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Synthesis and structure-activity relationships of a novel and selective bone morphogenetic protein receptor (BMP) inhibitor derived from the pyrazolo[1.5-*a*]pyrimidine scaffold of Dorsomorphin: The discovery of ML347 as an ALK2 versus ALK3 selective MLPCN probe

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Abstract

A structure-activity relationship of the 3- and 6-positions of the pyrazolo[1,5-*a*]pyrimidine scaffold of the known BMP inhibitors dorsomorphin, **1**, LDN193189, **2**, and DMH1, **3**, led to the identification of a potent and selective compound for ALK2 versus ALK3. The potency contributions of several 3-position substituents were evaluated with subtle structural changes leading to significant changes in potency. From these studies, a novel 5-quinoline molecule was identified and designated an MLPCN probe molecule, ML347, which shows >300-fold selectivity for ALK2 and presents the community with a selective molecular probe for further biological evaluation.

Keywords

ALK2 kinase; Bone morphogenetic receptor; Pyrazolo[1,5-*a*]pyrimidine; Selectivity; ML347

The bone morphogenetic protein (BMP) signaling pathway plays critical, diverse roles in embryonic pattern formation, and a number of disease processes.[1] BMP ligands bind and activate the type-I and type-II BMP receptors, a family of serine-threonine kinases belonging to the TGF-β receptor superfamily, which then activate downstream mediators Smad1/5/8 by phosphorylation.[2] Activated Smad1/5/8 translocate to the nucleus to turn on BMP target genes. Because there are more than 20 distinct BMP ligands, a number of extracellular antagonists, three type-II receptors (BMP type II receptor, BMPRII; Activin type II receptor; ActRIIa and ActIIb), and four BMP type-I receptors (activin-receptor like kinase 1, ALK1; ALK2, ALK3 and ALK6), the role of targeting an individual component in various signaling contexts is unclear.

Recently, in a chemical genetic screen for compounds that perturb zebrafish embryonic axis, we discovered dorsomorphin (DM), **1**, the first small molecule inhibitor of the BMP pathway which directly targets the type-I receptor.[3] DM, **1**, and its analog LDN-193189, **2**, have been instrumental in demonstrating the therapeutic potential of BMP inhibitors for anemia, Duchenne Muscular Dystrophy, atherosclerosis and heterotopic ossification syndromes.[1,4] However, the early generation compounds DM, **1**, LDN-193189, **2**, and DMH1, **3**[5], do not discriminate between ALK1, ALK2, ALK3 and ALK6 (Figure 1)[5], and long-term consequences of pharmacological inhibition of all BMP signals are unknown. The issue of subtype selectivity is particularly germane to fibrodysplasia ossificans progressiva (FOP), a rare congenital disease of progressive soft tissue ossification, since it is caused by dysregulated BMP signaling due to a highly recurrent mutation (R206H) in ALK2.[6,7] Although LDN-193189, **2**, could blunt ectopic ossification in a mouse model expressing a constitutively active form of ALK2 (Q207D)[8], inhibitors with greater subtype selectivity might be more desirable as lead compounds for FOP. Such inhibitors might also be useful chemical probes to interrogate the biology of BMP signaling at the subtype resolution. Moreover, because DM, **1**, and LDN-193189, **2**, have important off-target effects, including AMP-activated kinase (AMPK), platelet-derived growth factor receptor-β (PDGFRβ) and vascular endothelial growth factor type-II receptor (VEGFR-2/KDR), validity of some of their *in vivo* effects has been challenged.[5,9] Therefore, we undertook a synthetic effort to develop compounds with greater subtype selectivity, centered around the central pyrazolo[1.5-a]pyrimidine scaffold which has culminated in the discovery of an ALK2 selective compound, ML347.

The first round of SAR was designed by keeping the 6-(4-methoxyphenyl) moiety constant and varying the 3-position (R^1). To that end, compounds **7a-m** were synthesized as outlined in Scheme 1. Starting with the commercially available 2-(4-methoxyphenyl)malonaldehyde, **4**, and condensing with 1*H*-pyrazol-5-amine, **5**, under acidic conditions afforded the 6-(4-methoxyphenyl)pyrazolo[1,5-*a*]pyrimidine in 92% yield[10], which was then iodinated (NIS, DMF) to give **6**.[11] The final compounds **7a-m** were synthesized either by converting **6** to the boronate ester (diboronpinacol ester, Pd(dppf)Cl_2*DCM, KOAc, DMF, 100 °C, 16 h) followed by Suzuki-Miyaura cross-coupling[12] with the appropriate aryl halide (ArX, Pd(dppf)Cl_2*DCM, K_3PO_4) or direct Suzuki-Miyaura cros-coupling with an appropriate aryl boronic acid ($R^1B(OH)_2$, Pd(dppf)Cl_2*DCM, K_3PO_4).

