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Design and synthesis of 5-[(2-chloro-6-fluorophenyl)acetylamino]-3-(4-fluorophenyl)-4-(4-pyrimidinyl)isoxazole (AKP-001), a novel inhibitor of p38 MAP kinase with reduced side effects based on the antedrug concept

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ABSTRACT

Inhibitors of p38 mitogen-activated protein (MAP) kinase, which are closely involved in the production of inflammatory cytokines, are considered promising curative drugs for chronic inflammatory disorders. However, there is also a growing concern regarding its systemic side effects. To reduce the occurrence of side effects, we have identified a novel p38 MAP kinase inhibitor that shows properties of an antedrug, which imparts its effect solely on the inflammatory site and is metabolically inactivated right after. We have designed isoxazole derivatives through the addition of a fresh interacting fourth site to the structure of the prototypical p38 MAP kinase inhibitor that harbors three point interactive sites. The derivative **26d** (AKP-001) shows excellent p38 MAP kinase inhibitory activity and a high selectivity for various kinases. Its rapid metabolism has been confirmed in rats. Moreover, **26d** has been shown to be effective in animal models of inflammatory bowel disease.

1. Introduction

Inflammatory bowel disease (IBD) is a collective name for intestinal disorders that include Crohn's disease (CD) and ulcerative colitis (UC). These intestinal inflammatory and intractable diseases of unknown etiology are characterized by repetitive periods of activity and remission. The incidence of IBD has steadily increased in recent years, predominantly affecting young adults, thus resulting in the deterioration of their quality of life (QOL) during the acute period and forcing diet regulations even during remission. Treatment regimens for IBD include 5-aminosalicylic acid (5-ASA) formulations such as mesalazine,[1] which is used mainly during the remission period, and steroids such as prednisolone, which is generally administered during the active period. In case of the former, however, a high dose is required to observe an effect, whereas the latter is associated with various side effects or may result in drug dependency. Tumor necrosis factor-α (TNF-α) inhibitors such as infliximab and adalimumab have been clinically used and have shown excellent results,[2,3] although these are relatively costly and their route of administration has been described as not very ideal. Based on these conditions, there is a need to develop a small molecule drug of novel function that shows excellent efficacy and reduced side effects.

Mitogen-activated protein kinases (MAPKs) represent a class of serine/threonine kinases that are widely distributed across different cell types and play an important role in various cell functions. Of these, p38 MAP kinase is involved in the signaling pathway that regulates cellular responses to cytokines or stress. Its activation is responsible for the formation of inflammatory cytokines such as TNF-α or interleukin 1β (IL-1β). Accordingly, the p38 MAPK inhibitor acts as a therapeutic drug for the treatment of chronic inflammatory diseases such as rheumatic arthritis (RA) by regulating the secretion of cytokines. Many inhibitors have been reported in literature. Based on its structure and interaction with p38 MAP kinase, these inhibitors can be divided into 3 types (Fig. 1).[4] Compounds that competitively interact with the ATP-binding site are classified as prototypes (teardrop binders) such as SB203580,[5] and

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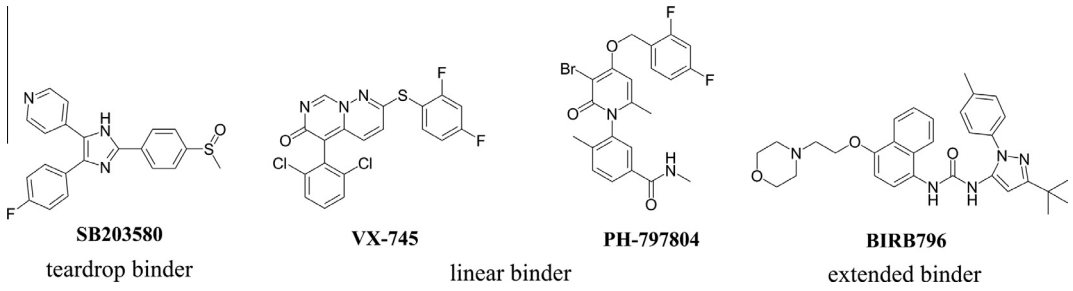


Figure 1. Typical inhibitors of p38 MAP kinase.

linear binders such as VX-745,[6,7] and PH-797804.[8–10] Teardrop binders carry a 5-membered heterocyclic ring as the nucleus and characteristically forms hydrogen bonds with Lys53. Extended binders, represented by BIRB796,[11] undergo allosteric binding with a p38 MAP kinase that results in an inactive DFG-out conformation. p38 MAP kinase is involved not only in the production of inflammatory cytokines, but also in cell differentiation and proliferation, as well as cell-cycle regulation or apoptosis. It is ubiquitously expressed in various systems of the body and thus, long-term exposure to inhibitors may result in toxicity.[12,13] Although not all cases are class effects, adverse events such as hepatotoxicity, cardiac toxicity, and disorders involving the central nervous system have been reported, prompting authorities to terminate its development.[14] Leftheris et al. have previously reported that p38 MAP kinase inhibitors contain a triazine structure that has a short duration of action, which suggests that its adverse effects can possibly be reduced.[15] Recently, Pfizer released the p38 MAPKs inhalant drug, PF-03715455, for the treatment of respiratory diseases.[16] Although the site of drug application was different, we agree with the study that minimizing the exposure of the rest of the body by using a drug with high clearance and low absorption is extremely imperative. Most anti-inflammatory drugs that have been designed with reduced side effects by limiting its site or period of action are steroids that are commonly used in the area of dermatology. After imparting its effect on the disease site, steroids are rapidly inactivated during metabolism. This reaction has served as the foundation of the concept of an antedrug.[17] We believe that anti-inflammatory drugs showing the properties of an antedrug may be applicable to not only the skin or specific respiratory regions, but also to inflammatory bowel disease. Therefore, we have attempted to design p38 MAP kinase inhibitors that exclusively follows a topical action.

2. Design concept

The X-ray crystal structure of SB203580, which is a representative of the prototypical p38 MAP kinase inhibitor, has been previously reported (Fig. 2).[18] SB203580 interacts with the ATP-binding site of p38 MAP kinase, and a hydrogen bond is formed between the nitrogen of the imidazole 3-position and Lys53. Its *p*-fluorophenyl group interacts with a lipophilic pocket (hydrophobic region I) consisting of Thr106 (gatekeeper residual group), Leu75, Leu86, and Leu104 through the formation of van der Waals bonds. In addition, the nitrogen atom from the pyridyl group interacts with the main chain of Met109 through the formation of hydrogen bonds. According to the same report, SB218655 has a cyclopropylmethyl group at the 1-position of the imidazole structure, and the cyclopropylmethyl group interacts with pockets formed by Val30, Tyr35, and Val38, and that is the cavity of the phosphate-binding ribbon. SB220025, which carries a piperidine ring, also interacts with Tyr35 of the phosphate-binding ribbon and Asp168 at the C terminal position. Moreover, Wilson et al. has also shown that VK-19911 interacts with p38 MAP kinase in a similar manner to them.[19] Relatively larger pockets (hydrophobic region II) formed by Met109, Ala111, Asp112, Ala157, and Leu167 also exist relatively close to the phosphate-binding ribbon with which the 1-position side chain interacts.[20–22] Based on this information, by considering the presence of amino acid residues that have the capability of interacting and a definite spatial tolerance at a position corresponding to the direction of the 1-substitution position of imidazole of these compounds in p38 MAP kinase, new compounds were designed to further increase the inhibition and adapted for application as antedrugs. Thus, compound **A** was designed on the basis of the pharmacophore obtained by adding one fresh point-of-interaction site to the prototypical structure of p38 MAP kinase that has a 5-membered ring as its core and represented by SB203580, which possesses 3-point interaction sites (presumed interaction sites: Met109, Lys53, and hydrophobic region I) (Fig. 3). The concept of this compound **A** is as follows: (1) once the drug reaches the inflammation site through an enteric-coated preparation, an excellent topical anti-inflammatory effect showing high inhibitory activity is exercised by a strong interaction mainly at the 4 points, with p38 MAP kinase produced in the intestines; and (2) once the drug is absorbed and circulates through the blood or reaches the liver, the part of the structure containing the fourth interaction site undergoes cleavage and releases metabolite B, resulting in the suppression of the side effects originating from p38 MAP kinase because of a considerable reduction in its inhibitory activity. By using pyrazole and isoxazole as basic structures, a substituent group was introduced into the site that was equivalent to the imidazole 1-position of the compound previously mentioned or into the 5-position of the individual structures.

3. Materials and methods

3.1. Chemistry

Melting points were determined on a micro hot plate melting point apparatus of Mettler-Toledo (Central Processor: Mettler FP80HT, Hot Stage: Mettler FP82HT) and were uncorrected. Proton NMR (^{1}H NMR) spectra were recorded on a JEOL JNM-ECP 400. Chemical shifts (δ) are expressed in parts per million using tetramethylsilane as the internal standard. Mass spectra (MS) were obtained on a Shimadzu GC/MS QP-5000 mass spectrometer system, with electrospray ionization methodology. Infrared spectra were recorded on JASCO FT/IR-470. High-resolution mass spectra (ESI-HRMS) were obtained on Waters XevoQ-TOF. For normal pressure and flash column chromatography, Wakogel® C-200 (100–200 mesh) was used. The purity of the final compounds was determined by HPLC on Shimadzu HPLC system (Liquid chromatograph: LC-10AD, System Controller: SCL-10A, Chromatopac: C-R7Aplus, UV Spectrophotometric detector: SPD-10A, Autoinjector: SIL-10A).

3.1.1. 3-(4-Fluorophenyl)-4-(4-pyridyl)pyrazole (2)

To a solution of 3-dimethylamino-1-(4-fluorophenyl)-2-(4-pyridyl)-2-propen-1-on (**1**) (3.21 g, 11.9 mmol) in ethanol (60 mL) was added hydrazine monohydrate (2.99 g, 2.9 mL, 59.7 mmol)

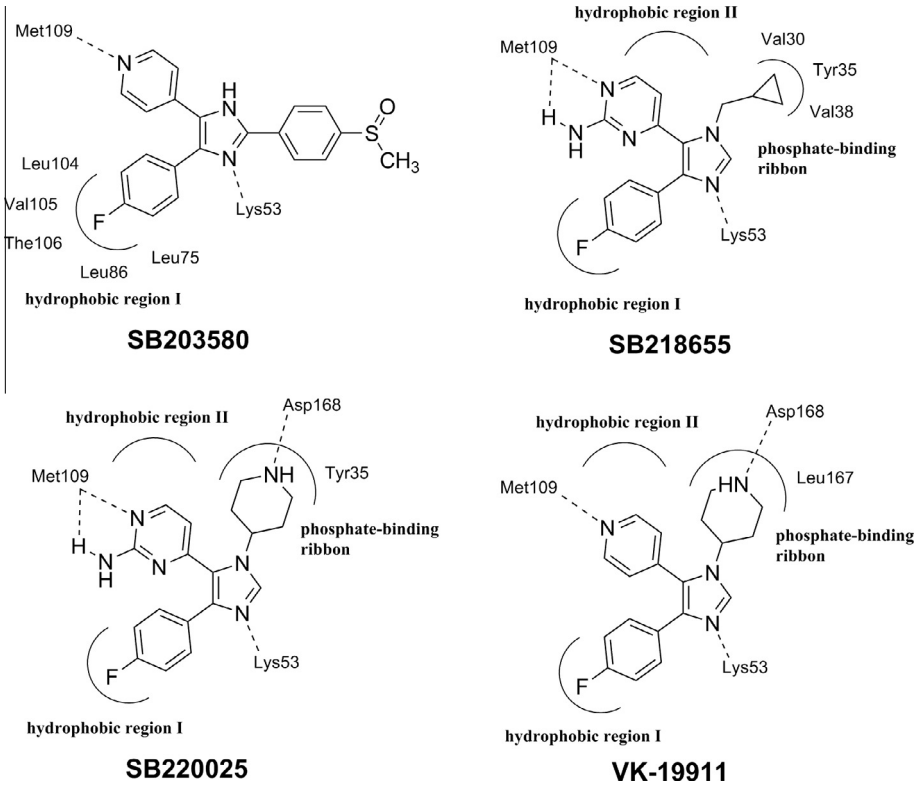


Figure 2. Important interactions between the prototypical p38 MAP kinase inhibitor and the ATP-binding site of p38: SB203580, SB218655, SB220025 and VK-19911.

