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Identification of pyrrolo[2,1-*f*][1,2,4]triazine-based inhibitors of Met kinase

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Abstract—An amide library derived from the pyrrolo[2,1-*f*][1,2,4]triazine scaffold led to the identification of modest inhibitors of Met kinase activity. Introduction of polar side chains at C-6 of the pyrrolotriazine core provided significant improvements in in vitro potency. The amide moiety could be replaced with acylurea and malonamide substituents to give compounds with improved potency in the Met-driven GTL-16 human gastric carcinoma cell line. Acylurea pyrrolotriazines with substitution at C-5 demonstrated single digit nanomolar kinase activity. X-ray crystallography revealed that the C-5 substituted pyrrolotriazines bind to the Met kinase domain in an ATP-competitive manner.

Over the last decade, a paradigm shift has occurred in cancer therapy. While cytotoxic agents have traditionally been employed to combat aberrant cellular growth, the advent of targeted therapies such as sunitinib[1] (VEGFR, PDGFR), erlotinib[2] (EGFR), and dasatinib[3] (Bcr-Abl, Src) has provided new treatment options. These new therapies function through targeted inhibition of protein kinases whose normal activity has become deregulated. For example, protein kinase activity can be altered due to ligand-dependent activation (autocrine or paracrine), gene amplification, gene mutation, or cross-talk with other receptors (heterodimerization).[4]

The Met receptor, also known as hepatocyte growth factor receptor, is a glycosylated, heterodimeric receptor tyrosine kinase, predominately expressed in the epithelium and endothelium.[5] Met kinase was first identified and derived its name as an activated oncogene in *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-treated human osteosarcoma cells.[6] The ligand for the Met receptor, hepatocyte growth factor (HGF) also known as scatter factor (SF), is a disulfide-linked heterodimer which can activate Met via autocrine or paracrine mechanisms. HGF–Met signaling has been shown to trigger a variety of cellular responses including cell growth, migration and invasion, tumor metastasis, angiogenesis, wound healing, and tissue regeneration. Deregulated Met kinase signaling has been implicated in a variety of cancers including prostate[7] (ligand-dependent activation), gastric[8] (gene amplification/overexpression), and hereditary papillary renal cell carcinoma[9] (activating mutation).

The successful identification of pyrrolo[2,1-*f*][1,2,4]triazine-based drug candidates in our VEGFR-2[10] and EGFR/pan-Her[11] programs prompted us to utilize this versatile scaffold in search of Met kinase inhibitors (Fig. 1). Early pyrrolotriazine leads were obtained from focused amide libraries. Computer assisted molecular modeling of these leads in the Met kinase domain suggests they bind in the ATP-binding site. The pyrrolotriazine moiety mimics the adenine ring of ATP in forming critical hydrogen bonds to the hinge region of the Met protein. Substitution of the pyrrolotriazine core at C-5 was believed to provide access to the ribose pocket while substitution at C-6 was expected to lead to surface-exposed protein, consistent with previously reported

Keywords: Met kinase; Hepatocyte growth factor receptor; HGFR; Pyrrolotriazine; Gastric carcinoma.
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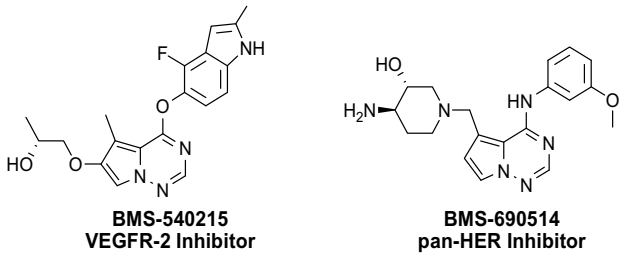


