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Structure–activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors

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ABSTRACT

A structure–activity relationship study of dorsomorphin, a previously identified inhibitor of SMAD 1/5/8 phosphorylation by bone morphogenetic protein (BMP) type 1 receptors ALK2, 3, and 6, revealed that increased inhibitory activity could be accomplished by replacing the pendent 4-pyridine ring with 4-quinoline. The activity contributions of various nitrogen atoms in the core pyrazolo[1,5-*a*]pyrimidine ring were also examined by preparing and evaluating pyrrolo[1,2-*a*]pyrimidine and pyrazolo[1,5-*a*]pyridine derivatives. In addition, increased mouse liver microsome stability was achieved by replacing the ether substituent on the pendent phenyl ring with piperazine. Finally, an optimized compound **13** (LDN-193189 or DM-3189) demonstrated moderate pharmacokinetic characteristics (e.g., plasma $t_{1/2}$ = 1.6 h) following intraperitoneal administration in mice. These studies provide useful molecular probes for examining the in vivo pharmacology of BMP signaling inhibition.

Bone morphogenetic proteins (BMPs) are a group of >25 protein ligands that comprise a subset of the transforming growth factor β (TGF-β) family. BMPs modulate a multitude of biological processes, including bone and cartilage formation during embryogenesis.[1a] However, they are also intimately involved with numerous non-osteogenic developmental and physiological processes throughout adulthood as well as several pathological conditions.

BMPs bind to two classes of cell surface bone morphogenetic protein receptors (BMPR-I and BMPR-II).[1a] The BMPR-I receptor class consists of three receptor types, activin receptor-like kinase-2 (ALK-2 or ActR-IA), ALK-3 (BMPR-IA) and ALK-6 (BMPR-IB). The BMPR-II receptor class is comprised of three receptor types, BMPR-II, ActR-IIA and ActR-IIB. Binding of BMPs results in the formation of heterotetrameric complexes containing two type I and two type II receptors. In addition to an extracellular binding domain, each BMP receptor contains an intracellular serine/threonine kinase domain. Following binding of BMPs, constitutively active type II receptor kinases phosphorylate type I receptor kinase domains that in turn phosphorylate BMP-responsive SMADs 1, 5, and 8, which can enter the cell nucleus and function as transcription factors.[1b] Phosphorylation of these specific SMADs results in various cellular effects, including growth regulation and differentiation. Signaling via BMP receptors may also activate other pathways, including mitogen activated protein kinase (MAPK).[1c]

Several diseases are known to arise from inborn defects in the BMP signaling pathway, including idiopathic pulmonary arterial hypertension,[2] hereditary hemorrhagic telangiectasia syndrome and juvenile familial polyposis syndrome,[3] all of which involve loss-of-function mutations in BMP receptors or co-receptors. Acquired defects in the BMP signaling pathway are thought to contribute to metastasis of prostate carcinoma[4] and renal cell carcinoma.[5] Other disorders, such as fibrodysplasia ossificans progressiva (FOP)[6] and anemia of chronic disease[7] may result from increased BMP signaling. For conditions where increased BMP signaling contributes to disease pathogenesis, inhibitors may offer therapeutic benefit.

Inhibition of BMP signal transduction could be envisioned to occur through various mechanisms, including antagonizing BMP binding to BMPRs or inhibition of the intracellular BMP receptor kinase activity.[8] Numerous endogenous protein antagonists that sequester BMP ligands preventing engagement with BMP receptors are known, including noggin, follistatin, chordin and gremlin. Small molecule antagonists of the BMP ligand-receptor interaction have not been identified, possibly due to difficulties antagonizing this protein–protein interaction.[9] In addition, the structural diversity of BMP receptors and ligands, and functional redundancy of both systems might pose a challenge for effective blockade of extracel-

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lular domains. However, inhibition of SMAD phosphorylation by BMPR-I intracellular kinase domains with small molecules may provide more efficient signal transduction pathway inhibition. This latter approach has been used to identify inhibitors (i.e., SB-431542) of the TGF-β1 receptor kinase ALK5.[10]