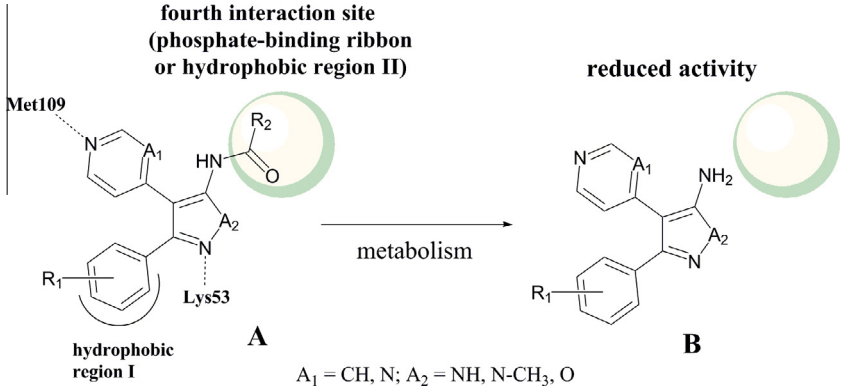


Figure 3. Strategy for antedrugs of prototypical p38 MAP kinase inhibitors.

and the mixture was heated to reflux for 2 h. Then cooled to room temperature, the solvent was removed in vacuo, the residue was added water and extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated. The crude product was recrystallized from ethyl acetate to give the title compound **2** (2.48 g, 86%). Mp: 208.5–209 °C; ^{1}H NMR ($CDCl_3$) δ: 8.51 (dd, J = 1.5 and 4.5 Hz, 2H), 7.82 (s, 1H), 7.5–6.9 (m, 6H); IR (KBr) 2840, 1606, 1518, 1222, 834, 814 cm^{-1}; MS (EI): m/z 239 (M^+).

3.1.2. 3-(4-Fluorophenyl)-4-(4-pyridyl)-1-(1-pyrrolidinomethyl) pyrazole (3)

To a solution of 3-(4-fluorophenyl)-4-(4-pyridyl)pyrazole (**2**) (1.66 g, 6.94 mmol) and 37% formaldehyde solution (0.42 g, 1.05 mL, 14.0 mmol) in ethanol (20 mL) was added pyrrolidine (1.00 g, 1.17 mL, 14.0 mmol), the resulting mixture was heated to reflux for 5 h. Then cooled to room temperature, the solvent was removed in vacuo, the residue was added water and extracted with ethyl acetate. The organic layer was washed with water, followed by brine and then dried over $MgSO_4$. The solvent was removed under vacuum to give the title compound **3** (2.17 g, 97%). ^{1}H NMR ($CDCl_3$) δ: 8.49 (dd, J = 1.6 and 4.5 Hz, 2H), 7.70 (s, 1H), 7.90–7.60 (m, 6H), 5.08 (s, 2H), 2.90–2.50 (m, 4H), 2.0–1.6 (m, 4H); IR (KBr) 1602, 1222, 1142 cm^{-1}; MS (EI): m/z 239 (M^+–83); HRMS (ESI) calcd for $C_{14}H_{11}FN_3$ $[M+H]^+$ 240.0937, found 240.0948.

3.1.3. 3-(4-Fluorophenyl)-5-(1-hydroxy-3-phenylpropyl)-4-(4-pyridyl)pyrazole (4)

3-(4-Fluorophenyl)-4-(4-pyridyl)-1-(1-pyrrolidinomethyl)pyrazole (**3**) (3.89 g, 12.1 mmol) was dissolved in dry THF (100 mL). To stirred the solution, 1.6 M n-butyllithium in hexane (8.3 mL, 13.3 mmol) was added dropwise while keeping the temperature at −70 °C or lower. The solution was stirred for 30 min followed by addition 3-phenylpropionaldehyde (1.77 g, 13.2 mmol) in THF

(15 mL) at the same temperature, then the resulting solution was stirred while rising to room temperature gradually. After 1 h, 2 M HCl was added to the solution and stirred for 10 min, then saturated $NaHCO_3$ aqueous solution was added until alkaline and separated the organic layer. After the aqueous layer was extracted with ethyl acetate, those organic layers were combined and washed with brine, dried over $MgSO_4$, then evaporated. The resulting residue was purified by column chromatography (eluent: ethyl acetate) to afford the title compound **4** (2.27 g, 51%). ^{1}H NMR ($CDCl_3$) δ: 8.43 (dd, J = 1.5 and 4.6 Hz, 2H), 7.4–6.8 (m, 11H), 5.0–4.7 (m, 1H), 5.0–4.0 (br s, 1H), 2.69 (t, J = 7.3 Hz, 2H), 2.2–1.9 (m, 2H); IR (KBr) 3160, 2880, 1602, 1518, 1220, 968, 834 cm^{-1}; MS (EI): m/z 373 (M^+); HRMS (ESI) calcd for $C_{23}H_{21}FN_3O$ $[M+H]^+$ 374.1669, found 374.1666.

3.1.4. 3-(4-Fluorophenyl)-5-(3-phenyl-1-propenyl)-4-(4-pyridyl) pyrazole (5)

A mixture of 3-(4-fluorophenyl)-5-(1-hydroxy-3-phenylpropyl)-4-(4-pyridyl)pyrazole (**4**) (0.373 g, 1.00 mmol), 4-toluenesulfonic acid monohydrate (0.399 g, 2.10 mmol) and toluene (10 mL) was heated to reflux for 24 h. After cooling, a saturated $NaHCO_3$ aqueous solution was added until alkaline and extracted with a mixture of chloroform and methanol (9:1). The organic layer was washed with brine, dried over $MgSO_4$, then evaporated. The resulting residue was purified by column chromatography (eluent: chloroform), then recrystallized from ethyl acetate to give the title compound **5** (0.160 g, 45%). Mp: 204–205.5 °C; ^{1}H NMR ($CDCl_3$) δ: 8.55 (dd, J = 1.6, 4.5 Hz, 2H), 7.5–6.7 (m, 11H), 6.4–6.1 (m, 2H), 3.52 (d, J = 5.1 Hz, 2H); IR (KBr) 3220, 1600, 1516, 1442, 1220, 974, 838, 828 cm^{-1}; MS (EI): m/z 355 (M^+); HRMS (ESI) calcd for $C_{23}H_{21}FN_3$ $[M+H]^+$ 356.1563, found 356.1556.

3.1.5. 3-(4-Fluorophenyl)-5-(3-phenylpropyl)-4-(4-pyridyl) pyrazole (6)

To a solution of 3-(4-fluorophenyl)-5-(3-phenyl-1-propenyl)-4-(4-pyridyl)pyrazole (**5**) (0.100 g, 0.28 mmol) in ethanol (30 mL) was added 5% palladium on carbon (50 mg). The mixture was stirred under hydrogen atmosphere at 1 atm and room temperature for 15 h. The catalyst was removed by filtration through Celite and the filtrate was evaporated in vacuo. To the resulting residue was added diethyl ether, the formed precipitate was collected by filtration to afford the title compound **6** (60 mg, 60%). Mp: 155.5–156.5 °C; ^{1}H NMR ($CDCl_3$) δ: 8.52 (dd, J = 1.5, 4.4 Hz, 2H), 7.4–6.8 (m, 11H), 2.9–2.5 (m, 4H), 2.2–1.7 (m, 2H); IR (KBr) 2920, 1602, 1510, 1226, 830 cm^{-1}; MS (EI): m/z 357 (M^+); HRMS (ESI) calcd for $C_{23}H_{21}FN_3$ $[M+H]^+$ 358.1720, found 358.1728.

3.1.6. *tert*-Butyl 1-methylhydrazinecarboxylate (8)

Methylhydrazine (**7**) (26 g, 564 mmol) and sodium hydroxide (23 g, 575 mmol) was dissolved in methanol (500 mL). To the solution, di-*tert*-butyl dicarbonate (123 g, 564 mmol) in methanol (500 mL) was added dropwise over a period of 2 h at 0 °C. Then the reaction solution was stirred at room temperature for 2 h, the deposit was removed by filtration through Celite and the filtrate was evaporated in vacuo. The oily residue was added water and neutralized with aqueous solution of ammonium chloride. The solution was extracted with dichloromethane, dried over $MgSO_4$, then evaporated to give the title compound **8** (79 g, 97%). ^{1}H NMR ($CDCl_3$) δ: 4.06 (br, 2H), 3.05 (s, 3H), 1.47 (s, 9H); IR (neat) 1694, 1365, 1154 cm^{-1}.

3.1.7. 3-(4-Fluorophenyl)-3-oxo-2-(4-pyridyl)propionitrile (11)

To a solution of 4-pyridylacetonitrile (**9**) (9.6 g, 81.3 mmol) and 2,5-dioxopyrrolidinyl 4-fluorobenzoate (**10**) (19.3 g, 81.4 mmol) in dimethylformamide (200 mL) was added potassium carbonate (11.2 g, 81.0 mmol), then stirred at room temperature for 24 h. The reaction solution was filtered off by Celite and the filtrate was evaporated in vacuo. The residue was added water and neutralized with aqueous solution of HCl. The formed precipitate was collected by filtration and washed with water, followed by diethyl ether and dried in a circulation dryer to afford the title compound **11** (16.3 g, 84%). Mp: 220.0–223.0 °C; ^{1}H NMR (CD_3OD) δ: 8.23–7.97 (m, 4H), 7.80–7.61 (m, 2H), 7.12 (t, J = 8.9 Hz, 2H); IR (KBr) 2180, 1636, 1608, 1542, 1492, 1408, 1374, 1342, 1222, 1202, 1156, 830 cm^{-1}; MS (EI): m/z 240 (M^+).

3.1.8. 5-Amino-3-(4-fluorophenyl)-4-(4-pyridyl)pyrazole (12)

A mixture of 3-(4-fluorophenyl)-3-oxo-2-(4-pyridyl)propionitrile (**11**) (11.24 g, 46.8 mmol) and phosphorus oxychloride (181 g, 110 mL, 1180 mmol) was stirred at 100 °C for 1 h. After excess phosphorus oxychloride was removed in vacuo, toluene was added to the residue, and evaporated. Then the residue was dissolved in ethanol (80 mL). To the solution was added hydrazine monohydrate (7.2 g, 7.0 mL, 144 mmol) and the mixture was heated to reflux. After 3.5 h, the mixture was evaporated in vacuo, and sat. $NaHCO_3$ was added to the residue. The solution was extracted with a mixture of chloroform and methanol. The organic layer was dried over $MgSO_4$, and evaporated. The resulting residue was washed with ethyl acetate to give the title compound **12** (6.40 g, 54%). Mp: 267.0–272.0 °C; ^{1}H NMR (DMSO-d_6) δ: 8.41 (d, J = 5.8 Hz, 2H), 7.37–7.03 (m, 6H), 5.26 (br s, 1H), 4.73 (br s, 1H); MS (EI): m/z 254 (M^+); HRMS (ESI) calcd for $C_{14}H_{12}FN_4$ $[M+H]^+$ 255.1046, found 255.1049.

3.1.9. 3-(4-Fluorophenyl)-5-phenylacetylamino-4-(4-pyridyl)pyrazole (13)

To a suspension of 5-amino-3-(4-fluorophenyl)-4-(4-pyridyl)pyrazole (**12**) (0.254 g, 1.0 mmol) in THF (20 mL) was added triethylamine (0.306 g, 3.0 mmol). After phenylacetyl chloride (0.464 g, 3.0 mmol) in THF (5 mL) was added dropwise to the suspension, the mixture was stirred at room temperature for 3 h. Then water was added to the mixture and the aqueous solution was extracted with ethyl acetate. The organic layer was washed with water, dried over $MgSO_4$, then evaporated. After the resulting residue was dissolved in a mixed solution 2 M NaOH aqueous solution (2 mL) and methanol (20 mL), the mixture was stirred at room temperature for 1 h. The formed precipitate was collected by filtration and suspended in a mixed solution 2 M NaOH aqueous solution (2 mL) and methanol (20 mL) once again, the mixture was heated at reflux for 5 h. After cooling, the reaction mixture was evaporated under reduce pressure. The residue was purified by column chromatography (eluent: chloroform/methanol = 30:1) to give the title compound **13** (0.080 g, 22%). Mp: 289.2–290.4 °C; ^{1}H NMR (DMSO-d_6) δ: 13.25 (br s, 1H), 9.93 (br s, 1H), 8.34 (d, J = 5.9 Hz, 2H), 7.50–7.06 (m, 9H), 7.01 (d, J = 5.9 Hz, 2H), 3.56 (s, 2H); IR (KBr) 1694, 1600, 1586 cm^{-1}; MS (EI): m/z 372 (M^+); HRMS (ESI) calcd for $C_{22}H_{18}FN_4O$ $[M+H]^+$ 373.1465, found 373.1458.

