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Identification of potent and selective VEGFR receptor tyrosine kinase inhibitors having new amide isostere headgroups

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ABSTRACT

A novel series of malonamide-type dual VEGFR2/c-Met inhibitors in which one of the amide bonds was replaced by an amide isostere—a trifluoroethylamine unit, was designed, synthesized, and evaluated for their enzymatic and cellular inhibition of VEGFR2 and c-Met enzymes. Optimization of these molecular entities resulted in identification of potent and selective inhibitors of VEGFR2 enzyme.

The development of anticancer drugs inhibiting angiogenesis has been an area of extensive research in the past decade.[1] The vascular endothelial growth factor receptor-2 (VEGFR2 or KDR)[2] and the hepatocyte growth factor (HGF) receptor tyrosine kinase c-Met[3] are expressed at abnormally high levels in a large variety of human solid tumors. Dysregulation of these receptor tyrosine kinases (RTKs) affects cell proliferation, survival and motility, leading to tumor growth, angiogenesis, and metastasis. Inhibition of this class of RTK by small molecules is a promising approach for cancer therapy.

We have recently disclosed the synthesis and the evaluation of malonamides, exemplified by the general structure **1**, as potent c-Met/VEGFR2 multitargeted kinase inhibitors (Fig. 1).[4]

With the aim to further explore one of the key recognition elements of the kinase inhibitors, which are presumed to bind into the hydrophobic back pocket of the kinase active site, we envisioned the replacement of one of the amide bonds of the malonyl moiety by an amide isostere to provide the general structures **2** and **3**, respectively. The replacement of one amide bond of the malonyl moiety by an amide isostere such as the trifluoroethylamine unit has been first explored by Zanda and co-workers[5] and extended by Ongeri and co-workers[6] in the synthesis of partially modified retropeptides in order to improve the biostability, bioavailability, and selectivity of these types of synthetic peptides. Subsequently, the same concept was successfully applied to the design of potent and selective cathepsin K inhibitors[7] where one amide bond was replaced by a chiral trifluoroethylamine unit. This type of amide isostere replacement is now generally accepted as one in which the hydrogen bond donor character of the amide functionality is retained by the non-basic character of the trifluoroethylamine unit.[7] Likewise, starting with the assumption that both the hydrogen bond donor character of the left-hand-side and the hydrogen bond acceptor character of the right-hand-side amide moieties of malonamides **4** and **5** (Table 1) were required for the favorable interactions with the kinase active site, we hypothesized that the trifluoroethylamine unit as an amide isostere, as shown in general structures **2** and **3** (Fig. 1) would be accepted by the hydrophobic back pockets of the active sites of our target RTK's.

Hence, in this study we disclose our efforts towards the design, the synthesis and the in vitro evaluation of new RTK inhibitors based on the thieno[3,2-*b*]pyridine, quinazoline, quinoline and pyrimidine scaffolds in which one of the amide bonds of the malonyl moiety has been replaced by an amide isostere.[8] This work culminated in the discovery of a new class of potent VEGFR inhibitors.

We first studied what changes were tolerated by the VEGFR2 and c-Met enzymes when keeping the left-hand-side of compound **4** unchanged and alternatively replacing one amide moiety of the

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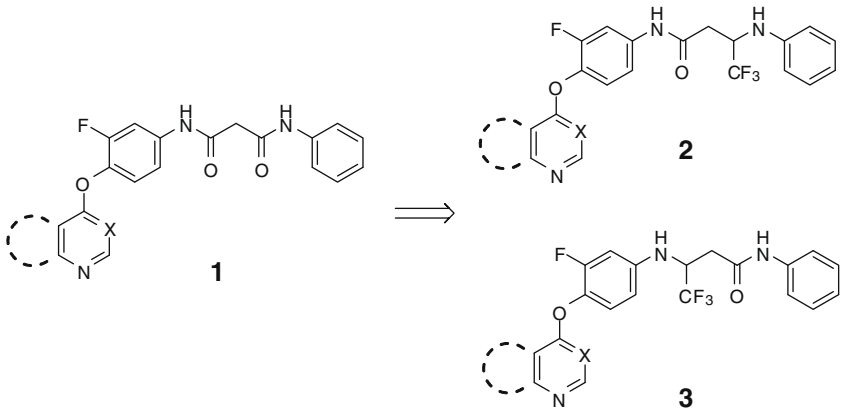
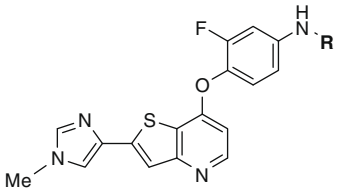