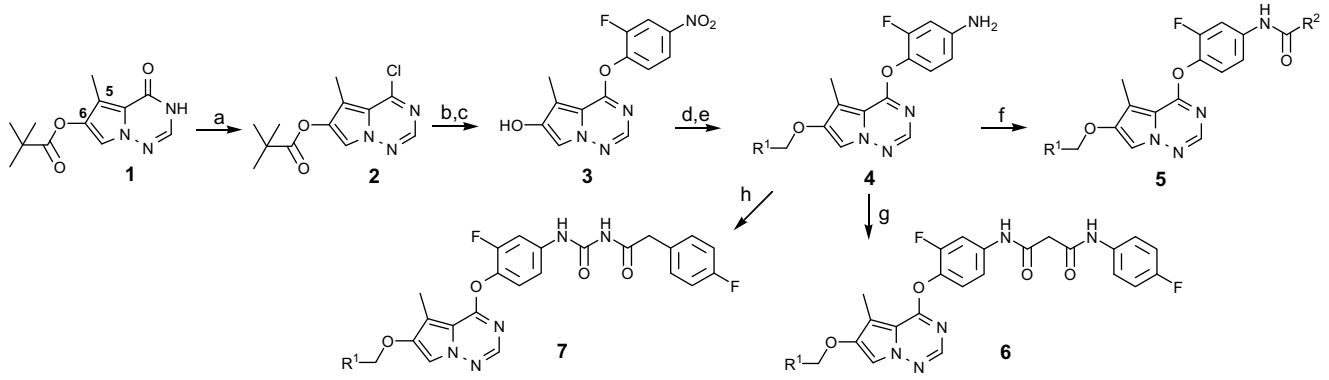
Figure 1. Pyrrolo[2,1-*f*][1,2,4]triazine-based kinase inhibitors.

observations.[10,11] This paper describes our efforts to access the ribose pocket and surface-exposed protein of the Met receptor using the pyrrolotriazine core as an ATP mimetic.

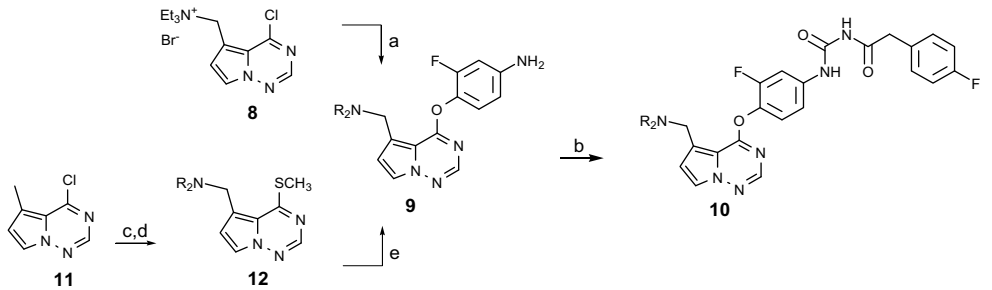
SAR at the pyrrolotriazine C-6 position was explored using the chemistry sequence shown in Scheme 1 wherein the protected hydroxyl group in **1**[12] served as a handle for introduction of polar substituents. Treatment of compound **1** with phosphorous oxytrichloride and Hunig's base gave rise to chloroimidate **2** in 78% yield. Displacement of the chlorine atom at C-4 of the pyrrolotriazine with 2-fluoro-4-nitrophenol took place using K_2CO_3 to yield the coupled product. Subsequent hydrolysis of the pivaloyl-protecting group at the C-6 position was achieved in 85% yield to give **3**. The resulting alcohol was derivatized using Mitsunobu conditions. Reduction of the nitro group proceeded smoothly with Zn/NH_4Cl to afford aniline **4** in 51–95% yield. Finally, amide formation was achieved using PyBOP to give the desired compounds **5**. Alternatively, intermediate **4** could be treated with 3-(4-fluorophenyl-amino)-3-oxo-propanoic acid or 2-(4-fluoro-phenyl)acetyl isocyanate[13] to afford malonamides **6** and acylureas **7**, respectively.

Analogs with solubilizing basic amines at C-5 were prepared via one of two routes (Scheme 2). In the first approach, tetraalkylammonium salt **8**[11] was reacted with commercially available 4-amino-2-fluorophenol followed by a secondary amine to rapidly yield intermediate **9**. Acylurea formation as described above gave the desired compounds **10**.