3.1.10. 5-Amino-3-(4-fluorophenyl)-1-methyl-4-(4-pyridyl)pyrazole (14)

A mixture of 3-(4-fluorophenyl)-3-oxo-2-(4-pyridyl)propionitrile (**11**) (2.0 g, 8.33 mmol) and phosphorus oxychloride (16.45 g, 10 mL, 107 mmol) was stirred at 100 °C for 1 h. After excess phosphorus oxychloride was removed in vacuo, the residue was dissolved in ethanol (150 mL). To the solution was added *tert*-butyl 1-methylhydrazinecarboxylate (**8**) (3.5 g, 20.5 mmol) and the mixture was heated to reflux. After 1.5 h, the reaction solution was added trifluoroacetic acid (4.44 g, 3 mL, 38.9 mmol) and the mixture was stirred at 100 °C. After cooling, the mixture was evaporated in vacuo, then water was added to the residue and neutralized with $NaHCO_3$. The solution was extracted with chloroform and dried over $MgSO_4$, then evaporated. The resulting residue was

purified by column chromatography (eluent: chloroform/methanol = 50:1–20:1) to give the title compound **14** (1.5 g, 62%). Mp: 155.2–157.9 °C; ^{1}H NMR ($CDCl_3$) δ: 8.53 (dd, J = 1.5, 4.4 Hz, 2H), 7.50–6.83 (m, 4H), 7.08 (dd, J = 1.5, 4.4 Hz, 2H), 3.77 (br s, 2H), 3.77 (s, 3H); IR (KBr) 1598, 1212 cm^{-1}; MS (EI): m/z 268 (M^+); HRMS (ESI) calcd for $C_{15}H_{14}FN_4$ $[M+H]^+$ 269.1202, found 269.1199.

3.1.11. 5-(4-Fluorophenyl)-1-methyl-3-phenylacetylamino-4-(4-pyridyl)pyrazole (15)

To a solution of 5-amino-3-(4-fluorophenyl)-1-methyl-4-(4-pyridyl)pyrazole (**14**) (0.398 g, 1.48 mmol) in THF (20 mL) was added triethylamine (0.165 g, 1.63 mmol). After the mixture was added dropwise phenylacetyl chloride (0.240 g, 1.55 mmol), the solution was stirred at room temperature overnight. Then water was added to the mixture, and extracted with chloroform. The organic layer was washed with water and dried over $MgSO_4$, then evaporated. The resulting residue was purified by column chromatography (eluent: chloroform/methanol = 40:1) and recrystallized from *n*-hexane/ethyl acetate to afford the title compound **15** (0.200 g, 35%). Mp: 164.9–166.5 °C; ^{1}H NMR ($CDCl_3$) δ: 8.40 (d, J = 5.9 Hz, 2H), 7.50–6.66 (m, 12H), 3.74 (s, 2H), 3.74 (s, 3H); IR (KBr) 1602, 1220 cm^{-1}; MS (EI): m/z 386 (M^+); HRMS (ESI) calcd for $C_{23}H_{20}FN_4O$ $[M+H]^+$ 387.1621, found 387.1619.

3.1.12. 4-Fluorobenzaldoxime (17)

To a solution of 4-fluorobenzaldehyde (**16**) (25.0 g, 201 mmol) and hydroxylamine hydrochloride (15.4 g, 222 mmol) in ethanol (47 mL) was added ice water (137 mL). Then 50% NaOH aqueous solution (42.8 mL, 1621 mmol) was added dropwise to the solution while keeping the internal temperature at 25–30 °C and the mixture was stirred at room temperature for 1 h. After the reaction solution was washed with diethyl ether, the aqueous layer was neutralized with concd HCl and extracted with chloroform. The organic extracts was washed with brine and dried over $MgSO_4$, then evaporated. The resulting residue was recrystallized from *n*-hexane/diethyl ether to afford the title compound **17** (24.3 g, 87%). Mp: 86.6–88.0 °C; ^{1}H NMR ($CDCl_3$) δ: 8.11 (s, 1H), 7.75 (s, 1H), 7.70–7.40 (m, 2H), 7.23–6.90 (m, 2H); MS (EI): m/z 139 (M^+).

3.1.13. 4-Fluoro-*N*-hydroxybenzimidoyl chloride (18a)

To a solution of 4-fluorobenzaldoxime (**17**) (19.4 g, 139 mmol) in DMF (140 mL) was added portion wise *N*-chlorosuccinimide (NCS) (18.9 g, 142 mmol) while keeping the internal temperature at 40 °C or lower. After the mixture was stirred at room temperature for 1.5 h, the reaction solution was poured into ice water, and extracted with diethyl ether. The extracts was washed with water and dried over $MgSO_4$, then evaporated. The resulting residue was recrystallized from *n*-hexane/diethyl ether to afford the title compound **18a** (23.4 g, 97%). Mp: 67.4–70.1 °C; ^{1}H NMR ($CDCl_3$) δ: 8.08 (s, 1H), 7.87 (dd, J = 2.2, 6.8 Hz, 1H), 7.14 (dd, J = 2.2, 6.8 Hz, 1H), 7.04 (dd, J = 2.2, 6.8 Hz, 1H); MS (EI): m/z 175 (M^++2).

3.1.14. 5-Amino-3-(4-fluorophenyl)-4-(4-pyridyl)isoxazole (19)

Sodium (2.92 g, 127 mmol) was dissolved in anhydrous ethanol (200 mL). To stirred the solution, 4-pyridylacetonitrile (**9**) (15 g, 127 mmol) in THF (200 mL) was added dropwise, then a solution of 4-fluoro-*N*-hydroxybenzimidoyl chloride (**18a**) (22.06 g, 127 mmol) in ethanol (200 mL) was added dropwise at 0 °C. The mixture was stirred at room temperature for 1 h. After the reaction solution was concentrated under reduced pressure, water was added to the residue, the formed precipitate was collected by filtration and dried under reduced pressure. The precipitate was washed with diethyl ether to yield the title compound **19** (31.86 g, 98%). Mp: 192.5–194.5 °C; ^{1}H NMR ($CDCl_3$) δ: 8.55 (dd, J = 1.8, 4.4 Hz, 2H), 7.50–6.90 (m, 4H), 7.05 (dd, J = 1.8, 4.4 Hz, 2H), 4.83 (br s, 2H); IR (KBr) 3460, 1644, 1606 cm^{-1}; MS (EI): m/z 255 (M^+); HRMS (ESI) calcd for $C_{14}H_{11}FN_3O$ $[M+H]^+$ 256.0886, found 256.0877.

3.1.15. 3-(4-Fluorophenyl)-4-(4-pyridyl)isoxazole (20)

Compound **20** was prepared from **19** according to the Laufer's method.[23]

3.1.16. Typical procedure for the preparation of 5-acylamino-3-(4-fluorophenyl)-4-(4-pyridyl)isoxazole (21)

3.1.16.1. 5-Acetylamino-3-(4-fluorophenyl)-4-(4-pyridyl)isoxazole (21a). To a solution of 5-amino-3-(4-fluorophenyl)-4-(4-pyridyl)isoxazole (**19**) (0.15 g, 0.59 mmol) and triethylamine (0.143 g, 0.197 mL, 1.41 mmol) in dichloromethane (10 mL) was added acetyl chloride (0.102 g, 0.092 mL, 1.30 mmol) at 0 °C, the mixture was stirred at 0 °C for 2 h. Then water was added to the solution and extracted with chloroform, dried over $MgSO_4$, then evaporated. After the resulting residue was dissolved in methanol (10 mL), 2 N NaOH aqueous solution (1 mL, 2.0 mmol) was added to the solution and stirred at room temperature for 30 min. The mixture was evaporated in vacuo, the residue was added water and neutralized with 2 N HCl aqueous solution. Then the formed precipitate was collected by filtration, washed with water, and dried under reduced pressure to afford the title compound **21a** (0.109 g, 63%). Mp: 189.5–194.0 °C; ^{1}H NMR (DMSO-d_6) δ: 11.00 (br s, 1H), 8.54 (d, J = 5.9 Hz, 2H), 7.54–7.00 (m, 6H), 2.05 (s, 3H); IR (KBr) 1722, 1630, 1440, 1225 cm^{-1}; MS (EI): m/z 297 (M^+); HRMS (ESI) calcd for $C_{16}H_{13}FN_3O_2$ $[M+H]^+$ 298.0992, found 298.0990.

3.1.16.2. Compounds 21b–21e (except for 21c). Those title compounds were prepared according to the same method as described above for **21a** using each the corresponding reagent.

3.1.16.3. 5-Benzoylamino-3-(4-fluorophenylamino)-4-(4-pyridyl)isoxazole (21b). Yield: 76%; mp:178.5–180.5 °C; ^{1}H NMR ($CDCl_3$) δ: 8.54 (dd, J = 1.5, 4.4 Hz, 2H), 8.38 (br s, 1H), 7.90–7.73 (m, 2H), 7.70–7.25 (m, 5H), 7.25–6.90 (m, 4H); IR (KBr) 1708, 1276 cm^{-1}; MS (EI): 359 (M^+); HRMS (ESI) calcd for $C_{21}H_{15}FN_3O_2$ $[M+H]^+$ 360.1148, found 360.1139.

3.1.16.4. 3-(4-Fluorophenyl)-5-(3-phenylpropionylamino)-4-(4-pyridyl)isoxazole (21d). Yield: 25%; mp: 184.0–187.0 °C; ^{1}H NMR ($CDCl_3$) δ: 8.50 (dd, J = 1.5, 4.4 Hz, 2H), 7.60 (br s, 1H), 7.50–6.85 (m, 11H), 3.15–2.90 (m, 2H), 2.85–2.60 (m, 2H); IR (KBr) 1722, 1505, 1224 cm^{-1}; MS (EI): m/z 387 (M^+); HRMS (ESI) calcd for $C_{23}H_{19}FN_3O_2$ 388.1461, found 388.1468.

3.1.16.5. 3-(4-Fluorophenyl)-5-(4-phenylbutanoylamino)-4-(4-pyridyl)isoxazole (21e). Yield: 57%; mp: 132.7–135.1 °C; ^{1}H NMR ($CDCl_3$) δ: 8.56 (dd, J = 1.5, 4.4 Hz, 2H), 7.63 (br s, 1H), 7.46–6.95 (m, 11H), 2.68 (t, J = 7.5 Hz, 2H), 2.50–2.34 (m, 2H), 2.19–1.91 (m, 2H); IR (KBr) 1722, 1628, 1610, 1442 cm^{-1}; MS (EI): m/z 401 (M^+); HRMS (ESI) calcd for $C_{24}H_{21}FN_3O_2$ $[M+H]^+$ 402.1618, found 402.1615.

3.1.16.6. 3-(4-Fluorophenyl)-5-phenylacetylamino-4-(4-pyridyl)isoxazole (21c). Imidazole (1.36 g, 20.0 mmol) and DBU (3.04 g, 20.0 mmol) were dissolved in THF (50 mL). To stirred the solution, phenylacetyl chloride (3.09 g, 20.0 mmol) in THF (10 mL) was added dropwise at 0 °C, then 5-amino-3-(4-fluorophenyl)-4-(4-pyridyl)isoxazole (**19**) (2.55 g, 10.0 mmol) and DBU (3.04 g, 20.0 mmol) in THF (20 mL) was added dropwise. The mixture was stirred at room temperature for 5 h. Then water was added to the reaction solution and extracted with ethyl acetate. The organic extracts was washed with water and dried over $MgSO_4$, then evaporated. The resulting residue was purified by

column chromatography (eluent: chloroform/methanol = 40:1) to give the title compound **21c** (3.06 g, 82%). Mp: 164.5–165.5 °C; ^{1}H NMR ($CDCl_3$) δ: 8.48 (dd, J = 1.7, 4.5 Hz, 2H), 7.69 (br s, 1H), 7.50–6.97 (m, 9H), 6.89 (dd, J = 1.7, 4.5 Hz, 2H), 3.75 (s, 2H); IR (KBr) 1712, 1630, 1602, 1436 cm^{-1}; MS (EI): 373 (M^+); HRMS (ESI) calcd for $C_{22}H_{17}FN_3O_2$ $[M+H]^+$ 374.1305, found 374.1301.

3.1.17. (*E*)-*N*,*N*-Dimethyl-2-(4-pyrimidinyl)ethenamine (23)

A mixture of 4-methylpyrimidine (**22**) (10 g, 106 mmol), *N*,*N*-dimethylformamide dimethylacetal (DMFDMA) (38 g, 319 mmol) and DMF (46.6 g, 637 mmol) was stirred in a sealed-tube at 140 °C for 24 h. After cooling, the solution was evaporated in vacuo to yield the title compound **23** (15.08 g, 95%). ^{1}H NMR ($CDCl_3$) δ: 8.73 (br s, 1H), 8.22 (d, J = 5.5 Hz, 1H), 7.77 (d, J = 12.9 Hz, 1H), 6.72 (dd, J = 5.5, 12.9 Hz, 1H), 5.00 (d, J = 12.9 Hz, 1H), 2.96 (s, 6H).

