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Novel thienopyrimidine and thiazolopyrimidine kinase inhibitors with activity against Tie-2 in vitro and in vivo

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ABSTRACT

The SAR and improvement in potency against Tie2 of novel thienopyrimidine and thiazolopyrimidine kinase inhibitors are reported. The crystal structure of one of these compounds bound to the Tie-2 kinase domain is consistent with the SAR. These compounds have moderate potency in cellular assays of Tie-2 inhibition, good physical properties, DMPK, and show evidence of in vivo inhibition of Tie-2.

There is an ongoing need for novel approaches to treating human tumours and one of the most promising new approaches is blocking angiogenesis.[1] Tumours require a blood supply to grow to a significant size and it is believed that blocking specific angiogenic factors will prevent the development of this vascular network. The endothelial cell growth factor VEGF is a potent stimulus for angiogenesis and recent clinical trials have shown efficacy of the antibody drug bevacizumab[2] which binds to VEGF. There is considerable work underway directed towards other angiogenesis inhibitors and VEGFr inhibitors are the most advanced kinase inhibitors in this field. Of the other kinases thought to be involved in angiogenesis in human tumours, Tie-2, an endothelium specific receptor tyrosine kinase, promotes tumour angiogenesis through interaction with angiopoietin[3] and plays an important role in stabilizing the immature endothelial cell network, attracting pericytes and maintaining vessel integrity.[4] Although the details of Tie receptor biology are still emerging[5] it has attracted considerable interest, although to date only a small number of inhibitors have been reported outside of patent literature.[6]

We wish to report here work leading to the identification of bicyclic imidazoles as a novel class of kinase inhibitors which inhibit Tie-2 in vitro, in cells and in vivo.

We recently described[7] the SAR developed around an imidazole vinyl pyrimidine HTS hit leading to **1** which had good potency against Tie-2 in cells, lead-like properties and oral bioavailability. However, these compounds have poor photostability and react with glutathione so we sought more stable analogues. As both of these stability issues appeared to be associated with the alkene linker we attempted to replace this with other groups but this led to loss of potency or no improvement in glutathione reactivity.[7] In the work described here we explored conceptually fusing the alkene with the pyrimidine ring to give bicyclic compounds (Fig. 1). The directly fused compounds **2** were not easily accessible synthetically but the analogues **3**, where one of the pyrimidine nitrogens had been moved, were. This re-positioning of the pyrimidine nitrogen was well tolerated in the imidazole vinyl pyrimidine series.[7]

A range of different bicyclic pyrimidines was synthesized but the thienopyrimidine and thiazolopyrimidine were most potent. The thienopyrimidine **7** was synthesized by reaction of the imine derived from thienopyrimidine **4**[8] with phenyl-TosMIC reagent **8** and then oxidation of the SMe of **6** with MCPBA and displacement with ammonia (Scheme 1). Full synthetic details for the compounds reported in this paper have been described elsewhere.[8] Thiazolopyrimidine **12** was prepared from phenyl-TosMIC reagent **8** by formation of alcohol **9** and then by oxidation and conversion to the acid chloride **10**, followed by amide coupling to give **11** and

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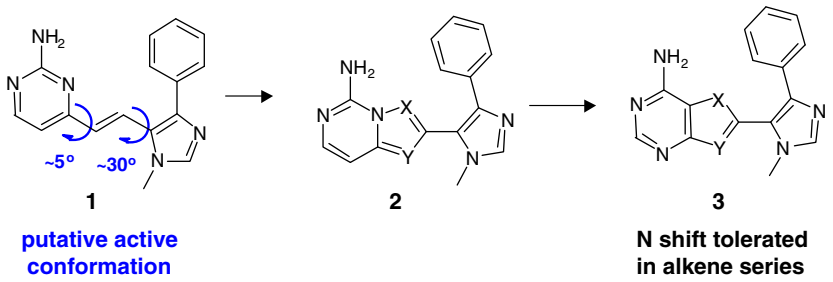
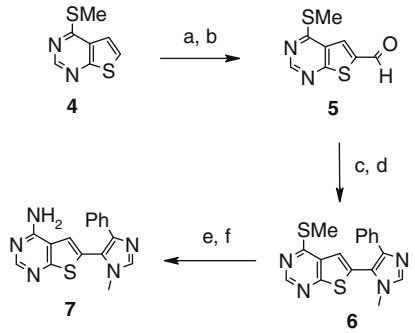


Figure 1. Design of bicyclic Tie-2 inhibitors.