The SAR of the 3-position of the pyrazolo[1,5-*a*]pyrimidine scaffold is detailed in Table 1. Based on previous studies from our laboratories[5], and others[13], where it was shown that heterocycles with nitrogen in the 4-position were optimal, it was not surprising to see the substituted isoquinoline and 3- or 8-quinoline compounds inactive (**7a-c, e, f**). The 4-pyrazole compound (**7d**) was active (94.1 nM) as was the 5-quinoline (152 nM), which was not expected as this is the first compound without a nitrogen in the 4-position to show such potency. The most potent compound in the BMP4 cell assay was the 4-quinoline (**7i**, <1 nM), which is consistent with previous findings. Other nitrogen (**7j** and **7l**) or sulfur

compounds (**7m**) were inactive. However, subtle changes to the 3-postion substituent led to significant loss of activity. By changing the 4-quinoline (**7i**) to the 7-thieno[3,2-*b*]pyridine (**7n**) resulted in nearly complete erosion of activity (4571 nM) even though these compounds are similar in size and shape.

Having evaluated a number of 3-position substituents, we next looked at the 4-position of the 6-phenyl substituent. Previous results from our laboratory[5] evaluated substituted alkyl chain substituents (such as those in **1** and **3**) with much success and additional studies evaluated phenyl replacements (pyridinone[16], unpublished results); however, any phenyl replacements led to inactive compounds. Thus, the 6-phenyl moiety has remained intact for this study. The synthesis of these analogs follows the outlined steps in Scheme 2. Starting with 2-bromomalonaldehyde, **8**, and condensing with 1*H*-pyrazol-5-amine, **5**, under acidic conditions led to 6-bromopyrazolo[1,5-*a*]pyrimidine, **9**.[10] Next, an appropriately substituted 4-phenylboronate ester was reacted with **9** under Suzuki-Miyaura cross-coupling conditions ($Pd(dppf)Cl_2$*DCM, K_3PO_4) in good yields 56-73%. The resulting compound, **11**, was then iodinated in the 3-position (NIS, DMF, 73%) and the final compounds (**13a-r**) were realized after a final Suzuki-Miyaura cross-coupling[12] step with an appropriate boronic acid ($R^3B(OH)_2$, $Pd(dppf)Cl_2$·DCM, K_3PO_4).

The SAR of these compounds had two points of diversity on the molecule – with the 6-(4-phenyl)-position having molecules similar to that found in LDN193189, **2**; namely six-membered heterocycloalkyl groups (piperazine in LDN-193189). The first R^2 group evaluated was morpholine (**13a-g**) with the SAR tracking similarly to that seen in Table 1 and previously. Thus, the most active compounds were those containing heterocycles in the 4-position of the 3-substituent (4-pyrazole, **13a**, 141 nM; 2-chloro-4-pyridine, **13c**, 529 nM; 4-quinoline, **13d**, <1.0 nM; 3-benzo[*b*]thiophene, **13g**, 418 nM) (Table 2). These same R^3 groups were active when the R^2 group was changed to piperazine (**13h-n**) and 4-methylpiperazine (**13o-r**). In each case, the 4-quinoline or 4-pyrazole was the most potent of the R^3 groups. Although the 3-benzo[*b*]thiophene was active in the morpholine groups, the activity significantly dropped off in the piperazine group (**13n**, 2636 nM) and was inactive in the 4-methylpiperazine group (**13o**). Notably, the 5-quinoline compound (**13m**) was equipotent to **7g**. This SAR trends mimics that which was seen in our earlier work on this scaffold.[5]

Having identified a number of potent inhibitors in the functional BMP4 cell based assay, which with certain structural classes can be difficult to interpret due to promiscuity, we next sent a number of compounds for kinase selectivity to Reaction Biology Corp. (Malvern, PA). We tested our active compounds against 10 kinases (Table 3) and each of the assays were run in 10-dose IC_{50} mode with 3-fold serial dilution starting at 100 μM. The reactions were carried out at 10 μM ATP. The three reference compounds have been previously run in these selectivity assays and are shown in Table 3. DM, **1**, LDN-193189, **2**, and DMH1, **3**, are potent against ALK2 (ACVR1) however, all the compounds are equipotent or more potent against ALK3 (BMPR1A). In addition, these compounds have variable selectivity against the other kinases evaluated; however, DMH1, **3**, shows the most selectivity within these three compounds. Across the board, all compounds tested were equipotent against ALK1 (ACVRL1) and ALK2 (ACVR1), however, there were two compounds identified that displayed selectivity against ALK3 (BMPR1A) and each compound contain a 5-quinoline R^3 substituent (**7g** and **13m**). The more potent (and selective) compound, **7g**, has IC_{50}'s of 46 and 32 nM, respectively, against ALK1 and ALK2; however, the IC_{50} against ALK3 is 10,800 nM, >300-fold selective over ALK3. In addition, **7g** is completely inactive against all the other kinases tested (with weak activity against ALK6, 9830 nM and KDR (VEGFR2) 19,700 nM). It is interesting to note that it appears to be a combination of the 5-quinoline and 4-methoxyphenyl which gives rise to the selectivity profile, as **13m** still retains