3.1.18. 4-Pyrimidinylacetonitrile (24)

To a solution of (*E*)-*N*,*N*-dimethyl-2-(4-pyrimidinyl)ethenamine (**23**) (5 g, 33.5 mmol) in water (70 mL) was added hydroxylamine-*O*-sulfonic acid (9.48 g, 83.8 mmol), the mixture was stirred at 50 °C for 30 min. Then a saturated $NaHCO_3$ aqueous solution was added until alkaline at 0 °C and extracted ethyl acetate. The organic extracts was dried over $MgSO_4$ and evaporated in vacuo. The resulting residue was purified by column chromatography (eluent: chloroform/methanol = 30:1) to give the title compound **24** (1.56 g, 39%). ^{1}H NMR ($CDCl_3$) δ: 9.21 (d, J = 1.2 Hz, 1H), 8.80 (d, J = 5.2 Hz, 1H), 7.51 (dd, J = 1.2, 5.2 Hz, 1H), 3.93 (s, 2H).

3.1.19. Typical procedure for the preparation of 5-amino-3-aryl-4-(4-pyrimidinyl)isoxazole 25

3.1.19.1. 5-Amino-3-(4-fluorophenyl)-4-(4-pyrimidinyl)isoxazole (25a). To a solution of sodium methoxide (2.50 g, 46.3 mmol) in methanol (50 mL) was added dropwise 4-pyrimidinylacetonitrile (**24**) (5.0 g, 42.0 mmol) in THF (50 mL) and the mixture was stirred at room temperature for 30 min. Then a solution of 4-fluoro-*N*-hydroxybenzimidoyl chloride (**18a**) (7.29 g, 42.0 mmol) in methanol (50 mL) was added dropwise to the mixture and stirred at room temperature for 7 h. The reaction solution was concentrated under reduced pressure, then water was added to the residue. The formed precipitate was collected by filtration and washed with water, then dried under reduced pressure. The resulting residue was purified by column chromatography (eluent: chloroform/methanol = 50:1–30:1) and washed with diethyl ether to give the title compound **25a** (7.86 g, 73%). Mp:196.5–198.5 °C; ^{1}H NMR ($CDCl_3$) δ: 9.03 (d, J = 1.4 Hz, 1H), 8.32 (d, J = 5.6 Hz, 1H), 7.54–7.49 (m, 2H), 7.24–7.18 (m, 2H), 6.88 (br s, 2H), 6.70 (dd, J = 1.4, 5.6 Hz, 1H); MS (EI): m/z 256 (M^+); HRMS (ESI) calcd for $C_{13}H_{10}FN_4O$ $[M+H]^+$ 257.0839, found 257.0835.

3.1.19.2. Compounds 25b–25g. Those title compounds were prepared according to the same method as described above for **25a** using each the corresponding reagent.

*3.1.19.2.1. 5-Amino-3-(2-chlorophenyl)-4-(4-pyrimidinyl)isoxazole (**25b**).* Yield: 56%; mp:181.0–184.0 °C; ^{1}H NMR ($CDCl_3$) δ: 9.00 (d, J = 1.3 Hz, 1H), 8.29 (d, J = 5.6 Hz, 1H), 7.58–7.40 (m, 4H), 6.92 (br s, 2H), 6.41 (dd, J = 1.3, 5.6 Hz, 1H); MS (EI): m/z 272 (M^+); HRMS (ESI) calcd for $C_{13}H_{10}ClN_4O$ $[M+H]^+$ 273.0543, found 273.0536.

*3.1.19.2.2. 5-Amino-3-(4-chlorophenyl)-4-(4-pyrimidinyl)isoxazole (**25c**).* Yield: 58%; mp:203.0–206.5 °C; ^{1}H NMR ($CDCl_3$) δ: 9.02 (d, J = 1.2 Hz, 1H), 8.32 (d, J = 5.4 Hz, 1H), 7.51–7.45 (m, 4H), 6.88 (s, 2H), 6.70 (dd, J = 1.2, 5.4 Hz, 1H); MS (EI): m/z 272 (M^+); HRMS (ESI) calcd for $C_{13}H_{10}ClN_4O$ $[M+H]^+$ 273.0543, found 273.0542.

*3.1.19.2.3. 5-Amino-3-(2,4-difluorophenyl)-4-(4-pyrimidinyl)isoxazole (**25d**).* Yield: 65%; mp:185.5–190.0 °C; ^{1}H NMR ($CDCl_3$) δ: 9.02 (d, J = 1.2 Hz, 1H), 8.35 (d, J = 5.4 Hz, 1H), 7.51 (dt, J = 6.6, 8.5 Hz, 1H), 7.08–6.97 (m, 2H), 6.92 (br s, 2H), 6.60 (td, J = 1.2, 5.4 Hz, 1H); MS (EI): m/z 274 (M^+); HRMS (ESI) calcd for $C_{13}H_9F_2N_4O$ $[M+H]^+$ 275.0744, found 275.0744.

*3.1.19.2.4. 5-Amino-3-(2,6-difluorophenyl)-4-(4-pyrimidinyl)isoxazole (**25e**).* Yield: 88%; mp:170.0–176.5 °C; ^{1}H NMR ($CDCl_3$) δ: 9.02 (d, J = 1.5 Hz, 1H), 8.34 (d, J = 5.4 Hz, 1H), 7.58–7.50 (m, 1H), 7.11–7.07 (m, 2H), 6.94 (br s, 2H), 6.57–6.54 (m, 1H); MS (EI): m/z 274 (M^+); HRMS (ESI) calcd for $C_{13}H_9F_2N_4O$ $[M+H]^+$ 275.0744, found 275.0746.

*3.1.19.2.5. 5-Amino-3-(2,6-dichlorophenyl)-4-(4-pyrimidinyl)isoxazole (**25f**).* Yield: 56%; mp:202.5–207.0 °C; ^{1}H NMR ($CDCl_3$) δ: 9.00 (d, J = 1.4 Hz, 1H), 8.30 (d, J = 5.6 Hz, 1H), 7.50–7.43 (m, 3H), 7.00 (br s, 2H), 6.29 (dd, J = 1.4, 5.6 Hz, 1H); MS (EI): m/z 306 (M^+); HRMS (ESI) calcd for $C_{13}H_9Cl_2N_4O$ $[M+H]^+$ 307.0153, found 307.0160.

*3.1.19.2.6. 5-Amino-3-(3-methylphenyl)-4-(4-pyrimidinyl)isoxazole (**25g**).* Yield: 18%; mp:164.5–166.5 °C; ^{1}H NMR ($CDCl_3$) δ: 9.00 (d, J = 1.6 Hz, 1H), 8.28 (d, J = 5.6 Hz, 1H), 7.40–7.28 (m, 4H), 6.86 (br s, 2H), 6.73 (dd, J = 1.6, 5.6 Hz, 1H), 2.41 (s, 3H); MS (EI): m/z 252 (M+); HRMS (ESI) calcd for $C_{14}H_{13}N_4O$ $[M+H]^+$ 253.1089, found 253.1080.

*3.1.19.2.7. 5-Amino-3-(4-fluoro-3-methyl)-4-(4-pyrimidinyl)isoxazole (**25h**).* Yield: 43%; mp:161.0–165.0 °C; ^{1}H NMR ($CDCl_3$) δ: 9.02 (d, J = 1.5 Hz, 1H), 8.32 (d, J = 5.4 Hz, 1H), 7.37 (dd, J = 1.6, 7.3 Hz, 1H), 7.32–7.28 (m, 1H), 7.13 (t, J = 8.5 Hz, 1H), 6.89 (br s, 2H), 6.73 (dd, J = 1.2, 5.4 Hz, 1H), 2.33 (d, J = 1.5 Hz, 3H); MS (EI): m/z 270 (M^+); HRMS (ESI) calcd for $C_{14}H_{12}FN_4O$ $[M+H]^+$ 271.0995, found 271.0997.

3.1.20. Typical procedure for the preparation of 3-aryl-5-phenylacetylamino-4-(4-pyrimidinyl)isoxazole 26

3.1.20.1. 3-(4-Fluorophenyl)-5-phenylacetylamino-4-(4-pyrimidinyl)isoxazole (26a). Imidazole (0.43 g, 6.32 mmol) and DBU (1.90 g, 12.5 mmol) were dissolved in THF (40 mL). To stirred the solution, phenylacetyl chloride (0.97 g, 6.27 mmol) in THF (10 mL) was added dropwise at 0 °C, then 5-amino-3-(4-fluorophenyl)-4-(-4-pyrimidinyl)isoxazole (**25a**) (0.80 g, 3.12 mmol) in THF (40 mL) was added dropwise. The mixture was stirred at room temperature for 6 h. Then the reaction solvent was evaporated in vacuo, water was added to the residue and extracted with ethyl acetate. The organic extracts was dried over $MgSO_4$ and evaporated. The resulting residue was purified by column chromatography (eluent: chloroform/methanol = 100:1) and washed with diethyl ether to give the title compound **26a** (0.77 g, 82%). Mp: 159.5–162.0 °C; ^{1}H NMR ($CDCl_3$) δ: 11.39 (s, 1H), 8.49 (s, 1H), 8.36 (d, J = 5.6 Hz, 1H), 7.50–7.38 (m, 7H), 7.20 (t, J = 8.5 Hz, 2H), 6.73 (dd, J = 1.3, 5.6 Hz, 1H), 3.94 (s, 2H); MS (EI): m/z 374 (M^+); HRMS (ESI) calcd for $C_{21}H_{16}FN_4O_2$ $[M+H]^+$ 375.1257, found 375.1269.

3.1.20.2. Compounds 26f, 26i and 26k–26l. Those title compounds were prepared according to the same method as described above for **26a** using each the corresponding reagent.

*3.1.20.2.1. 3-(2-Chlorophenyl)-5-phenylacetylamino-4-(4-pyrimidinyl)isoxazole (**26f**).* Yield: 47%; mp: 149.5–153.0 °C; ^{1}H NMR ($CDCl_3$) δ: 11.50 (s, 1H), 8.47 (br s, 1H), 8.33 (d, J = 5.7 Hz, 1H), 7.55–7.40 (m, 9H), 6.46 (dd, J = 1.3, 5.7 Hz, 1H), 3.95 (s, 2H); MS (EI): m/z 390 (M^+); HRMS (ESI) calcd for $C_{21}H_{16}ClN_4O_2$ $[M+H]^+$ 391.0962, found 391.0974.

*3.1.20.2.2. 3-(2,6-Difluorophenyl)-5-phenylacetylamino-4-(4-pyrimidinyl)isoxazole (**26i**).* Yield: 77%; mp: 191.0–193.5 °C; ^{1}H NMR ($CDCl_3$) δ: 11.48 (s, 1H), 8.48 (d, J = 1.2 Hz, 1H), 8.38 (d, J = 5.4 Hz, 1H), 7.58–7.41 (m, 6H), 7.11–7.06 (m, 2H), 6.60 (dd, J = 1.2, 5.4 Hz, 1H), 3.95 (s, 2H); MS (EI): m/z 392 (M^+); HRMS (ESI) calcd for $C_{21}H_{15}F_2N_4O_2$ $[M+H]^+$ 393.1163, found 393.1180.

*3.1.20.2.3. 3-(2,6-Dichlorophenyl)-5-phenylacetylamino-4-(4-pyrimidinyl)isoxazole (**26j**).* Yield: 50%; mp:186.0–191.0 °C; ^{1}H NMR ($CDCl_3$) δ: 11.50 (s, 1H), 8.47 (s, 1H), 8.35 (d, J = 5.6 Hz, 1H), 7.52–7.42 (m, 8H), 6.34 (dd, J = 1.5, 5.6 Hz, 1H), 3.96 (s, 2H); MS (EI): m/z 424 (M^+); HRMS (ESI) calcd for $C_{21}H_{15}Cl_2N_4O_2$ $[M+H]^+$ 425.0572, found 425.0566.