Scheme 1. Thienopyrimidine synthesis. Reagents and conditions: (a) LDA, THF; (b) DMF, 51%; (c) 4 Å mol. sieves, NH_2Me, DCM, reflux, 100%; (d) PhTosMIC, piperazine, THF, 90%; (e) *m*CPBA, DCM, 56%; (f) H_3N, dioxane, 100%.

Table 1
Thienopyrimidine and thiazolopyrimidine overall properties.

Property	Thienopyrimidine **7**	Thiazolopyrimidine **12**
pTie-2 cell IC_{50}[a] (μM)	1.8	2.7
Tie-2 enzyme IC_{50}[a] (μM)	34	20
Flt-1, KDR enzyme IC_{50}[a] (μM)	10, 22	84, 4.4
MW, log *D*	307, n.d.	308, 2.7
Solubility at pH7 (μM)	84	160
Plasma protein binding (rat% bound)	95.5	94.8
PK (rat): *F* (%), $t_{1/2}$ (h), Vdss (L/kg), Cl (mL/min/kg)[b]	>100, 1.8 [c], 1.4, 39	>100, 1.6 [c], 1.2, 30.1

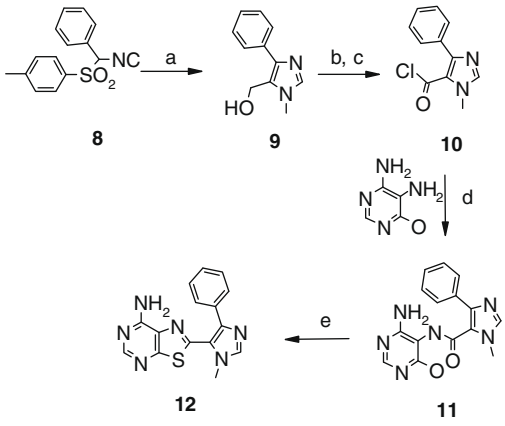
n.d.: not determined.
[a] Values are geometric means of two or more experiments with a standard deviation of < ±0.3 log units.
[b] Female rats dosed at 2 mg/kg iv and 5 mg/kg po.
[c] Half life after oral dosing.

incorporation of sulphur and cyclization with phosphorus pentasulphide to form **12** (Scheme 2).

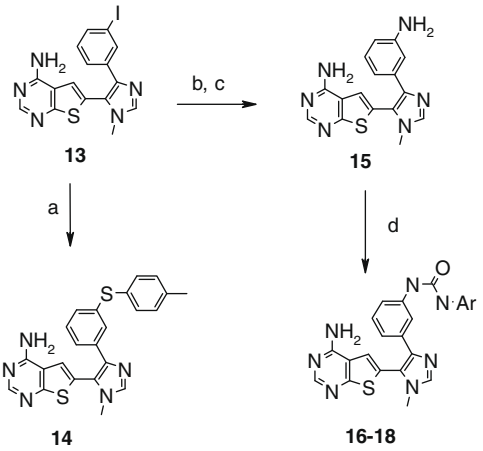
The thienopyrimidine **7** and thiazolopyrimidine **12** showed no reactivity with glutathione and were photostable (Hanau sun test). These compounds had an attractive overall balance of properties (Table 1). In a phospho Tie-2 cell assay[9] they were reasonably potent and selective. In contrast to their Tie-2 cell potency they were less potent in Tie-2 enzyme assay which we believe is due to insensitivity of this assay (assay conditions and further discussion on the sensitivity of the enzyme assay is contained in our previous paper[7]). They have good physical properties and good pharmacokinetics in the rat.

Our next aim was to improve the potency of these leads. We anticipated that the phenyl ring would be close to, but not in, the kinase selectivity pocket so we prepared analogues of **7** and **12** with substitution on this ring to try to position favourable groups in this pocket. Synthesis of the iodophenyl intermediate **13** (Scheme 3) was by the same route as used in Scheme 1. This intermediate was elaborated to the thiotoluene **14** and aniline **15**. Aniline **15** reacted with various isocyanates to give the aryl ureas **16–18**.

