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N^3-Arylmalonamides: A new series of thieno[3,2-*b*]pyridine based inhibitors of c-Met and VEGFR2 tyrosine kinases

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ABSTRACT

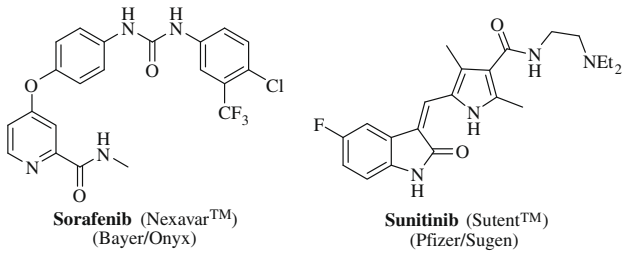
A family of thieno[3,2-*b*]pyridine based small molecule inhibitors of c-Met and VEGFR2 were designed based on lead structure **2**. These compounds were shown to have IC_{50} values in the low nanomolar range in vitro and were efficacious in human tumor xenograft models in mice in vivo.

When overexpressed or mutated, protein tyrosine kinases (PTKs) become potent oncoproteins that can cause deregulated cell growth, angiogenesis and metastasis.[1] Because of these characteristics, they are key targets for small molecule inhibitors in the treatment of cancer. Moreover, tumor survival could potentially be more efficiently reduced if multiple signaling pathways are interrupted either by multi-targeted agents or by combination of single targeted drugs.[2] Several PTK inhibitors have been found to have effective anti-tumor activity and some of them have been approved or are in clinical trials.[3] Recent FDA approved drugs Sutent™[4] and Nexavar™[5] are two such examples of multi-targeted agents (Fig. 1).

In particular, the simultaneous inhibition of the vascular endothelial growth factor (VEGF) receptors[6] and c-Met[7] is a promising approach for the treatment of cancer.

c-Met and its ligand the Hepatocyte Growth Factor/Scatter Factor (HGF/SF) are associated with the development of various human malignancies and are overexpressed or mutated in a number of malignancies while the VEGFRs play key roles in tumor angiogenesis.

In one of our previous Letters, the synthesis and biological evaluation of a novel family of potent thieno[3,2-*b*]pyridine based inhibitors of c-Met and VEGFR2 bearing a carbamothioyl-arylacetamide group were described, in which substitution at position 2 of the thieno[3,2-*b*]pyridine system was explored.[8a] To further understand the structure–activity relationship (SAR) of this novel series of compounds, certain surrogates of the carbamothioyl-arylacetamide fragment were investigated.[9] Modifications to the carbamothioyl-arylacetamide moiety had been reported previously by Kirin Brewery for a series of quinoline based inhibitors of c-Met[10] in which replacement of the sulfur atom with an oxygen atom of the



Sorafenib (Nexavar™) (Bayer/Onyx)

Sunitinib (Sutent™) (Pfizer/Sugen)

Figure 1.

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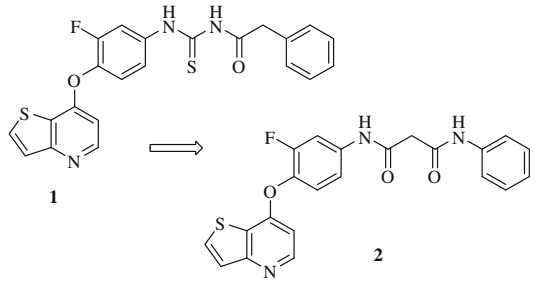


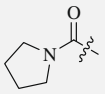
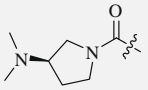
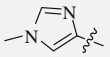
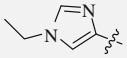
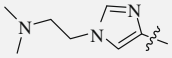
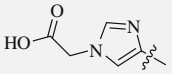
Figure 2.

thiocarbonyl unit and switching the CH and the NH groups had afforded N^3-arylmalonamides, possessing similar potency compared to the parent compounds. We were interested to see if such modifications were possible within our thieno[3,2-*b*]pyridine class of molecules[8a] (Fig. 2).

The chemistry described in Scheme 1 shows the synthetic route chosen to obtain **2** (R^1 = H). Thus, reaction of methyl 3-chloro-3-oxo-propionate with aniline afforded **3**[8b] which upon reaction with **4**[8a] using a BOP coupling procedure afforded **2.** The benefit of this protocol was the ease with which compound libraries could be made simply by using different amines (aromatic or aliphatic) or appropriately substituted amine **4**. A comparison of the activities of **1**[8a] and **2** against c-Met (116 nM and 270 nM, respectively) showed that the change was well tolerated. However, a similar comparison of activities against VEGFR2 (1.1 μM and 200 nM, respectively) showed a significant improvement of **2** over **1**. With this information in hand, a library of compounds was synthesized (Scheme 1) and the results are given in Table 1. Previously we have shown[8a] that the preferred substituent at the 2 position of thieno[3,2-*b*]pyridine was either a cyclic amide or an imidazole, as these tended to give molecules of higher potency against the target enzymes. Thus efforts were focused on these groups over other moieties (Table 1). The results show, as expected, both the amides and imidazole analogues were highly potent against both enzymes. The difference, however, was that inhibitors of the imidazole class were significantly more potent in the cellular assay. The only exception to this was **11** in which it appears the terminal carboxylic acid was detrimental to activity, possibly due to poor cellular penetration.