Alternatively, treatment of 5-methyl pyrrolotriazine **11**[12] with sodium thiomethoxide gave the corresponding thioether. Reaction of the intermediate thioether with one equivalent of NBS gave a benzylic bromide which could be displaced with various secondary amines. The resulting amine-substituted pyrrolotriazine **12** was then treated with 1 equiv of mCPBA to generate the corresponding sulfoxide. TFA was added to the oxidation to coordinate the basic amine, leaving the mCPBA free to perform its desired function. The resulting sulfoxide was then displaced with the sodium anion of 4-amino-2-fluorophenol to give aniline **9**, which was readily converted to compound **10**.



Scheme 1. Reagents and conditions: (a) $POCl_3$, DIPEA, toluene, 110 °C, 4 h, 78%; (b) 2-fluoro-4-nitrophenol, K_2CO_3, DMF, rt, 24 h, 44%; (c) NaOH, MeOH/THF, rt, 5 min, 85%; (d) R^1CH_2OH, DIAD, PPh_3, 0 °C to rt, 12 h, 19–82%; (e) Zn, NH_4Cl, MeOH/THF, rt, 7 h, 51–95%; (f) R^2CO_2H, PyBOP, DIPEA, DMF, 70 °C, 5 h; (g) 3-(4-fluorophenylamino)-3-oxo-propanoic acid, PyBOP, DIPEA, THF, rt, 24 h, 13–80%; (h) 2-(4-fluorophenyl)acetyl isocyanate, DCM, rt, 2 h, 15–46%.



Scheme 2. Reagents and conditions: (a) NaHMDS, 4-amino-2-fluorophenol, DMF, 2 h, then HNR_2, DIPEA, 130 °C, 4 h, 20–30%; (b) 2-(4-fluorophenyl)acetyl isocyanate, DCM, 1 h, 60%; (c) NaSMe, THF, 0 °C, 18 h, 90%; (d) NBS, AIBN, CCl_4, 80 °C, 1 h, then HNR_2, DIPEA, 18 h, 50–70%; (e) mCPBA (1.1 equiv), 1.5 equiv TFA, DCM, 0 °C, 1 h, then NaHMDS, 4-amino-2-fluorophenol, THF, 0 °C, 1 h, 30–50%.

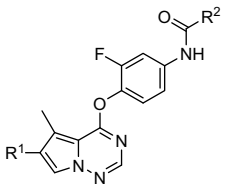
Finally, a conformationally constrained C-5 analog **17** was prepared as shown in Scheme 3. Alkenyl boronate ester **14** was prepared from the known ketone **13**[14] via ruthenium-catalyzed cross-metathesis.[15] Palladium-catalyzed Suzuki coupling with 5-chloro pyrrolotriazine **15**,[12] followed by acylurea formation, gave ketone **16**. Finally, reductive amination with ammonium acetate furnished analog **17**.[16]