Recently, dorsomorphin, **1**,[7a,11,12] was discovered as an inhibitor of SMAD 1/5/8 phosphorylation by BMP type 1 receptors (ALK2, 3, and 6) utilizing a phenotypic screen to identify compounds that perturb embryonic dorsoventral axis formation (see Fig. 1). Furthermore, this inhibition was shown to decrease BMP-regulated hepatic hepcidin gene transcription, leading to increased iron levels in vivo.[7a] However, **1** only demonstrated moderate inhibition of SMAD 1/5/8 phosphorylation by BMPR-I with an $IC_{50} \sim 0.5$ μM and lacks metabolic stability. Herein, we describe the results of a structure–activity relationship (SAR) study to optimize BMP signaling inhibition of SMAD 1/5/8 phosphorylation. In addition, we addressed the metabolic stability of this compound series and report a pharmacokinetic study for an optimized derivative.

The synthesis of substituted pyrazolo[1,5-*a*]pyrimidine derivatives was initially accomplished according to Scheme 1 (Method A). Arylacetonitriles, **2**, were allowed to react with dimethyformamide dimethylacetal (DMF-DMA) to give **3**. In the case of pyridine or quinoline acetonitrile salts, an equivalent of triethylamine was also added. Cyclization of **3** in the presence of hydrazine hydrobromide gave 2-amino-1H-pyrazoles **4a**. Condensation of **4a**–**c** with various malondialdehydes in acetic acid and ethanol either under conventional or microwave (MW)[13] heating yielded pyrazolo[1,5-*a*]pyrimidine derivatives **5a**–**c**. In the case of **5c**, palladium-mediated cross-coupling with arylboronic acids also gave **5a**. This reaction was useful for preparing derivatives where the corresponding

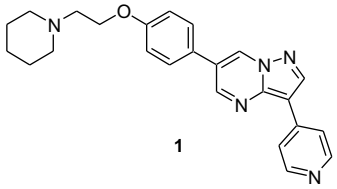

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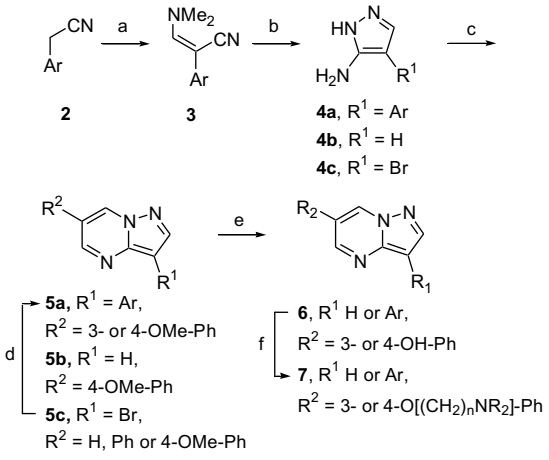
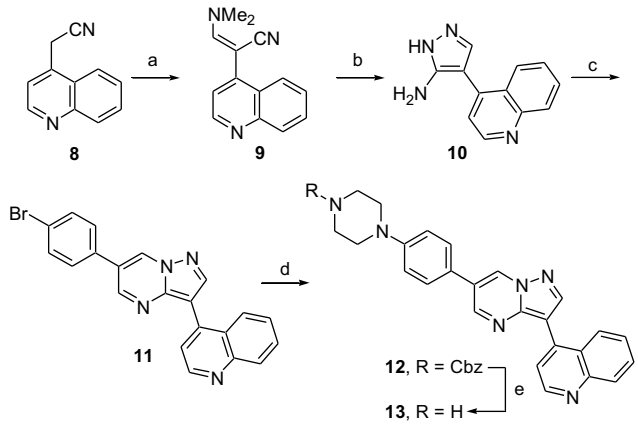
Figure 1. BMP signaling inhibitor of SMAD 1/5/8 phosphorylation.



Scheme 1. Method A. Reagents and conditions: (a) $(MeO)_2CHNMe_2$, Et_3N (for pyridine and quinoline salts), DMF, 110 °C, 4–6 h, 100%; (b) $NH_2NH_2{\cdot}HBr$, EtOH/H_2O, 110 °C, 6 h, 45–80%; (c) $ArCH(CHO)_2$, AcOH, EtOH, 110 °C, 6 h (or MW, 170 °C, 5 min); (d) $ArB(OH)_2$, $Pd_2(dba)_3$, 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl, K_3PO_4, *n*-BuOH, MW, 150 °C, 8 min, 84–90%; (e) HBr/HOAc, MW, 130 °C, 8 min, 65–86%; (f) $R_2N(CH_2)_nCl{\cdot}HCl$, Cs_2CO_3, NaI (cat), DMF, 60 °C, 3 h, (or MW, 140 °C, 6 min), 30–75% or $Cl(CH_2)_nCl$, K_2CO_3, DMF, MW, 140 °C, 6 min, then R_2NH, NaI (cat), DMF, MW, 150 °C, 10 min, 30–60%.