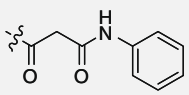
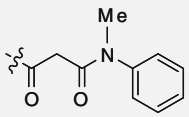
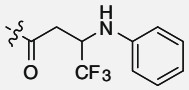
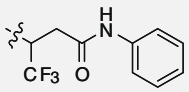
Figure 1. Replacement of one amide bond of the malonamide head group by a trifluoroethylamine unit (X = CH or N).

malonamide head group by a trifluoroethylamine unit (compounds **6 and 7**). The synthesis of compound **6** is shown is Scheme 1. The common intermediate **C** was prepared in three steps starting with the *Negishi* coupling reaction between 7-chlorothieno[3,2-*b*]pyridine and 4-iodo-1-methyl-1*H*-imidazole. Compound **A** was then heated at 180 °C in diphenyl ether in the presence of 2-fluoro-4-nitrophenol and potassium carbonate to afford **B**. The nitro group was reduced using nickel chloride and sodium borohydride in methanol to afford intermediate **C**. The racemic carboxylic acid **F** was synthesized in three steps starting from the aniline and following the procedure already described by Gong and Kato.[9] That material was then coupled to compound **C** using HATU reagent and Hunig's base in DMF to afford the desired compound **6**. The synthesis of the second regioisomer **7** is shown in Scheme 2. The same three-step linear sequence used to make intermediate **F** (Scheme 1) was followed to transform compound **C** into the racemic carboxylic acid **I**, which was subsequently coupled with aniline using HATU reagent.

In the first set of molecules (Table 1), we found that the replacement of the right-hand-side amide bond of the compound **4** by a trifluoroethylamine unit to give the compound **6** is not well tolerated. Indeed, compound **6** showed a 23-fold and an 11-fold decrease in activity for c-Met and VEGFR2, respectively, when compared to the parent malonamide **4**. The reduced activity against VEGFR2 and especially against c-Met by compound **6** could result from either a steric clash between the CF_3 group and the hydrophobic back pocket of the kinase active site and/or the loss of a strong hydrogen bond acceptor (carbonyl) present in the malonamides **4** and **5**. On the other hand, substitution of the left-hand-side amide bond by a trifluoroethylamine unit, giving rise to compound **7**, led to a fourfold decrease in potency against c-Met but, surprisingly, retained similar VEGFR2 inhibitory activity compared to the parent malonamide **4**. Thus, we were able to clearly determine which substitution pattern was required for the inhibition of our target RTK.[10,11]

Table 1
VEGFR2 and c-Met IC_{50} enzymatic assay for compounds **4**–**7** of general structure



