=6.0, 1H), 4.274 (d, *J*=10.5, 1H), 1.263–1.564 (m, 7H), 1.070–1.144 (m, 12H).

9-((3*aR*,3*bR*,4*aS*,5*R*,5*aS*)-3*b*-(((*tert*-Butyldiphenylsilyl)oxy)methyl)-2,2-dimethylhexahydrocyclopropa[3,4]cyclopenta[1,2-*d*][1,3]dioxol-5-yl)-*N*-(3-chlorobenzyl)-2-iodo-9*H*-purin-6-amine (12)

3-Chloro-benzylamine (145 g, 1.02 mol, 4.00 equiv.) and triethylamine (260 g, 2.57 mol, 10.0 equiv.) were added to a solution of compound **11** (180 g, 257 mmol) in methanol (2 L) in a 5-L three-necked round-bottom flask, and the mixture was stirred overnight at 28 °C. The reaction mixture was diluted with 3 L of ethyl acetate and washed with brine (4×1.0 L), dried over sodium sulfate and evaporated under vacuum. The residue was purified by silica gel column chromatography, eluting with ethyl acetate/petroleum ether (1:5–1:1) to provide compound **12** (160 g, 77 %) as off white solid.

((3*aR*,3*bR*,4*aS*,5*R*,5*aS*)-5-(6-((3-Chlorobenzyl)amino)-2-iodo-9*H*-purin-9-yl)-2,2-dimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-*d*][1,3]dioxol-3*b*(3*aH*)-yl)methanol (13)

TBAF (1 M in THF, 300 mL, 1.50 equiv.) was added to a solution of compound **12** (160 g, 198 mmol) in THF (1.6 L) in a 3-L three-necked round-bottom flask, and the mixture was stirred for 4 h at room temperature. The reaction mixture was diluted with ethyl acetate (2 L) and washed with brine (3×1 L), dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was purified by silica gel column chromatography, eluting with ethyl acetate/petroleum ether (1:3–1:0) to give compound **13** (80 g, 71 %) as a yellow solid.

(3*aR*,3*bS*,4*aS*,5*R*,5*aS*)-5-(6-((3-Chlorobenzyl)amino)-2-iodo-9*H*-purin-9-yl)-2,2-dimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-*d*][1,3]dioxole-3*b*(3*aH*)-carboxylic acid (14)

PDC (22.3 g, 59.3 mmol, 6.00 equiv.) was added to a solution of compound **13** (5.6 g, 9.86 mmol) in DMF (50 mL), and the mixture was heated at 40 °C overnight. The reaction mixture was quenched with 200 mL of ice-water, and the resulting solution was extracted with 3×100 mL of ethyl acetate. The combined organic layer was washed with 3×100 mL of water, dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was purified by silica gel column chromatography, eluting with ethyl acetate/petroleum ether (1:1–1:0) to give compound **14** (4.5 g, 78 %) as a yellow solid.

(3*aR*,3*bS*,4*aS*,5*R*,5*aS*)-5-(6-((3-Chlorobenzyl)amino)-2-iodo-9*H*-purin-9-yl)-*N*,2,2-trimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-*d*][1,3]dioxole-3*b*(3*aH*)-carboxamide (15)

EDC·HCl (1.78 g, 9.29 mmol, 1.20 equiv.), HOBt (1.25 g, 9.26 mmol, 1.20 equiv.), triethylamine (1.56 g, 15.5 mmol, 2.00 equiv.) and MeNH₂ solution (4.64 mL of 2 M solution in THF, 1.20 equiv.) were added to a solution of compound **14** (4.5 g, 7.73 mmol) in THF (60 mL), and the mixture was stirred for 4 h at room temperature. The reaction mixture was diluted with 100 mL ethyl acetate, washed with 3×50 mL of water, dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was purified by silica gel column chromatography, eluting with ethyl acetate (100 %) to give compound **15** (3 g, 65 %) as a yellow solid.

(3*aR*,3*bS*,4*aS*,5*R*,5*aS*)-5-(6-((3-Chlorobenzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9*H*-purin-9-yl)-*N*,2,2-trimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-*d*][1,3]dioxole-3*b*(3*aH*)-carboxamide (16)

4-Ethynyl-1,2-difluorobenzene **23** (1.6 g, 11.6 mmol, 2.50 equiv.), CuI (179 mg, 0.94 mmol, 0.20 equiv.), Pd(PPh₃)Cl₂ (659 mg, 0.94 mmol, 0.20 equiv.) and triethylamine (4.76 g, 47.1 mmol, 10.0 equiv.) were added to a solution of compound **15** (2.8 g, 4.71 mmol) in DMF (35 mL), and the mixture was stirred overnight at room temperature. The reaction mixture was diluted with 150 mL of ethyl acetate and washed with 4×50 mL of water, and the organic layer dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was purified by silica gel column chromatography, eluting with ethyl acetate (100 %) to give compound **16** (2.8 g, 98 %) as a yellow solid.

(1*S*,2*R*,3*S*,4*R*,5*S*)-4-(6-((3-Chlorobenzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9*H*-purin-9-yl)-2,3-dihydroxy-*N*-methylbicyclo[3.1.0]hexane-1-carboxamide (17)

A solution of compound **16** (20.8 g, 34.4 mmol) in methanol (250 mL) and HCl (200 mL, 6 M solution) was heated at 50 °C for 3 h. The reaction mixture was evaporated under vacuum and diluted with 300 mL water. The pH of the solution was adjusted to 7–8 with saturated sodium carbonate solution. The resulting solution was extracted with 400 mL of ethyl acetate, and the organic layer was washed with 3×150 mL of water, dried over anhydrous sodium sulfate, and concentrated under vacuum. The residue was purified by silica gel column chromatography, eluting with ethyl acetate/CH₃OH (100:1), and the solids resulting after reduction of volume were collected by filtration. The filter cake was washed with ethyl acetate/hexane=1:5 to afford the final

compound MRS5698 (**17**) (10.5 g, 54 %) as a yellowish white solid. MS: 565 $[M+H]^+$. H-NMR: (300 MHz, DMSO₃, ppm): δ 8.622 (1H, br), 8.200 (1H, s), 7.750–7.850 (1H, m), 7.500–7.700 (3H, m), 7.400–7.450 (1H, s), 7.250–7.400 (3H, m), 5.446–5.431 (1H, d, $J=4.5$ Hz), 5.008–4.961 (1H, m), 4.847–4.820 (1H, d, $J=8.1$ Hz), 4.750 (1H, s), 4.700–4.750 (2H, br), 3.931–3.895 (1H, m), 2.667–2.652 (3H, d, $J=4.5$ Hz), 1.865–1.836 (1H, m), 1.600–1.700 (1H, m), 1.321–1.340 (1H, m).

