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Discovery of imidazole vinyl pyrimidines as a novel class of kinase inhibitors which inhibit Tie-2 and are orally bioavailable

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ABSTRACT

Tie-2 is a receptor tyrosine kinase which is involved in angiogenesis and thereby growth of human tumours. The discovery and SAR of a novel class of imidazole-vinyl-pyrimidine kinase inhibitors, which inhibit Tie-2 in vitro is reported. Their synthesis was carried out by condensation of imidazole aldehydes with methyl pyrimidines. These compounds are lead-like, with low molecular weight, good physical properties and oral bioavailability.

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 blood supply to grow to a significant size and it is believed that blocking angiogenesis 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 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 the angiopoietins[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-2 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 our work leading to the identification of imidazole vinyl pyrimidines as a novel class of kinase inhibitors which inhibit Tie-2 in vitro.

A high-throughput screen (HTS) of the AstraZeneca compound collection identified a singleton hit, **1**, an ATP-competitive Tie-2 kinase inhibitor which inhibited the enzyme with an IC_{50} of 6 μM.[7] Tie-2 inhibitors need to be cell permeable to reach the intracellular target, therefore a phospho-Tie-2 cell-based ELISA assay[8] was utilized to test the compound's cellular potency. In this assay, compound **1** was active with an IC_{50} of 0.33 μM. This pattern of greater potency in the cell assay was common to most of the hits from the HTS, covering more than 20 distinct chemical series, and we suspect that it reflects a lack of sensitivity in the enzyme assay.[9] Compound **1** showed reasonable selectivity in a large panel of kinase assays, only inhibiting three other enzymes with an IC_{50} of less than 10 μM (p38, 1.6 μM; Flt-4, 5.5 μM; KDR, 7.0 μM). In contrast to Tie-2 the compound was inactive in VEGFr and p38 cellular assays suggestive of a greater drop in potency on going from enzyme to cell assays for these kinases. Compound **1** showed good oral exposure in a mouse cassette dosing experiment (C_{max} = 0.29 μM following a 1 mg/kg dose) and has a low molecular weight (319) and *C*log*P* (2.8). The novelty of the core structure as well as its good selectivity profile and lead-like properties motivated us to initiate hit to lead chemistry to explore the activity of this class of compounds as Tie-2 inhibitors.

We were aware of structural similarities of **1** and the imidazopyrimidine class of p38 inhibitors (e.g., **2**, SB203580),[10] but these compounds were not active in our Tie-2 assays, which presumably reflects different structural requirements for inhibition of Tie-2 and p38. Our initial assumption, based on docking in a Tie-2 homology model (Fig. 1), was that the pyrimidine N1 and NH2 were likely to form a hydrogen bond pair with the hinge domain of Tie-2 and that the phenyl ring would be directed towards the selectivity pocket. We wanted to test these assumptions and

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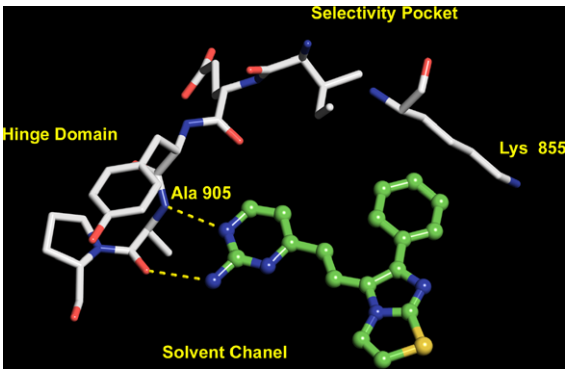
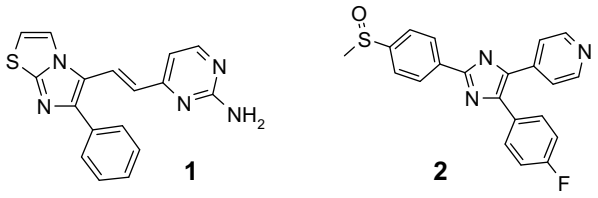


Figure 1. Proposed binding mode for compounds to Tie-2.

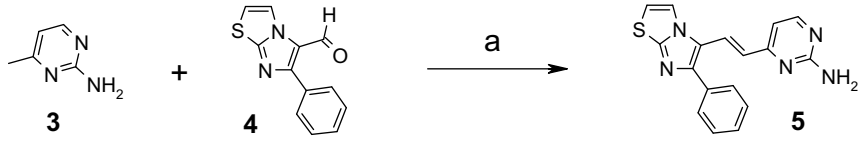
explore the importance of other structural features such as the imidazothiazole ring and the alkene linker.



