Journal of Medicinal Chemistry

Article

Scaffold hopping and Optimization of Maleimide based Porcupine Inhibitors

Soo Yei Ho, Jenefer Alam, Duraiswamy Athisayamani Jeyaraj, Weiling Wang, Grace Ruiting Lin, Shi Hua Ang, Eldwin Sum Wai Tan, May Ann Lee, Zhiyuan Ke, Babita Madan, David M Virshup, Li Jun Ding, Vithya Manoharan, Yun Shan Chew, Choon Bing Low, Vishal Pendharkar, Kanda Sangthongpitag, Jeffrey Hill, Thomas H. Keller, and Anders Poulsen

J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.7b00662 • Publication Date (Web): 03 Jul 2017

Just Accepted

ACS Publications

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Scaffold hopping and Optimization of Maleimide based Porcupine Inhibitors

Soo Yei Ho,[§] Jenefer Alam,[§] Duraiswamy Athisayamani Jeyaraj,[§] Weiling Wang,[§] Grace Ruiting Lin,[§] Shi Hua Ang,[§] Eldwin Sum Wai Tan,[§] May Ann Lee,[§] Zhiyuan Ke,[§] Babita Madan,[#] David M Virshup,[#] Li Jun Ding,[§] Vithya Manoharan,[§] Yun Shan Chew,[§] Choon Bing Low,[§] Vishal Pendharkar,[§] Kanda Sangthongpitag,[§] Jeffrey Hill,[§] Thomas H. Keller[§] and Anders Poulsen[§]**

[§]Experimental Therapeutics Centre, 31 Biopolis Way, #03-01 Nanos, 138669 Singapore

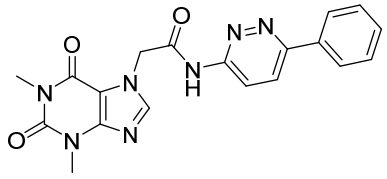
[#]Duke&NUS Graduate Medical School Singapore, 8 College Road, Singapore 169857

ABSTRACT. Porcupine is an O-acyltransferase that regulates Wnt secretion. Inhibiting porcupine may block the Wnt pathway which is often dysregulated in various cancers. Consequently porcupine inhibitors are thought to be promising oncology therapeutics. A high throughput screen against porcupine revealed several potent hits that were confirmed to be Wnt pathway inhibitors in secondary assays. We developed a pharmacophore model and used the putative bioactive conformation of a xanthine inhibitor for scaffold hopping. The resulting Maleimide scaffold was optimized to sub-nanomolar potency while retaining good physical druglike properties. A pre-clinical development candidate was selected for which extensive *in vitro* and *in vivo* profiling is reported.

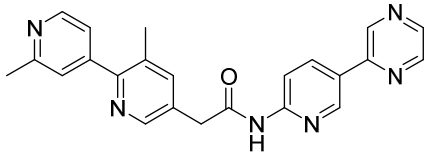
INTRODUCTION

Wnt signaling is important in many cellular functions, ranging from tissue homeostasis,[1,2] cell polarization,[3] cell differentiation[4] and apoptosis.[5] Aberrant Wnt signaling is found in a wide variety of cancers[6,7] as well as various diseases such as fibrosis,[8] metabolic diseases[9] and disorders in endocrine function and bone metabolism.[10] Hence various research groups have been active in finding therapeutic solutions to target components of the Wnt pathway.[11,12]

In recent years, there has been an interest in Porcupine (PORCN) as a molecular target.[13] Two porcupine inhibitors, **1** (ETC-159)[14] (ClinicalTrials.gov NCT02521844), and **2** (LGK974)[15] (ClinicalTrials.gov NCT01351103) are currently in Phase 1 clinical trials (Figure 1). PORCN, a membrane bound O-acyltransferase (MBOAT)[16] enzyme residing in the endoplasmic reticulum, is essential for the palmitoleation of the Wnt protein at the highly conserved serine residue 209. Palmitoleation is critical for the extracellular secretion of Wnt and for its binding to the Frizzled receptors.[17] Thus small molecule inhibitors of PORCN should halt the secretion of all Wnt ligands and therefore block Wnt signaling through both β-catenin dependent and independent pathways.



1 (ETC-159)



2 (LGK974)

Figure 1: Porcupine inhibitors in clinical development.

Our desire to find cancer therapeutics with new mechanisms of action prompted us to do a high throughput screen (HTS) to find leads that block the Wnt pathway by inhibiting PORCN.[18] We

employed a cellular pathway screen for hit finding and lead optimization as no suitable biochemical assays are available.[16] A HEK293-STF cell line expressing Wnt3A that contains a luciferase reporter for β-catenin mediated transcriptional activation was used. Hits that act downstream of surface receptor activation by Wnt were identified with a second assay and eliminated,[18] as were cytotoxic compounds. Porcupine inhibition was confirmed for selected hits using ω-alkynyl-palmitate and click chemistry[19] as well as a PORCN overexpression assay.[18] A number of hits with diverse scaffolds were obtained and a xanthine[20] and a phenoxy-acetamide scaffold[18] were found to be promising starting points and selected for lead optimization. In this manuscript we describe the xanthine structure activity relationship and the use of a pharmacophore model for the design of a novel scaffold as a follow-up compound.

CHEMISTRY

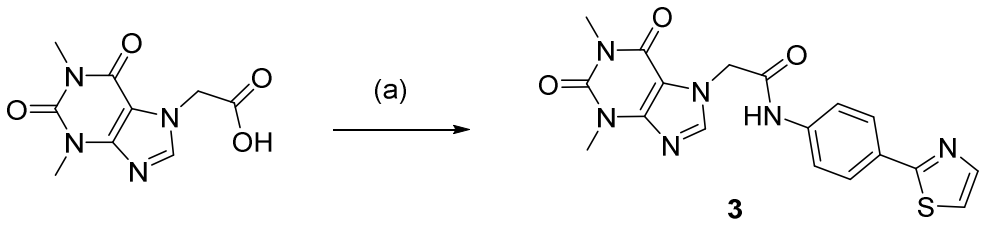
The anilines used for amide coupling described in this publication were either commercially available or synthesized via Suzuki reaction using the respective boronic acid with the corresponding aniline described in Schemes 1 and 2. Compound **3** was synthesized by amide coupling using commercially available theophylline-7-acetic acid with 4-(1,3-thiazol-2-yl)aniline.

The Wnt pathway modulator **5** was synthesized via amide coupling from its commercially available precursor, theophylline-7-acetic acid and 4-(thiophen-3-yl)aniline, **4**. Compounds **6** and **7** were also synthesized similarly using 2-(1H-imidazol-1-yl)acetic acid and 2-(1H-benzo[d]imidazol-1-yl)acetic acid respectively (Scheme 2).

Alkylation of 3,7-dihydro-1H-purine-2,6-dione with chloroacetic acid yielded the acid intermediate **8**. Compound **9** was obtained using amide coupling conditions. Compound **10** was

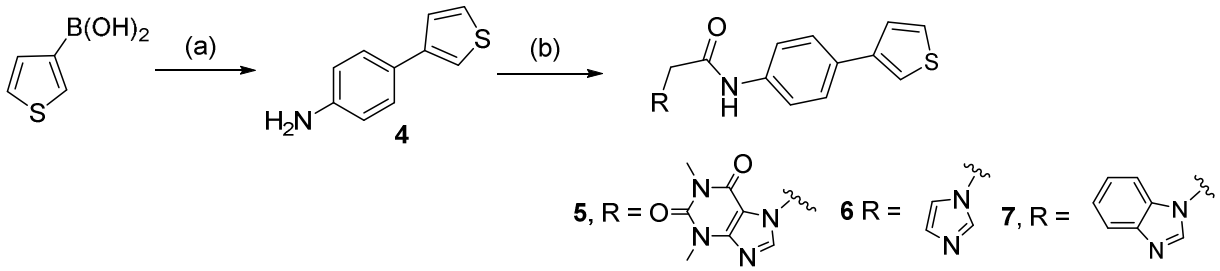
also synthesized in a similar manner by alkylation, followed by hydrolysis and amide coupling (Scheme 3).

Scheme 1. Synthesis of compound 3.[a]



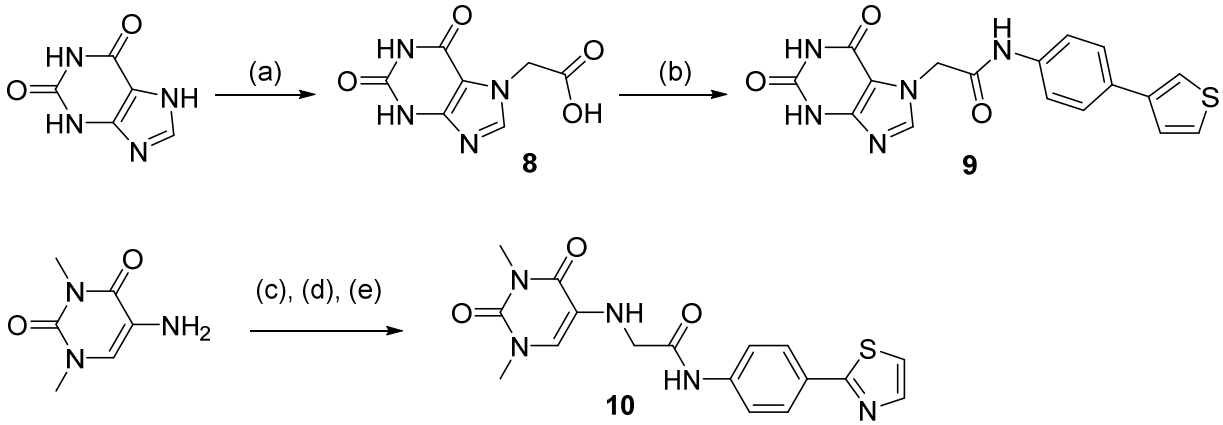
[a]Reagents and conditions: (a) 4-(1,3-thiazol-2-yl)aniline, HATU, Et_3N, DCM.

Scheme 2. Synthesis of compounds 5, 6 and 7.[a]



[a]Reagents and conditions: (a) 4-bromoaniline, Pd(dppf)Cl_2.DCM, K_2CO_3, 4:1 dioxane-H_2O, 100 ^oC. (b) respective acids, HATU, Et_3N, DCM.

Scheme 3. Synthesis of compounds 9 and 10.[a]



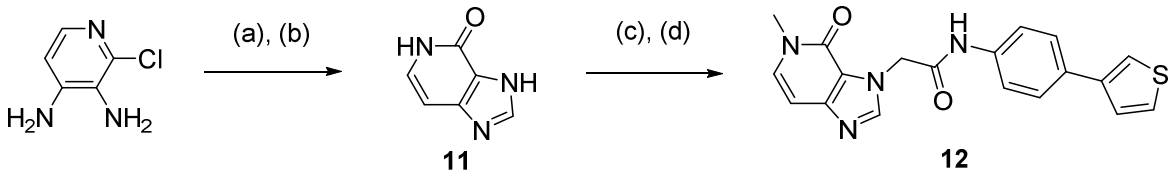
[a]Reagents and conditions: (a) $ClCH_2COOH$, NaOH, water, 100^oC. (b) 4-(thiophen-3-yl)aniline, EDCl, HOBT, DMF-DMSO (1:1). (c) $BrCH_2COOEt$, K_2CO_3, DMF, 80 ^oC. (d) LiOH, MeOH-THF-H_2O. (e) 4-(1,3-thiazol-2-yl) aniline, HATU, Et_3N, DCM.

Compound **12** was synthesized starting with 2-chloropyridine-3,4-diamine (Scheme 4). Alkylation followed by ring closure with trimethylorthoformate yielded 4-chloro-3H-

imidazol[4,5-c]pyridine which then underwent hydrolysis to form intermediate **11**. Alkylation of intermediate **11** with 2-chloro-N-(4-(thiophen-3-yl)phenyl)acetamide followed by methylation with methyl iodide yielded compound **12**.

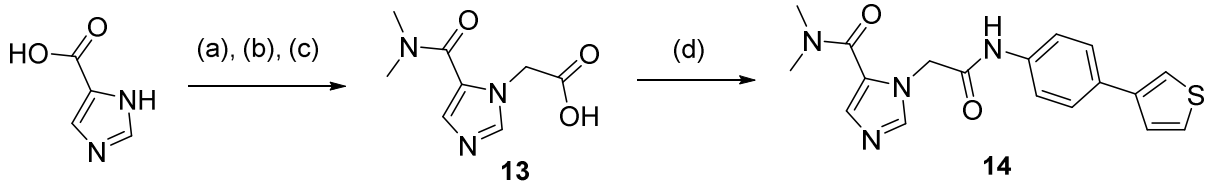
Compound **14** was synthesized starting from 1H-imidazole-5-carboxylic acid as described in Scheme 5. Amidation was carried out on the acid chloride of 1H-imidazole-5-carboxylic acid, followed by alkylation and hydrolysis to yield acid intermediate **13**. Compound **14** was obtained via amide coupling as described previously.

Scheme 4. Synthesis of Compound 12.[a]



[a]Reagents and conditions: (a) $CH(OMe)_3$, 70 ^oC. (b) HCl in MeOH. (c) 2-chloro-N-(4-(thiophen-3-yl)phenyl)acetamide, K_2CO_3, DMF. (d) MeI, K_2CO_3, DMF.

Scheme 5. Synthesis of Compound 14.[a]



[a]Reagents and conditions: (a) Me_2NH, $SOCl_2$, DCM. (b) $BrCH_2COOEt$, K_2CO_3, DMF, 70 ^oC. (c) LiOH, MeOH-THF-H_2O. (d) 4-(thiophen-3-yl)aniline, HATU, Et_3N, DCM.

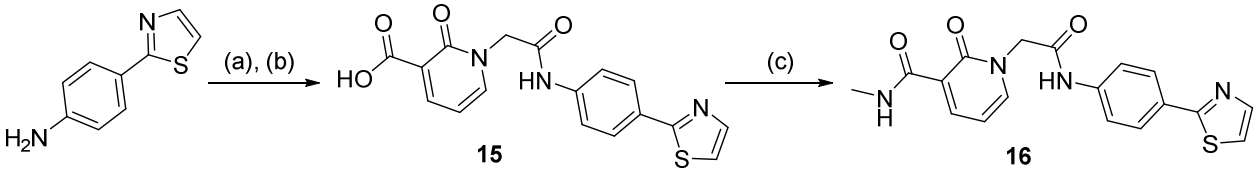
The synthesis of **16** started with an acylation of 4-(thiazol-2-yl)aniline with bromoacetylbromide, followed by N-alkylation with methyl 2-oxo-1,2-dihydro-3-pyridine carboxylate to form acid intermediate **15**. The acid then underwent amidation with methylamine to yield the final compound **16** as shown in Scheme 6.

Compound **19** was synthesized starting from 3-nitro-triazine as shown in Scheme 7. 3-nitro-triazine underwent alkylation with bromoethylacetate followed by hydrolysis to form the acid

intermediate **17**. Acid intermediate **17** was coupled with 4-(1,3-thiazol-2-yl)aniline using HATU as coupling reagent followed by nitro reduction using Fe in acidic conditions to yield intermediate **18**. Urea formation between **18** and 2,5-dioxopyrrolidin-1-yl methylcarbamate yielded the desired compound **19**.