Generally the SAR was rather flat in this area with substitution by relatively large groups such as the thiotoluene **14** and the phenyl urea **16** being tolerated but not beneficial (Table 2). Phenyl ureas with lipophilic *meta* or *para* substituents such as **17**, however, gave a significant improvement in potency. Unfortunately, the

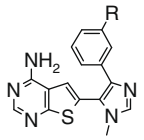


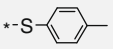
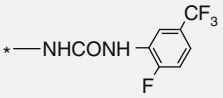
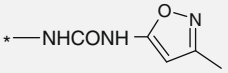
Scheme 2. Thiazolopyrimidine synthesis. Reagents and conditions: (a) $MeNH_2$, $(CHO–CHO)_2$, 80%; (b) $KMnO_4$, K_2CO_3, Me_2CO, H_2O; (c) $(COCl)_2$, DCM, DMF, 60%; (d) NMP, 24%; (e) P_2S_5, pyridine, 110 °C, 19%.



Scheme 3. Synthesis of phenyl substituted thienopyrimidines. Reagents and conditions: (a) NaSTol, CuI, 110 °C, DMF, 20%; (b) benzophenone imine, (Ph_2P)ferrocene, $(acac)_2Pd$, NaOtBu, 90 °C, dioxane; (c) 2 M HCl, THF, 50%; (d) Ar-NCO, THF, 67–90%.

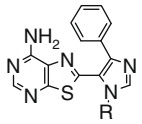
Table 2
Phenyl ring substitution

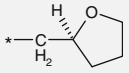
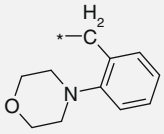


Compd	R	pTie-2 cells IC_{50}[a] (μM)
7	H	1.8
14		1.4
16		1.4
17		0.07
18		0.34

[a] Values are geometric means of two or more experiments with a standard deviation of <±0.3 log units.

Table 3
N1-imidazole substitution



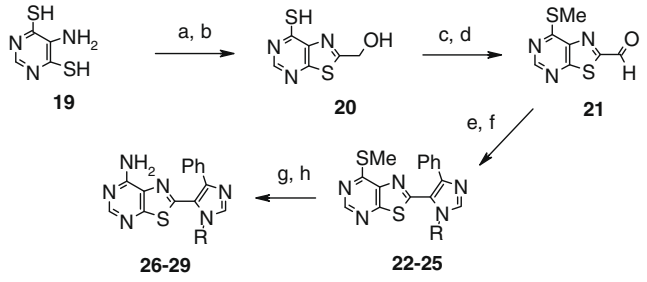
Compd	R	pTie-2 cells IC_{50}[a] (μM)	Solubility (μM)	Protein binding rat (% bound)
12	Methyl	2.7	160	94.6
26	Ethyl	3.0	171	n.d.
27	$-CH_2-$cyclohexyl	0.54	10	98.4
28	–Isobutyl	0.49	70	94.1
29		0.37	682	80
30		0.44	0.9	94.2

n.d.: not determined.
[a] Values are geometric means of two or more experiments with a standard deviation of <±0.3 log units.

physical properties of **17** were poor: aqueous solubility 0.5 μM and rat plasma protein binding 99.97%. By reducing the lipophilicity of the phenyl ring or its substituents the physical properties could be improved, but this also led to reduced potency. One of the best compromises between potency and properties was **18**, which had solubility of 7.2 μM and rat plasma protein binding of 99.85%.

We next explored substitution on the N1 of the imidazole. To provide an efficient route we first prepared the aldehyde **21** from the amino-dithiopyrimidine **19** by cyclization to form the thiazole ring, followed by methylation and oxidation (Scheme 4). The imines formed from aldehyde **21** and amines reacted with phenyl-TosMIC reagent to give the imidazoles **22–25**. Oxidation of the thiomethyl group and then displacement with ammonia led to compounds **26–29**.