A range of imidazole vinyl pyrimidines were synthesized according to the sequence outlined in Scheme 1. [11] Treatment of the methyl pyrimidine **3** with the commercially available aldehyde **4** under either basic or acidic conditions gave the imidazole vinyl pyrimidine **5**. In some cases, additional functionality was introduced using known chemical transformations (vide infra). The compounds were difficult to separate into the geometric isomers but were predominantly assigned as the E-isomers by ^{1}H NMR studies.

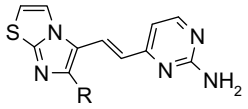
First, we examined the role of the phenyl ring by replacing it with Me or Cl. In both cases a significant drop in potency was observed. However, simple substituents such as *para*-fluoro were well tolerated (Table 1). These results are consistent with the proposed binding mode. Next, we modified the amino substituent of the pyrimidine ring. Small substituents such as NHMe or NHAc are well tolerated, but larger substituents or groups lacking an NH donor were not (Table 2). Results for isomers of the pyrimidine showed good potency for the two isomers with a nitrogen para to the point of attachment of the alkene linker but much reduced activity for the compound without a nitrogen there (Table 3). Again all these results are consistent with the proposed binding mode.

Next, we turned our attention to the imidazothiazole ring. Groups with good H-bond acceptors in a position equivalent to the N of the imidazothiazole were well tolerated but groups lacking this were not (Table 4). These results are consistent with this



Scheme 1. General synthetic route. Reagents and conditions: (a) (LDA or nBuLi, 0 °C, then TFA) or (H_2SO_4, AcOH, 50–100 °C).

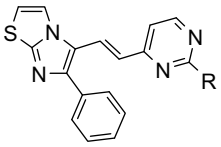
Table 1
Results for variation of the phenyl position



Compound	R	Ratio *E*:*Z*	Tie-2 IC_{50}[a] (μM)	Flt-1 IC_{50}[a] (μM)	KDR IC_{50}[a] (μM)
1	Ph–	>95:5	6.0	30	7.0
6	*p*-Fluoro-Ph	>95:5	13	nd	nd
7	Me–	>95:5	>100	>100	70
8	Cl–	>95:5	>100	>100	31

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

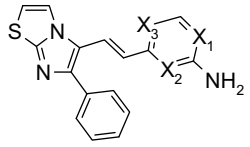
Table 2
Results for variation of the amino position



Compound	R	Ratio *E*:*Z*	Tie-2 IC_{50}[a] (μM)	Flt-1 IC_{50}[a] (μM)	KDR IC_{50}[a] (μM)
1	NH_2	>95:5	6.0	77	8
9	NHMe	7:1	14	84	17
10	NHCHMePh	3:1	>100	>100	>100
11	NHPh	>95:5	68	>100	>100
12	NHAc	7:3	11	100	4.5
13	$NHCO^tBu$	3:2	41	96	13
14	Cl	>95:5	55	66	>100
15	H	>95:5	27	>100	77

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

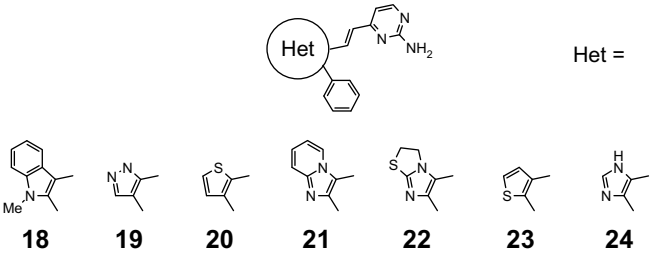
Table 3
Results for variation of the pyrimidine ring



Compound	$X^1/X^2/X^3$	Ratio *E*:*Z*	Tie-2 IC_{50}[a] (μM)	Flt-1 IC_{50}[a] (μM)	KDR IC_{50}[a] (μM)
1	N/N/CH	>95:5	6.0	77	8
16	CH/N/N	3:2	>100	>100	>100
17	N/CH/N	2:1	5.2	>100	35