significant ALK3 activity (539 nM). Due to the potency of **7g** against the BMP4 cell assay, ALK1 and ALK2 and the significant selectivity against the other kinases, **7g**, has been declared a probe molecule in the MLPCN and redesignated ML347.[17]

In order to further the BMP community as to the utility of ML347, we evaluated this molecule in our Tier 1 *in vitro* pharmacokinetic assays (Table 4). These studies are useful in order to evaluate the metabolic stability and predicted clearance in a number of species in order to inform on possible dosing routes. Utilizing rapid equilibrium dialysis, the protein binding of ML347 was determined in human, rat and mouse plasma. The results were similar in all three species with ML347 displaying high plasma protein biding (F_u ~0.01-0.015). ML347 was also assessed for its intrinsic clearance in hepatic microsomes. This measure will help predict the *in vivo* clearance in the same three species (CL_{HEP}). ML347 was unstable to oxidative metabolism – possibly due to the labile methoxy group[19] – and therefore was predicted to display high clearance in human and mouse, and moderate-to-high clearance in the rat. Going forward, the intrinsic clearance is predicting high clearance after oral dosing, a more appropriate dosing paradigm might be intraperitoneal dosing for this compound. Further *in vivo* experiments, including PK, will be reported in due course.

In conclusion, SAR studies of the 3- and 6-positions of the pyrazolo[1,5-*a*]pyrimidine scaffold revealed a potent and selective inhibitor of ALK2 versus ALK3. These studies further validated that 4-phenyl substituents of the 6-position on the pyrazolo[1,5-*a*]pyrimidine scaffold allowed a wide range of substituents, from ethers to cycloheteroalkyl (morpholine, piperazine, 4-methylpiperazine). These studies also revealed that subtle changes of the 3-position substituents can drastically influence the BMP activity (e.g., **7i** vs. **7n**). These SAR studies culminated in the discovery of a highly selective ALK2 inhibitor, ML347, which shows >300-fold selectivity for ALK2 vs. ALK3. ML347 is potent in the BMP4 cell assay (152 nM) as well as the in vitro kinase assay for ALK1 (46 nM) and ALK2 (32 nM) and is devoid of activity in a number of related kinases. Further studies are planned for this selective inhibitor in a number of *in vivo* animal disease models, such as FOP, and results will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scadden DT, Peterson RT, Bloch KD, Yu PB. Blood. 2011; 117:4915. [PubMed: 21393479] (c) Derwall M, Malhotra R, Lai CS, Beppu Y, Aikawa E, Seehra JS, Zapol WM, Bloch KD, Yu PB. Arterioscler. Thromb. Vasc. Biol. 2012; 32:613. [PubMed: 22223731] (d) Saeed O, Otsuka F, Polavarapu R, Karmali V, Weiss D, Davis T, Rostad B, Pachura K, Adams L, Elliott J, Taylor WR, Narula J, Kolodgie F, Virmani R, Hong CH, Finn AV. Arterioscler. Thromb. Vasc. Biol. 2012; 32:299. [PubMed: 22095982] (e) Theurl I, Schroll A, Sonnweber T, Nairz M, Theurl M, Willenbacher W, Eller K, Wolf D, Seifert M, Sun CC, Babitt JL, Hong CC, Menhall T, Gearing P, Lin HY, Weiss G. Blood. 2011; 118:4977. [PubMed: 21730356] (f) Shi S, Hoogaars WMH, de Gorter DJJ, van Heiningen SH, Lin HY, Hong CC, Kemaladewi DU, Aartsma-Rus A, ten Dijke P, 't Hoen PAC. Neurobiol. Dis. 2011; 41:353. [PubMed: 20940052]