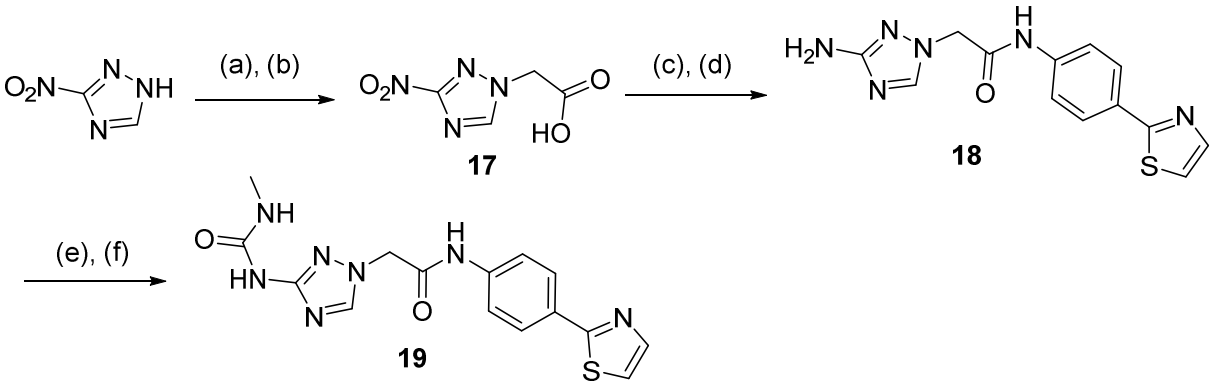
Compound **21** was synthesized via three steps as shown in Scheme 8. 1-methyl maleimide underwent mono-bromination to form 1-methyl-3-bromo maleimide which was subsequently treated with proline to yield acid intermediate **20**. Compound **21** was obtained from coupling acid **20** with 4-(1,3-thiazol-2-yl)aniline using amide coupling reagents.

Scheme 6. Synthesis of Compound 16.[a]



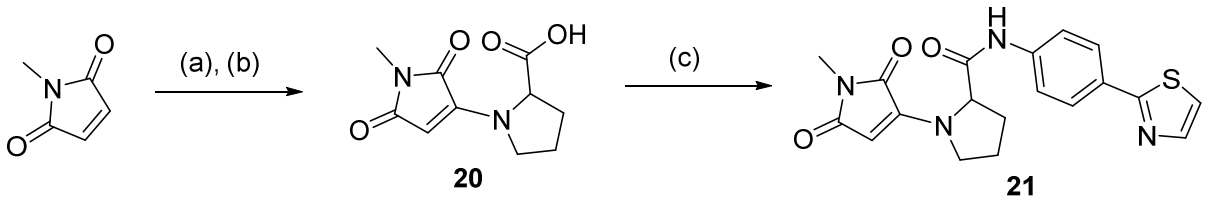
[a]Reagents and conditions: (a) Bromoacetylbromide, Hunig's base, DCM, -78 ^{o}C to r.t. (b) Methyl 2-oxo-1,2-dihydro-3-pyridine carboxylate, NaH, DMF. (c) Methylamine, HATU, Et_3N, DMF.

Scheme 7. Synthesis of Compound 19.[a]



[a]Reagents and conditions: (a) $BrCH_2COOEt$, NaOEt in EtOH, reflux. (b) LiOH, MeOH-THF-H_2O. (c) 4-(1,3-thiazol-2-yl)aniline, HATU, Et_3N, DCM, r.t. (d) Fe, AcOH, EtOH, reflux. (e) $NaHCO_3$, EtOAc. (f) 2,5-dioxopyrrolidin-1-yl methylcarbamate, pyridine, 70 ^{o}C

Scheme 8. Synthesis of compound 21.[a]



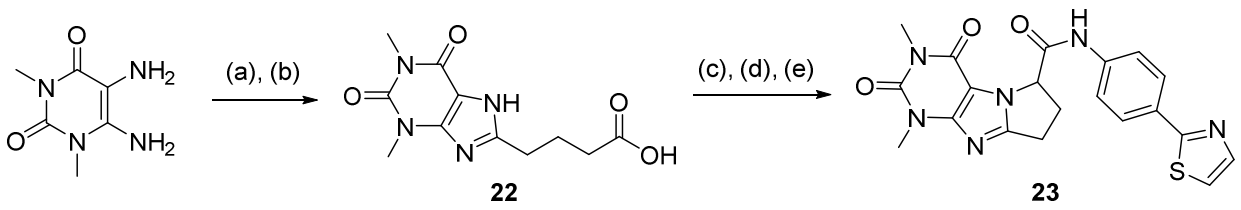
[a]Reagents and conditions: (a) Bromine, diethyl ether, 45°C. (b) L-proline, Et_3N, DMF. (c) 4-(1,3-thiazol-2-yl)aniline, HATU, Et_3N, DCM.

The tricyclic compound **23** was synthesized from 1,3-dimethyl,5,6-diamino uracil as described in Scheme 9. 1,3-dimethyl,5,6-diamino uracil underwent cyclization with dihydro-2H-pyran-2,6(3H)-dione to form intermediate **22**. Mono bromination was carried out at the carbon adjacent to the carboxylic acid followed by intramolecular N-alkylation to form the tricyclic precursor. Lastly, compound **23** was obtained after amidation of the tricyclic precursor with 4-(1,3-thiazol-2-yl)aniline.

The phthalimide compound **27** was obtained via a 7-step synthesis described in Scheme 10. 4-nitro phthalimide underwent methylation followed by nitro reduction to form the aniline intermediate **24**. **24** was subjected to iodination and Suzuki reaction yielded intermediate **25**. The acid **26** was obtained via the oxidation of **25** and desired compound **27** was obtained via amide coupling with 4-(1,3-thiazol-2-yl)aniline.

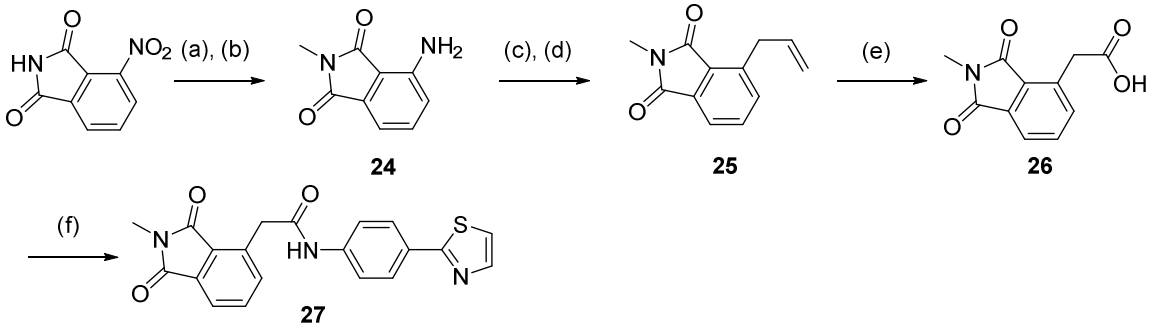
Compounds **30**-**44** were synthesized similarly from 2,3-pyridinedicarboxylic anhydride as described in Scheme 11. The derivatives of the maleimide scaffold were prepared using the corresponding amine, followed by hydrogenation to form the respective bicyclic intermediates **28** and **29**. The above-mentioned intermediates then underwent N-alkylation followed by hydrolysis to form the respective acid intermediates and subsequently amide coupling with the appropriate amines to yield the desired compounds.

Scheme 9. Synthesis of compound 23.[a]



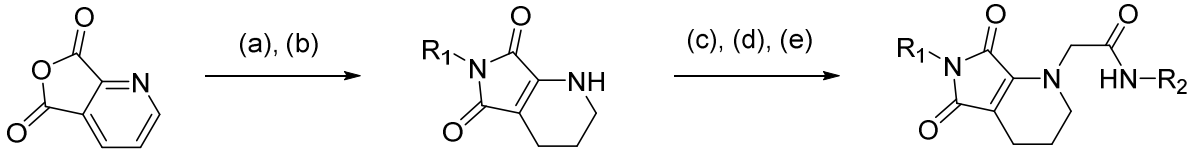
[a]Reagents and conditions: (a) dihydro-2H-pyran-2,6(3H)-dione, DMF. (b) 2M NaOH, reflux. (c) Br_2, PCl_3, dichloroethane, reflux. (d) K_2CO_3, ACN, 70 ^oC. (e) 4-(1,3-thiazol-2-yl)aniline, HATU, Et_3N, DCM.

Scheme 10. Synthesis of compound 27.[a]



[a]Reagents and conditions: (a) NaH, MeI, DMF. (b) Pd/C, H_2, MeOH. (c) aq. $NaNO_2$, 6M HCl, 0 ^oC. followed by KI. (d) 2-allyl-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, CsF, $Pd(PPh_3)_4$, 1,4-dioxane, 100 ^oC. (e) $RuCl_3.3H_2O$, $NaIO_4$, CCl_4-ACN-H_2O. (f) 4-(1,3-thiazol-2-yl)aniline, HATU, Et_3N, DCM.

Scheme 11. Synthesis of compounds 30-44.[a]



[a]Reagents and conditions: (a) R_1NH_2, toluene, 130 ^oC. (b) Pd/C, H_2, MeOH. (c) $BrCH_2COOEt$, K_2CO_3, ACN, 70 ^oC. (d) LiOH, MeOH-THF-H_2O. (e) R_2NH_2, HATU, Et_3N, DCM.

RESULTS AND DISCUSSION

Xanthine SAR. The xanthine development compound **1** was derived from HTS hit **3**. During the optimization it became very clear that both the biaryl substituent (green) and the amide linker

(blue) are absolutely required for activity on the PORCN O-acyltransferase.[21] In fact all known porcupine inhibitors share these features, i.e. compare with compounds **2**, **45** and **46** in Figure 2.

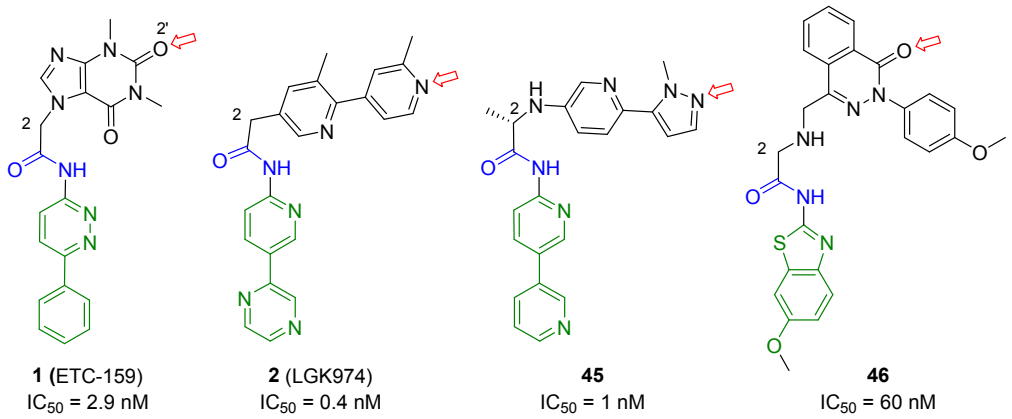
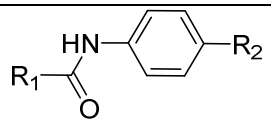
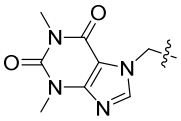
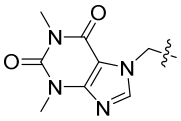
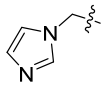
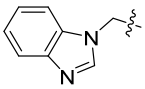
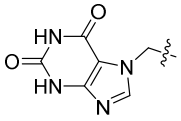
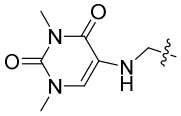
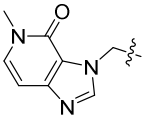


Figure 2. Porcupine inhibitors discussed in the literature. Compound **45**[18] and **46**[22] share common features with **1** and **2**. The red arrow depicts a ring hydrogen bond acceptor that is discussed in the text.[21]

However the heterocyclic substituent attached to the 2-acetamide carbon (black in Figure 2) varies considerably between the different PORCN inhibitors. The SAR in Table 1 shows that for compound **1** the xanthine is absolutely essential for activity. Removal of the uracil part from the xanthine moiety in **6** resulted in loss of activity. The inactive benzimidazole analogue **7** demonstrated that at least one of the carbonyl hydrogen bond acceptors is essential for activity. The inactive analogue **12** established that the carbonyl in the xanthine 2'-position (See Figure 2) is essential for biological activity.

Table 1. Xanthine SAR



Compound	R_1	R_2	STF3a IC_{50} (μM)[a]	Conformational Energy (kJ/mol)[b]
3			0.024	8.8
5			0.0004	10.4
6			<60% @ 100 μM	9.1
7			<60% @ 100 μM	10.2
9			<60% @ 100 μM	8.3
10			<60% @ 100 μM	61.2[c]
12			<60% @ 100 μM	10.2

a: data represent mean value of triplicate experiments. b: Energy difference between putative bioactive conformation and global energy minima calculated by the OPLS3 force field. c: Global energy minima is electrostatically collapsed and the force field energy is unlikely to be reliable.

All porcupine inhibitors have a ring hydrogen bond acceptor in the heterocyclic substituent attached to the 2-acetamide carbon (red arrow in Figure 2). Interestingly, when the putative bioactive conformations of xanthine **1** and **2** were superimposed, it became apparent that the ring hydrogen bond acceptor of **1** is about 2.8 Å from that of **2** (Figure 3). We therefore hypothesize

that **1** binds to the porcupine protein through a water molecule signified by the purple dot in Figure 3.

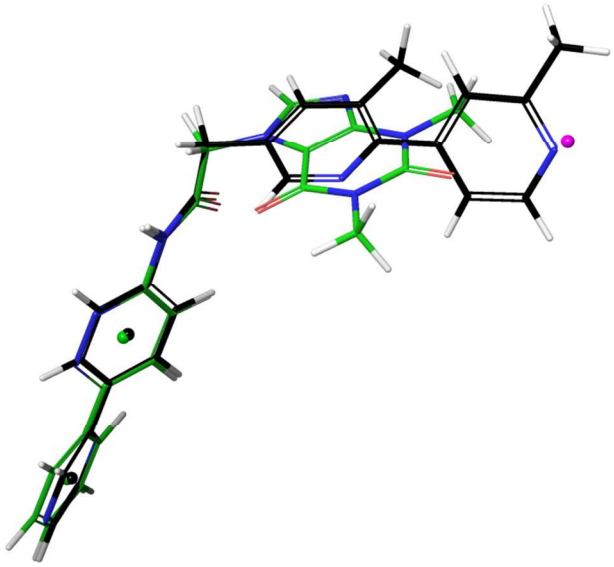
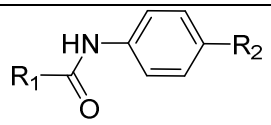
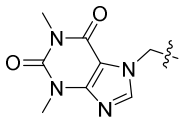
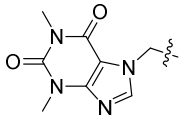
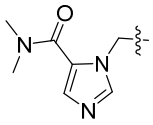
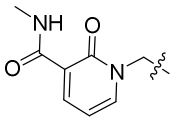
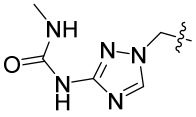
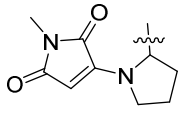
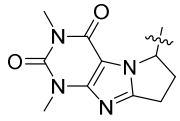
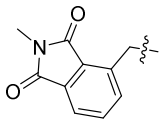
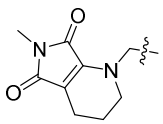


Figure 3. Superimposition of compound **1** (green carbon atoms) and **2** (grey carbon atoms) in their putative bioactive conformations. It was hypothesized that in compounds like **1**, the ring acceptor hydrogen bonds to the porcupine protein through a water molecule (purple ball).

Scaffold Hopping. Following the promotion of **1** to preclinical development[20] we started working on a back-up compound. Since no further hit was available from HTS screening, bioisosteric replacements for the xanthines were evaluated. The scaffolds were fitted to our pharmacophore model[21] and those with a compatible low energy conformation were synthesized (Table 2).