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

Table 4
Results for variation of the imidazothiazole ring



18 **19** **20** **21** **22** **23** **24**

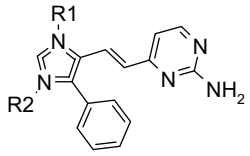
Compound	Ratio *E*:*Z*	Tie-2 IC_{50}[a] (μM)	Flt-1 IC_{50}[a] (μM)	KDR IC_{50}[a] (μM)	Tie-2 cell IC_{50}[a] (μM)
1	>95:5	6.0	77	8	0.33
18	>95:5	83	nd	nd	nd
19	>95:5	64	>100	>100	nd
20	19:1	>100	>100	>100	nd
21	4:1	5.6	79	13	nd
22	24:1	2.9	23	3.2	nd
23	>95:5	>100	>100	>100	nd
24	>95:5	8.4	68	9.8	0.37

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

nitrogen forming an important H-bond with the kinase as has been seen with the related p38 inhibitors[10] and was predicted to be possible from our Tie-2 homology model. The good potency of the simple imidazole analogue **24** was intriguing and we decided to explore this more thoroughly as it offered the advantage of reduced molecular weight and lipophilicity with no loss in potency.

First, we varied substituents on the imidazole ring nitrogens (Table 5). Methylation of the nitrogen distal to the alkene linkage **25** led to reduced potency, consistent with our proposal that this nitrogen forms an important H-bond acceptor. The other nitrogen was reasonably tolerant of substitution with the NMe compound **26** being of particular interest.

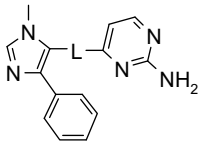
Table 5
Results for variation of the imidazole ring



Compound	R^1	R^2	Ratio *E*:*Z*	Tie-2 IC_{50}[a] (μM)	Flt-1 IC_{50}[a] (μM)	KDR IC_{50}[a] (μM)	Tie-2 cell IC_{50}[a] (μM)
24	H	—	>95:5	8.4	68	9.8	0.37
25	—	Me	>95:5	>100	>100	7.8	nd
26	Me	—	>95:5	3.8	34	3.2	0.34
27	CH2CO2H	—	>95:5	68	nd	nd	nd
28	CH2CH2-1-pyrollidine	—	>95:5	21	>100	32	nd
29	CH2Ph	—	>95:5	2.8	31	7.0	nd

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

Table 6
Results for variation of the linker group



Compound	L	Tie-2 IC_{50}[a] (μM)	Flt-1 IC_{50}[a] (μM)	KDR IC_{50}[a] (μM)
26	–CH=CH–	3.4	68	9.8
30	–NH–	>100	>100	>100
31	–CONH–	>100	>100	98
32	–NHCO–	>100	>100	>100
33	$–CH_2NH–$	>100	>100	>100
34	$NHCH_2CH_2–$	>100	>100	>100
35	– Cyclopropyl–	>100	>100	>100
36	–C≡C–	6.4	36	2.9

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

Replacements for the alkene linker were poorly tolerated with the exception of alkyne **36** (Table 6). However no oral exposure was seen with this compound.

Compound **26** had a particularly attractive overall balance of properties. In the phospho Tie-2 cell assay it was reasonably potent (IC_{50} 340 nM). Selectivity was maintained or improved with the greatest potency being against p38 (IC_{50} 12 μM) and a clean profile in an extended panel of 45 kinases (all <50% inhibition at 10 μM). In contrast to its Tie-2 cell potency, it was inactive (IC_{50} > 10 μM) in a HUVEC proliferation assay[12] in response to VEGF indicating no inhibition of VEGFRs in cells and it was also inactive in a p38 cell assay.[13] It had good physical properties: solubility 280 μM at pH 7.4, 12.5% free in mouse, 9.9% free in rat. It did not inhibit CyP450's significantly (IC_{50}'s > 5 μM) and showed only moderate turnover in in vitro rat and mouse microsome metabolism studies. The stability in microsomes was in stark contrast to the initial hit (**1**), which had shown high turnover. In a cassette dosed mouse PK experiment a 2 mg/kg dose gave a C_{max} of 0.46 μM and an AUC of 0.58 μM h. However this compound and many of these compounds suffered from photo instability with respect to cis–trans isomerization and also reacted with glutathione in the presence of glutathione *S*-transferase. Due to these stability issues we sought more stable analogues and this work will be described in a future paper.

In conclusion, imidazole alkene pyrimidines such as **26** are a new class of kinase inhibitors with good potency in a Tie-2 cellular assay and oral bioavailability.