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14. **BMP-responsive luciferase reporter assays.** BMP-responsive C2C12BRA cells, stably transformed with the Id1 promoter-firefly luciferase reporter; kind gift of D, Rifkin, NYU Medical Center) were seeded in 96-well plates, and incubated overnight with the compounds and BMP4 (50 ng/mL). The cells were then lysed, and cell extracts were then subjected to the firefly luciferase assay using Steady-Glo luciferase assay kit (Promega). The results were normalized to cell titers, as measured using Cell Titer-Glo luminescence assay (Promega).
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17. **7g**, VU0469381 (ML347) has been declared a probe via the Molecular Libraries Probe Production Centers Network (MLPCN) and is available through the network, see: http://mli.nih.gov.
18. **Kinase assay.** All kinase assays were conducted by Reaction Biology Corp (Malvern, PA), as previously reported. In brief, compounds were tested at 10 concentrations by 3-fold serial dilutions starting at 100 μM. In vitro kinase reactions were carried out in the presence of 10 μM (33P)γATP. Eleven human kinases tested were the BMP type-I receptors ALK-1/ACVRL1, ALK2/ACRV1, ALK3/BMPR1A and ALK6/BMPR1B, the TGFβ type-I receptors ALK4/ACVR1B and ALK5/TGFβR1, the BMP type-II receptor (BMPR2), the TGFβ type-II receptor (TGFβR2), VEGF type-II receptor (KDR/VEGFR2), AMP-activated protein kinase (AMPK-A1/B1/G1) and the human platelet-derived growth factor receptor-β (PDGFRβ).
19. LDN-193189, **2**, which has replaced the labile methoxy group with piperazine (similar to **13h-n**) was shown to be much more stable in liver microsome assay (see Ref. 13).

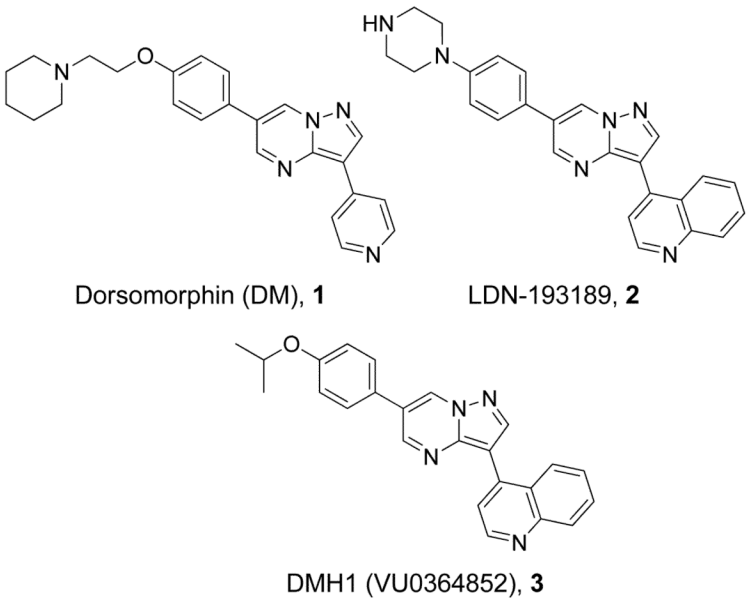
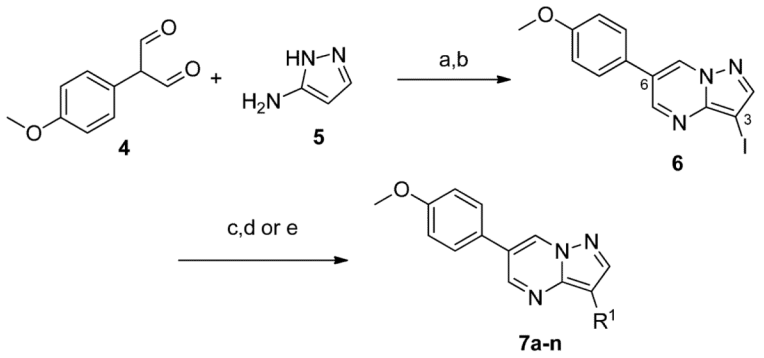
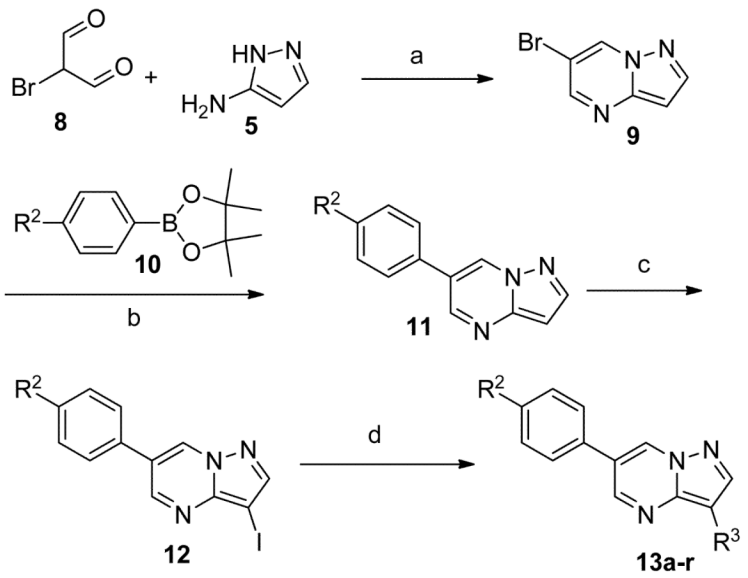


Figure 1.
Structures of previously disclosed BMP inhibitors, Dorsomorphin (DM), **1**, LDN-193189, **2**, and DMH1, **3**.