Table 2. Proposed xanthine bioisosters.



Compound	R_1	R_2	STF3a IC_{50} (μM)[a]	Conformational Energy (kJ/mol)[b]
3			0.024	8.8
5			0.0004	10.4
14			<60% @ 100 μM	13.3
16			<60% @ 100 μM	3.6
19			<60% @ 100 μM	10.3
21			<60% @ 100 μM	13.3
23			0.042	9.4
27			0.015	7.5
30			0.015	10.7

a: data represent mean value of triplicate experiments. b: Energy difference between putative bioactive conformation and global energy minima calculated by the OPLS3 force field.

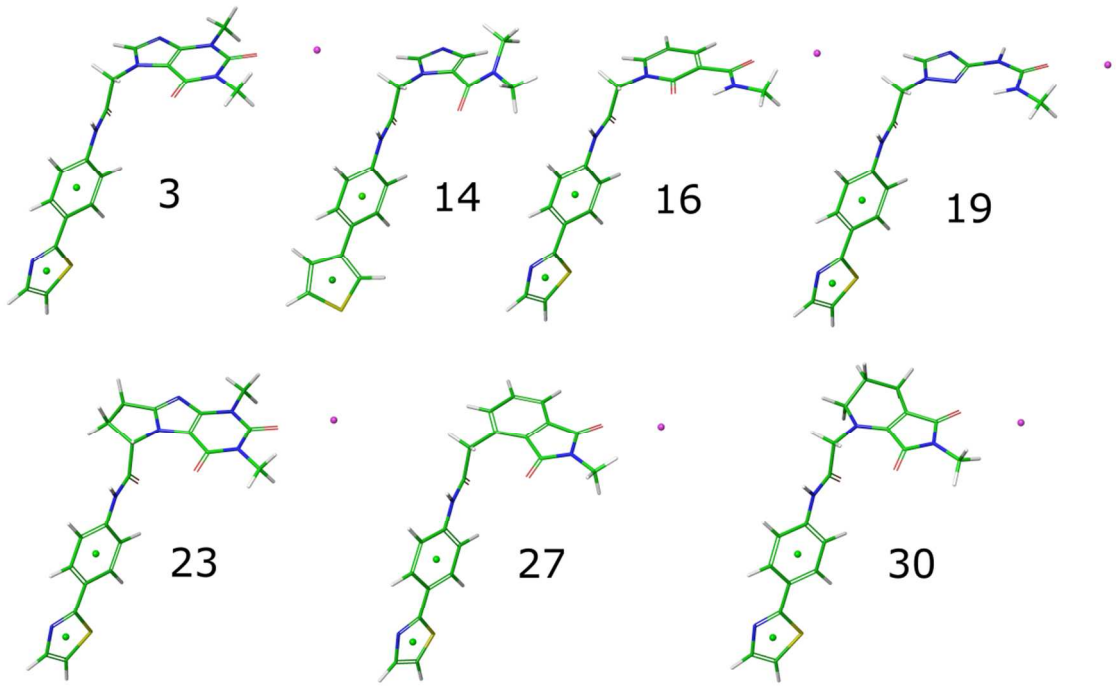


Figure 4. Putative bioactive conformations of some of the compounds in Table 2.

Figure 4 shows the putative bioactive conformations of some of these compounds. The imidazole-carboxamide compound **14** may be considered a xanthine in which the 2'-carbonyl has been removed and in agreement with our pharmacophore model this compound was found to be inactive.[21] In low energy conformations of the pyridone-amide **16** the amide donor is forming an intramolecular hydrogen bond with the pyridone carbonyl. The triazole-urea compound **19** has two low energy conformations with an internal hydrogen bond between the terminal hydrogen bond donor of the urea and either of the triazole acceptor nitrogens. One of these conformations may be considered equivalent to a xanthine in which one of the carbonyls has been deleted. All of the compounds **14**, **16**, **19** and **21** (Table 2) were found to be inactive even though they fitted the pharmacophore model with low energy conformations. It is likely that this is due to both

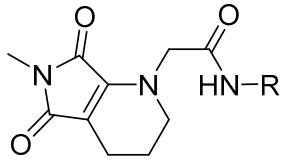
carbonyls of the xanthine being important for porcupine inhibition. The tricyclic xanthine compound **23** was our first successful attempt to make a rigid analogue of **3** and **5**. We have previously shown in a different scaffold that a proline ring can be used to rigidify the bioactive conformation.[18] Several of these tricyclic analogues of **23** were synthesized but the activities were generally lower than the analogous xanthines, e.g compare compounds **3** and **23** (table 2) for which there is a representative 2-fold difference.

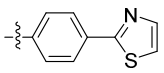
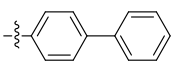
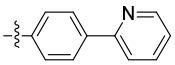
Our first successful design of a xanthine bioisostere was the phtalimide **27**. Both the carbonyls of the phthalimide superimpose well on those of the xanthine. Several of the phthalimides were synthesized and were generally more potent than the analogous xanthines but also less soluble, e.g. the solubility of **27** and **30** is 0.42 and 1.26 μg/ml, respectively. We hypothesized that the limited solubility may be due to stacking interactions of the flat aromatic groups in the solid state. The fused piperidine-maleimide compound **30** was an attempt to reduce the sp2 character of the phthalimide. Although the piperidine-maleimide compounds were found to be less potent than the phthalimides, they were more soluble and approximately equipotent with the xanthines. The physical properties of **30** were satisfactory (MW = 382.4 Dalton, cLogP = 2.2, PSA = 105Å2 and 4 rotatable bonds) and the scaffold seemed to be novel, which is why it was chosen for lead optimization.

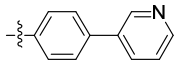
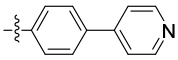
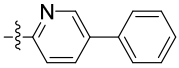
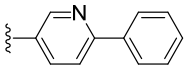
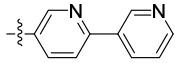
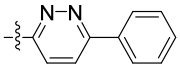
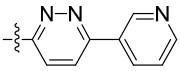
Lead Optimization. In vitro profiling of **30** showed a reasonable profile for a lead with Cytochrome P450 2D6 and 3A4 inhibition of >20 and 5.2 μM, respectively. Mice and human liver microsome (MLM and HLM) clearance was moderate at 65 and 24 μl/min/mg, while aqueous solubility at pH 7.4 and 4.0 was poor at 1.3 and 1.1 μg/ml. We first turned our attention to improving the metabolic stability and solubility of the compounds. One of the strategies pursued was to lower the lipophilicity of the compounds by introducing polar functionalities in

the form of aromatic nitrogens. Table 3 shows selected compounds from a nitrogen scan around the biphenyl of compound **31**. Nitrogen was tolerated in most positions although the activity decreased approximately 6-fold for compounds **32** and **34** with nitrogen in the ortho and para position of the terminal ring. In compound **35** with a nitrogen ortho to the amide group, the activity increased 10-fold compared to the biphenyl **31** but metabolic stability and solubility deteriorated. Introduction of a nitrogen meta to the amide as in **36** resulted in a metabolically very stable compound but solubility was poor. Unfortunately the introduction of a single nitrogen did not increase solubility compared to the biphenyl. A double nitrogen scan around the biphenyl was subsequently performed and selected compounds with in vitro profiling data are also shown Table 3. Introduction of two or more nitrogen in the biphenyl rings gives rise to improved metabolic stability with modest improvements in solubility as demonstrated in compounds **37** and **39**.

Table 3. Introduction of nitrogen atoms in the biaryl group.



Compound	R	STF3a IC_{50} (µM)[a]	MLM[b]/HLM[c] CL'int (µl/min/mg)	Sol. pH 7.4 (µg/ml)[d]
30		0.015	65/24	1.26
31		0.012	25/22	2.10
32		0.067	38/28	N.A.

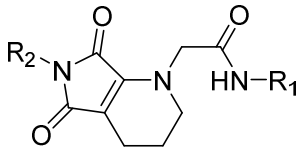
33		0.008	48/37	N.A.
34		0.072	44/31	N.A.
35		0.001	67/175	0.24
36		0.006	13/7	0.75
37		0.011	17/11	13.04
38		0.001	36/48	3.34
39		0.029	12/7	9.5

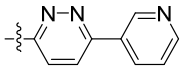
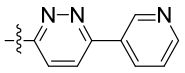
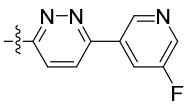
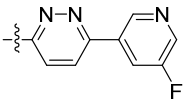
[a]data represent mean value of triplicate experiments; [b]mouse liver microsomes; [c]human liver microsomes; [d]thermodynamic solubility at pH 7.4.

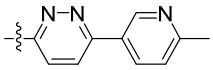
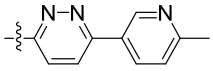
Selection of the preclinical candidate. We next synthesized a number of compounds with a combination of small substituents on the maleimide nitrogen and the biaryl system. Table 4 summarized the in vitro pharmacokinetic data for compounds **39**-**44**. Although in isolation the introduction of aromatic nitrogens and small substituents did not improve solubility and metabolic stability (data not shown), the combination produced compounds with improved properties. Addition of a third nitrogen at the meta position of the terminal aromatic ring in compounds **39**-**44** increased solubility without compromising metabolism. Since compounds **39**-**44** overall had an acceptable profile the in vivo pharmacokinetics were investigated at a dose of 5mg/kg in female ICR mice by oral gavage (Table 5). Generally, the cyclopropyl analogues had a better in vivo pharmacokinetic profile compared with the respective methyl analogues. There was a good correlation between MLM CL'int and in vivo clearance with an R^2 of 0.71. While the

half-life was relatively constant around 2 hours, the Tmax varied between 10 min and 2 hours. The Cmax was 750 to 4000 ng/mL where longer Tmax values resulted in a lower Cmax. An excellent correlation (R^2) of 0.84 was observed between Cmax and area under curve which varied from 800 to 15500. Compound **40** and **42** had the best absorption and exposures among the compounds investigated in vivo with Cmax greater than 3000 ng/ml and area under the curve greater that 10000 ng*h/ml. The biological activity of compounds **40** and **42** were comparable but compound **40** had a more desirable solubility compared to compound **42**. In addition, the free plasma fraction of **40** was three times higher than that of **42**. Consequently we chose compound **40** for efficacy studies in a mouse xenograft model and more extensive in vivo and in vitro profiling.

Table 4. In vitro pharmacokinetics for compounds **39**-**44**.



Compound	R_1	R_2	STF3a[a] IC_{50} (µM)	CYP 3A4/2D6[b] IC_{50}(µM)	MLM[c] / HLM[d] CL'int (µl/min/mg)	Sol. pH 7.4/4[e](µg/ml)	Plasma Protein Binding (%)
39		Me	0.029	6.2/>20	12/7	9.5/13.9	95.00
40		cPr	0.012	9.3/9.3	24/18	60/141	97.40
41		Me	0.009	7.1/>30	21/13	41/82	98.26
42		cPr	0.011	16/8.5	16/25	14.9/13.8	99.16

43		Me	0.029	>30/>30	8/8	35.5/>194	96.87
44		cPr	0.012	8/>30	15/23	11.1/>41	98.10

[a]data represent mean value of triplicate experiments; [b]Cytochrome P450 enzyme; [c]mouse liver microsomes; [d]human liver microsomes; [e]thermodynamic solubility at pH 7.4 and pH 4 respectively.

Table 5. In vivo PK for best compounds at a dose of 5 mg/kg PO in mice. See table 4 for structures.

Compound	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$(h)	CL (L/h/kg)	$AUC_{0\text{-inf}}$ (ng*h/mL)
39	759	2.00	1.32	1.01	4948
40	4031	0.50	1.48	0.39	12663
41	845	2.00	2.00	0.80	6465
42	3292	0.50	1.80	0.30	15509
43	1051	0.17	2.20	5.80	859
44	1189	0.50	2.10	4.00	1244

Profiling of preclinical candidate. To confirm that compound **40** is a porcupine inhibitor, we examined whether PORCN overexpression could blunt its effect on the Wnt/β-catenin reporter. HT1080 cells were treated with either a control plasmid or a murine PORCN containing plasmid in combination with a Wnt/β-catenin reporter plasmid and Wnt3a. Treatment of the wild-type cells with **40** inhibited luciferase activity in a dose dependent manner, but in cells overexpressing porcupine the luciferase activity was inhibited to a much lesser extent (Figure 5).

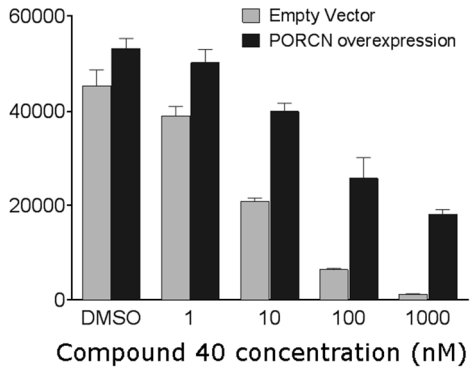


Figure 5. HT1080 cells were transfected with empty vector (EV) or mPORCN-D expression plasmids followed by treatment with **40** at indicated concentrations. Luciferase activity was measured after 24 hours. Error bars represent SD.

In vivo PK for an intravenous dose of 1 mg/kg of **40** in mice was obtained and the oral absolute bioavailability was calculated to be 100%. Oral PK in rats was established with a dose of 10 mg/kg. $T_{1/2}$ in rat were 1.6 hours, Cmax was 17273 ng/ml with an exposure of 81171 ng*h/ml. An intravenous dose of 2 mg/kg allowed us to calculate the oral bioavailability in rats to be 81%. The plasma concentration profile for both oral and intravenous administration is shown in figure 6 for mice (left) and rats (right).

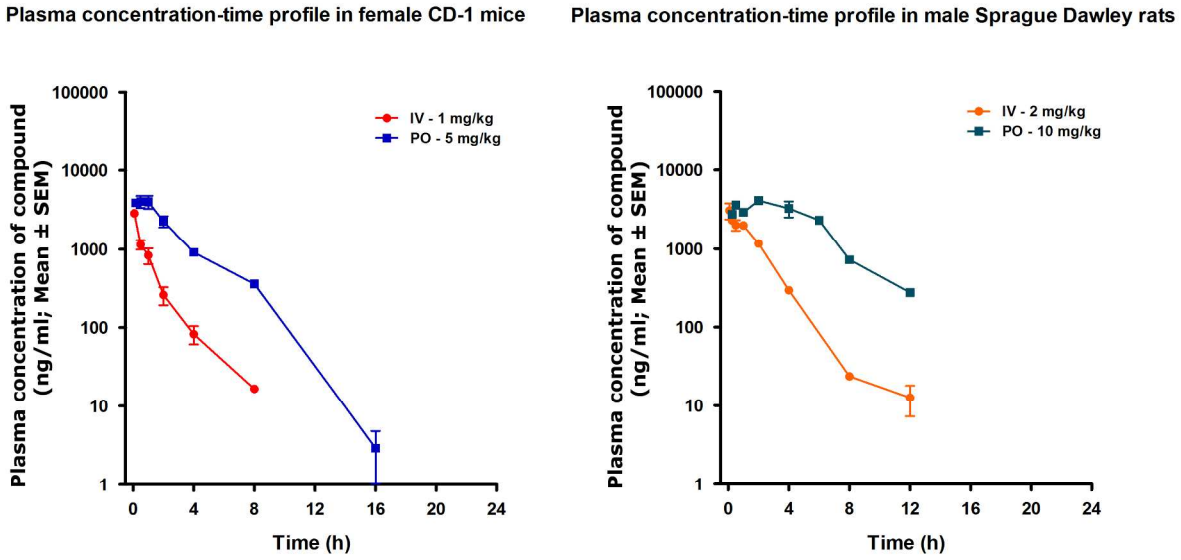


Figure 6. Plasma concentration profile of Compound **40** in mice (left) and rat (right).