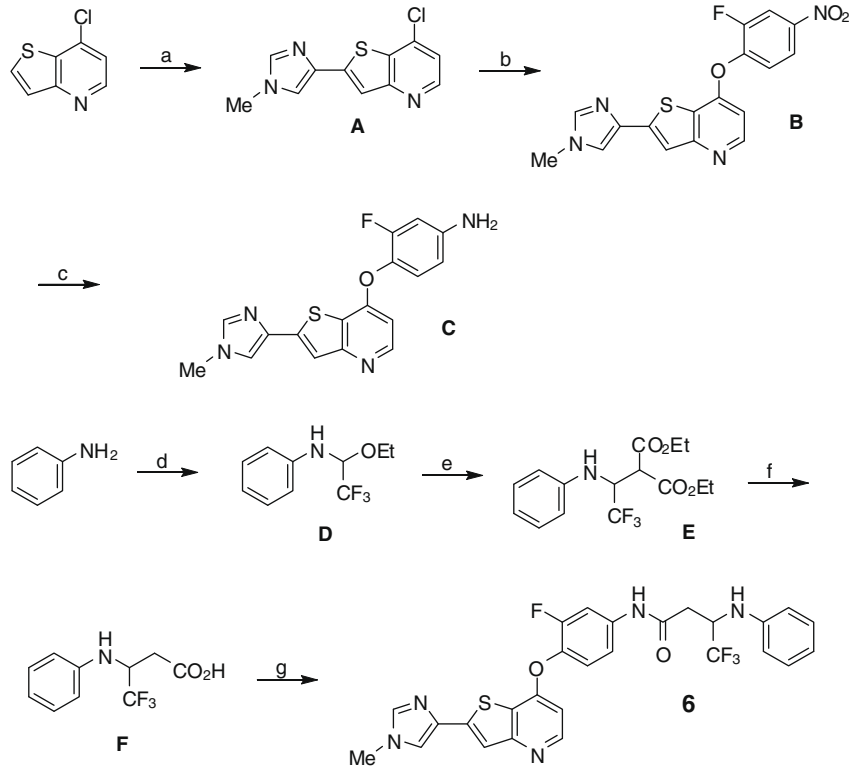
#	R	VEGFR2 IC_{50} (μM)	c-Met IC_{50} (μM)
4		0.02	0.03
5		0.01	0.04
6		0.21	0.70
7		0.01	0.12

We next explored the influence of the addition of a substituent on the terminal phenyl ring and also the removal of the fluorine from the middle phenyl ring (Table 2). Introduction of an *ortho*-methoxy substituent on the terminal phenyl ring (compound **8**) gave rise to a sevenfold decrease against c-Met when compared to the parent compound **7**, thus making compound **8** a selective VEGFR2 inhibitor. On the other hand, introduction of a *para*-fluoro substituent—compound **9**—allowed for improved activity against c-Met at the cellular level (0.4 μM in both wound healing and scattering assays, Table 2); and compound **9** was still equipotent to compound **7** for the inhibition of VEGFR2. Finally, removal of the fluorine substituent from the middle phenyl ring of compound **9** resulted in compound **10** with a slight decrease in both VEGFR2 and c-Met enzyme inhibition.

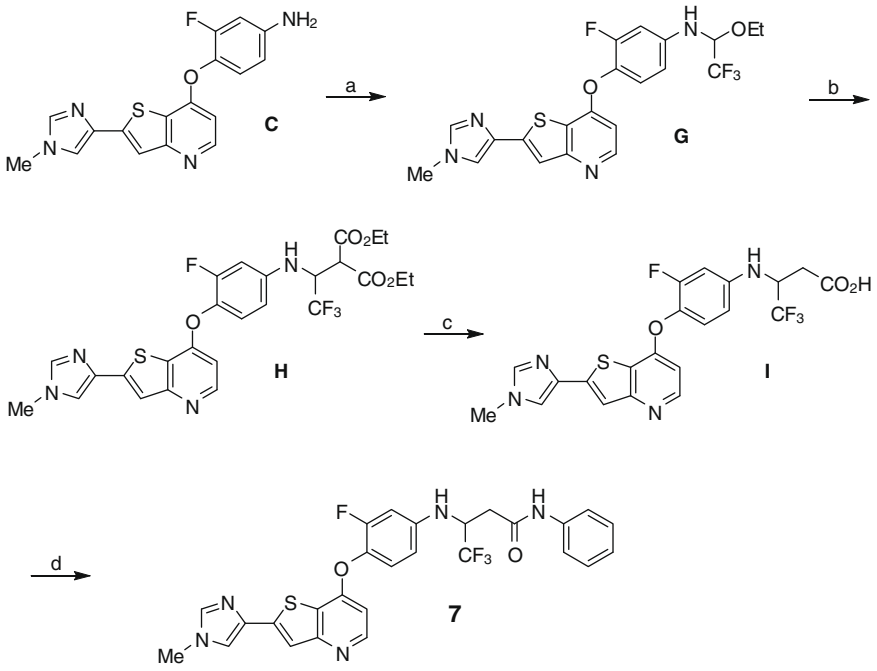
Compound **8** was profiled against a set of 27 kinases. As observed, compound **8** showed weak activity (>50% but <80% of inhibition) when tested at 0.1 μM against Flt-3, c-Met, RON, Tie-2, and TrkA and did not show significant activity against a representative panel of 19 human kinases.[12] However, compound **8** was very active (>95% of inhibition) for the other members of the VEGFR family-VEGFR1 and 3.