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7. Enzyme assays (Tie-2/Flt-1/KDR). The recombinant genes encoding the kinase domain containing fragments were cloned and expressed in standard baculovirus/Sf21 system. Lysates were prepared from the host insect cells following protein expression, by treatment with ice-cold lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA), plus protease inhibitors, and then cleared by centrifugation. Nunc MaxisorbTM 96-well immunoplates were coated with 100 μl of synthetic peptide Sigma P3899 (1 mg/ml stock solution in PBS diluted 1:500 in PBS prior to plate coating) and incubated at 4 °C overnight. Plates were washed in 50 mM Hepes, pH 7.4, at room temperature to remove any excess unbound synthetic peptide. Tie-2, KDR or Flt1 activities were assessed by incubation of the appropriate freshly diluted lysates (1:200, 1:400, and 1:1000, respectively) in peptide coated plates for 60 min (Tie-2) or for 20 min (KDR, Flt) at room temperature in 100 mM Hepes, pH 7.4, adenosine trisphosphate (ATP) at 5 μM for the respective enzyme, 10 mM $MnCl_2$, 0.1 mM Na_3VO_4, 0.2 mM DL dithiothreitol (DTT), 0.1% Triton X-100 together with the test compound dissolved in DMSO (final concentration of 2.5%) with final compound concentrations ranging from 0.05 to 100 μM. Reactions were terminated by the removal of the liquid components of the assay followed by washing of the plates with PBS T (phosphate buffered saline with 0.5% Tween 20) or an alternative equivalent wash buffer. The immobilized phospho peptide product of the reaction was detected by immunological methods. Firstly, plates were incubated for 4 h at room temperature with murine monoclonal anti-phosphotyrosin–HRP (Horseradish Peroxidase) conjugated antibodies (4G10 from Upstate Biotechnology UBI 16-105). Following extensive washing with PBS-T, HRP activity in each well of the plate was measured colorimetrically using 22′ Azino di [3 ethylbenzthiazoline sulfonate (**6**)] diammonium salt crystals ABTS (Sigma P4922) as a substrate incubated for 30–45 min to allow colour development, before 100 μl of 1 M H_2SO_4 was added to stop the reaction. Quantification of colour development and thus enzyme activity was achieved by the measurement of absorbance at 405nm on a Molecular Devices ThermoMax microplate reader.
8. Cellular Tie-2 autophosphorylation assay. Tie-2 cell potency was determined using CHOK1 cells stably transfected with human Tie-2. Cells were seeded at 6 × 104 cells/well in 250 μl DMEM, G418, 10% FCS into 96-well plates and grown for 3 days prior to assaying. On the day of assay media was removed and cells were dosed with compound in DMEM plus 1% FCS for 45 min at 37 °C. Cells were washed and lysed in 100 μl lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 50 mM NaF, 0.1% SDS, 1% NP40, 0.5% DOC, 1 mM orthovanadate, 1 mM EDTA, 1 mM PMSF, 30 ml/ml Aprotinin, 10 mg/ml Pepstatin, 10 mg/ml Leupeptin) on ice for 5 min. Lysates were transferred to Quantikine Immunoassay kit for human Tie-2, (R&D systems), and shaken for 2 h at room temperature. Unbound cell lysate was removed by washing four times with the supplied wash buffer, prior to addition of anti-phosphorlyated Tie-2 antibody (4221B Cell Signalling Technologies). Plates were placed on a shaker for 2 h at room temperature. Unbound secondary antibody was removed by washing four times with the supplied wash buffer. P Tie-2 antibody was detected using goat anti-rabbit HRP conjugated antibody (P0448 Dako). Following 2 h of incubation at RT, plates were washed a further four times and the degree of Tie-2 phosphorylation was determined by addition of the colour reagent supplied by R&D Systems. The reaction was stopped after 30 min by addition of stop reagent and optical density was read at 450 nm.
9. Competitor compounds were also less potent in our Tie-2 enzyme assay than reported in the literature, for example, CP-547,632 has a reported Tie-2 enzyme IC_{50} of 0.048 μM,[14] but in our Tie-2 enzyme assay it had an IC_{50} of 0.62 μM. Protein digestion and mass spectroscopy studies indicated that the enzyme was phosphorylated on Y897, perhaps due to its expression in insect cells, which has been suggested to lead to low specific activity.[15]
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