In vivo efficacy of **40** was established in a Wnt-driven tumor model. MMTV-Wnt1 tumor fragments were implanted in the mammary fat pads of Balb/c nude mice. After 17 days, mice bearing MMTV-Wnt1 tumors were selected and divided into 5 groups. These were treated with either vehicle or 1, 3 or 10 mg/kg of compound **40** once daily by oral gavage for 14 days. The last group was treated with 3 mg/kg of our reference compound **2** which have previously been shown to be efficacious in this model.[23] Compound **40** was found to be equipotent with **2** inhibiting tumor growth in a dose-dependent manner as shown in figure 7. Tumor growth inhibition for **40** ranged between 24% at 1 mg/kg to 97% at 10 mg/kg and found to be statistical significant with a p value < 0.0001 for 2 doses (3 mg/kg and 10 mg/kg). No significant bodyweight loss was observed in this model (See supplementary material).

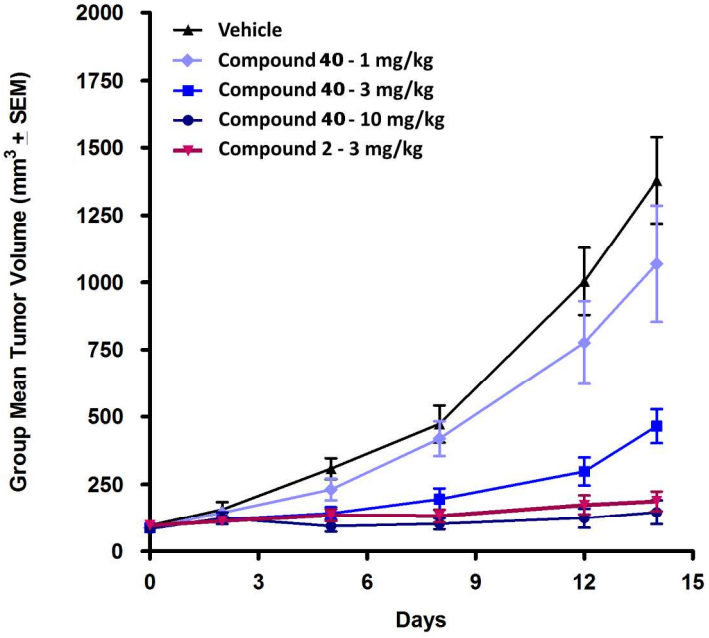


Figure 7. Inhibition of growth of MMTV-Wnt1 tumors for **40** and reference compound **2** (Figure 1). Tumor growth inhibition for **40** was 24% at 1 mg/kg, 72% at 3 mg/kg, and 97% at 10 mg/kg. Tumor growth inhibition compared with vehicle was statistically significant for the two highest doses with a p value of <0.0001.

Compound **40** was then tested in enzyme and receptor panels. No significant inhibition was found at 10 μM in Eurofins 87 receptor panel. In a cytochrome panel with 8 isoforms Cyp3A4, Cyp2D6 and Cyp1A2 were inhibited with IC_{50} values between 8.4 and 19.4 μM while no inhibition of the other isoforms were found. This is in contrast to the Cyp3A4 assay used during lead optimization where **40** had an IC_{50} of 2.1 μM. In the KINOMEscan panel with 456 wild type and mutated kinases, compound **40** was found to inhibit MEK5 and RIOK2 with K_d's of 1.3 and 7.4 μM. No other kinases were significantly inhibited at 10 μM which resulted in the S1 selectivity score of 0. **40** was also found to be AMES negative while it had marginal hERG inhibition in a patch clamp assay with an IC_{50} value of 48μM. A summary of the data for **40** is provided in the supporting materials.

CONCLUSION

Inhibition of the Wnt pathway is an attractive approach for developing new therapeutics for the treatment of various types of fibrosis and cancers. However the pathway is very complex with 19 human Wnt ligands that act on 10 Frizzled (Fzd) receptors. Our strategy was to find a point of intervention that blocks signaling through all Frizzled receptors. This is accomplished through inhibition of porcupine, an enzyme that palmitoleates Wnt ligands, thereby blocking Wnt excretion.

We designed a novel porcupine inhibitor scaffold using a previously developed pharmacophore model[19] and scaffold hopping. The new piperidine-maleimide scaffold had reasonable physical properties except for limited solubility. Medicinal chemistry optimization led to compounds with nanomolar activity in our cell based assay for porcupine inhibition. The solubility problem was solved by a combination of the addition of small substituents that may disrupt solid state interactions and substitution of aromatic carbon atoms for nitrogen.

Our inhibitors have good drug-like properties and good in vitro PK which generally translated into good oral PK in mice and high oral bioavailability. We selected a pre-clinical development candidate, compound **40** that was extensively profiled in cytochrome and receptor panels. This compound has high efficacy in a Wnt-driven murine tumor model.

EXPERIMENTAL

General. All reagents were purchased from commercial sources and used as received. Reaction progress was monitored by TLC using Merck silica gel 60 F254 coated glass plates with UV detection at 254 nM. Shimadzu LC-20AD and LCMS-2020 was used for LC−MS analysis with a Phenomenex Kinetex 2.6 μm, 50 × 2.10 mm column. Proton nuclear magnetic resonance (1H NMR) spectra were obtained using a Bruker Ultrashield 400 PLUS/R system, operating at 400 MHz. All resonance bands were referenced to tetramethylsilane (internal standard). Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad peak. The compounds' purities were ≥95% determined by a VARIAN ProStar HPLC instrument.

Compound Synthesis.

General Synthetic procedure for amide coupling. A stirred solution of 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.21 mmol.) in DCM (5 ml) was treated with HATU (0.252 mmol), TEA (0.42 mmol) and the corresponding amine (0.21 mmol) and stirred at r.t for 16 h. Upon reaction completion, water was added to the reaction mixture and extracted with DCM. The combined organics was washed with brine and dried over Na_2SO_4 and concentrated in vacuo. The crude compound was purified by column chromatography to afford the desired product.

2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-(thiazol-2-yl)phenyl)acetamide (3). The titled compound was obtained as a white solid (yield, 39 %) with the general amide coupling procedure using 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)acetic acid and 4-(thiazol-2-yl)aniline . ^{1}H NMR (400 MHz; DMSO-d_6) δ 10.67 (s, 1H), 8.08 (s, 1H), 7.92 (m, 2H), 7.88 (d, *J = 3.2* Hz, 1H), 7.72 (m, 2H), 7.70 (s, 1H), 5.24 (s, 2H), 3.47 (s, 3H), 3.20 (s, 3H); MS (ESI) *m/z* 397.1 $[C_{18}H_{16}N_6O_3S + H]^+$.

4-(thiophen-3-yl) aniline (4). To a stirred solution of 4-bromoaniline (5.75 mmol) in 1,4-dioxane (40 ml) and water (8 ml) was added thiophen-3-ylboronic acid (8.62 mmol) and K_2CO_3 (11.49 mmol) at r.t and passed nitrogen gas through reaction mixture for 15 minutes. Pd(dppf)Cl_2.DCM (0.287 mmol) was added to the reaction mixture and degassed for another 15 min. The reaction mixture heated to 100 ^{o}C for 4 h. After completion, the reaction mixture was cooled to r.t, added water and extracted with ethyl acetate. The combined organic layers were washed with brine solution, dried over anhydrous Na_2SO_4, filtered and dried under vacuum to afford the crude product. The crude product was purified by column chromatography to afford 4-(thiophen-3-yl) aniline as a pale yellow solid (yield, 48 %). ^{1}H NMR (400 MHz; $CDCl_3$) δ 7.41

(d, *J* = 8.4 Hz, 2H), 7.33 (d, *J* = 3.1 Hz, 1H), 7.32 (d, *J* = 0.9 Hz, 1H), 7.31 (s, 1H), 6.71 (d, *J* = 8.4 Hz, 2H), 3.7 (br s, 2H); MS (ESI) *m/z* 176.0 $[C_{10}H_9NS + H]^+$.

2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-(thiophen-3-yl)phenyl) acetamide (5). The titled compound was obtained as a white solid (yield, 68 %) with the general amide coupling procedure using theophylline-7-acetic acid and 4-(thiophen-3-yl) aniline, **4**. ^{1}H NMR (400 MHz; DMSO-d_6) δ 10.43 (s, 1H), 8.07 (s, 1H), 7.78 (br s, 1H), 7.68 (m, 2H), 7.61 (m, 3H), 7.52 (m, 1H), 5.22 (s, 2 H), 3.47 (s, 3H), 3,21 (s, 3H); MS (ESI) *m/z* 396.0 $[C_{19}H_{17}N_5O_3S + H]^+$.

2-(1H-imidazol-1-yl)-N-(4-(thiophen-3-yl)phenyl)acetamide (6). The titled compound was obtained as a white solid (yield, 42 %) with the general amide coupling procedure using 2-(1H-imidazol-1-yl)acetic acid (0.5 mmol) and 4-(thiophen-3-yl) aniline (0.5 mmol), **4**. ^{1}H NMR (400 MHz; DMSO-d_6) δ 10.51 (s, 1H), 8.60 (s, 1H), 7.80 (s, 1H), 7.69 (m, 2H), 7.61 (m, 3H), 7.54 (m, 2H), 7.42 (s, 1H), 5.11 (s, 2H); MS (ESI) *m/z* 396.0 $[C_{15}H_{13}N_3OS + H]^+$.

2-(1H-benzo[d]imidazol-1-yl)-N-(4-(thiophen-3-yl)phenyl)acetamide (7). The titled compound was obtained as a white solid (yield, 39 %) with the general amide coupling procedure using 2-(1H-benzo[d]imidazol-1-yl)acetic acid and 4-(thiophen-3-yl) aniline, **4**.^{1}H NMR (400 MHz, DMSO-d_6) δ 10.53 (s, 1H), 8.23 (s, 1H), 7.80 (d, *J* = 2.0 Hz, 1H), 7.70-7.60 (m, 6H), 7.53 (t, *J* = 8.4 Hz, 2H), 7.27-7.19 (m, 2H), 5.19 (s, 2H); MS (ESI) *m/z* 334.3 $[C_{19}H_{15}N_3OS +H]^+$.

2-(2,6-dioxo-2,3-dihydro-1H-purin-7(6H)-yl)acetic acid (8). 3,7-dihydro-1H-purine-2,6-dione (6.57 mmol) in water (4.2 ml) at r.t was added 2M NaOH solution (10.4 ml) and the resulting solution was stirred for 30 min and chloroacetic acid (6.57 mmol) was added. The resulting

reaction mixture was refluxed for 5 h before cooling to r.t and stirred overnight. The precipitated solid was removed by filtration and water was acidified with concentrated HCl. The solid collected by filtration, washed with hot EtOH to afford the titled compound as white solid (yield, 23 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 13.35 (br s, 1H), 11.59 (s, 1H), 10.88 (br s, 1H), 7.91(s, 1H), 5.0 (s, 2H); MS (ESI) m/z 211.1 $[C_7H_6N_4O_4 + H]^+$.

2-(2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-(thiophen-3-yl)phenyl)acetamide (9). A stirred solution of **8** (0.476 mmol) in DMF (5 ml) and DMSO (5 ml) was added EDCI (0.57 mmol), HOBT (0.57 mmol) and stirred at r.t for 15 min followed by the addition of **4** (0.476 mmol). The reaction mixture was stirred at r.t for 16 h. After completion, the reaction mixture was concentrated under vaccum, water was added to the reaction mixture the precipitated solid was collected by filtration to give the titled compound as an off-white solid (yield, 40 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 11.60 (s, 1H), 10.88 (br s, 1H), 10.43 (br s, 1H), 7.95 (s, 1H), 7.79 (d, J = 2.0 Hz, 1H), 7.68 (d, J = 8.8 Hz, 2H), 7.61-7.59 (m, 3H), 7.53 (d, J = 4.8 Hz, 1H), 5.14 (s, 2H); MS (ESI) m/z 366.10 $[C_{17}H_{13}N_5O_3S + H]^+$.

2-((1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)amino)-N-(4-(thiazol-2-yl)phenyl)acetamide (10). To a solution of 2,4-pyrimidinedione, 5-amino-1,3-dimethyl (0.645 mmol) in DMF (3.2 ml) was added methyl bromoacetate (0.838 mmol) and K_2CO_3 (0.968 mmol). The reaction was allowed to stir at 80 ^{o}C for 16 h. Upon completion of reaction, the reaction mixture was dried in vacuo. Water was added and the aqueous was extracted with $CHCl_3$. The combined organics was washed with brine, dried over anhydrous Na_2SO_4 and dried in vacuo to afford the crude product methyl (1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)glycinate which is carried on to the next step. MS (ESI) *m/z* 228.2 $[C_9H_{13}N_3O_4 + H]^+$.

To a stirred solution of methyl methyl (1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)glycinate (0.484 mmol) in THF (5 ml), MeOH (1 ml) and water (1 ml) was added $LiOH.H_2O$ (0.968 mmol) and stirred at r.t for 5 h. Upon completion of reaction, the reaction mixture was concentrated. The residue was dissolved in water, washed with EtOAc, acidified with aq.$KHSO_4$ and the product was extracted with 10 % MeOH/$CHCl_3$. The combined organics was dried over Na_2SO_4 and concentrated to give (1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)glycine as white solid. MS (ESI): *m/z* 212.2 $[C_8H_{11}N_3O_4 - H]^+$.

The titled compound was obtained as a white solid (yield, 52 %) with the general amide coupling procedure using (1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)glycine (0.484 mmol) and 4-(1,3-thiazol-2-yl) aniline (0,484 mmol). ^{1}H NMR (400 MHz; DMSO-d_6) δ 10.18 (s, 1H), 7.88 (m, 3H), 7.73 (m, 3H), 6.71 (s, 1H), 4.95 (t, *J* = 6.8 Hz, 1H), 3.77 (d, *J* = 6.4 Hz, 2H), 3.26 (s, 3H), 3.23 (s, 3H); MS (ESI) *m/z* 372.4 $[C_{17}H_{17}N_5O_3S + H]^+$.

3H-imidazo[4,5-c]pyridin-4(5H)-one (11). 2-chloropyridine-3,4-diamine (3.496 mmol) in trimethylorthoformate (10 ml) was heated at 70 ^oC for 16 h. After completion, the reaction mixture was cooled to r.t, and the reaction mixture was concentrated under vacuum. The residue was purified by column chromatography to give 4-chloro-3H-imidazo[4,5-c]pyridine as a white solid (yield, 58 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 13.20 (br s, 1H), 8.47 (s, 1H), 8.11 (d, *J* = 5.6 Hz, 1H), 7.62 (d, *J* = 5.2 Hz, 1H); MS (ESI) *m/z* 154.1 $[C_6H_4ClN_3 + H]^+$.

4-chloro-3H-imidazo[4,5-c]pyridine (1.633 mmol) in methanolic HCl (20 ml) was stirred at r.t for 24 h. Upon completion, the reaction mixture was concentrated under vacuum. The crude residue was washed with diethyl ether to give the titled compound as a light brown solid (yield,

95 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 11.88 (br s, 1H), 9.11 (s, 1H), 7.39-7.37 (m, 1H), 6.66 (d, *J* = 7.2 Hz, 1H); MS (ESI) *m/z* 136.0 $[C_6H_5N_3O + H]^+$.