1-(2-Amino-6-chloro-9H-purin-9-yl)-ethan-1-one (19)

Acetic anhydride (1.2 kg, 11.8 mol, 2.00 equiv.) was added to solution of 2-amino-6-chloro-purine **18** (1 kg, 5.90 mol, 1.00 equiv.) in DMF (10 L) in a 50-L three-necked round-bottom flask, and the mixture was heated at 80 °C overnight. The reaction mixture was cooled to room temperature, and ice-water (20 L) was added into it. The resulting solids were filtered and washed with water (3×3 L) and propanol (2 L) to give compound **19** (1.2 kg, 96 %) as a white solid.

6-Chloro-2-iodo-9H-purine (20)

Iodine (1.1 kg, 4.36 mol, 0.77 equiv.) and amyl nitrite (242 g, 20.7 mol, 3.64 equiv.) were added to a solution of compound **19** (1.2 kg, 5.67 mol) in THF (12 L) under a nitrogen atmosphere, and the mixture was heated at 60 °C overnight. The reaction was quenched by the addition of 15 % of Na₂SO₃ solution (5 L), and the resulting solution was extracted with ethyl acetate (3×10 L). The combined organic layer was washed 5 % sodium hydroxide solution (3×8 L). The pH value of the aqueous solution was adjusted to 4–5 by the addition of 36 % HCl solution. The resulting solution was extracted with ethyl acetate (3×10 L) and combined organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was washed with 3 L of ethyl acetate/petroleum ether (1:5) to give compound **20** (900 g, 57 %) as a yellow solid.

((3,4-Difluorophenyl)ethynyl)trimethylsilane (22)

Ethynyltrimethylsilane (127.4 g, 1.30 mol, 1.30 equiv.), Pd(PPh₃)₂Cl₂ (35 g, 50.0 mmol, 0.05 equiv.), CuI (10.5 g, 55.3 mmol, 0.055 equiv.) and triethylamine (404 g, 4.00 mol, 4.00 equiv.) were added to a solution of 4-bromo-1,2-difluorobenzene **21** (192 g, 995 mmol, 1.00 equiv.) in ethyl acetate (1.6 L), and the mixture was heated at 50 °C overnight. The solids were filtered; filtrate was evaporated under vacuum and residue was purified on silica gel column with petroleum ether (100 %) to give compound **22** (130 g, 62 %) as a brown oil.

4-Ethynyl-1,2-difluorobenzene (23)

K₂CO₃ (35 g, 251 mmol, 0.50 equiv.) was added to a solution of compound **22** (105 g, 499 mmol, 1.00 equiv.) in ethanol (800 mL), and the mixture was stirred for 4 h at room temperature. The solids were filtered, and the pH of the filtrate was adjusted to 5–6 by the addition of HCl (1.0 M). The resulting solution was extracted with hexane (2×500 mL), and the combined organic layer was washed with brine (4×1 L), dried over anhydrous sodium sulfate and concentrated under vacuum below 25 °C to give compound **23** (50 g, 73 %) as a brown oil.

ADME-Tox testing

Chemical stability

A 10 mM solution of MRS5698 in DMSO (10 μ L) was diluted in appropriate buffer (190 μ L) and small aliquots injected (15 μ L) to an analytical LC/MS (5 injections over 24 h). The stability of the compound was monitored by plotting area under the curve (AUC) vs. time.

Microsomal stability

The metabolic stability of MRS5698 in pooled human liver microsomes (HLM), male rat liver microsomes (MRLM) and male mouse liver microsomes (MMLM) was determined in duplicate. All substrates were incubated at 37 °C, pH 7.4. The reaction mixture consisted of 50 μ L of a 5 mg protein/mL suspension of liver microsomes (equivalent to approximately 0.5 mg/mL of microsomal protein in the final reaction mixture), 50 μ L of 50 mM MgCl₂ solution, 250 μ L of 200 mM phosphate buffer, and 95 μ L of ultra-pure water. The reaction was started with the addition of 50 μ L of 10 mM NADPH solution at a final concentration of 1 mM and carried out at 37 °C. Fifty microliters of ultra-pure H₂O was used instead of the NADPH solution in the negative control. Aliquots of 50 μ L were taken from the reaction solution at 0, 15, 30, 45, and 60 min. The reaction was stopped by the addition of three volumes of cold methanol with IS (200 nM imipramine, 200 nM labetalol, and 2 μ M ketoprofen) at the designated time points. Samples were centrifuged at 16,000g for 10 min to precipitate protein. Aliquots of 100 μ L of the supernatant were used for LC/MS/MS analysis. All calculations were carried out using Microsoft Excel. Peak areas were determined from extracted ion chromatograms. The slope value, k , was determined by linear regression of the natural logarithm of the remaining percentage of the parent drug vs. incubation time curve.

The in vitro half-life (in vitro $t_{1/2}$) was determined from the slope value:

$$\text{in vitro } t_{1/2} = -(0.693/k)$$

Conversion of the in vitro $t_{1/2}$ (in min) into the in vitro intrinsic clearance (in vitro CL_{int} , in $\mu\text{L}/\text{min}/\text{mg}$ proteins) was done using the following equation (mean of duplicate determinations):

$$\text{in vitro } CL_{\text{int}} = \left(\frac{0.693}{(t_{1/2})} \right) * \left(\frac{\text{volume of incubation}(\mu\text{L})}{\text{amount of proteins}(\text{mg})} \right)$$

The control compound (verapamil) was included in the assay. Any value of the compounds that was not within the specified limits was rejected, and the experiment was repeated. The negative control was used to exclude the misleading factor that would result from instability of the chemical itself.

Plasma protein binding

The mouse and rat plasma protein binding of MRS5698 was determined in duplicate at 1 μM concentration using a rapid equilibrium dialysis method. A DMSO stock solution of MRS5698 was prepared at a concentration of 30 mM and diluted in series into 50 % DMSO/ H_2O (v/v), and then mouse or rat plasma was spiked to achieve a final concentration of 1 μM MRS5698. The final percent volume of organic solvents was less than 0.5 %. One hundred microliters of spiked plasma sample and 300 μL of buffer (pH 7.4) were added to the buffer chamber. The loaded dialysis plate was covered with sealing tape and put on an orbital shaker at approximately 100 rpm and incubated at 37 °C for 4 h. At the end of dialysis, 50 μL aliquots of plasma were collected from the plasma chamber and transferred to microcentrifuge tubes containing 50 μL of blank buffer. Three hundred microliters of precipitation buffer (methanol) was added to the sample to precipitate protein and release the compound. The sample was centrifuged for 15 min, and the supernatant was analyzed by LC/MS/MS.