Scheme 1.

Reactions and conditions: (a) AcOH, EtOH, 170 °C, 10 min., μW, 92%; (b) NIS, DMF; (c) diboronpinacol ester, Pd(dppf)Cl_2·DCM, KOAc, DMF, 100 °C, 16 h; (d) R^1X, Pd(dppf)Cl_2·DCM, K_3PO_4, 1,4-dioxane, H_2O, 120 °C, 30 min., μW, 17–27% (3 steps); (e) $R^1B(OH)_2$, Pd(dppf)Cl_2·DCM, K_3PO_4, 1,4-dioxane, H_2O, 120 °C, 30 min., μW, 10-65% (2 steps).

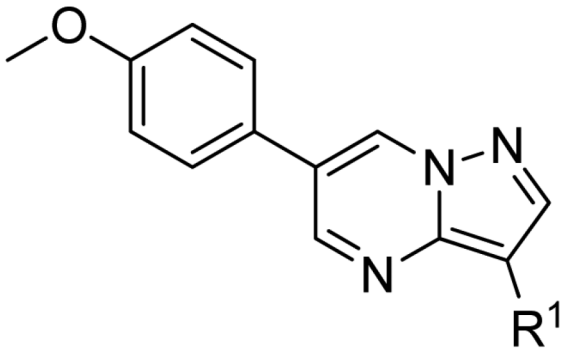


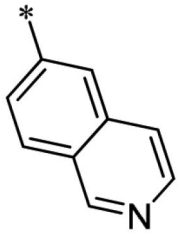
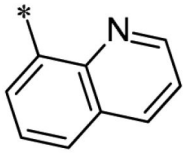
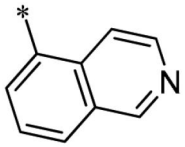
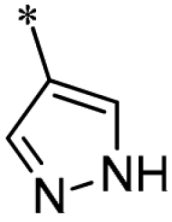
Scheme 2.

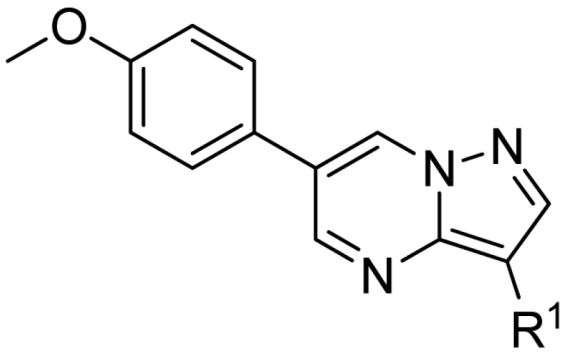
Reactions and conditions: (a) AcOH, EtOH, reflux; (b) $Pd(dppf)Cl_2 \cdot DCM$, K_3PO_4, 1,4-dioxane, H_2O, 150 °C, 30 min., μW, 56-73% (2 steps); (c) NIS, DMF, rt, 73%; (d) $R^3B(OH)_2$, $Pd(dppf)Cl_2 \cdot DCM$, K_3PO_4, 1,4-dioxane, H_2O, 120 °C, 30 min., μW, 14-52%.

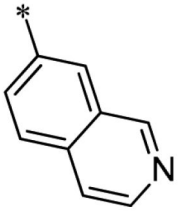
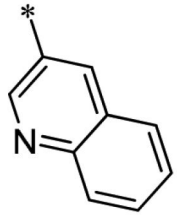
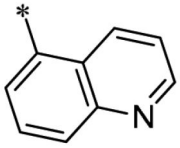
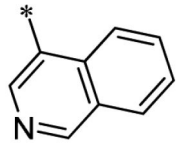
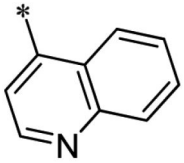
Table 1

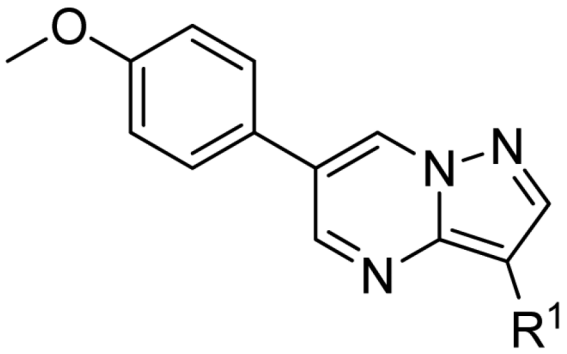
SAR of the 3-position of pyrazolo[1,5-*a*]pyrimidine scaffold (**7a-m**).