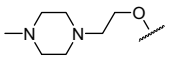
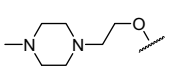
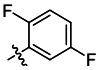
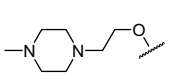
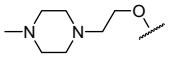
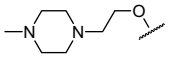
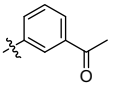
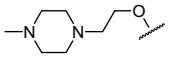
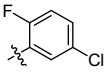
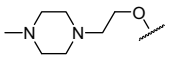
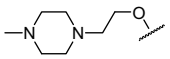
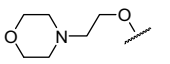
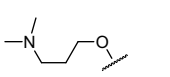
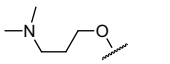
Initial efforts were directed at exploring SAR of the benzamide moiety of a C-6 unsubstituted pyrrolotriazine via the synthesis of a small library.[12] Unfortunately, Met kinase inhibitory activity of these analogs was disappointing with IC_{50}'s in the low to mid micromolar range (for example, **5a**, Table 1). Dramatic improvement in potency could be achieved upon addition of a polar substituent to C-6 of the pyrrolotriazine core. For example, *N*-methyl piperidine was appended to C-6 of the pyrrolotriazine core to give compound **5b**. This analog demonstrated an 8-fold improvement in activity with an IC_{50} of 0.09 μM as compared to the unsubstituted compound **5a**. The appendage of a polar group such as a basic amine also benefited from a significant increase in aqueous solubility, a common problem encountered in ATP-competitive kinase inhibitor programs. The 2,5- and 2,6-difluorobenzamides **5c** and **5d** gave improved potency relative to the 2-fluoro analog with IC_{50}'s of 0.04 and 0.03 μM, respectively. This trend mirrors that observed in the C-6 unsubstituted series of benzamides (data not shown). The 2-fluoro-5-methyl benzamide **5e** and 3-acetyl benzamide **5f** showed good activity against Met kinase each with an IC_{50} of 0.09 μM. While the 3-chloro-6-fluorobenzamide analog **5g** was also potent with an IC_{50} of 0.07 μM, heteroarylamide analogs **5h** and **5i** were relatively less potent. The heteroaryl analogs compare favorably to the unsubstituted phenyl compound where $R^1 = H$ and $R^2 = Ph$ ($IC_{50} > 2$ μM, data not shown).

The C-6 solubilizing group was varied and the morpholine analog **5j** was found to be less potent against Met kinase than the corresponding piperazine **5e** (0.51 vs 0.09 μM). On the other hand, a 3-dimethylamino group gave compounds with excellent potency. For example, analog **5k** possessing a 2,6-difluorophenyl amide gave an IC_{50} of 0.03 μM.

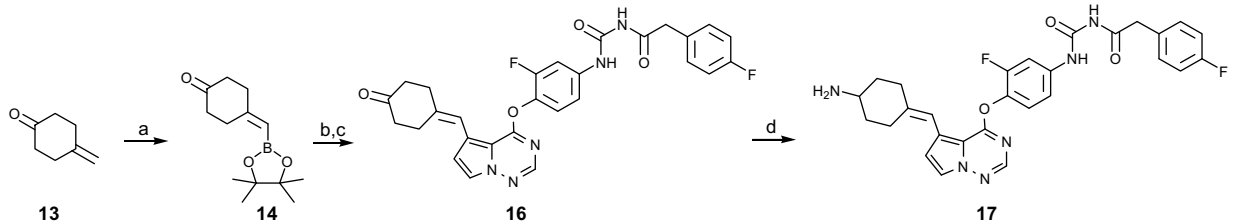
Next, we sought to replace the benzamide moiety with malonamides and acylureas (Table 2).[17] Malonamide **6a** was only marginally potent in the Met kinase assay (IC_{50} = 0.78 μM). Fortunately, as seen with the benzamides, incorporation of a polar substituent at the pyrrol-

Table 1. Pyrrolo[2,1-*f*][1,2,4]triazine benzamides[a]



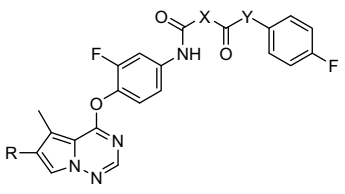
Compound	R^1	R^2	Met IC_{50} (μM)
5a	H		0.76
5b			0.09
5c			0.04
5d			0.03
5e			0.09
5f			0.09
5g			0.07
5h			0.16
5i			0.19
5j			0.51
5k			0.03
5l			0.07

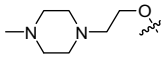
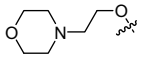
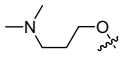
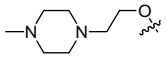
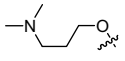
[a] For assay conditions see Ref. 23.