3.1.20.3. Typical procedure for the preparation of 3-aryl-4-(4-pyrimidinyl)-5-substituted phenylacetylaminoisoxazole 26. *3.1.20.3.1. 3-(4-Fluorophenyl)-5-[(2-fluorophenyl)acetylamino]-4-(4-pyrimidinyl)isoxazole (**26b**).* To a solution of 2′-fluorophenylacetic acid (0.12 g, 0.78 mmol) in THF (5 mL) was added 1,1′-carbonyldiimidazole (CDI) (0.126 g, 0.78 mmol) and the mixture was stirred at room temperature for 1 h. Then a solution of DBU (0.237 g, 1.56 mmol) and 5-amino-3-(4-fluorophenyl)-4-(4-pyrimidinyl)isoxazole (**25a**) (0.100 g, 0.39 mmol) in THF (5 mL) was added to the mixture and stirred at room temperature for 11 h. After the reaction solvent was evaporated in vacuo, water was added to the residue and extracted with ethyl acetate. The organic extracts was dried over $MgSO_4$ and evaporated. The resulting residue was purified by column chromatography (eluent: chloroform/methanol = 100:1) and washed with *n*-hexane/diethyl ether to afford the title compound **26b** (0.090 g, 59%). Mp:160.0–163.0 °C; ^{1}H NMR ($CDCl_3$) δ: 11.57 (s, 1H), 8.62 (s, 1H), 8.39 (d, J = 5.7 Hz, 1H), 7.50–7.40 (m, 4H), 7.30–7.17 (m, 4H), 6.76 (dd, J = 1.6, 5.7 Hz, 1H), 3.97 (s, 2H); MS (EI): m/z 392 (M^+); HRMS (ESI) calcd for $C_{21}H_{15}F_2N_4O_2$ $[M+H]^+$ 393.1163, found 393.1172.

3.1.20.4. Compounds 26c–26e, 26g–26h and 26k–26l. Those title compounds were prepared according to the same method as described above for **26b** using each the corresponding reagent.

*3.1.20.4.1. 5-[(2-Chlorophenyl)acetylamino]-3-(4-fluorophenyl)-4-(4-pyrimidinyl)isoxazole (**26c**).* Yield: 48%; mp:171.0–173.5 °C; ^{1}H NMR ($CDCl_3$) δ: 11.45 (br s, 1H), 8.54 (s, 1H), 8.38 (d, J = 5.7 Hz, 1H), 7.55–7.38 (m, 6H), 7.20 (t, J = 8.7 Hz, 2H), 6.75 (dd, J = 1.3, 5.7 Hz, 1H), 4.06 (s, 2H); MS (EI): m/z 408 (M^+); HRMS (ESI) calcd for $C_{21}H_{15}F_2N_4O_2$ $[M+H]^+$ 409.0868, 409.0859.

*3.1.20.4.2. 5-[(2-Chloro-6-fluorophenyl)acetylamino]-3-(4-fluorophenyl)-4-(4-pyrimidinyl)isoxazole (**26d**).* Yield: 79%; mp:181.0–182.0 °C; ^{1}H NMR ($CDCl_3$) δ: 11.55 (s, 1H), 8.64 (s, 1H), 8.40 (d, J = 5.7 Hz, 1H), 7.51–7.45 (m, 2H), 7.43–7.34 (m, 2H), 7.26–7.13 (m, 3H), 6.78 (dd, J = 1.3, 5.7 Hz, 1H), 4.14 (s, 2H); MS (EI): m/z 426 (M^+); HRMS (ESI) calcd for $C_{21}H_{14}ClF_2N_4O_2$ $[M+H]+$ 427.0773, found 427.0770.

*3.1.20.4.3. 5-[(2,5-Dimethylphenyl)acetylamino]-3-(4-fluorophenyl)-4-(4-pyrimidinyl)isoxazole (**26e**).* Yield: 95%; mp:180.5–182.5 °C; ^{1}H NMR ($CDCl_3$) δ: 11.32 (s, 1H), 8.39 (d, J = 1.4 Hz, 1H), 8.35 (d, J = 5.4 Hz, 1H), 7.49–7.43 (m, 2H), 7.23–7.14 (m, 5H), 6.72 (dd, J = 1.4, 5.4 Hz, 1H), 3.88 (s, 2H), 2.39 (s, 3H), 2.31 (s, 3H); MS (EI): m/z 402 (M^+); HRMS (ESI) calcd for $C_{23}H_{20}FN_4O_2$ $[M+H]^+$ 403.1570, found 403.1562.

*3.1.20.4.4. 3-(4-Chlorophenyl)-5-[(2-chlorophenyl)acetylamino]-4-(4-pyrimidinyl)isoxazole (**26g**).* Yield: 49%; mp:209.0–211.5 °C; ^{1}H NMR ($CDCl_3$) δ: 11.44 (br s, 1H), 8.53 (s, 1H), 8.39 (d, J = 5.6 Hz, 1H), 7.54–7.39 (m, 8H), 6.76 (dd, J = 1.5, 5.6 Hz, 1H), 4.06 (s, 2H); MS (EI): m/z 424 (M+); HRMS (ESI) calcd for $C_{21}H_{15}Cl_2N_4O_2$ $[M+H]^+$ 425.0572, found 425.0536.

*3.1.20.4.5. 5-[(2-Chlorophenyl)acetylamino]-3-(2,4-difluorophenyl)-4-(4-pyrimidinyl)isoxazole (**26h**).* Yield: 49%; mp:172.5–175.5 °C; ^{1}H NMR ($CDCl_3$) δ: 11.49 (br s, 1H), 8.53 (s, 1H), 8.41 (d, J = 5.4 Hz, 1H), 7.54–7.40 (m, 5H), 7.09–7.04 (m, 1H), 6.98 (dt, J = 2.3, 8.5 Hz, 1H), 6.67 (td, J = 1.5, 5.4 Hz, 1H), 4.07 (s, 2H); MS (EI): m/z 426 (M+); HRMS (ESI) calcd for $C_{21}H_{14}ClF_2N_4O_2$ $[M+H]^+$ 427.0773, found 427.0788.

*3.1.20.4.6. 5-[(2-Chlorophenyl)acetylamino]-3-(3-methylphenyl)-4-(4-pyrimidinyl)isoxazole (**26k**).* Yield: 73%; mp:143.5–145.5 °C; ^{1}H NMR ($CDCl_3$) δ: 8.52 (s, 1H), 8.34 (d, J = 5.4 Hz, 1H), 7.54–7.20 (m, 8H), 6.78 (dd, J = 1.5, 5.4 Hz, 1H), 4.07 (s, 2H), 2.39 (s, 3H); MS (EI): m/z 404 (M^+); HRMS (ESI) calcd for $C_{22}H_{18}ClN_4O_2$ $[M+H]^+$ 405.1118, found 405.1109.

*3.1.20.4.7. 3-(4-Fluoro-3-methylphenyl)-5-[(2-methylphenyl)acetylamino]-4-(4-pyrimidinyl)isoxazole (**26l**).* Yield: 82%; mp:193.5–195.0 °C; ^{1}H NMR ($CDCl_3$) δ: 11.35 (br s, 1H), 8.41 (d, J = 1.4 Hz, 1H), 8.34 (d, J = 5.4 Hz, 1H), 7.41–7.30 (m, 5H), 7.24–7.20 (m, 1H), 7.11 (t, J = 9.3 Hz, 1H), 6.74 (dd, J = 1.4, 5.4 Hz, 1H), 3.92 (s, 2H), 2.35 (s, 3H), 2.30 (d, J = 1.9 Hz, 3H); MS (EI): m/z 402 (M^+); HRMS (ESI) calcd for $C_{23}H_{20}FN_4O_2$ $[M+H]^+$ 403.1570, found 403.1547.

3.2. Docking studies

All docking studies were performed on Discovery Studio Ver. 3.5 system (Accelrys Software Inc.). Three crystal structures of p38α MAP kinase (PDB ID: 1bl7, 2ewa, 1ouk) were selected from the Protein Data Bank (PDB) as docking templates by ligand similarity to **26d**. Each crystal structure was modified using the Automatic Preparation on default configuration. Compound **26d** was docked into each crystal structure using the docking program CDOCKER on default configuration (Top Hits: 10, Random Conformations: 10, Orientations to Refine: 10). Because the best docking poses of each crystal structure was relatively similar in the interaction between **26d** and p38α, the docking result for 1bl7 was only presented in context.

3.3. Biological methods

3.3.1. Animals

All animal studies were approved by the Animal Research Committee of ASKA Pharmaceutical Co., Ltd and were conducted in compliance with the Law Concerning the Protection and Control of Animals (Japanese Law 105, October 1, 1973; revised on June 22, 2005). Female BALB/c mice and male Sprague-Dawley (SD) rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animals were housed in temperature- and humidity-controlled rooms, allowed to take standard rodent chow pellets and tap water ad libitum., and were acclimatized for at least 7 days.

3.3.2. Inhibition of p38α MAP kinase and kinase isoform selectivity assays

This study was conducted by Carna Biosciences, Inc. (Kobe, Japan).

For details, refer to the URL; (http://www.carnabio.com/output/pdf/ProfilingProfilingBook_en.pdf).

Inhibition of p38α MAP kinase were measured by Off-chip Mobility Shift Assay (MSA). The enzyme activities of various kinases except for p38α of **26d** and **25a** were measured by optimized ELISA or IMAP™ assay methods. IC_{50} values of test compounds against each isoform of p38 and JNK were estimated. Protein kinases evaluated **26d** were; p38β, p38γ, p38δ, JNK1, JNK2, JNK3, CSK, LCK, AKT2, CRIK, ROCK1, PKACα, PDK1, PKCα, RSK2, CaMK4, CaMK2α, CHK1, DAPK1, MAPKAPK2, PIM1, CHK2, CDK2/cyclinA, GSK3β, SRPK1, AurA, IKKβ, NEK2, TTK, IRAK4, PHKG1, CK1δ, PKD2, Erk1, Erk2, Erk5, MAP3K5 and RAF1. Protein kinases evaluated **25a** were; p38β, p38γ, p38δ, JNK1, JNK2, JNK3.

3.3.3. Inhibition of TNF-α production in THP-1 cells

This study was conducted by SB Drug Discovery (Scotland). Cell from the human monocytic cell line, THP-1, were cultured in RPMI media supplemented with 10% fetal calf serum (FCS). Cell were seeded out in 96-well cell culture plates at a density of 100,000 cells per well. Test compounds were diluted in 100% DMSO to yield working stock concentrations of 1 mM. Subsequent dilutions were carried out in cell culture media/10% DMSO. The final

concentration of DMSO across the plate did not exceed 1%. Unstimulated and Stimulated Control wells received an identical volume of cell culture media/DMSO at this point. Test compounds were added to the cells and 30 min later the cells were stimulated with LPS (*Escherichia coli* 055 B55, Sigma, 1 g/mL). Two hours after addition of LPS, plates were centrifuged to pellet the cells and the concentration of TNF-α in the supernatant was determined by ELISA (human TNF-α ELISA kits, BD Bioscience).

3.3.4. Metabolic stability in human liver S9

Test compounds were spiked at a final concentration of 1 μM to 50 mM phosphate buffer (pH 7.4), containing 3.3 mM $MgCl_2$, 1.0 mM β-$NADP^+$, 2.5 mM glucose 6-phosphate, and 2 U/mL glucose 6-phosphate dehydrogenase, and to 0.5 mg protein/mL liver S9. Metabolic reaction was terminated after incubation for 5 and 15 min at 37 °C, by addition of a four-fold volume of ice-cold acetonitrile including 0.1 μM internal standard. The samples were centrifuged at 2000×*g* for 10 min, and the supernatant was subjected to LC–MS/MS. Intrinsic clearance of test compounds in human liver S9 was calculated according to a following equation: $CL_{int} = K_e \times V/protein_{S9}$, where K_e is the elimination rate constant of test compound, V is the initial incubation volume, and $protein_{S9}$ represents the protein concentration in the incubation mixture.

3.3.5. Pharmacokinetic studies

Compound **26d** was suspended in 0.5% Tween 80 (w/v) to prepare oral dosing formulations. For intravenous dosing, Compound **26d** was dissolved in a mixed solution of DMSO, polyethylene glycol-400, and saline (5:65:30). Three male SD rats received doses by oral gavage or intravenous injection via the femoral vein, respectively. Blood samples were collected via the tail vein over 24 h after administration. Plasma was separated by centrifugation (12,000×*g*, 3 min at 4 °C), and was stored at −20 °C until analysis.

3.3.6. LC–MS/MS analysis

The LC–MS/MS system consisted of an Alliance 2795 separation module (Waters, Milford, MA) and a Quattro Ultima Pt triple quadrupole mass spectrometer (Micromass, Manchester, UK) with an electron spray ionization interface. Aliquots of plasma samples (50 μL) were added and vortex-mixed, followed by addition of the internal standard solution, 0.5 mL of 0.5 M sodium hydrogen carbonate solution, and 2 mL of diethyl ether. After vigorously shaking for 10 min and centrifugation (2000×*g*, 5 min), the organic layer was evaporated under a nitrogen gas stream and were reconstituted with 100 μL of acetonitrile. Analytes (10 μL) were separated on a Discovery HSF5 column (2.1 mm I.D. × 50 mm, 3 μm) at 40 °C with a linear gradient program consisting of 10 mM ammonium formate (pH 3) and acetonitrile. Intra- and interday precision and accuracy for these compounds were within 15%.