Compd	R^1	BMP4 Cell IC_{50} (nM)[14,15]
7a		Inactive
7b		Inactive
7c		Inactive
7d		94.1



Compd	R^1	BMP4 Cell IC_{50} (nM)[14,15]
7e		Inactive
7f		Inactive
7g		152
7h		6,732
7i		<1



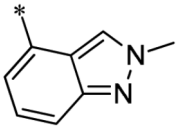
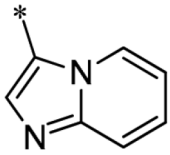
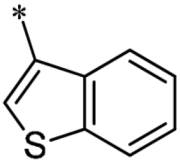
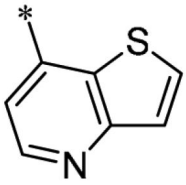
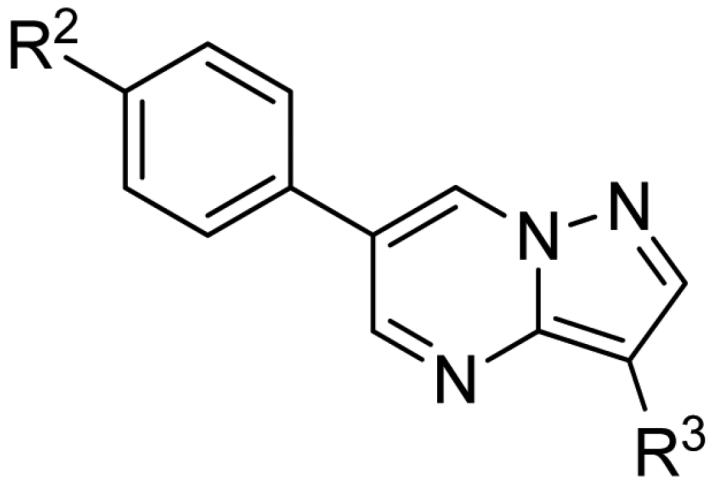
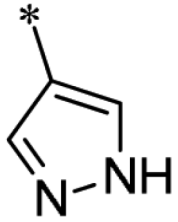
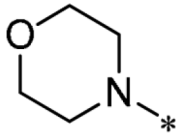
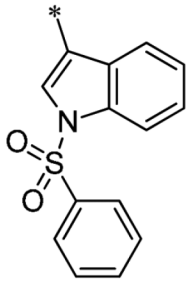
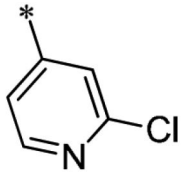
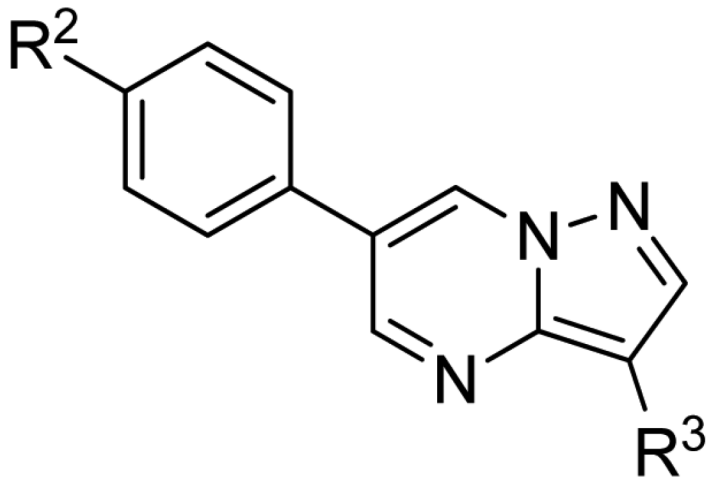
Compd	R^1	BMP4 Cell IC_{50} (nM)[14,15]
7j		Inactive
7k	H	Inactive
7l		Inactive
7m		Inactive
7n		4,571