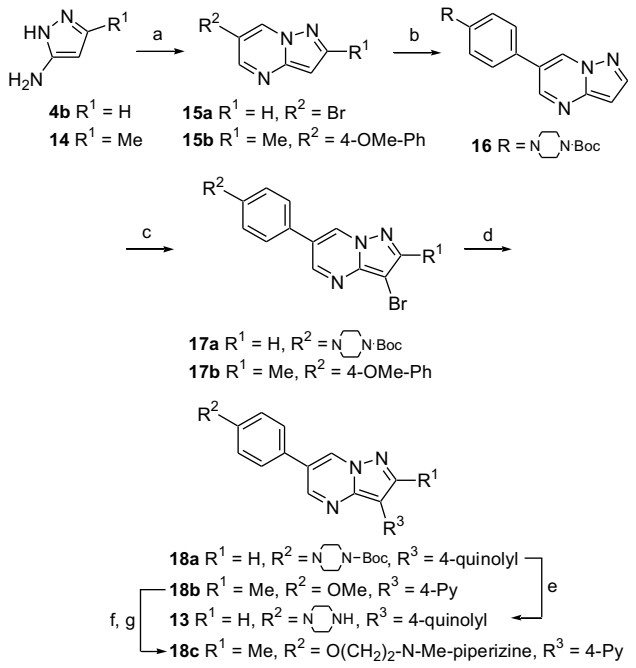
Scheme 2. Method B. Reagents and conditions: (a) $(MeO)_2CHNMe_2$, 110 °C, 16 h, 100%; (b) $NH_2NH_2{\cdot}HBr$, EtOH/H_2O, 110 °C, 4 h, 80%; (c) 4-BrPhCH(CHO)$_2$, AcOH, EtOH, MW, 170 °C, 5 min, 54%; (d) *N*-Cbz-piperazine, $Pd_2(dba)_3$, (2-biphenyl)di-tert-butylphosphine, KOBu-t, DME, 100 °C, 20 h, 20–30%; (e) H_2 (1 atm), 5% Pd/C (57% H_2O), MeOH/CH_2Cl_2, rt, 4 h, 86%.

arylacetonitriles were not readily available. Dealkylation of the 3- or 4-methoxy ethers on the pendent phenyl rings was accomplished with hydrobromic acid in acetic acid with microwave heating to give **6**. Finally, alkylation in one step with $R_2N(CH_2)_nCl$ or in two steps with $Cl(CH_2)_nCl$ followed by amine addition gave **7**.

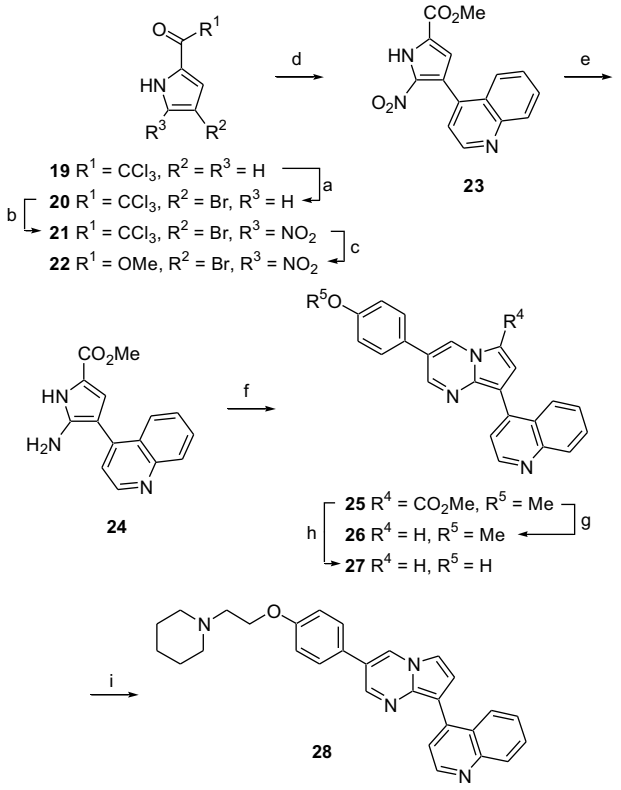
Two other routes were subsequently developed for the synthesis of pyrazolo[1,5-*a*]pyrimidine derivative **13**[14] and other analogs that contained an amine on the 3- or 4-position of the pendent phenyl ring. The first alternate route, depicted in Scheme 2 (Method B), began in a similar manner as previously described starting with **8**,[15] except that 2-(4-bromophenyl)malondialdehyde was used to generate **11**. Next, a palladium-mediated cross coupling with *N*-Cbz-piperazine yielded **12**. Removal of the benzyl carbamate by hydrogenation (1 atm) in the presence of 5% Pd/C gave **13**.