Scheme 3. Reagents and conditions: (a) (*Z*)-4,4,5,5-tetramethyl-2-(prop-1-enyl)-1,3,2-dioxaborolane, Ru catalyst (Ref. 15), DCM, reflux, 6 h, 24%; (b) 4-(5-chloropyrrolo[2,1-*f*][1,2,4]triazin-4-yloxy)-3-fluoroaniline **15**, $Pd(OAc)_2$, X-Phos, K_3PO_4, *t*-BuOH, 80 °C, 8 h, 18%; (c) 2-(4-fluorophenyl)acetyl isocyanate, DCM, rt, 1 h, 38%; (d) NH_4OAc, $NaCNBH_3$, MeOH, THF, 0 °C, 1 h, 19%.

Table 2. SAR of malonamides/acylureas[a]



Compound	X	Y	R	Met IC_{50} (μM)
6a	CH_2	NH	H	0.78
6b	CH_2	NH		0.04
6c	CH_2	NH		0.13
6d	CH_2	NH		0.14
6e	CH_2	NH		0.03
7a	NH	CH_2		0.05
7b	NH	CH_2		0.15
7c	NH	CH_2		0.04

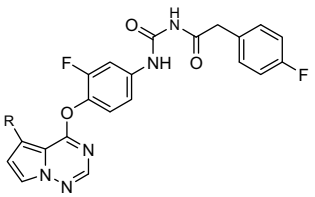
[a] For assay conditions see Ref. 23.

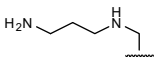
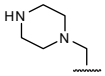
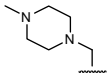
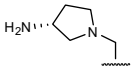
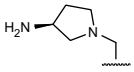
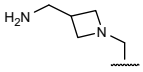
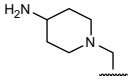
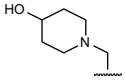
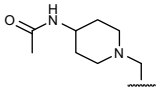
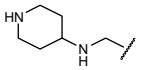
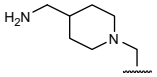
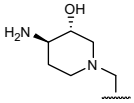
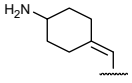
otriazine C-6 position gave dramatically improved results. For example, malonamide **6b** with a piperazine group attached at C-6 of the pyrrolotriazine core demonstrated almost 20-fold improvement in potency in the Met kinase assay (IC_{50} = 0.04 μM). As observed in the amide series, morpholine substitution also improved the potency of Met kinase inhibition, but less efficiently than with a piperazine (compound **6c**, IC_{50} = 0.13 μM). The acyclic 2-dimethylaminoethoxy analog **6d** had similar potency to morpholine compound **6c** (IC_{50} = 0.14 μM). Homologation of the basic amine gave improved potency with the 3-dimethylaminopropoxy analog **6e** affording an IC_{50} of 0.03 μM. Replacement of the malonamide with an acylurea substituent was examined and the SAR was found to parallel that of the malonamide series. Selected compounds from this effort are shown in Table 2. As observed in the malonamide series, the 2-piperazine derivative **7a** and the 3-dimethylamino-propoxy derivative **7c** were almost equipotent, while the 2-dimethylaminoethoxy analog **7b** was inferior in inhibiting Met kinase (IC_{50} = 0.15 μM).

With compounds of increased potency, we examined their ability to inhibit proliferation of the GTL-16 human gastric carcinoma cell line.[18] This cell line contains an amplified gene locus for the Met receptor, resulting in expression of high levels of constitutively-activated Met kinase. Disappointingly, compound **5b** had only marginal activity in the GTL-16 assay (IC_{50} = 0.84 μM) and compounds **5c** and **5d** had IC_{50}'s greater than 1.0 μM. Compounds **5g** and **5k** showed good cellular potency in the GTL-16 assay with IC_{50}'s of 0.27 and 0.17 μM, respectively. Malonamide **6e** also demonstrated good potency with an IC_{50} of 0.27 μM. Finally, acylurea **7c** gave the best cellular activity with an IC_{50} of 0.068 μM. Unfortunately, these compounds were deemed unsuitable for further development as they possessed high rates of metabolism in mouse- and human-liver microsomes (data not shown).