2-(5-methyl-4-oxo-4,5-dihydro-3H-imidazo[4,5-c]pyridin-3-yl)-N-(4-(thiophen-3-yl)phenyl)acetamide (12). To a solution of 3H-imidazo[4,5-c]pyridin-4(5H)-one, **11** (1.555 mmol) in DMF (5 ml) was added K_2CO_3 (1.711 mmol) and 2-chloro-N-(4-(thiophen-3-yl)phenyl)acetamide (1.555 mmol) and stirred at r.t for 16 h. Upon reaction completion, the reaction mixture was poured into water and precipitated solid was collected by filtration, washed with excess water and diethyl ether before drying under vacuum to afford 2-(4-oxo-4,5-dihydro-3H-imidazo[4,5-c]pyridin-3-yl)-N-(4-(thiophen-3-yl)phenyl)acetamide as a light brown solid (yield, 55 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 11.27 (s, 1H), 10.45 (s, 1H), 8.14 (s, 1H), 7.79 (s, 1H), 7.69-7.61 (m, 5H), 7.53 (d, *J* = 4.8 Hz, 1H), 7.09 (t, *J* = 6.0 Hz, 1H), 6.58 (d, *J* = 6.8 Hz, 1H), 5.36 (s, 2H); MS (ESI) *m/z* 351.1 $[C_{18}H_{14}N_4O_2S + H]^+$.

To a solution of 2-(4-oxo-4,5-dihydro-3H-imidazo[4,5-c]pyridin-3-yl)-N-(4-(thiophen-3-yl)phenyl)acetamide(1.287 mmol) in DMF (5 mL) at r.t was added K_2CO_3 (1.711 mmol) and stirred for 30 min. Methyl iodide (0.571 mmol) was added at r.t and stirred for 3 h. Upon completion of reaction, the reaction mixture was poured into water and precipitated solid was collected by filtration, washed with excess water and diethyl ether then dried under vacuum. The solid compound was further purified by column chromatography to afford the titled compound as an off-white solid (yield, 29 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 10.45 (s, 1H), 8.14 (s, 1H), 7.79 (d, *J* = 1.6 Hz, 1H), 7.69-7.67 (m, 2H), 7.62-7.61 (m, 3H), 7.53 (d, *J* = 5.2 Hz, 1H), 7.42 (d, *J* = 7.2 Hz, 1H), 6.62 (d, *J* = 7.6 Hz, 1H), 5.36 (s, 2H), 3.47(s, 3H), 3.31(s, 3H); MS (ESI) *m/z* 365.1 $[C_{19}H_{16}N_4O_2S + H]^+$.

2-(5-(dimethylcarbamoyl)-1H-imidazol-1-yl)acetic acid (13). A stirred solution of 1H-imidazole-5-carboxylic acid (4.464 mmol) in $SOCl_2$ (8 ml) was heated to reflux for 5 h, before cooling to r.t. The reaction mixture was added to toluene and the solid was collected by filtration and dried to give the corresponding acid chloride. The acid chloride in DCM (20 ml) was added dimethylamine in THF (2 ml, 2M solution) and stirred at r.t for 16 h. Upon completion of reaction, the reaction mixture was concentrated and washed with diethyl ether to give N,N-dimethyl-1H-imidazole-5-carboxamide as a yellow solid (yield, 80 %). MS (ESI) *m/z* 140.6 $[C_6H_9N_3O + H]^+$.

To a solution of N,N-dimethyl-1H-imidazole-5-carboxamide(3.597 mmol) in DMF (10 ml) was added K_2CO_3 (4.316 mmol) at r.t and heated to 100 ^oC for 1 h and cooled to 70 ^oC; bromoethylacetate (5.395 mmol) was added to reaction mixture and stirred at 70 ^oC for 16 h. After completion of reaction, the reaction mixture was cooled to r.t and diluted with water and extracted with EtOAc. The combined organics were dried over Na_2SO_4 and concentrated under vacuum. The crude compound was purified by column chromatography to give ethyl 2-(5-(dimethylcarbamoyl)-1H-imidazol-1-yl)acetate as a yellow solid (yield, 25 %). ^1H NMR (400 MHz; $CDCl_3$) δ 7.55 (s, 1H), 7.46 (s, 1H), 4.69 (s, 2H), 4.26 (q, J = 7.2 Hz 2H), 3.48 (s, 3H), 3.08 (s, 3H), 1.30 (t, J = 7.2 Hz, 3H); MS (ESI) *m/z* 225.8 $[C_{10}H_{15}N_3O_3 + H]^+$.

To a stirred solution of 2-(5-(dimethylcarbamoyl)-1H-imidazol-1-yl)acetate (0.888 mmol) in THF (10 ml), MeOH (10 ml) and water (5 ml) was added $LiOH.H_2O$ (1.333 mmol) and stirred at r.t for 2 h. The reaction mixture was concentrated, the residue was dissolved in water, washed with EtOAc and acidified with aq.KHSO4; product was extracted with EtOAc. The combined organics was washed with brine, dried over Na_2SO_4 and concentrated in vacuo to provide the titled compound as an off-white solid (yield, 80 %). ^1H NMR (400 MHz, DMSO-d_6) δ 13.20 (br

s, 1H), 7.63 (s, 1H), 7.59 (s, 2H), 3.40 (s, 3H), 2.92 (s, 3H); MS (ESI) *m/z* 198.2 [$C_8H_{11}N_3O_3$ + H]$^+$.

N,N-dimethyl-1-(2-oxo-2-(4-(thiophen-3-yl)phenylamino)ethyl)-1H-imidazole-5-carboxamide (14). The titled compound was obtained as a white solid (yield, 60 %) with the general amide coupling procedure using 2-(5-(dimethylcarbamoyl)-1H-imidazol-1-yl)acetic acid (0.71 mmol) and 4-(thiophen-3-yl) aniline, **4** (0.71 mmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.39 (s, 1H), 7.80 (s, 1H), 7.70-7.61 (m, 7H), 7.53 (s, 1H), 4.97 (s, 2H), 3.42 (s, 3H), 2.94 (s, 3H); MS (ESI) *m/z* 355.5 [$C_{18}H_{18}N_4O_2S$+ H]$^+$.

2-oxo-1-(2-oxo-2-((4-(thiazol-2-yl)phenyl)amino)ethyl)-1,2-dihydropyridine-3-carboxylic acid (15). To a solution of 4-(1,3-thiazol-2-yl) aniline (1.6 mmol) and Hunig's base (1.6 mmol) in anhydrous DCM (8 ml) at -78 °C was added bromoacetyl bromide (1.6 mmol) dropwise. The reaction mixture was stirred at -78 °C for 1 h, warmed to r.t and stirred further for 1 h. Upon completion of reaction, the reaction mixture was diluted with ethyl acetate, washed with H_2O and brine, dried over Na_2SO_4 and concentrated in vacuo. The crude was purified by column chromatography to afford 2-bromo-N-(4-(thiazol-2-yl)phenyl)acetamide as a yellow solid (yield, 34%). ^{1}H NMR (400 MHz; MeOD) δ 7.91- 7.93 (m, 2H), 7.83 (d, *J* = 3.2 Hz, 1H), 7.70 – 7.73 (m, 2H), 7.56 (d, *J* = 3.2Hz, 1H), 3.99 (s, 2H); MS (ESI) *m/z* 298.2 [$C_{11}H_9BrN_2OS$ + H]$^+$.

Methyl 2-oxo-1,2-dihydro-3-pyridine carboxylate (0.5 mmol) and 2-bromo-N-(4-(thiazol-2-yl)phenyl)acetamide (0.544 mmol) were taken up in anhydrous DMF (10 ml) and cooled to 0 °C using an ice-bath. NaH (2 mmol) was added carefully into the reaction. The mixture was left to warm up to r.t under N_2 for 4 h. Upon completion of reaction, the reaction mixture was diluted with DCM and quenched with 1N HCl. The reaction mixture was dried with Na_2SO_4, filtered and

concentrated in vacuo. The crude material was then purified by column chromatography to afford the titled compound as a white solid (yield, 18 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 14.19 (s, 1H), 10.74 (s, 1H), 8.45 (dd, J_1 = 2.0 Hz, J_2 = 7.2 Hz, 1H), 8.24 (dd, J_1 = 2.0 Hz, J_2 = 7.2 Hz, 1H), 7.92 – 7.94 (m, 2H), 7.88 (d, J = 3.2 Hz, 1H), 7.72 – 7.73 (m, 2H), 7.70 (d, J = 3.2 Hz, 1H), 6.79 (t, J = 6.8 Hz, 1H), 5.04 (s, 2H); MS (ESI) *m/z* 356.2 $[C_{17}H_{13}N_3O_4S + H]^+$.

N-methyl-2-oxo-1-(2-oxo-2-((4-(thiazol-2-yl)phenyl)amino)ethyl)-1,2-dihydropyridine-3-carboxamide (16). The titled compound was obtained as a white solid (yield, 62 %) with the general amide coupling procedure using **15** (0.09 mmol) and methylamine (0.09 mmol). ^{1}H NMR (400 MHz; DMSO-d_6) δ 10.67 (s, 1H), 9.43 – 9.45 (m, 1H), 8.38 (dd, J_1 = 2.4 Hz, J_2 = 7.2 Hz, 1H), 8.03 (dd, J_1 = 2.4 Hz, J_2 = 7.2 Hz, 1H), 7.91 – 7.93 (m, 2H), 7.88 (d, J = 3.2 Hz, 1H), 7.72 – 7.73 (m, 2H), 7.70 (d, J = 3.2 Hz, 1H), 6.57 (t, J = 6.8 Hz, 1H), 4.92 (s, 2H), 2.80 (d, J = 4.8 Hz, 1H); MS (ESI) *m/z* 369.2 $[C_{18}H_{16}N_4O_3S + H]^+$.

2-(3-nitro-1H-1,2,4-triazol-1-yl)acetic acid (17). Freshly prepared sodium ethoxide (10.526 mmol) was added to the stirred solution of 3-nitro-1H-1,2,4-triazole (8.771 mmol) in ethanol (10 ml) at 0 ^{o}C and the reaction was stirred at 0 ^{o}C temperature for 30 min. Bromo ethyl acetate (10.526 mmol) was added to the reaction mixture. The reaction mixture underwent reflux for 6 h. After completion the reaction mixture was quenched with ice water and concentrated under vacuum. The crude compound was purified by column chromatography to give ethyl 2-(3-nitro-1H-1,2,4-triazol-1-yl)acetate as white solid (yield, 68 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 8.32 (s, 1H), 5.08 (s, 2H), 4.32 (q, 2H), 1.35 (t, J = 6.4 Hz, 3H); MS (ESI) *m/z* 201.1 $[C_6H_8N_4O_4 + H]^+$.

A stirred solution of 2-(3-nitro-1H-1,2,4-triazol-1-yl)acetate (3.5 mmol) in THF (20 ml), MeOH (10 ml) and water (5 ml) was added $LiOH.H_2O$ (7 mmol) and allowed to stir for 3 h. Upon reaction completion, the reaction mixture was concentrated and the residue was dissolved in water and washed with EtOAc. The aqueous layer was acidified with aq. $KHSO_4$ and product was extracted with EtOAc, washed with brine, dried over Na_2SO_4 and concentrated to afford the titled compound as white solid (yield, 83 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 13.10 (br s, 1H), 8.87-8.86 (s, 1H), 5.29 (s, 2H); MS (ESI) *m/z* 171.1 $[C_4H_4N_4O_4 + H]^+$.

2-(3-amino-1H-1,2,4-triazol-1-yl)-N-(4-(thiazol-2-yl)phenyl)acetamide (18). 2-(3-nitro-1H-1,2,4-triazol-1-yl)-N-(4-(thiazol-2-yl)phenyl)acetamide was obtained as a white solid (yield, 42 %) with the general amide coupling procedure using **17** (2.906 mmol) and 4-(1,3-thiazol-2-yl) aniline (2.906 mmol). ^{1}H NMR (400 MHz; DMSO-d_6) δ 10.76 (s, 1H), 8.92 (s, 1H), 7.94 (d, *J* = 8.8 Hz, 2H), 7.90 (d, *J* = 3.6 Hz, 1H), 7.75-7.70 (m, 3H), 5.38 (s, 2H); MS (ESI) *m/z* 331.0 $[C_{13}H_{10}N_6O_3S + H]^+$.

The stirred solution of 2-(3-nitro-1H-1,2,4-triazol-1-yl)-N-(4-(thiazol-2-yl)phenyl)acetamide (0.909 mmol) in ethanol was added iron powder (9.09 mmol) and AcOH (1 ml) and heated to reflux for 5 h. After completion, the reaction mixture was concentrated in vacuo and purified by column chromatography to afford the titled compound as an off-white solid (yield, 68 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 10.57 (s, 1H), 8.01 (s, 1H), 7.93 (d, *J* = 8.0 Hz, 1H), 7.89 (d, *J* = 2.8Hz, 1H), 7.75-7.71 (m, 3H), 5.30 (s, 2H), 4.84 (s, 2H); MS (ESI) *m/z* 301.0 $[C_{13}H_{12}N_6OS + H]^+$.

2-(3-(3-methylureido)-1H-1,2,4-triazol-1-yl)-N-(4-(thiazol-2-yl)phenyl)acetamide (19). To a solution of **18** (0.033 mmol) in ethyl acetate (2 ml) and water (1 ml), was added sodium

hydrogen carbonate (0.396 mmol). The reaction mixture was stirred at r.t overnight. Following which, the solvent was evaporated and the aqueous phase was extracted with EtOAc. The combined organics was dried over Na_2SO_4, filtered and concentrated and taken for the next step. To a solution of crude product in pyridine (0.5 ml) was added 2,5-dioxopyrrolidin-1-yl methylcarbamate (1 mmol) and heated at 70 °C overnight. Upon completion of reaction, the reaction was concentrated in vacuo and purified by column chromatography to afford the titled compound as an off-white solid (yield, 17 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 10.68 (s, 1H), 9.46 (s, 1H), 8.41 (s, 1H), 7.96 (d, *J* = 8.8 Hz, 2H), 7.91 (d, *J* = 3.2 Hz, 1H), 7.75 (d, *J* = 2.8 Hz, 2H), 7.73 (d, *J* = 6.8 Hz, 2H), 5.07 (s, 2H), 2.75 (d, *J* = 4.4 Hz, 3H); MS (ESI) *m/z* 358.1 $[C_{15}H_{15}N_7O_2S + H]^+$.

1-(1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)pyrrolidine-2-carboxylic acid (20). 1-methyl-1H-pyrrole-2,5-dione (9 mmol) in diethyl ether (20 ml) at 0 ^{o}C was added bromine (9.9 mmol). The reaction mixture was heated at 45 ^{o}C for 2 h. After completion of reaction, the reaction mixture was extracted with diethyl ether, dried over Na_2SO_4 and concentrated to give 3-bromo-1-methyl-1H-pyrrole-2,5-dione as light brown solid (yield, 75 %).^{1}H NMR (400 MHz; $CDCl_3$) δ 6.88 (s, 1H), 3.08 (s, 3H); MS (ESI) *m/z* 190.0 $[C_5H_4BrNO_2 + H]^+$.

3-bromo-1-methyl-1H-pyrrole-2,5-dione (7.894 mmol) and L-proline (7.894 mmol) in DMF (30 ml) at 0 ^{o}C was drop wise added TEA (15.788 mmol). The resulted reaction mixture was stirred at r.t for 2 h. Upon reaction completion, the reaction mixture was concentrated to afford the titled compound. The crude compound was taken to next step without purification. MS (ESI) *m/z* 223.0 $[C_{10}H_{12}N_2O_4 - H]^+$.