In the N1 position, small substituents such as ethyl **26** had little effect on potency (Table 3). Larger lipophilic groups were beneficial such as cyclohexylmethyl **27**, isobutyl **28**, methyl-THF **29**, and, benzyl morpholine **30**. The methyl-THF compound **29** was particularly interesting as it retained potency and had good solubility and free drug levels in rat plasma. The cyclohexylmethyl compound **27** was crystallized with Tie-2 kinase domain[10] (Fig. 2). This confirmed the expected binding mode with the pyrimidine forming two H-bonds to the hinge region of the kinase and the cyclohexyl group in the ribose binding pocket. As expected the phenyl ring is just outside the selectivity pocket.



Scheme 4. Synthesis of N1-imidazole substituted thiazolopyrimidines. Reagents and conditions: (a) acetoxyacetyl chloride, pyridine; (b) 2 N HCl, reflux, 97%; (c) Me–I, 2 N NaOH, 53%; (d) MnO_2, 100 °C, dioxane, 91%; (e) RNH_2, THF; (f) PhTosMIC, morpholine, ~58%; (g) *m*CPBA, DCM; (h) NH_3, 25–64%,

With improvements in Tie-2 cell potency in hand we investigated the DMPK properties of these new leads. The isoxazole urea **18** gave very low levels on dosing orally in the rat (Cmax 0.009 μM from a 1 mg/kg dose). Compound **30** had no oral bioavailability in the rat but was evaluated using iv dosing in a haemodynamic model[12] for Tie-2 activity. This assay, in the anaesthetized rat, tests the ability of compounds to reverse kinase signalling-induced hypotension following stimulation by vasoactive ligands such as VEGF and Ang-1. The methodology for this model has previously been described for a similar VEGF response[13] and has been shown to predict the haemodynamic outcome in conscious animals to dosing by oral gavage.[14] Dosing **30** at 20 mg/kg iv produced complete reversal of Ang-1 induced hypotension but, as expected, did not re-

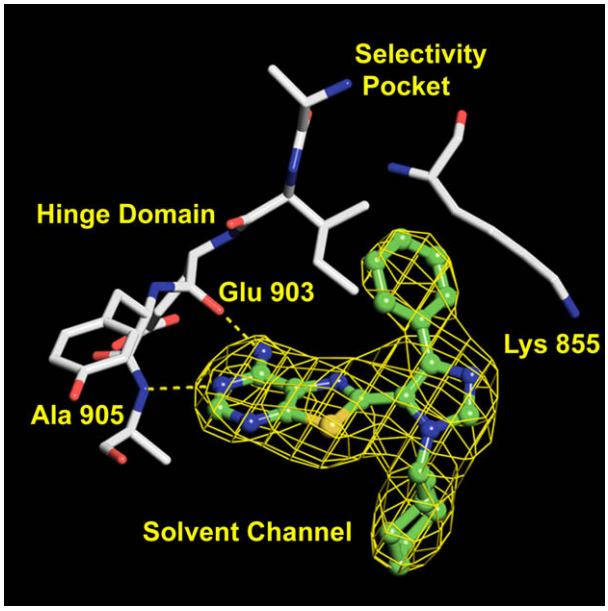


Figure 2. Crystal structure[10] of human Tie2 kinase domain complexed with **27** showing final $2F_o - F_c$ electron density (yellow, 1.0σ level). Selected nearby protein residues are shown. Hydrogen bonding interactions with the protein are indicated as dashed yellow lines. The figure was prepared using PyMol.[11]