Moving from the thieno[3,2-*b*]pyridine scaffold to either quinazoline (compound **11**) or quinoline (compound **12**) series showed similar enzymatic results when compared to compound **6**. On the other hand, quinoline **13**, having the left-hand-side trifluoroethylamine unit, exhibited a more pronounced selectivity in favor of the inhibition of VEGFR2 when compared to compound **7** (Table 3).

The same observations were made with compounds based on the pyrimidine scaffold: the regioisomer **14** was inactive against both c-Met and VEGFR2 while compound **15** turned out to be highly selective and potent against VEGFR2 (Table 4).



Scheme 1. Synthesis of compound **6**. Reagents and conditions: (a) (i) *n*-BuLi, THF, −78 °C; (ii). $ZnCl_2$, THF, −78 °C to rt; (iii) $Pd(PPh_3)_4$, 4-iodo-1-methyl-1*H*-imidazole, THF, reflux; (b) 2-fluoro-4-nitrophenol, K_2CO_3, Ph_2O, 180 °C; (c) $NiCl_2$, $NaBH_4$, MeOH; (d) trifluoroacetaldehyde ethyl hemiacetal, PTSA, EtOH, reflux; (e) diethylmalonate, NaH, THF, reflux; (f) (i) NaOH, EtOH/water; (ii) toluene, reflux; (g) C, HATU, DIPEA, DMF.

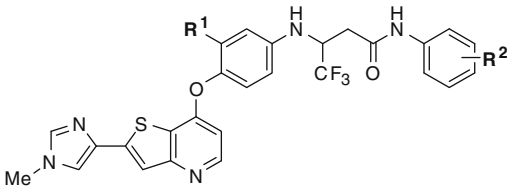


Scheme 2. Synthesis of compound **7**. Reagents and conditions: (a) trifluoroacetaldehyde ethyl hemiacetal, PTSA, EtOH, reflux; (b) diethylmalonate, NaH, THF, reflux; (c) (i) NaOH, EtOH/water; (ii) toluene, reflux; (d) aniline, HATU, DIPEA, DMF.

Compound **15** was profiled against a set of eight kinases. As expected, compound **15** showed weak activity against Tie-2 and did not show significant activity against c-Met, Ron, Flt-3 and Aurora A. Again like compound **8**, compound **15** turned out to be highly selective for the other members of the VEGFR family-VEGFR1 and 3.

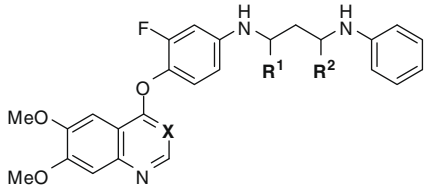
Compound **15** was also tested in two VEGF-dependent cellular assays (Table 5). It impeded the VEGF-dependent proliferation of

Table 2
VEGFR2 and c-Met IC_{50} enzymatic assay and cell-based assay for compounds **7**–**10** of general structure



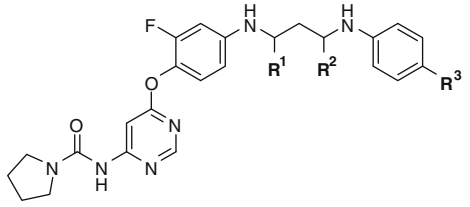
#	R^1	R^2	VEGFR2 IC_{50} (μM)	c-Met IC_{50} (μM)	Phospho-TPR-Met Elisa IC_{50} (μM)	A549 wound healing inh. IC_{50} (μM)	DU145 scattering inh. IC_{50} (μM)
7	F	H	0.01	0.12	0.60	2	2
8	F	*o*-OMe	0.02	0.84	1.80	2	10
9	F	*p*-F	0.02	0.09	0.37	0.4	0.4
10	H	*p*-F	0.02	0.18	0.88	2	2