1-(1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-N-(4-(thiazol-2-yl)phenyl)pyrrolidine-2-carboxamide (21). The titled compound was obtained as a white solid (yield, 7 %) with the general amide coupling procedure using **20** (6.696 mmol and 4-(1,3-thiazol-2-yl) aniline (6.696 mmol). ^{1}H NMR (400 MHz; DMSO-d_6) δ 10.39 (s, 1H), 7.91-7.87 (m, 3H), 7.73-7.69 (m, 3H), 5.03 (m, 1H), 5.00 (s, 1H), 3.31 (t, *J* = 6.4 Hz, 2H), 2.77 (s, 3H) 2.33-2.30 (m, 1H), 2.12-2.07 (m, 1H), 1.98-1.95 (m, 2H); MS (ESI) *m/z* 383.2 $[C_{19}H_{18}N_4O_3S + H]^+$.

4-(1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)butanoic acid (22). To a solution of 5,6-diamino-1,3-dimethylpyrimidine-2,4(1H,3H)-dione (20.58 mmol) in DMF (35 ml) was added glutamic anhydride (30.882 mmol). The reaction mixture was stirred at r.t for 16 h. Upon reaction completion, the reaction mixture was concentrated, washed with diethyl ether to give 5-(5-amino-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-ylamino)-5-oxopentanoic acid as brown solid. The crude compound was taken to next step without purification. MS (ESI) *m/z* 282.9 $[C_{11}H_{16}N_4O_5 - H]^+$.

5-(5-amino-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-ylamino)-5-oxopentanoic acid (10.563 mmol) in 2M NaOH (60 ml) was heated to reflux for 4 h. After completion, the reaction mixture was concentrated and neutralized with 6N HCl. The precipitated solid was collected by filtration and washed with minimum amount of water and dried to give the titled compound as light brown solid (yield, 46 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 13.20 (br s, 1H), 12.09 (br s, 1H), 3.32 (s, 3H), 3.22 (s, 3H), 2.72 (t, *J* = 7.2 Hz, 2H), 2.27 (t, *J* = 7.2 Hz, 2H), 1.91 (q, *J* = 7.2 Hz, 2H); MS (ESI) *m/z* 267.1 $[C_{11}H_{14}N_4O_4 + H]^+$.

1,3-dimethyl-2,4-dioxo-N-(4-(thiazol-2-yl)phenyl)-2,3,4,6,7,8-hexahydro-1H-pyrrolo[1,2-f]purine-6-carboxamide (23). To a solution of **22** (0.751 mmol) in 1,2-dichloroethane (5 ml)

was added bromine (1.12 mmol) and PCl_3 (0.05 ml) at r.t, following which the reaction mixture was heated to reflux for 6 h. Upon completion of reaction, the reaction mixture was concentrated to afford 2-bromo-4-(1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)butanoic acid. The intermediate was taken to the next step without purification. MS (ESI) *m/z* 347.0 $[C_{11}H_{13}BrN_4O_4+ H]^+$.

To a solution of 2-bromo-4-(1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)butanoic acid (0.579 mmol) in acetonitrile (20 ml) was added K_2CO_3 (0.579 mmol) and reaction mixture was heated at 70 ^oC for 16 h. After completion, the reaction mixture was concentrated and water was added to reaction mixture and extracted with EtOAc. The combined organics was washed with brine, dried over Na_2SO_4 and concentrated in vacuo to afford 1,3-dimethyl-2,4-dioxo-2,3,4,6,7,8-hexahydro-1H-pyrrolo[1,2-f]purine-6-carboxylic acid as a brown solid (yield, 52 %). MS (ESI) *m/z* 265.2 $[C_{11}H_{12}N_4O_4+ H]^+$.

The titled compound was obtained as a white solid (yield, 66 %) with the general amide coupling procedure using1,3-dimethyl-2,4-dioxo-2,3,4,6,7,8-hexahydro-1H-pyrrolo[1,2-f]purine-6-carboxylic acid (0.3 mmol) and 4-(1,3-thiazol-2-yl) aniline (0.3 mmol). ^1H NMR (400 MHz; DMSO-d_6) δ 10.60 (s, 1H), 7.91 (d, *J* = 8.8 Hz, 2H), 7.83 (d, *J* = 3.6 Hz, 1H), 7.69 (d, *J* = 8.8 Hz, 2H), 7.29 (d, *J* = 3.2 Hz, 1H), 5.24 (t, *J* = 8.8 Hz, 1H), 3.59 (s, 3H) 3.50 (s, 3H), 3.47-3.46 (m, 1H), 3.25-3.18 (m, 1H), 3.02-2.95 (m, 1H), 2.82-2.76 (m, 1H); MS (ESI) *m/z* 423.1 $[C_{20}H_{18}N_6O_3S + H]^+$.

4-amino-2-methylisoindoline-1,3-dione (24). A stirred solution of NaH (46.87 mmol) in DMF (50 ml) was added 4-nitroisoindoline-1,3-dione (31.25 mmol) portion wise at 0 ^oC. After 20 min, MeI (46.87 mmol) was added to reaction mixture drop wise. The reaction mixture was warmed

to r.t and stirred for 2 h. After completion, reaction mixture was treated with ice cold water and extracted with EtOAc. The combined organics was washed with water, brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. The crude product was purified by column chromatography to afford 2-methyl-4-nitroisoindoline-1,3-dione as a light yellow solid (yield, 44 %). ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.13 (m, 2H), 7.93 (m, 1H), 3.23 (s, 3H); MS (ESI) *m/z* 207.0 $[C_9H_6N_2O_4+ H]^+$.

To a solution of 2-methyl-4-nitroisoindoline-1,3-dione (9.7 mmol) in methanol (130 ml) was added a slurry of 10 % Pd/C (2.425 mmol) in methanol under nitrogen. The reaction mixture was hydrogenated using H_2 (balloon) at r.t for 10 h. The reaction mixture was filtered through celite bed and washed with 20 % methanol in chloroform. The filtrate was concentrated in vacuo to afford the titled compound as a light yellow solid (yield, 68 %). ^{1}H NMR (400 MHz; DMSO-d_6) δ 7.43 (t, *J* = 7.9 Hz, 1H), 6.96 (d, *J* = 3.1 Hz, 1H), 6.94 (s, 1H), 6.43 (s, 2H) 2.95 (s, 3H); MS (ESI) *m/z* 177.0 $[C_9H_8N_2O_2+ H]^+$.

4-allyl-2-methylisoindoline-1,3-dione (25). A stirred solution of **24** (6.53 mmol) in 6 N HCl (10 ml) at 0 ^{o}C was added aq. $NaNO_2$ (13.06 mmol) drop wise at 0 ^{o}C. The reaction mixture was stirred at 0 ^{o}C for 1 h before KI (1.6 g, 9.79mmol) was added. The reaction mixture was warmed to r.t and stirred for 3 h. After completion, the reaction mixture was diluted with water, washed with saturated $NaHCO_3$ solution and extracted with EtOAc. The combined organic layers were washed with water, brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo to afford the crude product. The crude product was purified by column chromatography to afford 4-iodo-2-methylisoindoline-1,3-dione as a light brown solid (yield, 41 %). ^{1}H NMR (400 MHz; $CDCl_3$) δ 8.10 (d, *J* = 7.9 Hz, 1H), 7.86 (m, 1H), 7.38 (t, *J* = 7.9 Hz, 1H), 3.19 (s, 3H); MS (ESI) *m/z* 287.9 $[C_9H_6INO_2+ H]^+$.

A mixture of 4-iodo-2-methylisoindoline-1,3-dione (2.65 mmol), 2-allyl-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (4.77 mmol) and cesium fluoride (5.3 mmol) in 1,4-dioxane (26 ml) under inert atmosphere. $Pd(PPh_3)_4$ (0.26 mmol) was added and again degassed with argon for 10 min and the reaction mixture was heated at 100 ^oC for 16 h. The reaction mixture was diluted with water and extracted with EtOAc. The combined organics was washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo to obtain the crude product. The crude product was purified by column chromatography to afford the titled compound as a light yellow solid (yield, 88 %). ^1H NMR (400 MHz; $CDCl_3$) δ 7.70 (d, *J* = 7.5 Hz, 1H), 7.62 (t, *J* = 7.9 Hz, 1H), 7.51(d, *J* = 7.9 Hz, 1H), 6.03 (m, 1H), 5.14 (m, 2H) 3.90 (d, *J* = 6.6 Hz, 2H); MS (ESI) *m/z* 202.1 $[C_{12}H_{11}NO_2+ H]^+$.

2-(2-methyl-1,3-dioxoisoindolin-4-yl)acetic acid (26). A 1.35 M aqueous solution of $NaIO_4$ (9.35 mmol) was added to 0.47 M solution of **25** (2.34 mmol) in CCl_4:ACN (1:1) containing $RuCl_3.3H_2O$ (0.047 mmol). The reaction mixture was stirred at r.t for 12 h. Upon completion, the reaction mixture was diluted with water, washed with saturated $NaHCO_3$ solution and extracted with EtOAc. The aqueous layer was acidified with saturated $KHSO_4$ solution and extracted with EtOAc. The combined organics was washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo to obtain the crude product. The crude product was purified by column chromatography to afford the titled compound as an off-white solid (yield, 47 %). ^1H NMR (400 MHz; DMSO-d_6) δ 9.30-9.15 (br s, 1H), 7.73-7.63 (m, 3H), 4.00 (s, 2H), 3.00 (s, 3H); MS (ESI) *m/z* 220.0 $[C_{11}H_9NO_4+ H]^+$.

2-(2-methyl-1,3-dioxoisoindolin-4-yl)-N-(4-(thiazol-2-yl)phenyl)acetamide (27). The titled compound was obtained as a white solid (yield, 66 %) with the general amide coupling procedure using **26** (0.456 mmol) and 4-(1,3-thiazol-2-yl) aniline (0.456 mmol). ^1H NMR (400

MHz; $CDCl_3$) δ 9.11 (s, 1H), 8.87 (dd, J_1 = 0.8 Hz, J_2 = 1.6 Hz, 1H), 8.20-8.21 (m, 2H), 7.87(d, J = 3.2 Hz,1H), 7.80 (dd, J_1 = 1.6 Hz, J_2 = 6.4 Hz, 1H), 7.67-7.73 (m, 2H), 7.35 (d, J = 3.2 Hz, 1H), 4.21(s, 2H), 3.21(s, 3H); MS (ESI) *m/z* 379 $[C_{20}H_{15}N_3O_3S + H]^+$.

6-methyl-3,4-dihydro-1H-pyrrolo[3,4-b]pyridine-5,7(2H,6H)-dione (28). To a solution of furo[3,4-b]pyridine-5,7-dione (67.11 mmol) in toluene (250 ml) was added methyl amine (134.22 mmol, 2M solution in THF) and fitted with dean stark setup and heated at 130 ^oC for 16 h. After completion, the reaction mixture was cooled to r.t and toluene was decanted. The solid was washed with toluene, the combined toluene layers were concentrated, purified by column chromatography to afford 6-methyl-5H-pyrrolo[3,4-b]pyridine-5,7(6H)-dione as a white solid (yield, 46 %). ^1H NMR (400 MHz, DMSO-d_6) δ 8.98-8.96 (m, 1H), 8.19-8.17 (m, 1H), 7.65-7.62 (m, 1H), 3.26 (s, 3H); MS (ESI) *m/z* 163.2 $[C_8H_6N_2O_2 + H]^+$.

To a solution of 6-methyl-5H-pyrrolo[3,4-b]pyridine-5,7(6H)-dione (30.46 mmol) in THF (100 ml) was added 10 % Pd/C (10 % w/w) and reaction was stirred at r.t for 24 h. Upon completion of reaction, the reaction mixture was filtered through Celite and the filtrate was concentrated in vacuo. The crude compound was purified by column chromatography to afford the titled compound as a yellow solid (yield, 24 %). ^1H NMR (400 MHz, DMSO-d_6) δ 7.69 (br s, 1H), 3.22 (t, J = 6.0 Hz, 2H), 2.77 (s, 3H), 2.16 (t, J = 6.4 Hz, 2H), 1.73-1.67 (m, 2H); MS (ESI) *m/z* 167.2 $[C_8H_{10}N_2O_2 + H]^+$.

2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid. To a solution of 6-methyl-3,4-dihydro-1H-pyrrolo[3,4-b]pyridine-5,7(2H,6H)-dione (7.22 mmol) in ACN (50 ml) was added K_2CO_3 (10.843 mmol) and ethyl bromoacetate (10.843 mmol) and resulting reaction mixture was heated at 70 ^oC for 16 h. Upon completion of the reaction, the

reaction mixture was concentrated, water was added and extracted with EtOAc. The combined organics was washed with brine solution, dried over Na_2SO_4 and concentrated in vacuo. The crude compound was purified by column chromatography to afford ethyl 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetate as a yellow solid (yield, 88 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.46 (s, 2H), 4.20 (q, J = 7.2 Hz, 2H), 3.28 (t, J = 5.2 Hz, 2H), 2.91 (s, 3H), 2.35 (t, J = 6.2 Hz, 2H), 1.96-1.90 (m, 2H), 1.28 (t, J = 7.2 Hz, 3H); MS (ESI) *m/z* 253.2 $[C_{12}H_{16}N_2O_4 + H]^+$.

To a stirred solution of ethyl 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetate (1.984 mmol) in THF (20 ml), MeOH (20 ml) and water (10 ml) was added $LiOH.H_2O$ (2.976 mmol) and stirred at r.t for 2 h. After completion of reaction, the reaction mixture was concentrated. The residue was dissolved in water, washed with EtOAc followed by aq.$KHSO_4$ and the product was extracted with EtOAc. The combined organics was dried over Na_2SO_4, concentrated in vacuo to afford the titled compound as a yellow solid (yield, 81 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.85 (br s, 1H), 4.38 (s, 2H), 3.28 (t, J = 5.2 Hz, 2H), 2.77 (s, 3H), 2.19 (t, J = 6.0 Hz, 2H), 1.83-1.75 (m, 2H); MS (ESI) *m/z* 224.2 $[C_{10}H_{12}N_2O_4 + H]^+$.

6-cyclopropyl-1,2,3,4-tetrahydro-5H-pyrrolo[3,4-b]pyridine-5,7(6H)-dione (29). To a solution of furo[3,4-b]pyridine-5,7-dione (33.55 mmol) in toluene (100 ml) was added cyclopropyl amine (67.11 mmol, 2 M solution in THF) and fitted with dean stark setup and heated at 130 ^{o}C for 16 h. After completion, the reaction mixture was cooled to r.t and toluene was decanted and solid was washed with toluene. The combined toluene layers were concentrated, purified by column chromatography to afford of 6-cyclopropyl-5H-pyrrolo[3,4-b]pyridine-5,7(6H)-dione as a white solid (yield, 27 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.97 (dd, J_1 = 1.2 Hz, J_2 = 3.6 Hz, 1H), 8.15

(dd, J_1 = 1.2 Hz, J_2 = 6.4 Hz, 1H), 7.63-7.60 (m, 1H), 2.80-2.75 (m, 1H), 1.12-1.01 (m, 4H); MS (ESI) *m/z* 189.1 $[C_{10}H_8N_2O_2+ H]^+$.

To a solution of 6-cyclopropyl-5H-pyrrolo[3,4-b]pyridine-5,7(6H)-dione (18.51 mmol) in THF (60 ml) was added 200 mg of 10 % Pd/C (12.5 % w/w) and reaction was stirred at r.t for 16 h. Upon reaction completion, the reaction mixture filtered through Celite and filtrate was concentrated in vacuo. The crude compound was purified by column chromatography to afford the titled compound as a yellow solid (yield, 26 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.94 (br s, 1H), 3.38-3.35 (m, 2H), 2.45-2.42 (m, 1H), 2.33-2.30 (m, 2H), 1.89-1.83 (m, 2H), 0.91-0.80 (m, 4H); MS (ESI) *m/z* 193.3 $[C_{10}H_{12}N_2O_2 + H]^+$.