The second alternate route to **13**, depicted in Scheme 3 (Method C), began with 2-amino-1H-pyrazole, **4b**, which was allowed to react with 2-bromomalondialdehyde to give 6-bromopyrazolo[1,5-*a*]pyrimidine, **15a**. A palladium-mediated cross-coupling with 4-4-(*tert*-butoxycarbonyl) piperazin-1-ylphenylboronic acid pinacol ester yielded **16**. Next, a regioselective bromination of the C-3 carbon with *N*-bromosuccinimide (NBS) in dichloromethane at room temperature gave **17a** in 79% yield. Palladium-mediated cross-coupling of this aryl bromide with quinoline-4-boronic acid produced **18a** in a moderate 46% yield. Finally, deprotection of the *tert*-butyl carbamate with 4N HCl in a mixture of 1,4-dioxane and methanol gave **13** as the hydrochloride salt. This method was also used to prepare several other derivatives, including **18c** that contains a C-2 substituent.

The synthesis of pyrrolo[1,2-*a*]pyrimidine derivatives is illustrated in Scheme 4 (Method D). 2-Trichloromethylketopyrrole, **19**, was regioselectively brominated affording **20**.[16] Next, regioselective nitration with concentrated nitric acid gave **21**.[17] This compound was allowed to react with sodium methoxide in methanol producing the methyl ester **22**. Palladium-mediated cross-coupling of this pyrrole bromide with quinoline-4-boronic acid generated **23** in 60% yield.[12a] Reduction of the nitro group with hydrogen (1 atm) in the presence of 10% Pd/C gave **24**, which was used immediately without purification. Condensation with 2-(4-methoxyphenyl)malondialdehyde in acetic acid and ethanol yielded pyrrolo[1,2-*a*]pyrimidine derivative **25**. Heating this material at 110 °C in aqueous sulfuric acid for 2 h gave **26** via ester hydrolysis and subsequent decarboxylation.[18] Prolonged heating of **25** for 2 days resulted in ether hydrolysis producing **27**. Finally, alkylation of the phenol afforded **28**.



Scheme 3. Method C. Reagents and conditions: (a) $BrCH(CHO)_2$ (or 4-OMe-$PhCH(CHO)_2$ for **15b**) AcOH, EtOH, 80 °C, 7 h, 49%; (b) $B(O[C(CH_3)_2]_2O)$-4-Ph-*N*-Boc-piperazine, $Pd(PPh_3)_4$, K_2CO_3, dioxane/H_2O, MW, 150 °C, 8 min, 90% (or 110 °C, 3 h, 86%); (c) NBS, CH_2Cl_2, rt, 5 h, 79%; (d) quinoline-4-boronic acid, $Pd_2(dba)_3$, 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl, K_3PO_4, *n*-BuOH, MW, 150 °C, 15 min, 46%; (e) 4 N HCl in 1,4-dioxane, MeOH, rt, 24 h, 95%; (f) HBr/HOAc, MW, 130 °C, 8 min, 81%; (g) $Cl(CH_2)_2Cl$, K_2CO_3, DMF, MW, 140 °C, 6 min, then *N*-Me-piperizine, NaI (cat), DMF, MW 150 °C, 10 min, 57%.