Attention was turned next to investigate the SAR around the malonamide unit. Using the same approach as in Scheme 1 except replacing aniline for an appropriately substituted amine, a series of compounds was made (Table 2). It was decided to make analogues of **9** as it had shown good activity against both enzymes (enzymatically and cellularly) and was easily synthesized in bulk. Replacement of the phenyl group (**9**) by a cyclohexyl group (**12**) caused a small decrease in c-Met and VEGFR2 enzymatic inhibitory activity as well as its cellular potency. Addition of fluorine to the malonamide unit in either the *ortho* (**13**), *meta* (**14**) or *para* (**15**) positions essentially had no effect on the potency of the molecules when compared with **9**. However, compound **14**, was less potent in the c-Met cellular assay than either **13** or **15**. Introduction of electron donating and electron withdrawing groups at the *ortho*-position of the phenylmalonamide ring were also investigated. *ortho*-Methoxy substitution was well tolerated by both enzymes, while substitution with a trifluoromethyl group caused a ~5-fold decrease in potency against c-Met (compound **9** vs compound **17**) but retained good activity against VEGFR2. Interestingly the 2-hydroxy analogue **18**, which is essentially a demethylated version of compound **16**, had almost identical potency enzymatically against both enzymes but the cellular activity was significantly less, again possibly due to decreased cellular penetration.

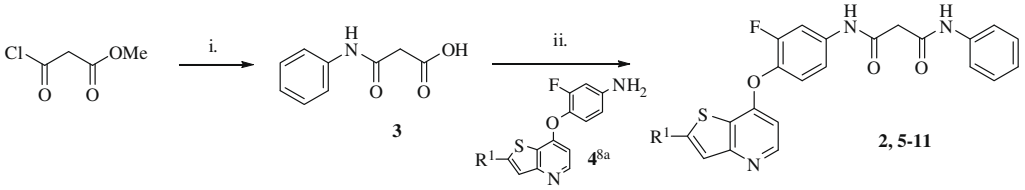
Table 1
In vitro[13,14] profile of compounds **2**, **5**–**11**

Compd	R^1	c-Met, IC_{50} (nM)	VEGFR2, IC_{50} (nM)	Phospho-TPR-Met, IC_{50} (nM)
2	H–	270	200	nt
5		40	4	2 μM
6		65	89	560
7		36	50	40
8		25	30	340
9		19	5	180
10		20	6	190
11		36	19	>5 μM

It has been shown previously that substitution at the C-2 position or the N-terminal amide atom of the head group was tolerated by c-Met or VEGFR2 in a series of quinoline based inhibitors[10,11] and the question was raised as to whether this would be tolerated within the thieno[3,2-*b*]pyridine class of molecules as well. The chemistry used to obtain these novel molecules is shown in Scheme 2. The N-substituted analogues were synthesized using the chemistry shown in Scheme 1 by using the appropriate N-substituted anilines.

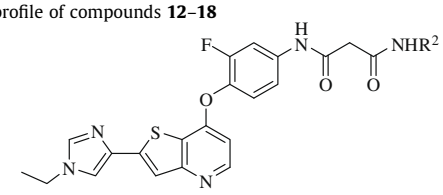
N-Methylation of the malonamide was well tolerated, as compound **22** was equipotent to compound **9** against both enzymes. A further improvement in cell-based properties, presumably at the level of cell permeability, was achieved with compound **21** from the introduction of a cyclopropyl moiety at the methylene unit of the malonamide fragment. It was found, however, that a combination of both the *N*-methyl group and a cyclopropyl group was detrimental to activity against both targets (data not shown) perhaps due to the conformation restrictions placed on such a molecule.[9]

Certain compounds that had reasonable solubility (data not shown) were further evaluated for their pharmacokinetic properties



Scheme 1. Reagents and conditions: (i) aniline, DCM, rt, 8 h, then LiOH, THF, water, rt, 1 h; (ii) BOP, DIPEA, DMF, rt, 2 h.