Caco-2 permeability assay

Caco-2 permeability assay was performed by Pharmaron, Inc. (Beijing, China) using the Caco-2 epithelial monolayers. Caco-2 cells, grown in tissue culture flasks, were trypsinized, suspended in media, and plated in 96-well plates to be grown for 3 weeks; the proper formation of cell monolayer was determined by monitoring the transepithelial electrical resistance (TEER). All assays were performed with compounds at a concentration of 5 μM for 2 h. For apical to basolateral

(A→B) permeability, compounds were added on the apical side (A), and permeation determined on the basolateral side (B), where the receiving buffer was removed for analysis by LC/MS/MS using an API4000Qtrap mass spectrometer (ESI, MRM mode) coupled with a Waters UPLC. The buffers used contained 100 μM Lucifer yellow in transport buffer (10 mM HEPES, 1×Hank's balanced salt solution, pH 7.4) (apical side) and transport buffer, pH 7.4 (basolateral side). The apparent permeability (P_{app}) is expressed using the following equation: $P_{\text{app}} = (dQ/dt)/C_0A$, where dQ/dt is the rate of permeation, C_0 is initial concentration, and A is the monolayer area. For bidirectional permeability, the efflux ratio was defined as $P_{\text{app}} (\text{B} \rightarrow \text{A})/P_{\text{app}} (\text{A} \rightarrow \text{B})$; high efflux ratio (>3) indicates that a compound is a potential substrate for P-gp or other active transport systems.

CYP inhibition assay

The CYP inhibition assay of MRS5698 was performed against the three major human liver microsomal CYP enzymes: CYP2C9, CYP2D6, and CYP3A4. We did not evaluate the possibility of stimulatory activity of MRS5698 on these CYP isoforms.

For CYP2C9 inhibition, the following components were combined: 20 μL of 2 mg/mL human liver microsomes at a final concentration of 0.2 mg/mL and 1 μL of 40 mM specific drug substrate (tolbutamide) at a final concentration of 200 μM . For CYP2D6 inhibition, 20 μL of 2 mg/mL human liver microsomes at a final concentration of 0.2 mg/mL and 1 μL of 2 mM specific drug substrate (dextromethorphan) at the final concentration of 10 μM were added to the above solution. For CYP3A4 inhibition, 20 μL of 2 mg/mL human liver microsomes at a final concentration of 0.2 mg/mL and 1 μL of specific drug substrate (testosterone: 10 mM or midazolam: 1 mM) at the final concentration of 50 μM or 5 μM were added to the above solution. The microsomal test mixtures were incubated for 2 min, and the reactions were initiated at 37 °C upon addition of 20 μL of 10 mM NADPH solution at a final concentration of 1 mM. The reaction was stopped by the addition of three volumes of methanol at the designated time points (tolbutamide, 20 min; dextromethorphan, 20 min; midazolam, 5 min). Samples were centrifuged at 16,000g for 10 min to precipitate protein. Aliquots of 200 μL of the supernatant were used for LC/MS/MS analysis. All experiments were performed in duplicate. The % inhibition was calculated for each time point with respect to control values (without inhibitor) at the same time point.

Cytotoxicity study in HepG2 cells

The cytotoxicity potential of MRS5698 was determined in an assay using HepG2 hepatocytes with XTT, a colorimetric reagent for assessing cell viability. HepG2 cells were treated

with the compound for 72 h over a 9-point 3-fold dilution series, ranging from 100 to 0.015 μM . Following the incubation, the relative viable cell number was determined using Infinite M200 (Tecan). Each plate contained 6 replicates of vehicle-treated cells, which served as vehicle controls, and 6 wells without cells as blank. The maintenance of the HepG2 cells followed the recommendations of the ATCC (<http://www.atcc.org/>). Cells were passaged as needed, harvested from flasks using 0.25 % trypsin-EDTA, and maintained for no more than 20 passages. On the day of the assay, the HepG2 cells were harvested as previously indicated and the concentration was adjusted to $7 \times 10^4/\text{mL}$ cells/mL in complete growth medium supplemented with 1 % penicillin/streptomycin. One hundred microliters or approximately 7000 cells were dispensed to each well in the 96-well plate. Solutions of MRS5698 or carrier control (DMSO) were diluted to $2 \times$ in complete growth medium supplemented with 1 % penicillin/streptomycin, and 100 μL was dispensed into each of the corresponding wells of 96-well tissue culture-treated plates. The DMSO concentration was maintained at 0.5 % final concentration. The plates were then incubated at 37 °C, 5 % CO_2 for 72 h prior to endpoint detection. Following the 72-h incubation period, the assay plates were equilibrated to room temperature for 10 min. The medium was then removed and 100 μL of XTT working solution (containing 0.3 mg/mL XTT and 0.00265 mg/mL PMS) was added to each well by pipetting. The plates were then incubated for an additional 2 h at 37 °C, 5 % CO_2 . At the end of the incubation, 450 nm absorbance was measured using a Tecan Infinite M200 microplate reader.

hERG K⁺ channel conventional patch-clamp assay

Cultured cells (1–7 days) were used for patch-clamp assay. The cells were cultured in DMEM/GlutaMax-1 + 10 % FBS and were planted on collagen-coated dishes at low density ($\sim 2 \times 10^4$ cells/dish). The cell was held at -80 mV. A 50-ms pulse to -40 mV was delivered to measure the leaking currents, which were subtracted from the tail currents online. Then, the cell was depolarized to $+20$ mV for 2 s, followed by a second pulse to -40 mV for 1 s to reveal the tail currents. This paradigm was delivered once every 5 s to monitor the current amplitude. After the current amplitude was stabilized, the compound was delivered to the extracellular medium by a rapid solution changer perfusion system. During superfusion, the cell was repetitively stimulated with the protocol described above, and the current amplitude was continuously monitored. The experimental conditions are described in Table 2. The degree of inhibition (%) was obtained by measuring the tail current amplitude before and after superfusion with MRS5698 (the difference current was normalized to control and multiplied by 100 to obtain the percent of inhibition). Concentration (log) response curves were fitted to a logistic equation to

generate estimates of IC_{50} . The concentration–response relationship of MRS5698 was constructed from the percentage reductions of current amplitude by sequential concentrations.