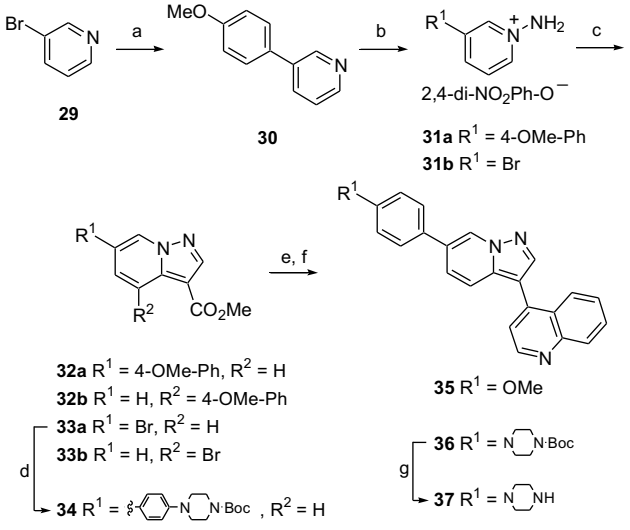


Scheme 4. Method D. Reagents and conditions: (a) Br_2, $CHCl_3$, 0 °C, 57%; (b) HNO_3 (70%), Ac_2O, −40 °C to rt, 40%; (c) NaOMe, MeOH, rt, 99%; (d) quinoline-4-boronic acid, $Pd(PPh_3)_4$, Na_2CO_3, 1,4-dioxane, reflux, 16 h, 60%; (e) H_2 (1 atm), 10% Pd/C, MeOH, rt, 0.5 h; (f) 4-MeOPhCH(CHO)$_2$, AcOH, EtOH, reflux, 16 h, 73%; (g) 40% aqueous H_2SO_4, 110 °C, 2 h, 91%; (h) 40% aqueous H_2SO_4, 110 °C, 2 days, 71%; (i) piperidyl-*N*-CH_2CH_2Cl·HCl, 60% NaH, DMF, rt, 24 h, 80%.

The synthesis of pyrazolo[1,5-*a*]pyridine derivatives is outlined in Scheme 5 (Method E). A palladium-mediated cross-coupling of 3-bromopyridine, **29**, with 4-methoxyphenylboronic acid produced **30** in 58% yield.[19] This pyridine derivative was converted to the 1-aminopyridinium salt **31a** utilizing *O*-(2,4-dinitrophenyl)hydroxylamine.[20] Cyclization of **31a** upon treatment with methyl propiolate gave regioisomers **32a** and **32b** in a 1:2 ratio and a combined yield of 33% over two steps.[21] In a similar manner, **29** was converted to **33a** and **33b** (1:2 ratio) in 37% yield, via intermediate **31b**. Compound **33a** was further converted to **34** via a palladium-mediated coupling. Then, **32a** and **34** were hydrolyzed with aqueous sodium hydroxide and the resulting carboxylic acids were subjected to metal-mediated decarboxylative coupling[22] with 4-bromoquinoline in the presence of $Pd(acac)_2$ and CuI producing **35** and **36**, respectively, albeit in low yields (10–22%). Finally, exposure of **36** to 4 N HCl in 1,4-dioxane resulted in removal of the *tert*-butyl carbamate yielding **37** as the hydrochloride salt.

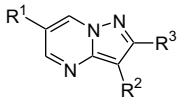
Evaluation of BMP4-induced phosphorylation of SMAD 1/5/8 was performed using a sensitive cytoblot cellular ELISA assay in the presence of varying concentrations of test compounds.[23a,b] Functional IC_{50} values were calculated for the inhibitory effects of test compounds on phosphorylation of SMAD 1/5/8 and are shown in Tables 1–3.[23c]

Introduction of an aminoether at the 4-position of the pendent phenyl ring on the pyridine derivatives (e.g., **1** and **39**–**41** vs **38**) increased activity three to fifteen fold in addition to improving aqueous solubility (Table 1). Introduction of a substituent on the 2-position of the pyrazolo[1,5-*a*]pyrimidine ring (**43**) abolished activity. In addition, activity was dramatically affected by the nature of the substituent on the 3-position of the pyrazolo[1,5-*a*]pyrimidine ring. Removal (**44**) or replacement of the 4-pyridyl in **1** with 3-pyridyl (**45**) or phenyl (**46**) resulted in complete loss of activity. Replacement of the pyridine ring with 3-fluoro-4-pyridyl (**47**) likewise resulted in reduced activity.