Table 2
In vitro[13,14] profile of compounds **12–18**



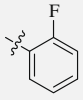
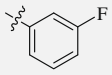
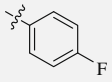
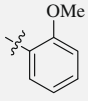
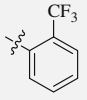
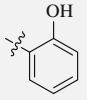
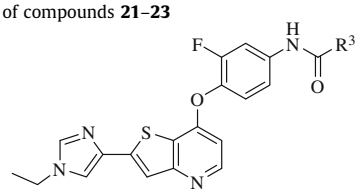
Compd	R^2	c-Met IC_{50} (nM)	VEGFR-2 IC_{50} (nM)	Phospho-TPR-Met, IC_{50} (nM)
9		19	5	180
12		52	10	590
13		32	18	35
14		52	10	870
15		24	5	23
16		25	6	44
17		91	13	150
18		26	7	600

Table 3
In vitro[13,14] profile of compounds **21–23**



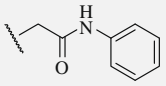
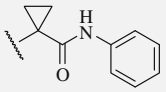
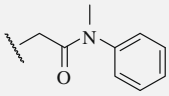
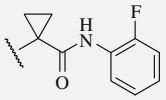
Compd	R^3	c-Met, IC_{50} (nM)	VEGFR-2 IC_{50} (nM)	Phospho-TPR-Met, IC_{50} (nM)
9		19	5	180
21		24	8	4
22		33	6	31
23		38	15	120

Table 4
Pharmacokinetic profile of selected compounds in rats[a]

Parameter	**9**	**21**	**22**
$t_{1/2}$ (h), iv	2.6	3.1	0.91
CL (L/h/kg)	0.33	0.99	0.52
Vss (L/kg)	0.82	2.2	0.33
T_{max} (h), po	5.7	2	0.5
C_{max} (μM/(mg/kg)), po	0.18	0.10	0.29
AUC (μM h/(mg/kg)), po	0.77	0.62	2.28
%F	12	33	60

[a] iv doses 2.17–2.86 mg/kg; po doses 4.28–4.98 mg/kg.

Table 5
Effect of compound **21** on c-Met cellular endpoints

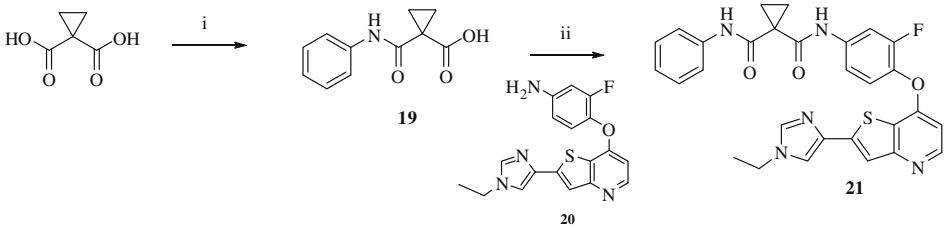
Compd	A549 wound healing, IC_{50} (nM)	DU145 cell scattering, IC_{50} (nM)
21	220	40

in the rat (Table 4). Compounds **9**, **21** and **22**—all showed an appreciable half-life, a reasonably low rate of clearance, a low steady-state volume of distribution and good oral bioavailability.

Compound **21** was further evaluated in additional in vitro assays as it had a reasonable overall PK profile and in addition was the most potent of the three compounds in the cellular assay (Table 3). Compound **21** potently inhibited HGF-induced epithelial cell migration and scattering (Table 5), showing that it is able to inhibit c-Met-dependent cell motility events.

Second, compound **21** was tested in a variety of VEGF-dependent cellular assays (Table 6). It potently inhibited the VEGF-induced phosphorylation of the downstream effector ERK, and also impeded the VEGF-dependent proliferation of human umbilical vein endothelial cells (HUVEC). In an in vitro angiogenesis assay, which measures the formation of tubules (Angiokit™; TCS Cellworks), compound **21** significantly affected tubule growth and was also able to almost completely inhibit tubule formation at the 100 nM dose (Table 6). These results show that compound **21** has a significant effect on both c-Met and VEGF-dependent functional activity in cells.

In addition, compound **21** was also profiled against a panel of kinases and was found to inhibit other therapeutically significant



Scheme 2. Reagents and conditions: (i) $SOCl_2$, THF, 0 °C, 30 min then aniline, triethylamine, 0 °C, 2 h; (ii) EDC, DMF, 3 h, rt.

Table 6
Effect of compound **21** on VEGFR2 cellular endpoints

Compd	ERK phosphorylation IC_{50} (nM)	HUVEC proliferation IC_{50} (nM)	Tubule length IC_{50} (nM)
21	1	0.3	<3

Table 7
The effect of oral dosage of **21** on various human tumor models in vivo at a dosage of 40 mg/kg once daily

Tumor model	Experiment duration (days)	% Tumor growth inhibition
A549 (lung)	14	54
U87MG (glioblastoma)	10	70
MKN45 (gastric)	12	101

kinases.[12] Compound **21** showed good efficacy in vivo when evaluated in several human tumor xenograft models, with daily oral administration of 40 mg/kg (Table 7).