Antimutagenicity assay (Ames test)

The antimutagenicity of MRS5698, in terms of its ability to reduce the number of revertant colonies induced by known mutagens, was evaluated according to Di Sotto et al. [17]. MRS5698 was tested from the maximum non-cytotoxic concentration of 56 μM and then serially diluted in DMSO by using a dilution factor of 3:1. The positive control compounds that were used in this study include ICR 191 (for TA97 in the absence of S9), 2-nitrofluorene (for TA98 in the absence of S9), sodium azide (for TA100 in the absence of S9), and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG, for TA102 in the absence of S9), 2-aminoanthracene (for TA97 and TA98 in the presence of S9), and aflatoxin B1 (for TA100 and TA102 in the presence of S9). The quantities used were as follows: ICR-191, 1 $\mu\text{g}/\text{plate}$ for TA97; 2-nitrofluorene 10 $\mu\text{g}/\text{plate}$ for TA98; sodium azide, 13.5 $\mu\text{g}/\text{plate}$ for TA100; MNNG, 10 $\mu\text{g}/\text{plate}$ for TA102, 2-aminoanthracene, 10 $\mu\text{g}/\text{plate}$ for TA97 and TA98; aflatoxin B1, 5 $\mu\text{g}/\text{plate}$ for TA100 and 100 $\mu\text{g}/\text{plate}$ for TA102. The mutagen concentrations, obtained from the linear part of the concentration–response curve of mutagens, were chosen as they induced a submaximal mutagenic effect (about 70 %). Plates containing the test substance alone were also included. Experiments were repeated at least twice, and each concentration was tested in triplicate. In order to highlight if the antimutagenicity could be due to desmutagenic or bioantimutagenic mechanisms, three different treatment protocols were applied, as described below.

In the pretreatment, the bacterial (overnight) culture (100 μL) was mixed with test substance (25 μL) and S9 mixture or phosphate buffer (0.1 M; 500 μL), gently vortexed in a sterile tube, and preincubated under shaking at 37 °C for 30 min. Thereafter, mutagen (25 μL) and top agar (2 mL) containing a 10 % v/v of 0.5 mM histidine/biotin (his/bio) or tryptophan (trp) solution were added; the mixture was vortexed and poured onto a minimal agar plate. In the cotreatment, the bacterial overnight culture (100 μL) was mixed with test substance (25 μL), mutagen (25 μL), and the S9 mixture or phosphate buffer (0.1 M) (500 μL); gently vortexed in a sterile tube; and incubated under shaking at 37 °C for 30 min. Then the tubes were added with top agar (2 mL), containing his/bio or trp solution (10 % v/v; 0.5 mM), vortexed, and poured onto a minimal agar plate. Finally, in the post-treatment, bacterial overnight culture (100 μL), mutagen (25 μL), and S9 mixture or phosphate buffer (0.1 M; 500 μL) were mixed; gently vortexed in a sterile tube; and incubated under shaking at 37 °C for 30 min. Thereafter, the tubes were added with different concentrations of 1 (25 μL) and with top

agar (2 mL), containing his/bio or trp solution (10 %v/v; 0.5 mM), vortexed, and poured onto a minimal agar plate. For all the protocols, the plates were incubated at 37 °C for 72 h and scored for histidine- or trp-independent revertant colonies and viable cells. The percentage of inhibition of the mutagenic effect was calculated according to Di Sotto et al. [18]. Antimutagenicity was considered moderate with an inhibitory effect in the range of 25–40 % and strong when the inhibition was higher than 40 %; when the inhibitory effect was lower than 25 %, it was considered weak and not recognized as a positive result [18].

Maximum tolerated dose

Maximum tolerated dose (MTD) and pharmacokinetic studies of MRS5698 in Sprague–Dawley rats were performed by Pharmaron, Inc. following one single intraperitoneal (i.p.) dose and four oral administration doses. Twelve male and 12 female Sprague–Dawley rats each weighing 200–400 g were used for the acute toxicity study. They were randomly distributed into four groups, containing six animals per group (3 M/3 F) and were on standard normal diet provided with water ad libitum. They were allowed to acclimatize for 7 days to the laboratory condition before the experiment. The first group received one dose volume (10 mL/kg) as an i.p. dose of 10 mg/kg MRS5698 formulated in 10 % DMSO and 30 % PEG 400 in water. Groups 2–4 were administered MRS5698 via oral gavage with 75 (7.5 mg/mL), 150 (15 mg/mL), and 200 (20 mg/mL) mg/kg doses in 40 % PEG300 and 10 % Solutol in “10 % HP- β -CD in water.” The animals were observed for at least a 1-day interval for abnormal clinical signs before deciding the next dose level. After each dose, there was a 3-day observation period. The rats were continuously observed for 72 h to detect any changes in autonomic or behavioral responses such as visual alertness, spontaneous activity, irritability, corneal reflex, urination, and salivation. Any mortality during the experimentation period of 7 days was also recorded. The percentage of mortality in each group was noted.

Pharmacokinetics

Preparation of dose solutions The dosing formulation of MRS5698 for mouse studies was prepared as 0.04 mg/mL (for i.v.), 0.2 mg/mL (for i.p.), and 0.3 mg/mL (for per oral, p.o.) solutions in 40 % PEG300 and 10 % Solutol in a solution of 10 % HP- β -CD in water. For example, for i.v. administration, 1.58 mg MRS5698 was dissolved in 1.580 mL PEG300 and vortexed, and sonication provided a clear solution of MRS5698 at 1 mg/mL. An aliquot of 0.1 mL of the above solution was added to a mixture of 0.9 mL PEG300 and 0.250 mL warm Solutol and then gradually added to 1.25 mL of a solution of 10 % HP- β -CD in water with

vortexing to obtain a concentration of 0.04 mg/mL of MRS5698 in solution. **Animals** Male CD-1 mice (6–8 weeks of age), weighing 20–22 g, were obtained from Beijing Huafukang Biological Technology Co., Ltd. (Beijing, China) and male Sprague–Dawley rats (6–8 weeks of age) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All animals were allowed to acclimate to this environment for 1 week prior to experimental manipulations.

Study design The CD1 mice were given a single dose of MRS5698 by body weight via intravenous (i.v.), i.p., or via oral gavage administration. The blood samples were collected at intervals (h) of 0, 0.083, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 24 ($n=3$, each time point). Blood (0.030 mL) was collected in the experiment using i.v. administration from the dorsal metatarsal vein at each time point. With i.p. and p.o. administration, 0.30 mL blood was collected from the heart at each time point. All blood samples were transferred into plastic microcentrifuge tubes containing 20 μ L of heparin sodium as anticoagulant and 30 μ L of 0.5 M NaF as stabilizer and refrigerated until processed for plasma.

Fifty-five microliters of standards, 55 μ L of quality control (QC) samples, or 55 μ L of unknown samples (50 μ L of plasma with 5 μ L of 50 vol% acetonitrile) were added to 200 μ L of acetonitrile (verapamil, 2 ng/mL and labetalol, 25 ng/mL) for protein precipitation, respectively. Then the samples were vortexed for 30 s. After centrifugation at 4°C, 4000 rpm for 10 min, the supernatant was diluted to twice the volume with water and 20 μ L of the diluted supernatant was injected to the analytical column for quantitation analysis. In this study, the samples obtained from i.v. administration were diluted 10-fold with blank plasma (5 μ L of the test plasma sample was added to 45 μ L of blank plasma).