3.3.7. In vivo assays

3.3.7.1. DSS-induced colitis in mice. Female BALB/c mice were used in this study. Colitis was induced by allowing the mice free access to 5% DSS (Sodium Dextran Sulfate) in their drinking water for 7 days. Vehicle or **26d** (3, 10, 30 and 100 mg/kg) were orally administrated to 8 mice per group twice daily during DSS-treatment. SASP (100 mg/kg) was administrated once daily to 8 mice during DSS-treatment. Mice were sacrificed 7 days after the start of DSS-treatment and the colon length was measured.

3.3.7.2. TNBS-induced colitis in rats. Male SD rats, fed an elemental liquid diet (Elental®; Ajinomoto Co., Inc.) for 7 days to remove the gastrointestinal contents, were used in this study. To induce colitis, 1 mL of TNBS solution (30 mg/mL in 40% ethanol/saline) was instilled into the lumen of the colon. From 2 days after the colitis induction, vehicle, **26d** (3, 10, 30 and 100 mg/kg) or prednisolone acetate (10 mg/kg) were orally administrated to 8–10 rats per group twice daily for 6 days. Rats were sacrificed 7 days after the induction of colitis, and the colonic damage was scored according to the Wallace Criteria.[24]

4. Results and discussion

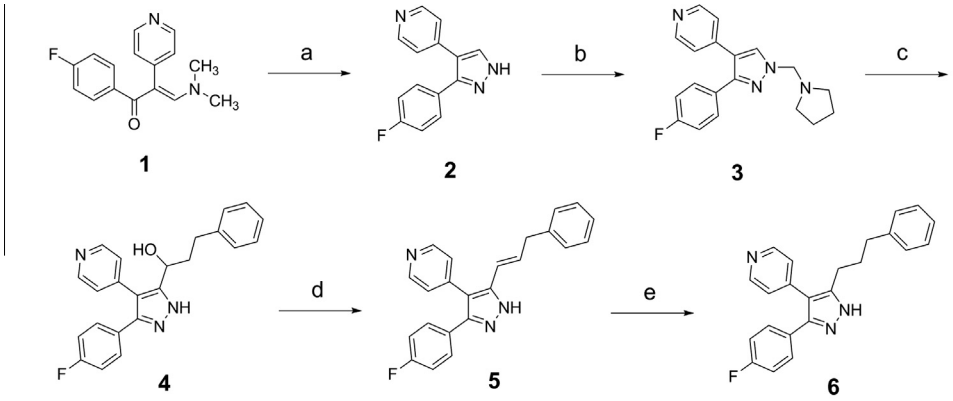
4.1. Chemistry

Although not fulfilling the conditions for the antedrug concept (Fig. 3), first, 5-phenylpropylpyrazole **6** was synthesized by using the procedure presented below to confirm the effect of the new fourth interaction site (Scheme 1). By the reaction of enamine **1** and hydrazine monohydrate, the pyrazole framework was constructed to generate **2**,[25] and then **3** was obtained through the reaction of pyrrolidine and formalin. After deprotonation of the 5-position using n-butyl lithium and introduction of the side chain through a reaction with phenylpropionaldehyde, **4** was obtained. A dehydration reaction resulted in compound **5** and finally, target compound **6** was synthesized by catalytic hydrogen reduction of compound **5**.

Pyrazoles **13** and **15**, which contain an amide structure as side chains at the 5-position, were synthesized using the procedure shown in Scheme 2. Pyridylacetonitrile **9** and activated ester **10** were condensed in the presence of a base to produce cyanoketone **11,** which upon chlorination of a carbonyl group, followed by a reaction with hydrazine monohydrate, produced 5-aminopyrazole **12**.[26] Condensation with phenylacetylchloride in the presence of a base produces 5-acylaminopyrazole **13**. Meanwhile, 1-methyl derivative **15** was synthesized by employing almost the same route as previously described by using *tert*-butoxycarbonyl (Boc) compound **8** derived from methylhydrazine **7**.

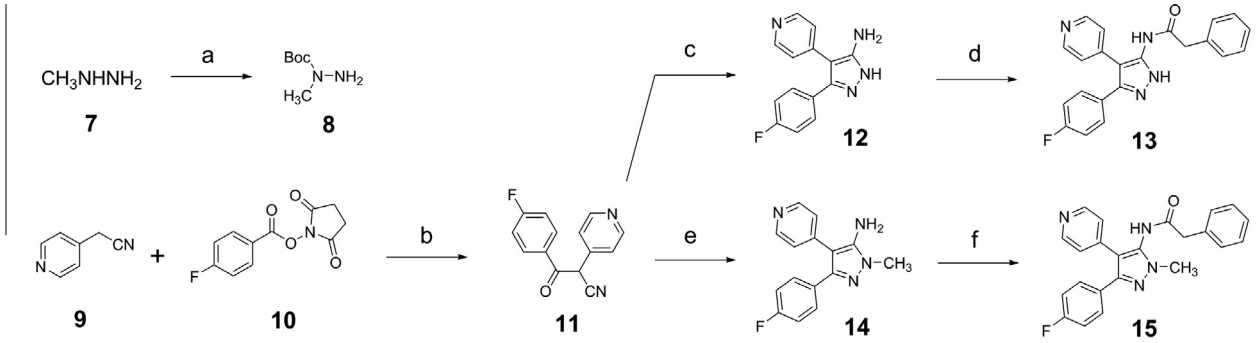
The synthesis of 4-(4-pyridyl)isoxazole derivatives is shown in Scheme 3. The key intermediate 5-aminoisoxazole **19** was synthesized using the method of Miller et al.[27] Aldehyde **16** was reacted with hydroxylamine to form oxime **17** and then hydroxamic acid chloride **18a** was obtained by using *N*-chlorosuccinimide (NCS). Through the reaction between **18a** and commercially available 4-pyridylacetonitrile **9** under basic conditions, an isoxazole framework was built, which produced 5-amino isoxazole **19**. Isoxazole **20**, which contained an unsubstituted 5-position, was synthesized by using the method of Laufer et al. and by deamination through the diazotization reaction of **19**.[23] Introduction of the side chain to the amino group at 5-position of **19** by condensation with a carboxylic acid could not proceed by using condensation reagents such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Therefore, this reaction was conducted through the diacyl form obtained from the reaction using two or more equivalent quantities of carboxylic acid chloride and then, under alkaline conditions, produced the monoacyl derivatives (**21a**, **21b**, **21d**, and **21e**). To simplify the reaction procedure and improve yield, condensation with imidazoleamide in the presence of 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU) was performed and deemed optimal and was therefore employed for the synthesis of **21c**.[28]

4-(4-Pyrimidinyl)isoxazole derivative **26** was also synthesized by using Scheme 4, which was basically similar to that for the generation of the 4-(4-pyridyl)isoxazole derivative. By reacting 4-methylpyrimidine **22** and *N*,*N*-dimethylformamide dimethyl acetal (DMFDMA), enamine **23** was obtained, and then 4-pyrimidinylacetonitrile **24** was obtained by using hydroxylamine-*O*-sulfonic acid. The target compound **26** was obtained via 5-aminoisoxazole **25** using a method similar to that previously described.



Reagents and conditions: (a) NH_2NH_2 H_2O, EtOH, reflux, 86%; (b) pyrrolidine, HCHO, EtOH, reflux, 97%; (c) *n*-BuLi, $PhCH_2CH_2CHO$, THF, -78°C to RT, 51%; (d) *p*-TsOH H_2O, toluene, reflux, 45%; (e) H_2, Pd-C, EtOH, RT, 60%.

Scheme 1. Synthesis of 5-aralkylpyrazole.



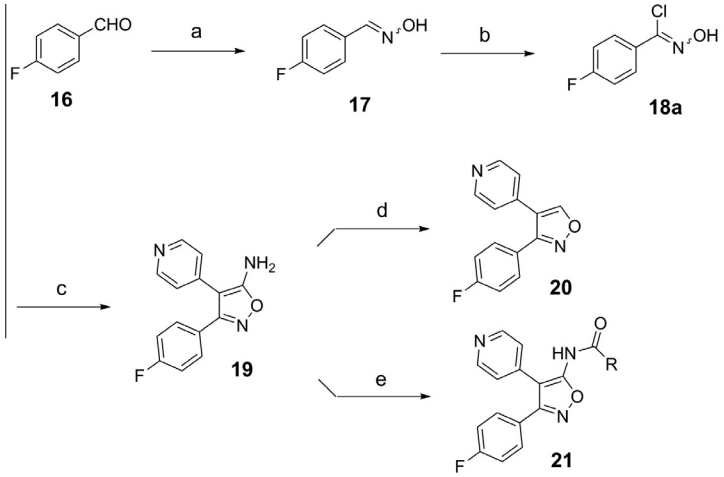
Reagents and conditions: (a) Boc_2O, MeOH, 0°C, 97%; (b) K_2CO_3, DMF, RT, 84%; (c) 1) $POCl_3$, 100°C, 2) NH_2NH_2 H_2O, EtOH, reflux, 54%; (d) $PhCH_2COCl$, Et_3N, THF, RT, 22%; (e) 1) $POCl_3$, 100°C, 2) **8**, EtOH, reflux, 3) TFA, 100°C, 62%; (f) $PhCH_2COCl$, Et_3N, THF, RT, 35%.

Scheme 2. Synthesis of 5-acylaminopyrazole.

4.2. Biological evaluation and discussion

For primary screening, measurements of the inhibitory activity of p38α MAP kinase, which is involved in the production of inflammatory cytokines in four isoforms (α, β, γ, and δ), was performed. TNF-α inhibitory activity in THP-1 cells was also measured as an index for inflammation. For the evaluation of its applicability as an antedrug, a metabolic stability test using human liver S9 was also carried out. Table 1 shows the results of biological activity of a series of pyrazole derivatives and a metabolic stability test. First, a comparison of a simple 3-(4-fluorophenyl)-4-(4-pyridyl)pyrazole (**2**)[25] not having any substituent, with 5-amino compound **12** and 5-amino-1-methyl compound (**14**) showed that all of these have p38α inhibitory activities and TNF-α inhibitory activities of almost the same magnitude despite the difference in these substituents. A comparison between the compounds having a side chain at the 5-position showed that the activity of the compounds from **6**, **13**, and **15** with side chains exceeds that of **2**, **12**, and **14** (i.e., prior to condensation). This finding indicates that according to our objective, the side chain at the 5-position effectively interacts with p38α. Derivative **6**, in which the phenylpropyl group was introduced at the 5-position, showed a particularly high activity (p38α: IC_{50} = 0.950 nM; TNF-α: IC_{50} = 3.8 nM) and from this it was predicted that in the case of the freshly added fourth interaction site, the substitution by a lipophilic group was favorable. The 5-phenylacetylamino groups from **13** and **15** were not as active as the phenylpropyl group of **6**. Nevertheless, an increase in activity of about one order of magnitude from each of **12** and **14** (before condensation) was observed. However, pyrazoles **13** and **15** were stable in human liver S9, (individual percentage of unchanged residue after 5 min: 90.4%, 84.4%, for **13** and **15**, respectively) and this metabolic stability is a desirable property for a drug to manifest its efficacy through a systemic action. However, for the antedrug, which is the goal of our present research, this may not be suitable.

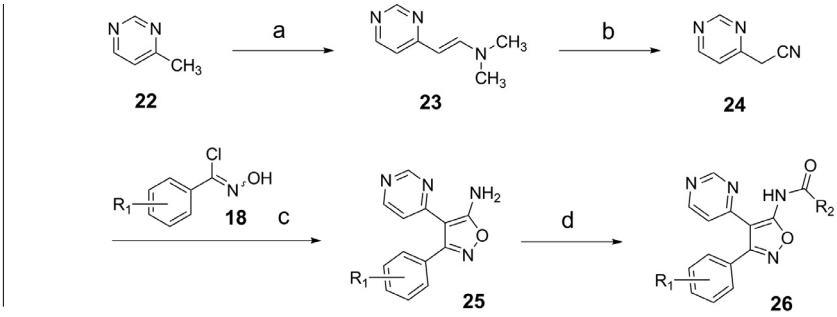
Table 2 shows the activity and metabolic stability of isoxazole derivatives. Comparison of activity of the 5-amino compound **19** that does not possess a fourth interaction site and an unsubstituted compound **20** with pyrazole **12** showed that **19** has a p38α inhibitory activity of the same order as that of **12** (105 nM vs 97.4 nM) and the activity of **20** was considerably low (871 nM) (in the report by Laufer et al., the inhibitory activities of **19** and **20** were almost of the same order[23]). This result differs from pyrazole, in which the unsubstituted compound **2** and 5-amino compound **12** have



21a: R = CH_3, **21b**: R = C_6H_5, **21c**: R = $C_6H_5CH_2$,
21d: $C_6H_5CH_2CH_2$, **21e**: $C_6H_5CH_2CH_2CH_2$

Reagents and conditions: (a) NH_2OH HCl, EtOH, H_2O, RT, 87%; (b) NCS, DMF, RT, 97%; (c) **9**, $NaOCH_3$, MeOH, THF, RT, 98%; (d) $NaNO_2$, AcOH, H_2O; (e) 1) RCOCl, Et_3N, CH_2Cl_2, 0°C, 2) NaOHaq., MeOH, RT, **21a**: 63%, **21b**: 76%, **21d**: 25%, **21e**: 57% or RCOCl, imidazole, DBU, THF, 0°C to RT, **21c**: 82%.