Table 3
VEGFR2 and c-Met IC_{50} enzymatic assay for compounds **11**–**13** of general structure



#	X	R^1	R^2	VEGFR2 IC_{50} (μM)	c-Met IC_{50} (μM)
11	N	=O	CF_3	0.86	>5
12	CH	=O	CF_3	0.36	0.68
13	CH	CF_3	=O	0.03	0.91

Table 4
VEGFR2 and c-Met IC_{50} enzymatic assay for compounds **14** and **15** of general structure



#	R^1	R^2	R^3	VEGFR2 IC_{50} (μM)	c-Met IC_{50} (μM)
14	=O	CF_3	F	1.2	>5
15	CF_3	=O	H	0.04	>5

human umbilical vein endothelial cells (HUVEC). In an in vitro angiogenesis assay, which measures the formation of tubules generated by a co-culture of endothelial and fibroblast cells (Angiokit™; TCS Cellworks), compound **15** significantly affected tubule growth and branch formation and was also able to almost completely inhibit tubule growth and junction formation at 100 nM dose where cells were supplemented with VEGF ligand (Table 5). Thus, these results demonstrate that compound **15** has a significant effect on VEGF-dependent angiogenic activity in endothelial cells.

In summary, replacement of the malonamide head group in the thienopyridine-based c-Met/VEGFR2 multitargeted kinase inhibitors **4** and **5** by a substituted trifluoroethylamine moiety as an amide isostere (as in compound **7** but not in compound **6**) can afford potent and selective VEGFR2 inhibitors with reduced activity against the c-Met enzyme—compounds **7**–**10**. The same modification within the quinoline series (compound **13**) has even more pronounced effect in favor of VEGFR2 enzyme while pyrimidine-based compounds become VEGFR2-selective (compound **15**). Hence, appropriately positioned substituted trifluoroethylamine moiety can be successfully used in the design and the synthesis of novel VEGFR inhibitors.

Table 5
Effect of compound **15** on VEGF-mediated cellular endpoints

#	VEGF-dept HUVEC proliferation IC_{50} (μM)	Angiokit, tubule length IC_{50} (μM)	Angiokit, tubule length with VEGF (% inh.)	Angiokit, tubule branching IC_{50} (μM)	Angiokit, tubule branching with VEGF (% inh.)
15	0.04	0.01	97	0.006	95

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Griffith, D. A.; Oballa, R. M.; Palmer, J. T.; Percival, M. D.; Riendeau, D.; Robichaud, J.; Rodan, G. A.; Rodan, S. B.; Seto, C.; Thérien, M.; Truong, V.-L.; Venuti, M. C.; Wesolowski, G.; Young, R. N.; Zamboni, R.; Black, W. C. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 923.

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10. *In vitro kinase assays (c-Met and VEGFR-2/KDR)*: Preparation of GST fusion proteins: recombinant baculovirus containing the catalytic domain of c-Met and of the VEGFR-2/KDR receptor fused to glutathione-*S*-transferase (GST) fusion genes were used to infect High five (c-Met) or Sf9 (VEGFR-2/KDR) 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 the manufacturer's instructions. Biochemical kinase assays for IC_{50} determination and kinetic studies: Inhibition of c-Met and VEGFR2/KDR 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/KDR).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/KDR). 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 (%).
11. A cellular clone of 293T kidney epithelial 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 wells 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.
12. Compound **8** showed no significant inhibitory activity (<50%) when tested at 0.1 μM against a representative panel of human kinases: ALK, Bmx, CHK1, cKit, c-Raf, EphB4, FAK, GSK3β, Haspin, IKKβ, JAK2, JAK3, LIMK1, MEK1, PDK1, PI3Kb, Pim-1, PKBα, Ret.