The desired serial concentrations of working solutions were achieved by diluting stock solutions of analytes with 50 vol% acetonitrile. Five microliters of MRS5698 was added to 50 μ L of respective (CD1 mouse or SD rat) blank plasma simultaneously to achieve calibration standards of 0.5–1000 ng/mL (0.5, 1, 2, 5, 10, 50, 100, 500, 1000 ng/mL) for MRS5698 in a total volume of 55 μ L. QC samples at 1 ng/mL (low, 1), 2 ng/mL (low, 2), 50 ng/mL (medium), and 800 ng/mL (high) were prepared. These QC samples were prepared on the day of analysis in the same way as calibration standards. Fifty-five microliters of standards, 55 μ L of QC samples, or 55 μ L of unknown sample (50 μ L of plasma with 5 μ L of 50 vol% acetonitrile) were added to 200 μ L of acetonitrile (verapamil, 10 ng/mL and labetalol, 25 ng/mL) for protein precipitation.

Twenty microliters of plasma samples were analyzed using LC/MS/MS (Shimadzu-DGU-20A5 integrated with AB API4000 LC/MS/MS instrument) with to determine plasma

concentrations of MRS5698. The mobile phase consisted of acetonitrile and water mixtures, solution A: 5 % acetonitrile (0.1 % formic acid) and solution B: 95 % acetonitrile (0.1 % formic acid) with gradient elution from 90 to 10 % solution A over 1.71 min. The flow rate was 0.7 mL/min. Phenomenex Luna 5 μ C18 (2.00 $\times$ 50 mm) column was used for the separation of the sample components. Mass information was obtained by selected ion monitoring of the $[M-H]^-$ pseudo-molecular ions of the analyte and internal standards.

Statistical analysis Non-compartmental model pharmacokinetic parameters were calculated using the WinNonlin pharmacokinetic software package, WinNonlin Program, Version 1.1 (Scientific Consulting Inc., Cary, NC) (Anonymous. WinNonlin Software Package. Apex, NC: Scientific Consulting Inc., 1995). Concentration data were weighted by $1/\text{concentration}$. The elimination half-life was estimated by linear regression analysis of the terminal phase of the plasma concentration profile. The area under the concentration time curve (AUC) was calculated by the trapezoidal rule between first (0 h) and last sampling time plus C/k , where C is the concentration of last sampling that provided the measurable concentration by LCMS and k is the elimination rate constant. Systemic clearance (CL) was determined by dividing the dose by the AUC. Bioavailability (F) was estimated using the equation $F(\%) = \text{AUC}_{i.p./p.o.} \times \text{dose}_{i.v.} / \text{AUC}_{i.v.} \times \text{dose}_{i.p./p.o.}$. Statistical analysis was performed using the Microsoft Excel. The significance of differences between pharmacokinetic parameters was analyzed using a two-tailed t test and P values <0.05 were considered significant.

In vivo efficacy studies

Animals and animal care Male Sprague–Dawley rats (220–230 g, Harlan Laboratories) were used for all studies. Animals were housed in a climate-controlled room on a 12-h light/dark cycle with food and water provided ad libitum. Procedures for the maintenance and use of animals were in accordance with the guidelines of the International Association for the Study of Pain (Seattle, MD) and conformed to the regulations of the Saint Louis University Committee on Animal Research. All experiments were conducted with the experimenters blinded to treatment conditions.

Stock solution of MRS5698 MRS5698 was prepared in a vehicle composed of a 1:1 solution of PEG300 in Solutol (4:1 v/v) and 10 % HP- β -CD in water. MRS5698 was administered by oral gavage (p.o., 0.4 mL dosing volume).

A stock solution of PEG300 and of warm Solutol (4:1 v/v) was prepared. Separately, a solution of 10 % HP- β -CD in water was prepared. Seventy-five milligrams of MRS5698 was dissolved in 5 mL of PEG300 + Solutol stock solution. The above mixture was vortexed and sonicated to obtain a

uniform solution of MRS5698. Gradually, 5 mL of the 10 % HP- β -CD solution was added, and the mixture vortexed to obtain the final stock solution of MRS5698 (10 mL of 7.5 mg/mL) for in vivo administration by gavage.

Pain model CCI was performed as previously reported [15, 16]. Animals were anesthetized with 3 % isoflurane/100 % O_2 inhalation and maintained on 2 % isoflurane/100 % O_2 . The left sciatic nerve of the rat was exposed proximal to the trifurcation and freed of adhering tissue. Three silk sutures (4–0) with about 1-mm spacing were tied around the nerve causing slight constriction. CCI-induced mechano-allodynia peaks at 7 days (D7) post-surgery, which was the time point used for pharmacological experiments (9, 14).

Behavioral testing Mechano-allodynia was defined as a significant ($P < 0.05$) reduction in mechanical mean paw withdrawal threshold (PWT, g) at forces that failed to elicit withdrawal responses on D0 before CCI. Mechano-allodynia was assessed using the von Frey up and down method [19]. All animals were allowed to acclimate for ~ 15 min on an elevated mesh platform in an enclosure. Calibrated von Frey filaments [Stoelting, Wood Dale, IL, ranging from 3.61 (0.407 g) to 5.46 (26 g) bending force] were applied to the midplantar surface of the hindpaw. The mean PWT (g) was reported for each group. Animals were tested at baseline (D0) prior to CCI and at maximal mechano-allodynia (D7).

Results and discussion

We have previously reported the design of a new series of highly potent and selective A_3AR agonists containing a (*N*)-methanocarba ring system with detailed SAR studies for the N^6 -arylalkyl and C2-arylethynyl groups [12]. These studies have identified MRS5698 as a potent and selective A_3AR agonist with $K_i \sim 3$ nM for both human and mouse A_3AR . Moreover, MRS5698 is efficacious in models of chronic and prolonged neuropathic pain in mice and rats [10, 15], including bone cancer pain. In order to assess its preclinical properties, we have developed a more practical synthetic route and further evaluated MRS5698 in vivo and in vitro.