Since the acylurea moiety gave the best cellular potency, it was held constant as substitution at the C-5 position was explored to gain access to the ribose pocket. As shown in Table 3, 5-chloro substituted pyrrolotriazine **10a** was found to have poor activity against Met kinase (IC_{50} > 2.0 μM). Replacement of the 5-chloro substituent with a phenyl ring gave a compound with a greater than 10-fold increase in potency (**10b**, IC_{50} = 0.26 μM). While these initial results were encouraging, the aryl and heteroaryl (data not shown) substituted pyrrolotriazines suffered from poor aqueous solubility (typically <1 μg/mL at pH 6.5). To address this issue, we sought to prepare analogs that would impart improved aqueous solubility in addition to enzyme potency through the incorporation of basic amines. To that end, the conformationally flexible diamine **10c** gave disappointing results (IC_{50} = 1.4 μM), however, the ring constrained analog **10d** gave encouraging activity (IC_{50} = 0.41 μM). Importantly, piperazine **10d** demonstrated dramatically improved aqueous solubility (290 μg/mL, pH 6.5) compared to the previous aryl/heteroaryl analogs. Removal of the piperazine N–H as in *N*-methyl analog **10e** (IC_{50} = 0.86 μM) and morpholine **10f** (IC_{50} = 0.99 μM) resulted in 2-fold loss of potency relative to **10d** suggesting a pivotal role for the amine. Contraction of the piperazine to give a 3-amino pyrrolidine ring was well tolerated (**10g** and **10h**). Absolute configuration of the pyrrolidine amine had little effect on enzyme potency with the (*S*)-enantiomer **10h** giving slightly improved Met kinase inhibitory activity (IC_{50} = 0.19 μM). Further ring contraction to give azetidine **10i** was also tolerated and gave potent Met activity (IC_{50} = 0.18 μM). Encouraged by the activity of primary amine analogs **10h** and **10i**, we explored extension to amino piperidines. Gratifyingly, 4-aminopiperidine **10j** gave dramatically improved enzyme potency (IC_{50} = 0.045 μM). Conversion of the amino to an alcohol (**10k**) or acetamide (**10l**) gave significantly reduced potency (IC_{50} = 0.90 and IC_{50} = 0.44 μM, respectively) again revealing the critical nature of this amine. The orientation of the piperidine substituent was reversed to give analog **10m** and this compound demonstrated a 3-fold reduction in activity relative to **10j**. Extension of the hydrogen bond donor by insertion of a methylene was not well tolerated (**10n**, IC_{50} = 1.0 μM). As observed in our pan-Her program,[11] addition of a second hydrogen bond donor as in amino alcohol **10o** resulted in slightly improved potency (IC_{50} = 0.037 μM) relative to **10j**.[19]

Table 3. SAR of 5-substituted pyrrolotriazines[a]



Compound	R	Met IC_{50} (μM)
10a	Cl	>2.0
10b	Ph	0.26
10c		1.4
10d		0.41
10e		0.86
10f		0.99
10g		0.44
10h		0.19
10i		0.18
10j		0.045
10k		0.90
10l		0.44
10m		0.15
10n		1.0
10o		0.037
17		0.0015

[a] For assay conditions see Ref. 23.

An X-ray crystal structure of compound **10j** complexed with the Met kinase domain demonstrated that pyrrolotriazine inhibitors bind in the ATP-binding site of the enzyme with the activation loop of the protein in an inactive, DFG-out conformation (Fig. 2).[20] The pyrrolotriazine core engages in key hydrogen-bonding interactions with the hinge region of the Met protein. For example, backbone NH of Met1160 donates a hydrogen bond to the pyrrolotriazine N1 nitrogen and backbone carbonyls of Pro1158 and Met1160 accept hydrogen bonds in the form of aromatic C–H interactions from the pyrrolotriazine C-2 and C-7 hydrogens, respectively. The piperidine ring occupies the ribose pocket and forms a key ionic interaction with Asp1164. The central fluorinated phenyl ring is flanked on one side by gatekeeper residue Leu1157 and on the opposite face by Phe1223 (DFG motif) in an edge-face π-stacking interaction. The acylurea portion of **10j** forms an intramolecular hydrogen bond. In addition, the terminal acylurea carbonyl hydrogen bonds with the backbone NH of Asp1222 and the terminal acylurea NH hydrogen bonds with the conserved Glu1127 of helix αC. Finally, the terminal 4-fluorophenyl ring resides in a mostly hydrophobic pocket and interacts with several aromatic residues including Phe1134 and Phe1200.