Scheme 5. Method E. Reagents and conditions: (a) 4-MeOPhB(OH)$_2$, $Pd(PPh_3)_4$, K_3PO_4, 1,4-dioxane, 100 °C, 18 h, 58%; (b) 2,4-di-NO_2PhONH$_2$, CH_3CN, 40 °C, 20 h; (c) HC≡CCO_2Me, K_2CO_3, DMF, rt, 33–37% over two steps (**32a**:**32b** and **33a**:**33b** ~ 1:2); (d) $B(O[C(CH_3)_2]_2O)$-4-Ph-*N*-Boc-piperazine, $Pd(PPh_3)_4$, K_2CO_3, 1,4-dioxane/H_2O, 110 °C, 5 h, 73%; (e) NaOH, EtOH/H_2O (6:1), Δ, 3 h; (f) 4-bromoquinoline, $Pd(acac)_2$, CuI, K_2CO_3, 1,10-phenanthroline, 4 Å MS, NMP, 165 °C, 24 h, 10–22% (over two steps); (g) 4 N HCl in 1,4-dioxane, MeOH, rt, 24 h.

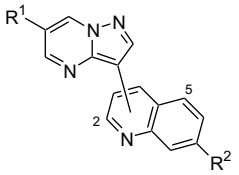
Table 1
IC_{50} determinations for inhibition BMP4-induced phosphorylation of SMAD 1/5/8



Compound	R^1	R^2	R^3	Method	IC_{50} (μM)
1	Pip-CH_2CH_2O-4-Ph	4-Py	H	—	0.43
38	4-MeO-Ph	4-Py	H	A	6.5
39	Morph-CH_2CH_2O-4-Ph	4-Py	H	A	2.0
40	Et_2N-CH_2CH_2O-4-Ph	4-Py	H	A	0.50
41	NMP-CH_2CH_2O-4-Ph	4-Py	H	A	0.45
42	NMP-CH_2CH_2O-3-Ph	4-Py	H	A	4.5
43	NMP-CH_2CH_2O-4-Ph	4-Py	Me	C	>20
44	Pip-CH_2CH_2O-4-Ph	H	H	A	>20
45	Pip-CH_2CH_2O-4-Ph	3-Py	H	A	>20
46	Pip-CH_2CH_2O-4-Ph	Ph	H	A	>20
47	NMP-CH_2CH_2O-4-Ph	3-F-4-Py	H	C	3.3

Pip, piperidinyl; Morph, morpholinyl; NMP, *N*-methylpiperazinyl; Py, pyridyl.

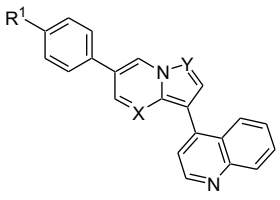
Table 2
IC_{50} determinations for inhibition BMP4-induced phosphorylation of SMAD 1/5/8



Compound	R^1	Position	R_2	Method	IC_{50} (μM)
48	4-MeO-Ph	6	H	C	>20
49	4-MeO-Ph	8	H	C	>20
50	4-MeO-Ph	5	H	C	3.0
51	4-MeO-Ph	3	H	C	>20
52	4-MeO-Ph	4	H	C	0.055
53	NMP-CH_2CH_2O-4-Ph	4	H	A	0.010
54	Pip-CH_2CH_2O-4-Ph	4	H	A	0.090
55	H	4	H	A	5.0
56	Ph	4	H	A	0.75
57	HO-4-Ph	4	H	A	0.25
13	Piperazinyl-4-Ph	4	H	A,B,C	0.0049
58	Piperazinyl-3-Ph	4	H	C	20
59	4-MeO-Ph	4	Cl	C	0.50
60	Piperazinyl-4-Ph	4	Cl	C	0.35

Pip, piperidinyl; NMP, *N*-methylpiperazinyl.

Table 3
IC_{50} determinations for inhibition BMP4-induced phosphorylation of SMAD 1/5/8



Compound	R^1	X	Y	Method	IC_{50} (μM)
25	MeO	N	CCO_2Me	D	>20
26	MeO	N	CH	D	>20
28	Pip-CH_2CH_2O	N	CH	D	2.6
35	MeO	CH	N	E	0.15
37	Piperazinyl	CH	N	E	0.005

Pip, piperidinyl.