Chemistry

MRS5698 was synthesized on a large scale by the route shown in Fig. 1. The methods used were similar to those reported [20–22], except that modifications of the procedures and reagents were introduced in order to adapt the process to a large scale. We have improved upon the synthetic route, showing that MRS5698 could be synthesized on a multi-gram scale from a less expensive starting material, D-ribose **1**, instead of L-ribose in the previous method [23]. However, the overall

Table 1 Chemical and physical properties of MRS5698

Property (calcd.) ^a	Value	Preferred
MW	565.0	<500
cLog P	2.65	1–3, 1–4.5
tPSA	121.9	<140 (oral), <90 (CNS)
Ligand efficiency (LE)	0.295	>0.29
Rotatable bonds	6	≤10
N + O (HBA)	6	≤10
NH + OH (HBD)	4	≤5

^a cLogP and rotatable bonds were calculated as described [28]. tPSA was calculated using ChemBioDraw14.0 (cLogP by this method was 3.37)

Table 3 Chemical stability of MRS 5698 in buffer solutions

Compound		Remaining percentage (%)		
		0 min	60 min	120 min
SGF	Erythromycin	100.0	56.5	22.4
	MRS5698	100.0	100.6	100.2
SIF	Chlorambucil	100.0	13.9	1.52
	MRS5698	100.0	100.3	104.4

MRS5698 was incubated in pH 1.6 stimulated gastric fluid (SGF) and pH 6.5 stimulated intestinal fluid (SIF) at 37 °C. At the appropriate time intervals, the depletion of MRS5698 was monitored by analyzing its concentration using LCMS/MS

yield for 14 steps starting from compound **1** was 0.45 %. Although our previous synthetic route has a similar overall

yield, the starting material was much more costly, and the synthesis was not suitable for scale-up.

Table 2 In vitro ADME-tox parameters

Property	Units	Value	Preferred
Solubility (pH 7.4, DPBS)	(μg/mL)	1.60	>60
Stability—microsomes (human)	t _{1/2} (min)	>60	>30
	CL _{int} (mL/min/mg)	0.00919	<10
	% remaining at 60 min	76.2	–
	t _{1/2} (min)	>60	>30
Stability—microsomes (male rat)	CL _{int} (mL/min/mg)	0.00919	<10
	% remaining at 60 min	77.8	–
	t _{1/2} (min)	>60	>30
	CL _{int} (mL/min/mg)	0.00	<10
Stability—microsomes (male mouse)	% remaining at 60 min	100	–
	t _{1/2} (min)	>120	>120
	CL _{int} , μL/min/10 ⁶ cells	N/A	<5
	% remaining at 2 h	100 %	>80 %
Stability—plasma (mouse, rat)	% remaining at 24 h	100 %	>80 %
Stability—solution (pH 7, aqueous)	% remaining at 4 h	100 %	>80 %
Stability—simulated intestinal fluid	% remaining at 2 h	100 %	>80 %
Stability—simulated gastric fluid	% inhibition at 3 μM	<10 %	<15 %
CYP450 inhibition (2C9, 2D6, 3A4 isozymes)	IC ₅₀ (μM)	22 (2C9), 40 (2D6), 47 (3A4)	>10
	C _{max} at MED/K _i	<0.01	<0.1
Plasma protein and tissue binding (mouse)	F _{u, plasma} (%)	99.88	
Plasma protein and tissue binding (rat)	F _{u, plasma} (%)	99.78	
Permeability—Caco-2 ^a	P _{app} (A–B, 10 ^{–6} cm/s)	0.08	>10
	P _{app} (B–A, 10 ^{–6} cm/s)	7.26	
	Efflux ratio	86.4	<3
	IC ₅₀ (μM)	11.9	>10
hERG—(fluorescence-based membrane potential, HEK cells) ^b	IC ₅₀ /free C _{max}	58	>30
Free C _{max} —plasma	Total C _{max} (μM) × F _{u, plasma}	6.02	
Ames screening	Positive/negative	Negative	Negative

^a Recovery from both chambers was >80 %

^b hERG ion channel inhibition assay performed by Psychoactive Drug Screening Program (PDSP, <http://pdspdb.unc.edu/>)

Table 4 Chemical stability of MRS5698 in rat and mouse plasma

Species	Compound	Remaining percentage (%)					
		0 min	15 min	30 min	45 min	60 min	120 min
Rat	Propantheline	100.0	100.3	97.7	100.5	96.2	83.0
	MRS5698	100.0	103.1	102.7	101.5	104.8	104.3
Mouse	Propantheline	100.0	77.4	50.1	30.2	19.7	3.95
	MRS5698	100.0	100.1	98.2	99.2	101.4	100.1

MRS5698 was incubated in SGF and SIF at 37 °C. At the appropriate time intervals, the depletion of MRS5698 was monitored by analyzing its concentration using LCMS/MS

The previous route used an intramolecular cyclopropanation of a diazo derivative to form the bicyclic ring system [23]. We presently use Zhan catalyst-1B (Hoveyda–Grubbs type catalyst) to convert **6** to **7** [24] by a ring closure metathesis reaction. This is followed subsequently by cyclopropanation using diethyl zinc in dichloromethane to form protected bicyclic intermediate **10**. A Mitsunobu reaction on **10** with using purine intermediate **20**, prepared as shown in Fig. 2, was performed to give the nucleoside precursor **11**. The *N*⁶-(3-chlorobenzyl) group was then introduced to give compound **12**, which was prepared in 10 steps from D-ribose. The TBDPS group was removed using tetrabutyl ammonium fluoride, and the resulting compound **13** was oxidized with PDC to give compound **14**. 2-Iodo 5'-carboxylate intermediate **14** was condensed with methylamine using a carbodiimide in THF in the presence of HOBt to provide a C2-iodo derivative of a (*N*)-methanocarba adenosine **15**. The arylethynyl group was introduced by a Sonogashira reaction of **15** with 3,4-difluorophenylacetylene **23** (Fig. 2) in the presence of PdCl₂(Ph₃P)₂, CuI, and triethylamine in DMF to give **16** in high yield. Finally, hydrolysis of the 2',3'-isopropylidene protecting group of **16** afforded the target nucleoside MRS5698 **17**.

In vitro ADME-Tox studies

MRS5698 was evaluated for its stability in simulated biological fluids (SIF at pH 1.6 and SGF at pH 6.5), mouse and rat plasma, microsomal, hepatocyte, plasma protein binding (mouse, rat), CYP450 inhibition (2C9, 2D6, 3A4), CaCO-2 permeability, human ether-à-go-go-related gene (hERG)

inhibition, pharmacokinetic profile, and GLP bacterial reversed mutation assay (Ames test).