2-(6-cyclopropyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid). To a solution of 6-cyclopropyl-1,2,3,4-tetrahydro-5H-pyrrolo[3,4-b]pyridine-5,7(6H)-dione (3.012 mmol) in acetonitrile (25 ml) was added K_2CO_3 (4.518 mmol) and ethyl bromoacetate (4.518 mmol) and resulting reaction mixture was heated at 70 ^{o}C for 16 h. Upon completion of reaction, the reaction mixture was concentrated; water was added and extracted with EtOAc. The combined organics was washed with brine solution, dried over Na_2SO_4 and concentrated in vacuo. The crude compound was purified by column chromatography to afford ethyl 2-(6-cyclopropyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetate as a yellow solid (yield, 69 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.45 (s, 2H), 4.27-4.19 (m, 2H), 3.28 (t, J = 5.6 Hz, 2H), 2.42-2.39 (m, 1H), 2.32 (t, J = 6.0 Hz, 2H), 1.95-1.89 (m, 2H), 1.31-1.26 (m, 3H) 0.89-0.78 (m, 4H); MS (ESI) *m/z* 279.1 $[C_{14}H_{18}N_2O_4 + H]^+$.

To a stirred solution of 2-(6-cyclopropyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetate (1.052 mmol) in THF (20 ml), MeOH (10 ml) and water (5 ml) was added

LiOH.H_2O (1.578 mmol) and stirred at r.t for 2 h. Upon completion of reaction, the reaction mixture was concentrated, the residue was dissolved in water, washed with EtOAc, acidified with aq.$KHSO_4$ and the product was extracted with EtOAc. The combined organics was dried over Na_2SO_4, concentrated to give the titled compound as yellow solid (yield, 58 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.60 (br s, 1H), 4.36 (s, 2H), 3.27 (t, *J* = 5.2 Hz, 2H), 2.39-2.35 (m, 1H), 2.16 (t, *J* = 6.0 Hz, 2H), 1.82-1.76 (m, 2H), 0.76-0.64 (m, 2H); MS (ESI) *m/z* 251.0 $[C_{12}H_{14}N_2O_4 + H]^+$.

2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)-N-(6-phenylpyridazin-3-yl)acetamide (30). The titled compound was obtained as a yellow solid (yield, 15 %) with the general amide coupling procedure using 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.223 mmol) and 4-(1,3-thiazol-2-yl) aniline (0.223 mmol). ^{1}H NMR (400 MHz, MeOD) δ 7.90 (d, *J* = 8.4 Hz, 2H), 7.83 (d, *J* = 3.6 Hz, 2H), 7.69 (d, *J* = 8.4 Hz, 2H), 7.55 (d, *J* = 3.6 Hz, 2H), 4.60 (s, 2H), 4.55 (br s, 2H), 3.39 (t, *J* = 5.6 Hz, 2H), 2.87 (s, 3H), 2.32 (t, *J* = 5.6 Hz, 2H), 1.97 (q, *J* = 5.6 Hz, 2H); MS (ESI) *m/z* 383.1 $[C_{19}H_{18}N_4O_3S + H]^+$.

N-([1,1'-biphenyl]-4-yl)-2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetamide (31). The titled compound was obtained as a yellow solid (yield, 30 %) with the general amide coupling procedure using 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.223 mmol) and 4-biphenylamine (0.223 mmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.19 (s, 1H), 7.61 – 7.67 (m, 6H), 7.42 – 7.45 (m, 2H), 7.30 – 7.34 (m, 1H), 4,54 (s, 2H), 3.31 (t, *J* = 5.6 Hz, 2H), 2.77 (s, 3H), 2.22 (t, *J* = 5.6 Hz, 2H), 1.85 (q, *J* = 5.6 Hz, 2H); MS (ESI) *m/z* 379.0 $[C_{22}H_{21}N_3O_3 + H]^+$.

2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)-N-(4-(pyridin-2-yl)phenyl)acetamide (32). The titled compound was obtained as a yellow solid (yield, 40 %) with the general amide coupling procedure using 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.223 mmol) and 4-(pyridin-2-yl)aniline (0.223 mmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.26 (s, 1H), 8.62 (d, J= 4.4 Hz, 1H), 8.05 (d, J= 8.8 Hz, 2H), 7.92-7.90 (m, 1H), 7.86-7.82 (m, 1H), 7.69 (d, J= 8.8 Hz, 2H), 7.31 -7.28 (m, 1H), 4.55 (s, 2H), 2.77 (s, 3H), 3.35-3.31 (m, 2H), 2.22 (t, J=6.0 Hz, 2H), 1.86-1.84 (m, 2H); MS (ESI) *m/z* 377.0 $[C_{21}H_{20}N_4O_3 + H]^+$.

2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)-N-(4-(pyridin-3-yl)phenyl)acetamide (33). The titled compound was obtained as a yellow solid (yield, 47 %) with the general amide coupling procedure using 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.223 mmol) and 4-(pyridin-3-yl)aniline (0.223 mmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.25 (s, 1H), 8.87 (d J=1.6 Hz, 1H), 8.53-8.52 (m, 1H), 8.06-8.03 (m, 1H), 7.70 (s, 4H), 7.47-7.44 (m, 1H), 4.55 (s, 2H), 3.35-3.31 (m, 2H), 2.77 (s, 3H), 2.22 (t, J=6.0 Hz, 2H), 1.88-1.84 (m, 2H); MS (ESI) *m/z* 377.0 $[C_{21}H_{20}N_4O_3 + H]^+$.

2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)-N-(4-(pyridin-4-yl)phenyl)acetamide (34). The titled compound was obtained as a yellow solid (yield, 43 %) with the general amide coupling procedure using 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.223 mmol) and 4-(pyridin-4-yl)aniline (0.223 mmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.30 (s, 1H), 8.59 (d, J=6.0 Hz, 2H), 7.81-7.78 (m, 2H), 7.73-7.67 (m, 4H), 4.55 (s, 2H), 3.35-3.31 (m, 2H), 2.77 (s, 3H), 2.22 (t, J=6.0 Hz, 2H), 1.86-1.84 (m, 2H); MS (ESI) *m/z* 377.0 $[C_{21}H_{20}N_4O_3 + H]^+$.

2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)-N-(5-phenylpyridin-2-yl)acetamide (35). The titled compound was obtained as a yellow solid (yield, 41 %) with the general amide coupling procedure using 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.223 mmol) and 5-phenylpyridin-2-amine (0.223 mmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.77 (s, 1H), 8.65 (s, 1H), 8.09 (br s, 2H), 7.69 – 7.71 (m, 2H), 7.46 – 7.49 (m, 2H), 7.36 – 7.40 (m, 1H), 4.60 (s, 2H), 3.42 (t, *J* = 6.0 Hz, 2H), 2.76 (s, 3H), 2.21 (t, *J* = 6.0 Hz, 2H), 1.84 (q, *J* = 6.0 Hz, 2H); MS (ESI) *m/z* 377.0 $[C_{21}H_{20}N_4O_3 + H]^+$.

2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)-N-(6-phenylpyridin-3-yl)acetamide (36). The titled compound was obtained as a yellow solid (yield, 35 %) with the general amide coupling procedure using 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.223 mmol) and 6-phenylpyridin-3-amine (0.223 mmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.43 (s, 1H), 8.79 (d, *J* = 2.4 Hz, 1H), 8.13-8.10 (m, 1H), 8.04-8.02 (m, 2H), 7.94-7.92 (m, 1H), 7.48-7.45 (m, 2H), 7.41-7.37 (m, 1H), 4.58 (s, 2H), 2.77 (s, 3H), 2.22 (t, *J* = 6.4 Hz, 2H), 1.88-1.82 (m, 2H); MS (ESI) *m/z* 377.0 $[C_{21}H_{20}N_4O_3 + H]^+$.

N-([2,3'-bipyridin]-5-yl)-2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetamide (37). The titled compound was obtained as a yellow solid (yield, 38 %) with the general amide coupling procedure using 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.223 mmol) and 5-phenylpyrazin-2-amine (0.223 mmol). ^{1}H NMR (400 MHz, MeOH-d_4) δ 9.14 (s, 1H), 8.84 (s, 1H), 8.55 – 8.57 (m, 1H), 8.39 – 8.42 (m, 1H), 8.21 – 8.24 (m, 1H), 7.92 – 7.94 (m, 1H), 7.52 – 7.56 (m, 1H), 4.63 (s, 2H), 3.41

(t, *J* = 5.6 Hz, 2H), 2.87 (s, 3H), 2.32 (t, *J* = 5.6 Hz, 2H), 1.98 (q, *J* = 5.6 Hz, 2H); MS (ESI) *m/z* 378.0 $[C_{20}H_{19}N_5O_3 + H]^+$.

2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)-N-(6-phenylpyridazin-3-yl)acetamide (38). The titled compound was obtained as a yellow solid (yield, 15 %) with the general amide coupling procedure using 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.223 mmol) and 6-phenylpyridazin-3-amine (0.223 mmol). ^{1}H NMR (400 MHz, MeOH-d_4) δ 8.47–8.49 (m, 1H), 8.10 – 8.13 (m, 1H), 8.00 – 8.02 (m, 2H), 7.49 – 7.55 (m, 3H), 4.70 (s, 2H), 4.55 (s, 1H), 3.42 (t, *J* = 5.6 Hz, 2H), 2.86 (s, 3H), 2.32 (t, *J* = 5.6 Hz, 2H), 1.98 (q, *J* = 5.6 Hz, 2H); MS (ESI) *m/z* 378.2 $[C_{20}H_{19}N_5O_3 + H]^+$.

2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)-N-(6-(pyridin-3-yl)pyridazin-3-yl)acetamide (39). The titled compound was obtained as a yellow solid (yield, 52 %) with the general amide coupling procedure using 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.223 mmol) and 6-(pyridin-3-yl)pyridazin-3-amine (0.223 mmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.47 (br s, 1H), 9.27 (s, 1H), 8.68 – 8.70 (m, 1H), 8.46 – 8.49 (m, 1H), 8.31 – 8.38 (m, 2H), 7.55 – 7.59 (m, 1H), 4.67 (s, 2H), 3.31 (t, *J* = 5.6 Hz, 2H), 2.77 (s, 3H), 2.22 (t, *J* = 5.6 Hz, 2H), 1.85 (q, *J* = 5.6 Hz, 2H); MS (ESI) *m/z* 379.4 $[C_{19}H_{18}N_6O_3 + H]^+$.

2-(6-cyclopropyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)-N-(6-(pyridin-3-yl)pyridazin-3-yl)acetamide (40). The titled compound was obtained as a yellow solid (yield, 68 %)with the general amide coupling procedure using 2-(6-cyclopropyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.21 mmol) and 6-(pyridin-3-

yl)pyridazin-3-amine (0.21 mmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.47 (s, 1H), 9.27 (d, J = 1.6 Hz, 1H), 8.70-8.68 (m, 1H), 8.48-8.46 (m, 1H), 8.38-8.31 (m, 2H), 7.58-7.55 (m, 1H), 4.65 (s, 2H), 3.34 (t, J = 5.2 Hz, 2H), 2.40-2.30 (m, 1H), 2.20-2.17 (t, J = 6.0 Hz, 2H), 1.85-1.82 (m, 2H), 0.75-0.71 (m, 2H), 0.69-0.68 (m, 2H); MS (ESI) *m/z* 405.1 $[C_{21}H_{20}N_6O_3 + H]^+$.

N-(6-(5-fluoropyridin-3-yl)pyridazin-3-yl)-2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetamide (41). The titled compound was obtained as a yellow solid (yield, 16 %) with the general amide coupling procedure using 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.223 mmol) and 6-(5-fluoropyridin-3-yl)pyridazin-3-amine (0.223 mmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.51 (s, 1H), 9.18 (s, 1H), 8.71 (d, J = 2.8 Hz, 1H), 8.14 (s, 1H), 8.38 (s, 2H), 4.68 (s, 2H), 3.60-3.55 (m, 2H), 2.77 (s, 3H), 2.22 (t, J = 6.0 Hz, 2H), 1.86 (t, J = 5.6 Hz, 2H); MS (ESI) *m/z* 397.1 $[C_{19}H_{17}FN_6O_3 + H]^+$.

2-(6-cyclopropyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)-N-(6-(5-fluoropyridin-3-yl)pyridazin-3-yl)acetamide (42). The titled compound was obtained as a yellow solid (yield, 33 %) with the general amide coupling procedure using 2-(6-cyclopropyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.21 mmol) and 6-(5-fluoropyridin-3-yl)pyridazin-3-amine (0.21 mmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.50 (s, 1H), 9.18 (s, 1H), 8.71 (d, J = 2.4 Hz, 1H), 8.41-8.38 (m, 3H), 4.66 (s, 2H), 3.34 (t, J = 4.8 Hz, 2H), 2.40-2.34 (m, 1H), 2.19 (t, J = 6.0 Hz, 2H), 1.85-1.82 (m, 2H), 0.77-0.65 (m, 2H); MS (ESI) *m/z* 423.1 $[C_{21}H_{19}FN_6O_3 + H]^+$.

2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)-N-(6-(6-methylpyridin-3-yl)pyridazin-3-yl)acetamide (43). The titled compound was obtained as a

yellow solid (yield, 36 %) with the general amide coupling procedure using 2-(6-methyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.223 mmol) and 6-(6-methylpyridin-3-yl)pyridazin-3-amine (0.223 mmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.45 (s, 1H), 9.13 – 9.14 (m, 1H), 8.33 – 8.37 (m, 2H), 8.27 – 8.29 (m, 1H), 7.41 – 7.43 (m, 1H), 4.67 (s, 2H), 3.35 (t, *J* = 6.0 Hz, 2H), 2.77 (s, 3H), 2.55 (s, 3H), 2.22 (t, *J* = 6.0 Hz, 2H), 1.85 (q, *J* = 6.0 Hz, 2H); MS (ESI) *m/z* 393.1 $[C_{20}H_{20}N_6O_3 + H]^+$.

2-(6-cyclopropyl-5,7-dioxooctahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)-N-(6-(6-methylpyridin-3-yl)pyridazin-3-yl)acetamide (44). The titled compound was obtained as a yellow solid (yield, 11 %) with the general amide coupling procedure using 2-(6-cyclopropyl-5,7-dioxo-2,3,4,5,6,7-hexahydro-1H-pyrrolo[3,4-b]pyridin-1-yl)acetic acid (0.21 mmol) and 6-(6-methylpyridin-3-yl)pyridazin-3-amine (0.21 mmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.68 (s, 1H), 9.11 (d, *J* = 2.0 Hz, 1H), 8.58 (d, *J* = 9.6 Hz, 1H), 8.33 (d, *J* = 8.4 Hz, 1H), 7.91 (d, *J* = 9.2 Hz, 1H), 7.35 (d, *J* = 8.0 Hz, 1H), 4.69 (s, 2H), 3.40 (t, *J* = 5.2 Hz, 2H), 2.68 (s, 3H), 2.46-2.41 (m, 1H), 2.37 (t, *J* = 6.0 Hz, 2H), 2.02-1.96 (m, 2H), 0.88-0.82 (m, 4H); MS (ESI) *m/z* 421.1 $[C_{22}H_{24}N_6O_3 + H]^+$.

Cell-basedAssays. The STF3A reporter assay, STF/Wnt3A conditioned medium assay and PORCN overexpression assay were described in Duraiswamy et al.[18]

In Vivo studies. All procedures used for animals studies including in vivo antitumor efficacy studies ad in vivo pharmacokinetic studies were approved by the BRC-IACUC committee (approval no. 110622) in Singapore. Female Balb/C nude mice of approximately 8-10 weeks old were purchased from Biolasco, Taiwan. CD-1 female mice were purchased from InVivos, Singapore. Food and water were given ad libitum.