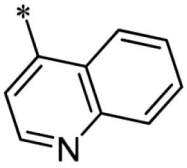
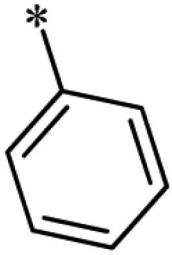
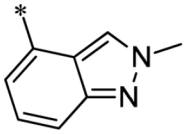
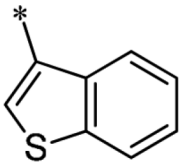
Table 2

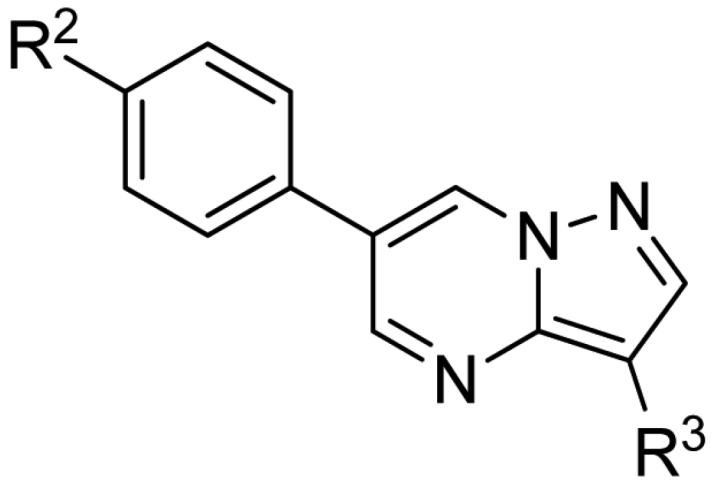
SAR of the 6- and 3-position of the pyrazolo[1,5-*a*]pyrimidine scaffold (**13a-r**).

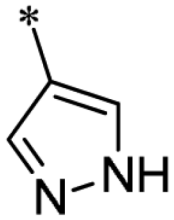
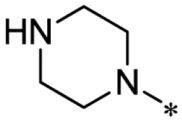
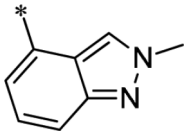
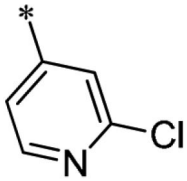


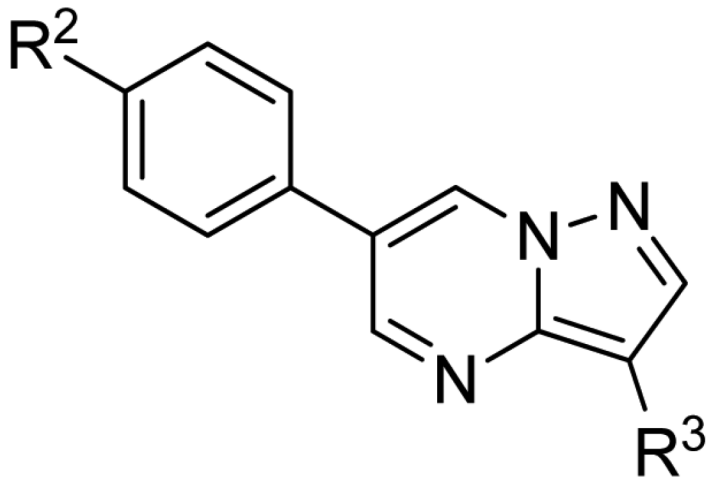
Compd	R^2	R^3	BMP4 Cell IC_{50} (nM)[14,15]
13a			141
13b			Inactive
13c			529

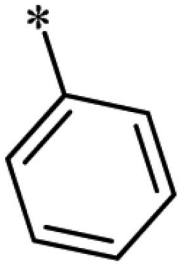
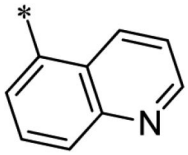
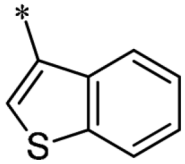
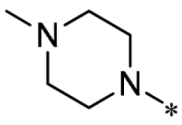
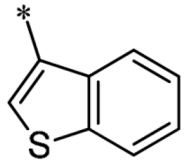


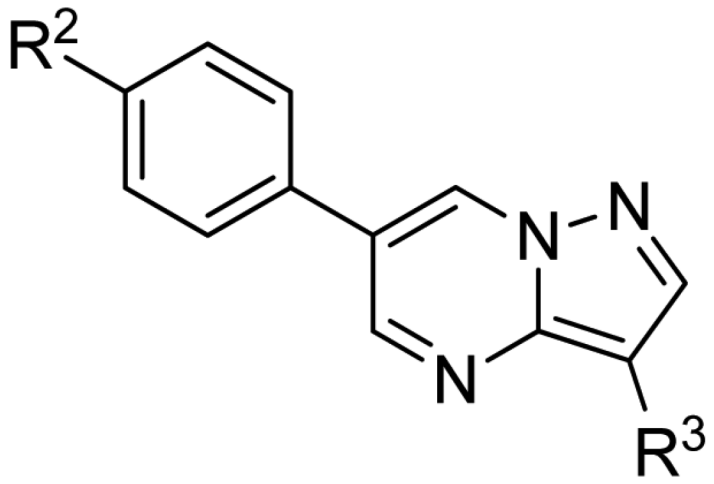
Compd	R^2	R^3	BMP4 Cell IC_{50} (nM)[14,15]
13d			<1.0
13e			inactive
13f			inactive
13g			418