Based on the X-ray crystal structure of **10j**, we postulated that a more conformationally rigid analog could optimize interactions in the ribose pocket. Indeed, constrained analog **17** gave dramatically improved results with an IC_{50} against Met kinase of 1.5 nM (Table 3). Compound **17** was tested in the GTL-16 gastric carcinoma cell proliferation assay and was determined to have an IC_{50} of 140 nM.[21]

In pharmacokinetic studies, compound **10j** demonstrated high stability in mouse-liver microsome (MLM = 0 nmol/min/mg protein) and mouse hepatocyte (2 pmol/min/10^6 cells) assays. The compound demonstrated low clearance after iv dosing (25 mL/min/kg) and 81% bioavailability from ip administration both at doses of 20 mg/kg, respectively. Low exposures were observed following a 50 mg/kg oral dose (AUC_{0-8h} = 1.1 μM h, C_{max} = 0.78 μM) and the poor PK was attributed to limited absorption. Consistent with this

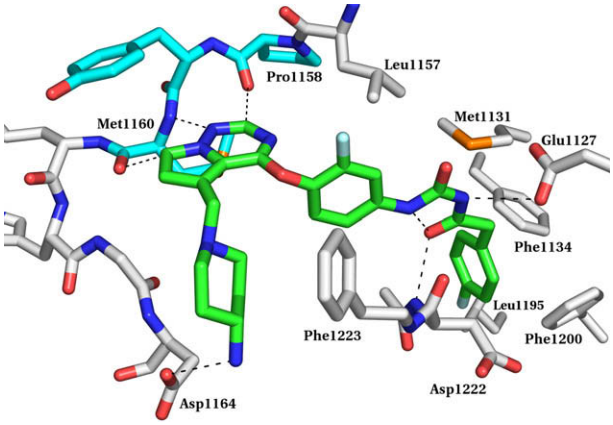


Figure 2. X-ray co-crystal structure of compound **10j** bound to the Met kinase domain (triple mutant). The hinge region of the Met protein is shaded blue.

hypothesis, **10j** demonstrated poor cell permeability in a Caco-2 assay (P_c < 15 nm/s).[22]

In summary, potent C-5 and C-6 substituted pyrrolotriazine inhibitors of Met kinase were identified. Initial studies explored benzamides with solubilizing groups at the pyrrolotriazine C-6 position. Acylureas and malonamides were found to be suitable benzamide replacements and provided compounds with excellent cellular potency. The series suffered from poor PK properties making the compounds unsuitable for in vivo efficacy studies. Further SAR studies to address liabilities of the present series of compounds will be discussed in due course.