In Vivo Efficacy studies. The primary mammary tumors (MMTV-Wnt1 #947-R#5) provided by DUKE-NUS (Dr David Virshup laboratory), Singapore, were passaged in the female Ncr nude mice prior to implantation. The tumor fragment utilized in this experiment originated from working tumor bank #947 R75. Each test mouse received a 3 mm^3 MMTV-Wnt1 fragment implanted in the mammary fat pad number 4 (MFP4). The growth of tumors was monitored using calipers and the tumor volume was calculated using the formula:

Tumor volume (mm^3) = (w^2 x l)/2

Where w = width and l = length in mm. 17 days after tumor implantation (designated as day 0 of the study), mice bearing established orthotopic MMTV-Wnt1 #947-R#5 tumors with tumors volumes between 63 to 172 mm^3, were selected and randomly distributed into 5 groups. Each group contained 8 mice. The group mean tumor volume ranged from 84 to 96 mm^3. After randomization, mice bearing established orthotopic MMTV-Wnt1 #947-R#5 tumors received vehicle (50% polyethylene glycol (PEG) 400 in deionised water), **2** or compound **40** once daily for 14 days (qd x 14) via oral gavage. The volume of oral administration was 10 ml/kg. The dosing level for **2** was 3 mg/kg and the dosing levels for compound **40** were 1, 3 and 10 mg/kg. The endpoint used to measure the response of the MMTV-Wnt1 #947-R#5 tumors after compound treatment was tumor growth inhibition at day 14 and was expressed as %TGI. The percentage of tumor growth inhibition (%TGI) was calculated as follows:

$$\%TGI = (C_{day\ a} - T_{day\ a})/ (C_{day\ a} - C_{day0}) \times 100$$

Where $C_{day\ a}$ = mean tumor volume of the vehicle control group at the indicated day a; $T_{day\ a}$ = mean tumor volume of the group treated with the test compound at the indicated day a; C_{day0}= mean tumor of the vehicle control group at day 0. GraphPad Prism version 5.03

(www.graphpad.com) was used for statistical analysis and graphic presentations (Figure 6). One-way ANOVA followed by Dunnett's Multiple Comparison Test was used to determine statistically significant differences between the tumor volumes of the vehicle control group and the tumor volumes of the group treated with compound **40** or **2**.

In Vivo PK studies. CD-1 female mice (6 - 8 weeks old) were weighed and those animals of 26 ± 3 g were selected for dosing. Three mice were randomly grouped per time point. Mice were administered a single dose of 1 mg/kg compound via tail vein injection or a single dose of 5 mg/kg compound via oral gavage. The volume of injection for intravenous and oral administration was 4 ml/kg and 8 ml/kg respectively. Mice were sacrificed using CO_2 gas at the predefined time points (pre-dose, post-dose at 5 or 10 min and 30 min, 1, 2, 4, 8, 16 and 24 h). Blood samples were taken from euthanized mice by cardiac puncture and placed into tubes containing K_3EDTA. Plasma samples were obtained by centrifugation of the blood samples at 15,700 × g, 4^{o}C, for 2 minutes, and were stored at -80^{o}Cuntil analysis. Standard curves of compound were prepared in blank mouse plasma matrices at the concentration ranging from 1 to 1000ng/ml. 10µl of the internal standard, carbamazepine (50ng/ml in the mixture of 50% acetronitrile and 0.1% formic acid) and 250µl of the extract solvent (70% acetronitrile and 0.1% formic acid in milli Q water) were added into 50µl of thestandards and the PK plasma samples. After extraction, the samples were centrifuged at 4500g for 20 min in a refrigerated centrifuge (Eppendorf 5415R) at 4°C. 80µl of the supernatants were transferred to a fresh 96 well-plate. Samples were analysed using the RRLC1200SL unit (Agilent Technologies, Singapore) coupled with a triple stage quadrupole MS unit (Thermo Quantum Ultra TripleQuadrupole, Thermo Scientific, Singapore). The samples were resolved on a Kinetex C18 column (50 x 2.1mm, Phenominex, USA) maintained at 25°C. The mobile phase A consisted of 0.1% formic acid in

deionised water and the mobile phase B consisted of acetonitrile. The gradient program was 5% B to 95% B from 0.0 to 0.6 min, maintaining 95% B from 0.6 min to 1.4 min, then switched back to 5% B from 1.45 to 2.5 min, with a constant flow rate of 0.4ml/min. The injection volume was 2µl. The total eluent from the liquid chromatography was injected directly into the Thermo Quantum Ultra TripleQuadrupole mass spectrometer for analysis. MS/MS detection was operated in electrospray ionization positive ion mode. The spray voltage was 4.0 KV and the vaporization temperature was set to 400°C. The capillary temperature was set at 350°C. The sheath gas pressure and the auxiliary gas pressure were set at 60 and 20 (arbitrary units), respectively. Quantitation was carried out using the multiple reaction monitoring (MRM) of the transitions. The MS parameters for compound **40** - MRM were *m/z* 405.09→*m/z* 205.1; collision energy, 24V; tube lens, 94.4. The MS parameters for carbamazepine - MRM were *m/z* 237.1→*m/z* 194.1;collision energy, 21 V; tube lens, 95.

Pharmacokinetic Analysis. Pharmacokinetic parameters, e.g. the elimination half-life ($T_{1/2}$), the systemic clearance (CL), area under the plasma concentration-time curve up to the last nonzero concentration (AUC0-t), area under the plasma concentration-time curve from time 0 to infinity (AUC0-inf), the time of maximum concentration in plasma (T_{max}), the maximum concentration in plasma (C_{max}) were calculated by the non-compartmental method using Phoenix WinNonlin 6.0 software (www.certara.com). The absolute oral bioavailability (F) was calculated using the following equation:

$$\%F = (AUC_{0\text{-inf, po}} / AUC_{0\text{-inf, iv}})*(dose, iv / dose, po)*(100).$$

Molecular Modeling. Detailed energy calculations, superimposition studies and physical properties calculations are described in Poulsen et al.[21] The conformational energies given in

Tables 1 and 2 are obtained using the procedure from Poulsen et al. Except that the newer OPLS3 force field was used. The final compounds were screened with a PAINS filter as build into Canvas version 3.2 (www.schrodinger.com) and all compounds passed.

AUTHOR INFORMATION

Corresponding Author

*Tel: +65 6478 8767. Fax: +65 6478 8768. E-mail: apoulsen@etc.a-star.edu.sg.

*Tel: +65 6478 0670. Fax: +65 6478 8768. E-mail: syho@etc.a-star.edu.sg.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

Any funds used to support the research of the manuscript should be placed here (per journal style).

Notes

All authors are current or former employees of the Experimental Therapeutics Centre or Duke-NUS Graduate Medical School Singapore. Both institutes have a commercial interest in the development of WNT secretion inhibitors.

ACKNOWLEDGMENT

We acknowledge the technical assistance provided by members of Experimental Therapeutics Centre including Jeff Hill, Shermaine Q Y Lim and Sifang Wang; Justina Fulwood and Horst

Flotow for performing the High Throughput Screening of our compound library. This work was supported in part by the Singapore Translational Research Investigator Award (D.M. Virshup), funded by the National Research Foundation and the National Medical Research Council of Singapore.

ABBREVIATIONS

Cyp, cytochrome P450 enzyme; ER, endoplasmic reticulum; Fzd, Frizzled; HLM, human liver microsomes; HTS, high throughput screening; MLM, mouse liver microsomes; MMTV, mouse mammary tumor virus; PK, pharmacokinetics; PSA, polar surface area; RB, rotatable bonds; RMSD, root mean square deviation; r.t, room temperature; SAR, structure activity relationships.

ASSOCIATED CONTENT

Supporting Information. Figure S1, Table S1, Smiles strings and activity data. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Logan, C. Y.; Nusse, R., The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **2004,** *20*, 781-810.

2. van Amerongen, R.; Nusse, R., Towards an integrated view of Wnt signaling in development. *Development* **2009,** *136* (19), 3205-3214.

3. Montcouquiol, M.; Crenshaw, E. B., 3rd; Kelley, M. W., Noncanonical Wnt signaling and neural polarity. *Annu. Rev. Neurosci.* **2006,** *29*, 363-386.

4. Ouyang, H.; Zhuo, Y.; Zhang, K., WNT signaling in stem cell differentiation and tumor formation. *J. Clin Invest.* **2013,** *123* (4), 1422-1424.

5. Pecina-Slaus, N., Wnt signal transduction pathway and apoptosis: a review. *Cancer Cell Int.* **2010,** *10*, 22-26.

6. Porfiri, E.; Rubinfeld, B.; Albert, I.; Hovanes, K.; Waterman, M.; Polakis, P., Induction of a beta-catenin-LEF-1 complex by wnt-1 and transforming mutants of beta-catenin. *Oncogene* **1997,** *15* (23), 2833-2839.

7. de La Coste, A.; Romagnolo, B.; Billuart, P.; Renard, C. A.; Buendia, M. A.; Soubrane, O.; Fabre, M.; Chelly, J.; Beldjord, C.; Kahn, A.; Perret, C., Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc. Natl. Acad. Sci. U.S.A.* **1998,** *95* (15), 8847-8851.

8. Dees, C.; Distler, J. H., Canonical Wnt signalling as a key regulator of fibrogenesis - implications for targeted therapies? *Exp. Dermatol.* **2013,** *22* (11), 710-713.

9. Chilosi, M.; Poletti, V.; Zamo, A.; Lestani, M.; Montagna, L.; Piccoli, P.; Pedron, S.; Bertaso, M.; Scarpa, A.; Murer, B.; Cancellieri, A.; Maestro, R.; Semenzato, G.; Doglioni, C., Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. *Am. J. Pathol.* **2003,** *162* (5), 1495-1502.

10. Boyden, L. M.; Mao, J.; Belsky, J.; Mitzner, L.; Farhi, A.; Mitnick, M. A.; Wu, D.; Insogna, K.; Lifton, R. P., High bone density due to a mutation in LDL-receptor-related protein 5. *N. Engl. J. Med.* **2002,** *346* (20), 1513-1521.

11. Kahn, M., Can we safely target the Wnt pathway? *Nat. Rev. Drug Discovery.* **2014,** *13* (7), 513-352.

12. Madan, B.; Virshup, D. M., Targeting Wnts at the source--new mechanisms, new biomarkers, new drugs. *Mol. Cancer Ther.* **2015,** *14* (5), 1087-1094.

13. Ho, S. Y.; Keller, T. H., The use of porcupine inhibitors to target Wnt-driven cancers. *Bioorg. Med. Chem. Lett.* **2015,** *25* (23), 5472-5476.

14. Ho, S. Y.; Blanchard, S. E.; Duraiswamy, A. J.; Alam, J.; Adsool, V. A. 19. Ho, S. Y.; Blanchard, S. E.; Duraiswamy, A. J.; Alam, J.; Adsool, V. A., Purine diones as Wnt pathway modulators. *WO2014189466*, **2014**.

15. Cheng, D.; Zhang, G.; Han, D.; Gao, W.; Pan, S., N-(hetero)aryl, 2-(hetero)aryl-substituted acetamides for use as wnt signaling modulators. *WO2010101849*, **2010**.

16. Masumoto, N.; Lanyon-Hogg, T.; Rodgers, U. R.; Konitsiotis, A. D.; Magee, A. I.; Tate, E. W., Membrane bound O-acyltransferases and their inhibitors. *Biochem. Soc. Trans.* **2015,** *43* (2), 246-252.

17. MacDonald, B. T.; Tamai, K.; He, X., Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev. Cell* **2009,** *17* (1), 9-26.

18. Duraiswamy, A. J.; Lee, M. A.; Madan, B.; Ang, S. H.; Tan, E. S.; Cheong, W. W.; Ke, Z.; Pendharkar, V.; Ding, L. J.; Chew, Y. S.; Manoharan, V.; Sangthongpitag, K.; Alam, J.; Poulsen, A.; Ho, S. Y.; Virshup, D. M.; Keller, T. H., Discovery and optimization of a porcupine inhibitor. *J. Med. Chem.* **2015,** *58* (15), 5889-5899.

19. Yap, M. C.; Kostiuk, M. A.; Martin, D. D.; Perinpanayagam, M. A.; Hak, P. G.; Siddam, A.; Majjigapu, J. R.; Rajaiah, G.; Keller, B. O.; Prescher, J. A.; Wu, P.; Bertozzi, C. R.; Falck, J.

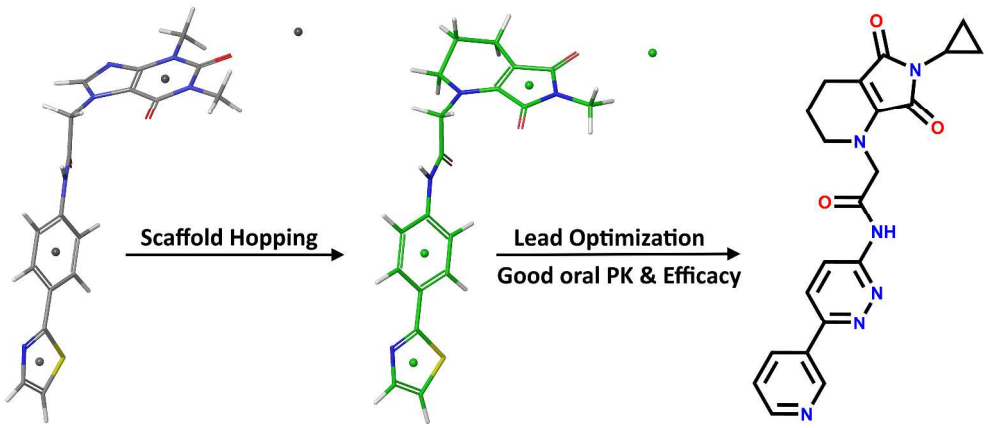
R.; Berthiaume, L. G., Rapid and selective detection of fatty acylated proteins using omega-alkynyl-fatty acids and click chemistry. *J. Lipid Res.* **2010,** *51* (6), 1566-1580.

20. Madan, B.; Ke, Z.; Harmston, N.; Ho, S. Y.; Frois, A. O.; Alam, J.; Jeyaraj, D. A.; Pendharkar, V.; Ghosh, K.; Virshup, I. H.; Manoharan, V.; Ong, E. H.; Sangthongpitag, K.; Hill, J.; Petretto, E.; Keller, T. H.; Lee, M. A.; Matter, A.; Virshup, D. M., Wnt addiction of genetically defined cancers reversed by PORCN inhibition. *Oncogene* **2016,** *35* (17), 2197-2207.

21. Poulsen, A.; Ho, S. Y.; Wang, W.; Alam, J.; Jeyaraj, D. A.; Ang, S. H.; Tan, E. S.; Lin, G. R.; Cheong, V. W.; Ke, Z.; Lee, M. A.; Keller, T. H., Pharmacophore model for Wnt/porcupine inhibitors and its use for drug design. *J. Chem. Inf. Model.* **2015**, *55*, 1435-1448.

22. Chen, B.; Dodge, M. E.; Tang, W.; Lu, J.; Ma, Z.; Fan, C. W.; Wei, S.; Hao, W.; Kilgore, J.; Williams, N. S.; Roth, M. G.; Amatruda, J. F.; Chen, C.; Lum, L., Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat. Chem. Biol.* **2009,** *5* (2), 100-107.

23. Proffitt, K. D.; Madan, B.; Ke, Z.; Pendharkar, V.; Ding, L.; Lee, M. A.; Hannoush, R. N.; Virshup, D. M., Pharmacological inhibition of the Wnt acyltransferase PORCN prevents growth of Wnt-driven mammary cancer. *Cancer Res.* **2013,** *73* (2), 502-507.



Graphical Abstract

1572x671mm (92 x 91 DPI)