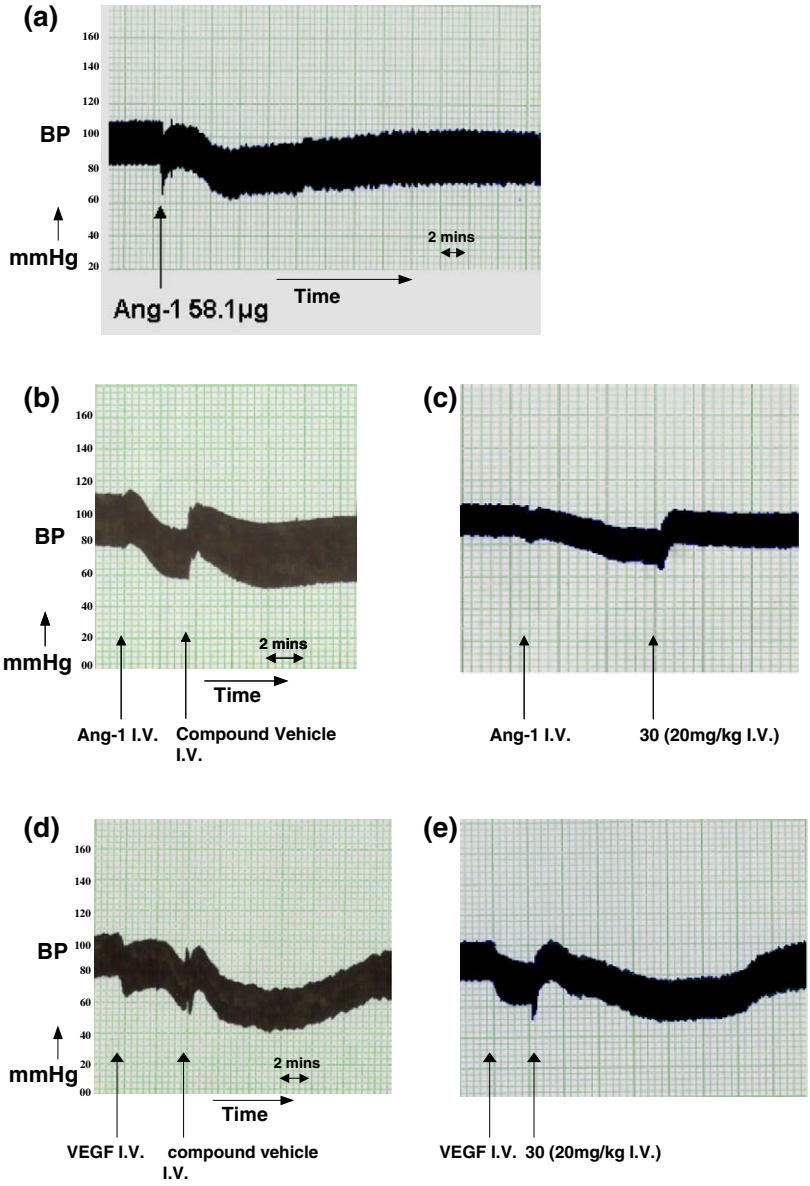


Figure 3. Hypotension assay[12] results for compound **30**. (a) Ang-1 (40–60 µg) induced a fall in blood pressure (BP) (upper line is systolic pressure, lower line is diastolic pressure); (b) compound vehicle was administered, showing an injection spike, but did not reverse the Ang-1 induced fall in blood pressure; (c) compound 30 reversed the Ang-1 induced fall in blood pressure; (d) VEGF (10 µg) induced a fall in blood pressure; (e) compound 30 did not reverse the VEGF induced fall in blood pressure.

verse VEGF induced hypotension (Fig. 3). These initial results are consistent with **30** selectively inhibiting Tie-2 activity and demonstrate that a methodology is available for assessing the in vivo efficacy and selectivity of other targeted compounds. However as the most potent compounds such as **30** were not orally bioavailable, and so were not considered drug candidates, we did not test them in further in vivo angiogenesis or tumour xenograft models but developed a new series of orally bioavailable compounds which will be reported in a further paper (in preparation).

In conclusion we have shown that the attractive, moderately potent, but unstable alkene imidazoles can be converted to bicyclic thieno- and thiazolopyrimidines with retention of potency and overall properties but with improved stability. The potency of these bicyclic compounds was increased by substituting on the N1 of the imidazole or the phenyl ring although this led to loss of oral bioavailability. The benzyl morpholine **30** was shown to reverse Tie-2 mediated hypotension in vivo following iv administration.