In conclusion, novel dual c-Met/VEGF receptor tyrosine kinase inhibitors based upon the thieno[3,2-*b*]pyridine scaffold were designed and synthesized. These compounds exhibit potent activities against target enzymes and in cell-based assays. Lead molecules possess favorable pharmacokinetic profiles and demonstrate significant oral anti-tumor activity in vivo. This work was part of the MethylGene kinase inhibitor research program, which led to the identification of the clinical candidate, **MGCD265**, currently in Phase II clinical development.

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12. Compound **21** at a concentration of 100 nM inhibited the following enzymes: Flt-3 (100%), Tie-2 (100%), PDGFRα (99%), c-Kit (99%), VEGFR1 (98%), VEGFR3 (97%), Aurora A (96%), EphB4 (95%), c-SRC (93%). Compound **21** had <5% inhibitory activity against the following enzymes CDK2/Cyclin E, EGFR, GSK3β, IKKβ, PKBα. Compound **21** was evaluated using the Kinaseprofiler™ Kinase Selectivity Screening Service (radiometric protein kinase assays) by Millipore.
13. *In vitro kinase assays (c-Met and VEGFR-2):* Preparation of GST fusion proteins: recombinant baculovirus containing the catalytic domain of c-Met and of the VEGFR-2 receptor fused to glutathione *S*-transferase (GST) fusion genes were used to infect High five (c-Met) or Sf9 (VEGFR-2) cells at a multiplicity of infection of 1 or 0.1, respectively. Cell lysates were prepared after ~72 h of infection in 1% Triton X-100, 2 μg of leupeptin/mL, and 2 μg of aprotinin/mL after ~72 h of infection in phosphate-buffered saline, and the fusion proteins were purified over glutathione agarose (Sigma) according to manufacturer's instructions. Biochemical kinase assays for IC_{50} determination and kinetic studies: Inhibition of c-Met and VEGFR2 was measured in a DELFIA™ assay (Perkin–Elmer). The substrate poly(Glu_4,Tyr) was immobilized onto black high-binding polystyrene 96-well plates (Nunc Maxisorp). The c-Met kinase reaction was conducted in 25 mM Hepes pH 7.5 containing 20 mM NaCl, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mg/mL bovine serum albumin (BSA) and 20 μM vanadate, while the VEGFR-2/KDR reaction was conducted in 60 mM Hepes pH 7.5 containing 3 mM $MgCl_2$, 3 mM $MnCl_2$, 1.2 mM β-mercaptoethanol, 0.1 mg/mL BSA and 3 μM vanadate. ATP concentrations in the assay were 10 μM for c-Met (5× the K_m) and 0.6 μM for VEGFR-2/KDR (2× the K_m). Enzyme concentration was 25 nM (c-Met) or 5 nM (VEGFR-2). The recombinant enzymes were pre-incubated with inhibitor and Mg-ATP on ice in polypropylene 96-well plates for 4 min, and then transferred to the substrate coated plates. The subsequent kinase reaction took place at 30 °C for 30 min (c-Met) or 10 min (VEGFR2). After incubation, the kinase reactions were quenched with EDTA and the plates were washed. Phosphorylated product was detected by incubation with Europium-labeled anti-phosphotyrosine MoAb. After washing the plates, bound MoAb was detected by time-resolved fluorescence in a Gemini SpectraMax reader (Molecular Devices). Inhibitors were tested at seven different concentrations each in triplicate. IC_{50}s were calculated in a four parameters equation curve plotting inhibition (%).
14. *Cellular assay conditions*: A cellular clone of 293T cells stably expressing TPR-Met (Park, M.; Dean, M.; Cooper, C. S.; Schmidt, M.; O'Brien, S. J.; Blair, D. G.; Vande Woude, G. F. *Cell* **1986**, *45*, 895), the activated mutated form of the receptor Met, under a CMV promoter was derived. Cells were treated with compounds dilutions for 150 min and lysate samples from treatment wells were transferred to high-binding white polysterene 96-well plates (Corning). TPR-Met autophosphorylated levels were detected by ELISA using the primary antibodies anti-phospho-Tyrosine (Millipore, 4G10) and a reporter antibody, anti-mouse-horseradish peroxidase (Sigma). Plates were washed on a plate washer (SkanWasher, Molecular Devises) and subsequently incubated with chemiluminescent substrate solution (ECL, Roche). Luminescence signal was captured on a Polar Star Optima apparatus (BMG LabTech). Average values of triplicate treatment points were used to prepare IC_{50} curves using a 4-parameter fit model. These curves were calculated using GRAFIT 5.0 software.