Due to these significant substituent effects on the 3-position of the pyrazolo[1,5-*a*]pyrimidine ring and the demonstrated influence of heterocyclic substituents on other TGF-β receptor type inhibitors,[24] quinolines attached through various positions were examined (Table 2). In compound **52**, where the pyrazolo[1,5-*a*]pyrimidine ring is connected to the 4-position of the quinoline, a significant increase in activity was observed. Introduction of an aminoether to the 4-position of the pendent phenyl ring (**53**) increased potency as was previously observed for the pyridine derivatives (vida supra). Replacing the aminoether with piperazine (**13**) was also well tolerated. However, transposing this substituent to the 3-position of the pendent phenyl (**58**) resulted in significant loss of activity. Likewise, introduction of a chloride to the 7-position of the quinoline ring (**59** vs **52** and **60** vs **13**) resulted in decreased activity.

Next, the contributions of the nitrogen atoms in the 1- and 4-positions of the pyrazolo[1,5-*a*]pyrimidine ring were examined (Table 3). The importance of the N-1, but not the N-4, nitrogen atoms was previously demonstrated for KDR kinase inhibition by pyrazolo[1,5-*a*]pyrimidine derivatives.[25] Similarly, in the present series of compounds the N-1 (**28** vs **54**) appears necessary for potent inhibition of BMP4-induced phosphorylation of SMAD 1/5/8, whereas the N-4 was not essential (**37** vs **13**).

Both the original lead compound **1** and a more potent derivative **53** demonstrated poor metabolic stability in mouse liver microsomes (**1**: half-life ($t_{1/2}$) of 10.4 min and intrinsic clearance (CL_{int}) of 133 ± 6.6 μL/min/mg protein; **53**: $t_{1/2}$ of 13.3 min and CL_{int} of 104 ± 3.4 μL/min/mg protein).[23b,26] However, replacement of the ether on the pendent phenyl ring with piperazine resulted in a significant increase in mouse liver microsome stability. For example, **13** demonstrated a $t_{1/2}$ of 82 min and CL_{int} of 16.9 ± 5.6 μL/min/mg protein. Based on the potency and metabolic stability of **13**, it was selected for in vivo pharmacokinetic analysis following a single bolus intraperitoneal (ip) administration of 3 mg/kg in male and female C57B16 mice.[23b] The results of this study are shown in Table 4. The pharmacokinetics of **13** was similar in both male and female mice. The average maximal plasma concentrations were slightly higher in males (1.54 μM) than in females (1.29 μM) and were reached quickly (<5 min) following administration. The plasma half-life (1.6 h) and the average AUC_∞ values (994 and 1030 ng h/mL) were similar in male and female mice.

In conclusion, a structure–activity relationship study of dorsomorphin, **1**, a previously identified inhibitor of SMAD 1/5/8 phosphorylation by BMP type 1 receptors ALK2, 3, and 6, revealed that increased inhibitory activity could be accomplished by replacing the pendent 4-pyridine ring with 4-quinoline. The nitrogen atom in the 1-position of the pyrazolo[1,5-*a*]pyrimidine ring was determined to be necessary for inhibitory activity. However, the nitrogen atom in the 4-position was not vital. In addition, increased mouse liver microsome stability was achieved by replacing the ether substituent on the pendent phenyl ring with piperazine. Finally, an optimized compound **13** (LDN-193189 or DM-3189) demonstrated moderate pharmacokinetic characteristics (e.g., plasma $t_{1/2}$ = 1.6 h) following intraperitoneal administration in mice. Evaluation of this inhibitor in various animal disease models in which BMP signaling has been hypothesized to play a role, such as FOP and anemia of chronic disease, are currently ongoing.

Table 4
Pharmacokinetic analysis of **13** in plasma following bolus intraperitoneal administration in mice (N = 3/sex)

Sex	Dose (mg/kg)	C_{max} (μM)	t_{max} (min)	$t_{1/2}$ (h)	AUC_∞ ng h/mL
Male	3.0	1.54	<5	1.6	994
Female	3.0	1.29	<5	1.6	1030

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.06.052.

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