Scheme 3. Synthesis of pyridylisoxazole derivatives.



18a, **25a**: R_1 = 4-F, **18b**, **25b**: R_1 = 2-Cl, **18c**, **25c**: R_1 = 4-Cl, **18d**, **25d**: R_1 = 2,4-F, **18e**, **25e**: R_1 = 2,6-F, **18f**, **25f**: R_1 = 2,6-Cl, **18g**, **25g**: R_1 = 3-CH_3, **18h**, **25h**: R_1 = 3-CH_3, 4-F, **26a**: R_1 = 4-F, R_2 = $C_6H_5CH_2$, **26b**: R_1 = 4-F, R_2 = 2-F-$C_6H_4CH_2$, **26c**: R_1 = 4-F, R_2 = 2-Cl-$C_6H_4CH_2$, **26d**: R_1 = 4-F, R_2 = 2-F, 6-Cl-$C_6H_3CH_2$, **26e**: R_1 = 4-F, R_2 = 2,5-CH_3-$C_6H_3CH_2$, **26f**: R_1 = 2-Cl, R_2 = $C_6H_5CH_2$, **26g**: R_1 = 4-Cl, R_2 = 2-Cl-$C_6H_4CH_2$, **26h**: R_1 = 2,4-F, R_2 = 2-Cl-$C_6H_4CH_2$, **26i**: R_1 = 2,6-F, R_2 = $C_6H_5CH_2$, **26j**: R_1 = 2,6-Cl, R_2 = $C_6H_5CH_2$, **26k**: R_1 = 3-CH_3, R_2 = 2-Cl-$C_6H_4CH_2$, **26l**: R_1 = 3-CH_3, 4-F, R_2 = 2-CH_3-$C_6H_4CH_2$

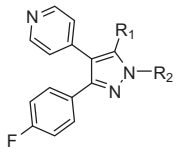
Reagents and conditions: (a) DMFDMA, DMF, 140°C, 95%, (b) H_2NOSO_3H, H_2O, 50°C, 39%, (c) **18**, $NaOCH_3$, MeOH, THF, RT, **25a**: 73%, **25b**: 56%, **25c**: 58%, **25d**: 65%, **25e**: 88%, **25f**: 56%, **25g**: 18%, **25h**: 43%, (d) RCOOH, CDI, DBU, THF, RT or RCOCl, imidazole, DBU, THF, RT, **26a**: 82%, **26b**: 59%, **26c**: 48%, **26d**: 79%, **26e**: 95%, **26f**: 47%, **26g**: 49%, **26h**: 49%, **26i**: 77%, **26j**: 50%, **26k**: 73%, **26l**: 82%.

Scheme 4. Synthesis of pyrimidinylisoxazole derivatives.

activities of almost the same order. In the case of isoxazole, the 5-amino group from **19** contributed to an increase in its activity and thus is due to the interaction of the 5-amino group itself. However, it supports the possibility of an increase in the affinity of the nitrogen atom at the 3-position with Lys53 because of an electron donation effect of the 5-amino group. In fact, **21a**, in which the electron-donating property has been reduced by acetylation of the 5-amino group, showed a significantly lower activity than **19**, but it is almost of the same order as that of the unsubstituted derivative **20**. Compounds **21b**–**21e** were synthesized to optimize the length of the linker portion of the 5-position side chain, which contains a benzene ring that can potentially become the fourth interaction site. As a result, phenylacetylamide **21c** and phenylpropionylamide **21d** had most excellent p38α inhibitory activities, with values approaching that of pyrazole **13**. **21c** and **21d** had almost equal p38α inhibitory activities (23.0 nM vs 23.4 nM), whereas **21c** showed a higher TNF-α inhibitory activity, which was at least 2-fold (IC_{50}: 54.7 nM vs 126.0 nM). Thus, the optimum 5-position substituent group is phenylacetylamide from **21c** in terms of the activity.

Next, in terms of metabolism, the series of isoxazole derivatives showed an increasing tendency of clearance in human liver S9 compared to the pyrazole derivatives and thus, it is suitable as an antedrug. However, **21c**, which showed the highest activity, was stable in isoxazole (CLint: 91.0 mL/min/mg) and thus needs further enhancement in metabolism.

Table 1
Biological activity and metabolic stability of pirazole derivatives



Compound	Structure		p38 α MAP kinase	TNF-α	Metabolic stability in human liver S9 (% of remaining)		Clint (mL/min/mg)
	R_1	R_2	IC_{50} (nM)	IC_{50} (nM)	5 min	15 min	
2	H	H	67.0	316.7	NA[a]	NA[a]	NA[a]
6	$C_6H_5(CH_2)_3$	H	0.950	3.8	81.5	50.3	81.8
12	NH_2	H	97.4	195.6	NA[a]	NA[a]	NA[a]
13	$C_6H_5CH_2CONH$	H	16.5	22.9	90.4	86.2	40.6
14	NH_2	CH_3	70.4	304.3	NA[a]	NA[a]	NA[a]
15	$C_6H_5CH_2CONH$	CH_3	5.72	11.9	84.4	65.0	68.1

[a] NA: not applicable. The data of metabolic stability of compounds without a side chain at the 5-position was not applicable, since it is not so important from a perspective of the concept of an antedrug.

Table 2
Biological activity and metabolic stability of isoxazole derivatives

Compound	Structure		p38α MAP kinase	TNF-α	Metabolic stability in human liver S9 (% of remaining)		CLint
	R	A	IC_{50} (nM)	IC_{50} (nM)	5 min	15 min	(mL/min/mg)
19	NH_2	CH	105	490.0	NA[a]	NA[a]	NA[a]
20	H	CH	871	4617.0	NA[a]	NA[a]	NA[a]
21a	CH_3CONH	CH	1310	4058.0	92.4	82.5	31.6
21b	C_6H_5CONH	CH	1210	2771.0	39.6	10.9	371
21c	$C_6H_5CH_2CONH$	CH	23.0	54.7	79.7	64.3	91.0
21d	$C_6H_5(CH_2)_2CONH$	CH	23.4	126.0	32.1	27.9	455
21e	$C_6H_5(CH_2)_3CONH$	CH	271	762.5	50.7	28.5	272
25a	NH_2	N	792	>10,000	NA[a]	NA[a]	NA[a]
26a	$C_6H_5CH_2CONH$	N	45.6	241.8	5.6	NT[b]	NT[b]

[a] NA: not applicable. The data of metabolic stability of compounds without a side chain at the 5-position was not applicable, since it is not so important from a perspective of the concept of an antedrug.
[b] NT: not tested.

Compound **25a** was obtained by changing the 4-position of **19** from the 4-pyridyl group to the 4-pyrimidinyl group. Through this change, p38α inhibitory activity decreased to about 1/8 (IC_{50}: 105 nM vs 792 nM) and TNF-α inhibitory activity to at least 1/20 (IC_{50}: 490.0 nM vs >10,000 nM). The compound **26a**, which was obtained by condensing the phenylacetyl group with compound **25a**, showed a significantly increased level of p38α inhibitory activity, as well as TNF-α inhibitory activity. In the pyrimidinylisoxazole form also, the 5-position substituent group effectively showed affinity toward p38α. However, its activity was reduced relative to that of pyridylisoxazole **21c**. Thus, its p38α inhibitory activity was reduced to about 1/2 (IC_{50}: 45.6 nM vs 23.0 nM) and TNF-α inhibitory activity to about 1/5 (IC_{50}: 241.8 nM vs 54.7 nM). However, surprisingly, in the metabolic stability test, pyrimidinylisoxazole **26a** showed a considerably improved metabolic profile with an unchanged drug residual percentage of 5.6% and because the activity of its anticipated metabolite **25a** was low, it was confirmed to be extremely suitable as an antedrug. To further increase its activity, optimization was carried out using **26a** as the lead compound.

The results of optimization using **26a** as the lead compound are presented in Table 3. First, analysis of the inhibitory effect of individual substituents revealed that the 4-fluoro group (**26a**–**26e**), 2,4-difluoro group (**26h**), 4-chloro group (**26g**), 3-methyl group (**26k**), and 4-fluoro-3-methyl group (**26l**) were preferable in the case of the isoxazole 3-position benzene ring (R_1). On the other hand, compounds with a halogen introduced only at the *ortho* position (**26f**, **26i**, and **26j**) showed a considerably low activity. This 3-position benzene ring was expected to interact with the hydrophobic region I, these substituent effects showed a similar tendency with that corresponding to the same site in the other inhibitors. On the other hand, the introduction of a chloro group (**26c**, **26d**, **26g**, **26h**, and **26k**) of the *ortho* position or methyl group (**26e**, **26l**) as substituent group (R_2) of the isoxazole 5-position side chain benzene ring was effective, showing higher activities than the unsubstituted compound **26a**. Of these, **26k** and **26l**, which carried a 3-methyl group at R_1, showed very high TNF-α inhibition activities, with their individual IC_{50} values exceeding p38α inhibitory activities. Detailed investigations have not been carried out, but this may be attributed

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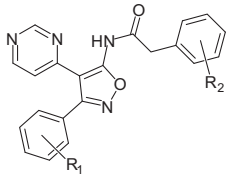
Table 3
Biological activity and metabolic stability of 4-pyrimidinylisoxazole derivatives



Compound	Structure		p38α MAP kinase	TNF-α	Metabolic stability in human liver S9
	R_1	R_2	IC_{50} (nM)	IC_{50} (nM)	(5 min, % of remaining)
26a	4-F	H	45.6	241.8	5.6
26b	4-F	2-F	33.2	100.6	10.0
26c	4-F	2-Cl	6.88	15.5	5.8
26d	4-F	2-F, 6-Cl	10.9	49.1	16.1
26e	4-F	2,5-CH_3	8.43	93.9	53.9
26f	2-Cl	H	141	522.8	NT[a]
26g	4-Cl	2-Cl	10.8	33.6	96.6
26h	2,4-F	2-Cl	2.73	22.9	20.4
26i	2,6-F	H	294	1293.0	NT[a]
26j	2,6-Cl	H	1010	4242.0	NT[a]
26k	3-CH_3	2-Cl	3.62	0.78	21.2
26l	3-CH_3, 4-F	2-CH_3	5.48	0.89	52.6

[a] NT: not tested.

to the lower selectivity of these compounds for other kinases [off target, such as c-Jun N-terminal kinase (JNK)] that regulate the production of inflammatory cytokines. From the perspective of metabolic stability, a comparison of compounds having high activity, obtained by introducing an *ortho* position substituent at R_2 (**26c**–**26e**, **26g**, **26h**, **26k**, and **26l**), showed a tendency of undergoing stable metabolism as compared to the unsubstituted compound (**26a**). All compounds, except **26g** (percentage of unchanged residues after 5 min: 96.6%), were metabolized to about half or even more in 5 min in human liver S9 and therefore, each of these compounds is at a level of fulfilling the concept of an antedrug. Among these, **26c**, **26d**, **26h**, and **26k** not only showed particularly excellent TNF-α inhibition (IC_{50}: <50 nM), but also excellent metabolic stability (percentage of unchanged residue after 5 min: <30%). Furthermore, after a comprehensive investigation of CYP inhibition (unpublished data), genotoxicity (negative Ames test), and drug efficacy, **26d** (AKP-001) was selected as the candidate for further development.[29]

To reduce the side effects of a p38 MAP kinase inhibitor, it is essential to eliminate the class effect by optimizing the compound to a profile that was suitable for an antedrug. At the same time, it is necessary to increase selectivity for various kinds of kinases other than p38α and prevent the off-target drug effects. Accordingly, the inhibitory effect of **26d** and the presumed metabolite **25a** against various isoforms of p38 MAP kinase and against various tyrosine/threonine kinases and serine/threonine kinases was evaluated (Table 4). Analysis of the inhibitory effect of **26d** against various isoforms of p38 MAP kinase showed that the activity of p38α was the highest (IC_{50}: 10.9 nM), followed by p38β, with about 1/30 of the activity, then p38γ and p38δ, which were inactive. In the case of JNK, which is a kinase involved in inflammation in a manner similar to that of p38 MAP kinase, an intermediate inhibitory activity was observed in JNK2 and JNK3. In addition, all were inactive against the 32 types of kinases and **26d** was confirmed as the compound showing relatively higher p38α selectivity. Furthermore, although selectivity of the anticipated metabolite **25a** for each of the isoforms of p38 and JNK showed an overall tendency that resembled **26d**, in general, the inhibitory activity was low compared to that of **26d**.