The Lipinski “Rule of 5” outlines the molecular properties preferred for oral bioavailability of experimental drugs based on the PK properties of known drugs [25]. These properties are: hydrogen bond donor <5, hydrogen bond acceptor <10, Log *P* <5, and molecular weight <500 daltons. Later, the predication criteria for better prediction of drug-likeness were extended; log *P* −0.4 to +5.6, molar refractivity of 40 to 130, number of atoms in the range of 20 to 70, and polar surface area <140 Å² [26]. Furthermore, Veber et al. analyzed the properties of 1100 drug candidates with oral bioavailability in rats and concluded that reduced molecular flexibility, as measured by the number of rotatable bonds and low polar surface area or total hydrogen bond count (sum of donors and acceptors) are found to be important predictors of good oral bioavailability, independent of molecular weight [27]. The commonly applied molecular weight cutoff at 500 does not itself significantly separate compounds with poor oral from acceptable bioavailability. Tables 1 and 2 summarize the chemical properties of the MRS5698, which satisfy most of the typical drug-like characteristic. Although it has molecular weight >500, we believed that MRS5698 with a relatively low clogP (2.65), number of rotatable bonds (6), polar surface area (121.9 Å²), and total hydrogen bond count (10) would be sufficient for an in vivo proof of concept (PoC) efficacy study. The solubility of MRS5698 was 1.6 µg/mL in pH 7.4 buffer. MRS5698 also showed an excellent chemical stability in SGF, SIF, and plasma (Tables 3 and 4). Furthermore, it was stable when incubated for 60 min at a concentration of 1 µM in the presence of rat, dog, or human liver microsomal fractions or hepatocytes (Table 5).

Table 5 Metabolic stability of MRS5698 in the presence of liver microsomes of three species

Compound	Remaining percentage at 60 min			CL _{int} (µL/min/mg proteins)		
	HLM	MRLM	MMLM	HLM	MRLM	MMLM
Verapamil	16.25	3.51	10.52	62.40	226.3	117.7
MRS5698	76.19	77.76	106.0	9.19	9.19	0.00*

10 µM of MRS5698 was incubated with pooled human and rat and mouse (male) liver microsomes (1 mg/mL) for 60 min

*No clearance detected

Table 6 Inhibition by MRS5698 of the activity of CYP450 isozymes

CYP	Substrate	IC ₅₀ MRS5698, μ M	% inhibition at 10 μ M MRS5698
2C9	Tolbutamide	22.19	28.42
2D6	Dextromethorphan	39.99	13.28
3A4	Midazolam	46.71	18.30

Reference compounds had the following IC₅₀ values (μ M): sulfaphenazole, 0.66 (2C9); quinidine, 0.11 (2D6); ketoconazole, 0.016 (3A4)

As shown in Table 6, MRS5698 was tested for inhibition of human liver microsomal CYP450s, and it exhibited no or poor inhibitory activities (IC₅₀ values) for the key CYP isoforms including CYP4A (47 μ M), CYP2C9 (22 μ M), and CYP2D6 (40 μ M). The compound needs to be evaluated in brain tissue. It was extremely stable in the presence of liver microsomes and was not toxic to liver cells in culture. Therefore, metabolite identification is not required at this point.

These results clearly predict the absence of clinically significant interactions between MRS5698 and other medicinal products that are metabolized by the above-mentioned human enzymes. MRS5698 was largely bound to plasma proteins (Table 7). CACO-2 bidirectional transport studies (Table 2 and supporting information) suggested intestinal efflux of MRS5698 (efflux ratio 86).

Since toxicity is one of the most common reasons for withdrawal of approved drugs, we used HepG2 cells for evaluation of cytotoxicity [29]. MRS5698 was evaluated in a large concentration range (1–100 μ M) for 72 h. An IC₅₀ value of 33 μ M suggested that these compounds do not trigger cell death pharmacologically relevant concentrations (Figs. 3 and 4). Other off-target effects of MRS5698 in the micromolar range at several receptors were reported [14]. We evaluated for inhibition of the hERG product in the hERG manual patch clamp functional assay. MRS5698 showed IC₅₀=12 μ M, Table 2), suggesting a low risk for cardiac arrhythmias via a prolongation of cardiac QT action potential, which is a major reason for compound attrition and withdrawal from the market. The mutagenic potential of MRS5698 was determined by the non-GLP bacterial reversed mutation assay (Ames test, Table S1). The test uses histidine-dependent strains of *Salmonella typhimurium* TA97, TA98, TA100, and TA102. TA97 and TA98 strains are used for the detection of frame-shift mutations; the TA100 strain is used for the detection of

base-pair substitution mutations, while the TA102 strain is used for transitions or transversions. The mutational frequency is expressed as the quotient of the number of revertant colonies in the presence of the test compound or a positive control over the number of revertant colonies in the presence of the negative control (DMSO). The mutational frequency is less than 1.6 at any given concentration of MRS5698 with TA97, TA98, TA100, and TA102 strains in the absence or presence of metabolic activation, and there is not an apparent dose–effect relationship. The data suggests that MRS5698 does not have any significant mutagenic potential against strains TA97, TA98, TA100, and TA102 under current test conditions.

In vivo pharmacokinetics, pharmacodynamics, and toxicology studies

The MTD and PK of MRS5698 were studied and its efficacy measured quantitatively in a standard model of prolonged neuropathic pain. For in vivo PK and efficacy studies, we have developed a suitable formulation (40 % PEG300 and 10 % Solutol in “10 % HP- β -CD in water”) for both oral and parenteral administration. We did not analyze possible metabolites of MRS5698, some of which might be biologically active.

Pharmacokinetic studies

The pharmacokinetic evaluation of MRS5698 administered at three different doses was carried out in CD1 mice (Fig. 5), specifically i.v. (0.2 mg/kg), i.p. (1.0 mg/kg), and p.o. (3.0 mg/kg) administrations. The plasma concentrations of MRS5698 were below the detection limit 2 h after the i.v. administration and 8 h after i.p. and p.o. administration. The plasma pharmacokinetic parameters were derived from the compound concentration profiles using a non-compartmental method. The pharmacokinetic parameters are shown in Table 2. The central volume of distribution (V_{ss_obs}) was 700 mL/kg for the i.v. administration. The in vivo compound half-lives (t_{1/2}) were on average 26 min for i.v. administration, 65.5 min for i.p. administration, and 75.5 min for p.o. administration. Bioavailability (*F*) was on average only 4.75 % for p.o. administration and 29.6 % for i.p. administration. A less than optimal pharmacokinetic profile of MRS5698 may be

Table 7 Binding of MRS5698 in plasma protein of two species

Compound	Mouse plasma		Rat plasma	
	% bound	% recovery	% bound	% recovery
Ketoconazole	99.10	91.01	99.42	99.51
MRS5698	99.88	92.93	99.78	101.11

The measured % protein binding and recovery results of MRS5698 and control compound ketoconazole in mouse and rat plasma

Fig. 3 HepG2 cells for evaluation of cytotoxicity of **a** MRS5698 compared to **b** paclitaxel

due to its poor solubility and intestinal permeability. Therefore, its structure is currently being optimized to improve these parameters, while maintaining the favorable *in vivo* potency and safety profile. Additionally, a formulation of MRS5698 will be developed that will improve oral absorption; alternatively, appropriate delivery technology independent of oral adsorption will be adopted for preclinical and clinical studies.