Compd	R^2	R^3	BMP4 Cell IC_{50} (nM)[14,15]
13h			<1.0
13i			Inactive
13j			246
13k		Br	inactive



Compd	R^2	R^3	BMP4 Cell IC_{50} (nM)[14,15]
13l			inactive
13m			117
13n			2,636
13o			Inactive



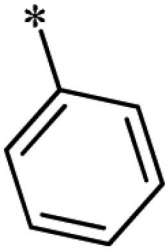
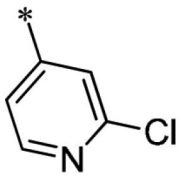
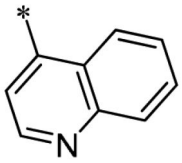
Compd	R^2	R^3	BMP4 Cell IC_{50} (nM)[14,15]
13p			Inactive
13q			523
13r			25.2

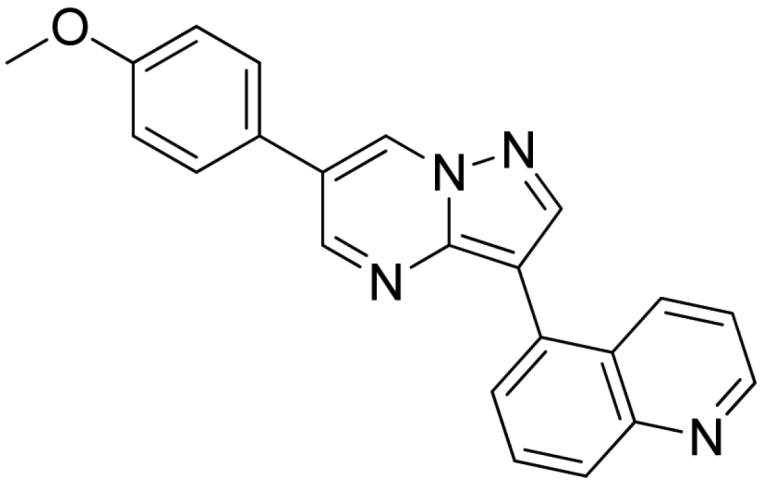
Table 3

Kinase selectivity data for selected compounds[5,18]

	IC_{50} (nM)									
Cmpd	**ALK1/ ACVRL1**	**ALK2/ ACVR1**	**ALK3/ BMPR1A**	**ALK4/ ACVR1B**	**ALK5/ TGFBR1**	**ALK6/ BMPR1B**	**BMPR2**	**TGFBR2**	**AMPK**	**KDR/ VEGFR2**
DM, 1	106.3	67.5	95	25740	17090	235	74	102.9	234.6	21.8
LDN-193189, 2	13.3	40.7	<5	1825	565	60	3845	140.4	1122	214.7
DMH1, 3	27	107.9	<5	9622	Inactive	47.6	inactive	Inactive	Inactive	Inactive
7d	52.4	110	41	Inactive	Inactive	102	24	16	44	13
7g	**46**	**32**	**10800**	**Inactive**	**Inactive**	**9830**	**Inactive**	**Inactive**	**Inactive**	**19700**
7i	26.5	33.3	6.78	1840	33300	56.9	Inactive	236	7260	4300
13a	42.1	53.4	10.4	5930	10900	60.9	15.9	1.37	39.9	1.68
13d	<5	<5	<5	326	178	<5	3640	26	2680	5330
13h	55	43	55	3420	3320	129	59	38	82	14
13m	14.4	26.2	539	4090	3960	92.9	695	327	2520	2040
13r	<5	<5	<5	183	75	<5	2360	15	960	1520

Table 4

In vitro pharmacokinetic properties of ML347



ML347
7g

MW		352.4	
cLogP		4.00	
TPSA		52.3	
In vitro PK Parameter	Human	Rat	Mouse
CL_{INT} (mL/min/kg)	516	148	617
CL_{HEP} (mL/min/kg)	20.2	47.5	78.5
PPB (% fu)	0.9	1.4	1.4
PBS Solubility		4 μM	