Table 4
Inhibitory effects of **26d** and the principal metabolite **25a** on p38 MAP kinase and JNK isoforms

Compound	Kinase isoform IC_{50} (nM)						
	p38α	p38β	p38γ	p38δ	JNK1	JNK2	JNK3
26d	10.9	326	NE[a]	NE[a]	NE[a]	198	572
25a	792	9714	NE[a]	NE[a]	NE[a]	1476	2199

To evaluate the selectivity profiles of **26d** against protein kinases, the inhibitory effects of **26d** against 32 distinct kinases were studied other than Table 4. As those kinases were; CSK, LCK, AKT2, CRIK, ROCK1, PKACα, PDK1, PKCα, RSK2, CaMK4, CaMK2α, CHK1, DAPK1, MAPMAPK2, PIM1, CHK2, CDK2/cyclinA, GSK3β, SRPK1, AurA, IKKβ, NEK2, TTK, IRAK4, PHKG1, CK1δ, PDK2, Erk1, Erk2, Erk5, MAP3K5 and RAF1, **26d** were inactive against them.

[a] NE: no effect.

4.3. Docking studies

Compound **26d** was docked to the ATP-binding site in the X-ray crystal structure (PDB ID: 1bl7) of p38α MAP kinase obtained from Protein Data Bank (PDB) by using the docking tool (CDOCKER) of Discovery Studio (Fig. 4). In the resulting binding structure, a 4-fluorophenyl site of **26d** was detected in hydrophobic region I, which consisted of Leu75, Leu86, Leu104, Val105, and Thr106. Moreover, the 4-position nitrogen atom of pyrimidine became the hydrogen bond acceptor of the Met109 principal chain N–H, and the nitrogen atom of the isoxazole ring interacted with the terminal amino group of Lys53 via hydrogen bonding. In the case of the most interesting 5-position substituent, the 2-fluoro-6-chlorophenyl terminal site was situated in the hydrophobic region II, which consisted of Met109, Ala111, Asp112, Ala157, and Leu167 and seem to be interacting with them by van der Waals forces. These results were in accordance to the initial concept. The compound 2-acylaminoimidazole derivative **27**, reported by Bracht et al.,[29] presented a structure that was relatively similar to that of **26d**. In their docking model, the acyl site of **27** interacts with the hydrophobic region II, and p38α inhibition increased 10-fold after the introduction of this acyl site. However, the suitability of **27** for antedrug was not tested.

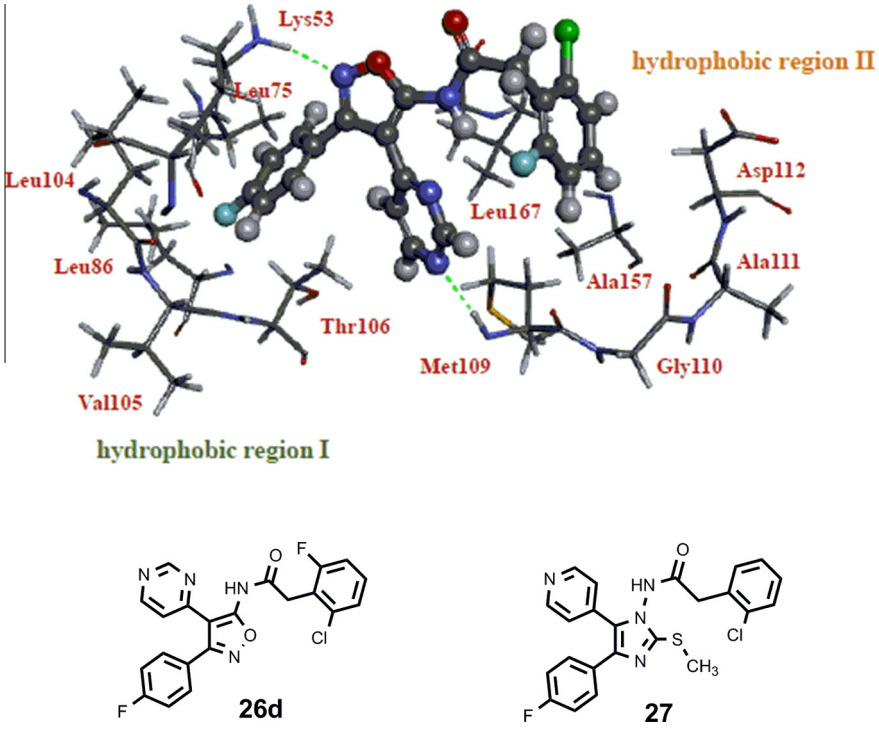


Figure 4. Docking results for **26d** in the p38α MAP kinase. X-ray structure 1bl7 is from the PDB database.

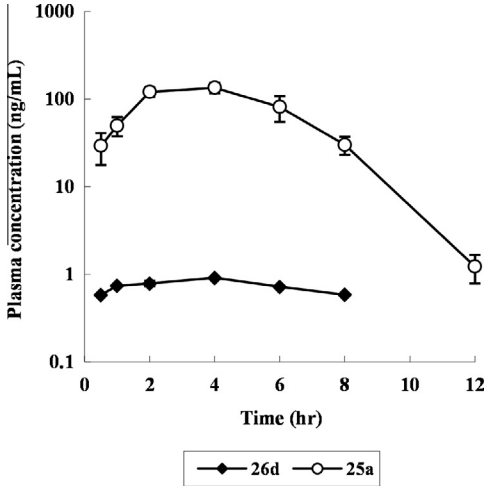


Figure 5. Plasma concentrations of **26d** and its metabolites **25a** after a single oral administration of **26d** (30 mg/kg) to male rats.

Table 5
PK parameters of **26d** and its metabolites **25a** in plasma after a single oral (30 mg/kg) or intravenous (3 mg/kg) administration of **26d** to male rats

Route	p.o.		iv	
Compound	**26d**	**25a**	**26d**	**25a**
C_{max} (ng/mL)	0.88 ± 0.08	136 ± 16.6	363 ± 89[a]	744 ± 111
T_{max} (h)	3.33 ± 1.15	3.33 ± 1.15	0.08 ± 0.0	0.5 ± 0.0
$t_{1/2}$ (h)	18.7 ± 17.9	0.98 ± 0.09	0.474 ± 0.0028	0.428 ± 0.037
$AUC_{0-\infty}$ (ng h/mL)	23.7 ± 17.4	760 ± 40.6	505 ± 37.9	924 ± 98.5
BA[b] (%)	<1	NA[c]	NA[c]	NA[c]

Each value represents the mean ± SD of three rats.
[a] Plasma concentration at 5 min.
[b] Oral bioavailability of **26d**.
[c] NA: not applicable.

4.4. Pharmacokinetics of 26d

The pharmacokinetics (PK) of an antedrug is particularly important because it is directly linked to the onset of side effects. Figure 5 shows the plasma concentrations of **26d** and its metabolites **25a** after a single oral administration of **26d** (30 mg/kg) to male rats. Table 5 shows the important PK parameters at that time, along with the data for intravenous administration. After oral administration, the maximum concentration of unchanged **26d** in plasma (C_{max}) was 0.88 ng/mL. This was extremely low and confirms that according to the strategy, it metabolizes rapidly into **25a** after absorption by cleavage of the 5-position amide bond. On the other hand, the plasma concentration of **25a** showed a relatively high value (C_{max}: 136 ng/mL), but with a short half-life ($t_{1/2}$) of 1 h or less, which indicates that **25a** is also rapidly metabolized. The bioavailability of **26d** after oral administration was 1% or less and its systemic exposure has been estimated to be extremely low. This confirms that **26d** considerably reduces the undesired side effects originating from p38α inhibition and this compound suits the concept of an antedrug.

4.5. Drug efficacy evaluation of 26d

Compound **26d** was evaluated by using animal models of two types of IBD. In the dextran sulfate sodium (DSS)-induced colitis mouse model, the shortening of the colon, which is a marker of inflammation, was significantly inhibited at dose of 10 mg/kg or more of **26d** (Fig. 6). In a 2,4,6-trinitrobenzene sulfonic acid sodium salt (TNBS)-induced colitis rat model, a dose of 10 mg/kg or more of **26d** significantly reduced the damage score (Fig. 7). These results clearly show that **26d** has a curative effect on colitis in animal models of IBD.

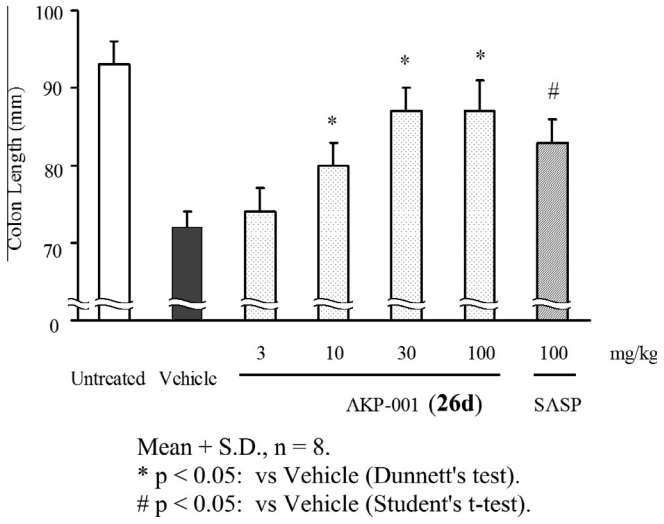


Figure 6. Inhibitory effects of **26d** and SASP on DSS-induced colitis in mice.

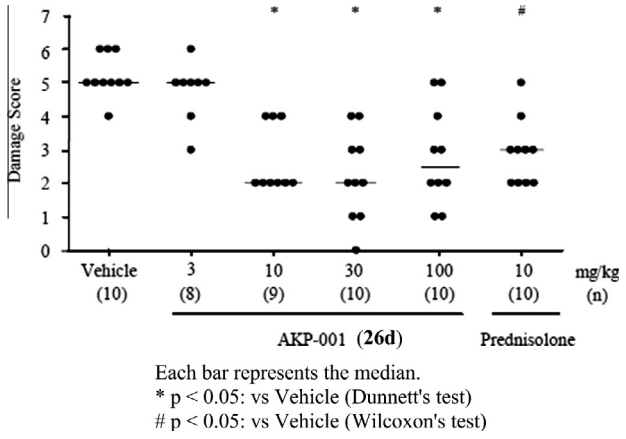


Figure 7. Inhibitory effects of **26d** and prednisolone on TNBS-induced colitis in rats.

5. Conclusions

In the present study, we have discovered a novel compound **26d** using isoxazole as the nucleus and a p38 MAP kinase inhibitor of prototypical structure. The fourth interaction site and 5-position substituent, which effectively interacts with p38α MAP kinase, was discovered and an antedrug was designed with reduced side effects due to its rapid inactivation during metabolism. Compound **26d** showed a high clearance in metabolic stability tests using human liver S9 cells. Furthermore, **26d** underwent rapid metabolism when orally administered to rats, and left almost no unchanged compound in the plasma, suggesting a reduction in systemic side effects that are attributable to the p38α inhibitor. Moreover, **26d** showed a significantly high drug efficacy in experimentally induced colitis in animals.

To date, various p38 MAP kinase inhibitors have been tested clinically, but the development of many of these drugs has been suspended. Owing to the low selectivity of the earlier compounds for various kinases, one reason may be the expression of their side effects.[14] In terms of drug efficacy, some p38 MAP kinase inhibitors such as BIRB796 or VX-702 resulted in a lowering of C-reactive protein levels (indicator of inflammation) for a definite period following their administration in clinical studies involving RA as the target illness. Upon continued administration, however, their efficacy was reduced and thus the so-called redundancy of MAP kinase pathway was indicated[30,31] On the other hand, although the reason remains unclear, PH-797804 did not show any reduction in anti-inflammatory action even after being administered over a long period and is currently undergoing phase III tests.[16] Moreover, losmapimod and dilmapimod also have completed phase II tests. The common factor among the 3 drugs is that chronic obstructive pulmonary disease (COPD) is their target illness, and the possibility of persistence of their anti-inflammatory effect was influenced by either the disease or by the treated body organs. In the case of BIRB796, a reduction in the anti-inflammatory effect against IBD or digestive organs was observed, but because of the lack of clinical cases as well as several unclear points, we are only including the clinical results of **26d**. We expect **26d** to be considered as a new and excellent anti-inflammatory drug with reduced side effects and can be used in various organs (including the respiratory organs).

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