In vivo toxicology

Following a single dose of MRS5698 in Sprague–Dawley rats via intraperitoneal administration at 10 mg/kg, or via oral administration at doses up to 200 mg/kg, there were no treatment-related, toxicologically significant effects on body weight, body weight gain, or clinical signs. The administered dose did not exceed 10 mg/kg (i.p.) and 200 mg/kg (p.o.) due to solubility limitation of the compound, and the MTD limit was not precisely defined.

Thus, the MTD of MRS5698 was considered to be ≥ 200 mg/kg, because no mortality was observed in the studies. Following a single dose of MRS5698 in

Sprague–Dawley rats at 10 mg/kg i.p. or oral administration up to 200 mg/kg, the treatment resulted in locomotor depression and other non-toxicologically significant transient clinical signs at 150 mg/kg. At the highest dose tested, 200 mg/kg, there were no changes in body weight or toxic clinical signs. Treatment with MRS5698 at 150 mg/kg p.o. resulted in increased activity at 10 min post-dose and decreased activity at 30 min post-dose in all animals, excessive scratching at 10 min post-dose in one male and one female, and weakness in one male.

We have earlier reported that MRS5698 showed excellent efficacy achieving complete reversal at its peak effect at 1 mg/kg (i.p.) in rats and 1.7 mg/kg (p.o.) in mice [15, 30], suggesting that MRS5698 has an acceptable therapeutic index.

In vivo efficacy studies

We have already reported that of the highly selective A_3AR agonist ($\geq 10^4$ -fold over human and mouse A_1AR or $A_{2A}AR$ [12]), MRS5698 reduces or prevents the development of chronic neuropathic pain in several animal models [10, 15,

Fig. 4 Efficacy of MRS5698 to reverse mechano-allodynia in the CCI model in male Sprague–Dawley rats, showing time-response curve for oral administration (100 mg/kg, p.o.), using the same vehicle as used for PK measurement (Fig. 5). Here, the agonist was given by oral gavage on day 7 after sciatic nerve ligation, i.e., the time when peak pain develops. The paw withdrawal threshold (PWT) in response to mechanical challenge on the ipsilateral hind paw was measured. At this time, a higher than control PWT indicated efficacy in the CCI model

Fig. 5 PK measurement of MRS5698 administered as solution doses in the CD1 mouse. The PK parameters were determined using the following doses: i.v. (0.2 mg/kg), i.p. (1.0 mg/kg), and p.o. (3.0 mg/kg). The PK parameters were calculated as follows: $t_{1/2}$ (h): 0.434 (i.v.), 1.09 (i.p.), 1.26 (p.o.); $AUC_{0-\infty, total}$ (h $\times$ ng/mL): 140 (i.v.), 213 (i.p.), 102 (p.o.); CL (mL/min/kg): 23 (i.v.); C_0 (ng/mL): 338 (598 nM); $C_{max, total}$ (ng/mL): 115 (204) for i.p. (T_{max} 1 h), 51.3 (91) for p.o. (T_{max} 0.5 h); %F: 29.6 % (i.p.), 4.75 % (p.o.)

30]. The efficacy of MRS5698 was measured quantitatively in a standard model of prolonged neuropathic pain. CCI in rats and mice produces mechano-allodynia as indicated by a reduction in the paw withdrawal threshold (PWT, g) in the ipsilateral paw as compared to baseline before surgery (D0). Previously, MRS5698 administered i.p. was shown to be efficacious in vivo in the mouse CCI model [16]. In the present study, we have selected a high dose of MRS5698 for in vivo efficacy testing, based on the high tolerability of the compound. Oral administration (100 mg/kg, 0.4 mL) at peak CCI-induced pain (D7) using the same vehicle as used for bioavailability studies (above) reversed established mechano-allodynia for 2 h before resolving by 5 h (Fig. 4). Contralateral paws did not differ from baseline at any time point (data not shown). The efficacy of a high dose of MRS5698 is significant not only in terms of the tolerability of the compound, because in inflammatory models other A₃AR agonists showed a bell-shaped dose–response curve [13].

These protective effects of A₃AR agonists were naloxone-insensitive and thus are not opioid receptor mediated [9]. MRS5698 was at least 1.6-fold more efficacious than morphine and over 5-fold more potent [15]. In addition, MRS5698 was equally efficacious to commonly used anti-neuropathic pain drugs, e.g., gabapentin (Neurontin®) or amitriptyline, but >350- and 75-fold more potent, respectively. Also, in the CCI model of hyperalgesia induced by cancer chemotherapeutic agents, A₃ receptor agonists both prevent the development of pain and reverse pain already present on day 7 [10]. Aside from its potent stand-alone ability to reverse established mechano-allodynia, MRS5698 significantly increased the anti-allodynic effects of all three analgesics. A₃AR agonists including MRS5698 have no effect on normal pain thresholds and are non-narcotic and non-addictive and so avoid abuse and other side effects common to opioid pain management. The protective effects of A₃ agonists did not wane upon repeated dosing or delivery of a steady concentration using an implanted minipump [15].

Conclusions

We are the first to document that A₃ agonists protect against neuropathic pain [9]. The novel concept of using A₃AR agonists such as MRS5698 for treating chronic neuropathic pain is very attractive but needs additional preclinical characterization prior to a proposed clinical trial. Nevertheless, based on our initial, well-defined pharmacological studies in animals, the selective activation of this subtype of ARs has dramatic effects on relieving neuropathic pain. Although the molecular weight is higher than generally preferred, MRS5698 displays good efficacy and bioavailability.

We have developed a synthetic route for MRS5698 that is suitable for scale-up production. We have also developed

analytical and bioanalytical methods for MRS5698 and chemistry for the 100 g scale, which can potentially be further optimized for GMP and non-GMP manufacturing. The data obtained thus far looks very promising for drug development being well tolerated in vivo and of low toxicity, but additional testing, including IND-directed toxicology, is needed. In the initial PK testing in mouse, the compound was found to be of relatively low bioavailable ($F=5\%$ p.o., 30% i.p.). Nevertheless, MRS5698 was highly efficacious in vivo when administered by oral gavage in the mouse, likely due to the nM potency of this agonist. Future suggested studies include the development of suitable formulations for human studies and for improving oral bioavailability, additional PK and ADME studies (especially in larger animals), IND-directed toxicology, and scaled-up manufacture of clinically directed supplies of MRS5698.

In conclusion, MRS5698 has shown an excellent safety profile in vitro and in vivo and protects against chronic neuropathic pain in vivo at a very high dose. We anticipate that the development of an effective formulation that improves intestinal permeability will enhance absorption of the drug and its oral bioavailability.

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