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9. The phospo Tie-2 cell assay used in this work has been described previously.[7] In the development of this assay we used an anti-Tie-2 antibody to show that, unlike phospho-Tie-2, total Tie-2 levels were not reduced by a diverse set of Tie-2 inhibitors.
10. *Protein and crystals were obtained as follows*: A baculovirus directing expression of 6His-TEV-Tie2(802-1124)D964 N was generated using the Bac-to-Bac method from Invitrogen. Protein was expressed in Sf9 insect cells infected with high titre baculovirus at an MOI of 2, and cultured in SF900II media (Invitrogen) for 48 h before harvesting by centrifugation and storage of the cell pellets at −80 °C. Protein was purified by immobilized metal affinity and size exclusion chromatography: frozen cell pellets were lysed by sonication in buffer A (50 mM Tris pH8, 300 mM NaCl, 5 mM DTT, containing Roche Complete EDTA-free protease inhibitor tablets), the lysate clarified by centrifugation for 30 min at 48,000*g*, and the lysate supernatant stirred overnight at 4 °C with Ni-NTA beads equilibrated with buffer A. The beads were loaded into a column, washed with buffer A, then buffer A containing 50 mM imidazole, and bound proteins eluted with buffer A containing 500 mM imidazole. The N-terminal His-tag was cleaved with rTEV protease, and the tag removed by subtractive NiNTA chromatography. Pooled fractions containing 6His-TEV-Tie2(802-1124)D964 N were concentrated using a YM30 membrane before loading on a Supderdex75 sizing column pre-equilibrated with buffer B (20 mM HEPES pH 7.5, 300 mM NaCl, 5 mM DTT). Co-crystals with compound **27** were grown by incubating purified protein at 5 mg/ml with 1 mM compound (1% DMSO) on ice for 30 min, and then setting up a hanging-drop vapour diffusion experiment at 293 K using a reservoir of 5% (w/v) PEG6000, 5% (v/v) MPD and 100 mM MOPS pH 7.5. The drop was composed of 5 μL protein + compound, 5 μL reservoir and 1 μL additive (1 M glycine from Hampton Research Additive Screen). Crystals were cryoprotected with 2,3-butanediol (17% v/v) in mother liquor and flash cooled in a cryostream at 100 K. Diffraction data were collected on beamline PX14.2 at the SRS, Daresbury, at 100 K. Data processing, data reduction and structure solution by molecular replacement were carried out using programs from the CCP4 suite.[15] Compound **27** was modelled into the electron density using QUANTA.[16] The protein-compound complex model was refined using CNX,[17] Refmac[18] and Buster[19] with intermediate rounds of model building in QUANTA[16] and Coot.[20] The final structure[21] has been deposited in the Protein Data Bank (2wqb) together with structure factors and detailed experimental conditions.
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12. *Hypotension assay*: Angiopoietin-1 (Ang-1) activates the tyrosine kinase receptor, Tie2 and this has been shown to influence the tone of blood vessels, inducing dilation and leading to a hypotensive response. Full experimental details for the procedure using VEGF are given in a previous publication[13] and the same methodology was used for the Ang-1 response. Ang-1 was expressed in insect cells using a baculovirus system in the same way[13] as for VEGF. Briefly, anesthesia was induced in male Alderley Park rats using α-chloralose (iv) and then maintained with thiopentone (ip). The carotid artery was cannulated to enable blood pressure recording using a pressure transducer. The jugular vein was cannulated to allow VEGF or Ang-1 administration as a bolus injection in 0.85% sodium chloride. To give the most comparable data the dose of Ang-1 used was adjusted to induce a consistent blood pressure fall of between 10 and 20 mmHg to control for slight differences in the level of functional Ang-1 across batches (40–60 μg of Ang-1 depending on the batch). Compound 30 or vehicle alone [25% (w/v) hydroxypropyl-β-cyclodextrin in Sorensons phosphate buffer (pH 5.5)] was administered iv and blood pressure recorded. Consistent effects on blood pressure were seen in repeat experiments.
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21. *Crystallographic statistics for the Tie2 K-compound **27** complex are as follows:* Space group $C222_1$, unit cell 80.1, 108.8, 101.6 Å, resolution 2.95 Å, 9451 reflections from 49,960 observations give 96.0% completeness with R_{merge} of 10% and mean $I/\sigma(I)$ of 3.4. The final model containing 2286 protein, 20 water, and 28 compound atoms has an *R*-factor of 19.5% (R_{free} using 5% of the data 25.8%). Mean temperature factors for the protein and the ligand are 42 and 46 $Å^2$, respectively.