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16. Representative characterization data: (a) **7c**: aq sol. (pH 6.5) = 23 μg/mL (crystalline material); ^{1}H NMR (CD_3OD) δ 10.77 (s, 1H), 7.80 (s, 1H), 7.69 (s, 1H), 7.40–7.23 (m, 5H), 7.11–7.02 (m, 3H), 4.15–4.12 (m, 2H), 3.71 (s, 2H), 3.41–3.36 (m, 2H), 2.97 (s, 6H), 2.44 (s, 3H), 2.31–2.22 (m, 2H); HRMS for $C_{27}H_{28}F_2N_6O_4$ $(M+H)^+$ Calcd 539.2218. Found: 539.2218; (b) **10j**, aq sol. (pH 6.5) = 1500 μg/mL (crystalline material); ^{1}H NMR (DMSO-d_6) δ 11.01 (s, 1H), 10.57 (s, 1H), 8.21–8.18 (m, 2H), 7.73 (dd, 1H, J = 12.8, 2.4 Hz), 7.48–7.33 (m, 4H), 7.18–7.11 (m, 3H), 4.57 (m, 1H), 3.69 (s, 2H), 3.44 (s, 2H), 3.59–3.48 (m, 2H), 3.13–3.06 (m, 2H), 2.05–1.98 (m, 2H), 1.71–1.55 (m, 2H); MS(ESI^+) *m/z* 535.29 $(M+H)^+$.
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18. Inhibition of GTL-16 cell growth was assessed by a tetrazolium-based colorimetric assay (490 nm) using a CellTiter 96 Aqueous Non-Radioactive Proliferation Assay kit from Promega. GTL-16 cells were inoculated into 96-well microtiter plates in 0.5% fetal calf serum and incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of drug. At the time of drug treatment, one plate of the cell line was processed using the above kit to represent a measurement of the cell population at the time of drug addition. Following drug treatment, the plates were incubated for an additional 72 h before processing, and measuring cell populations. Each compound was tested at 8 different concentrations in

triplicate, as was the untreated control sample. Growth inhibition of 50% (IC_{50}) is calculated by analysis of the data in Excel that uses a 4 parameter logistic equation with data fitted using the Levenburg Marquardt algorithm.

19. Compound **10j** was tested against a variety of protein kinases. The IC_{50} was determined to be greater than 5 μM for IGF1R, InsR, LCK, VEGFR2, CDK2, PKC, PKA, HER2, and MK2. Moderate activity was observed for TrkA (IC_{50} = 200 nM).
20. The structure was deposited in the PDB as 3C1X.
21. The discrepancy between enzymatic and cell activity is likely attributed to poor cell permeability (Caco-2 P_c < 15 nm/s).
22. For experimental details see Borzilleri, R. M.; Zheng, X.; Qian, L.; Ellis, C.; Cai, Z.-W.; Wautlet, B. S.; Mortillo, S.; Jeyaseelan, R.; Kukral, D. W.; Fura, A.; Kamath, A.; Vyas, V.; Tokarski, J. S.; Barrish, J. C.; Hunt, J. T.; Lombardo, L. J.; Fargnoli, J.; Bhide, R. S. *J. Med. Chem.* **2005**, *48*, 3991.
23. *Met kinase assay:* The entire cytoplasmic domain incorporating the enzymatic function of the human c-Met protein form was expressed as an *n*-terminal Glutathione-*S*-Transferase (GST) recombinant fusion protein in High Five insect cells using the Baculovirus Expression Vector System. Glutathione-*S*-Sepharose (Pharmacia Biotech) was used for rapid affinity purification to high homogeneity. Glutathione (Sigma Chemicals) eluted protein was further purified with buffer exchange using Centricon™ size exclusion filter centrifugal units (Amicon Bioseparations) and then stored at −80 °C. Kinase assays were performed in 96-well microtiter plates using the synthetic polymer poly(Glu4/Tyr) (Sigma Chemicals) as a phosphoacceptor substrate. The c-Met kinase reaction mixture contained 10 ng GST-Met enzyme, 100 μg/mL poly(Glu_4/Tyr), 1 μM ATP and 0.2 μCi [γ-^{33}P]ATP in 50 μl total reaction volume (kinase buffer: 20 mM Tris–HCl, pH 7.0, 1 mM $MnCl_2$, 0.5 mM dithiothreitol and 100 μg/mL BSA). Reaction mixtures were incubated for 60 min at 27 °C and stopped by the addition of cold trichloroacetic acid (TCA) to a 4% final concentration. TCA precipitates were collected onto GF/C unifilter plates (Packard Instrument Co., Meriden, CT) using a Filtermate™ universal harvester (Packard Instrument Co., Meriden, CT). Filters were quantitated using a TopCount 96/384-well liquid scintillation counter (Packard Instrument Co., Meriden, CT). Dose–response curves were generated to determine the concentration required to inhibit 50% of the kinase activity (IC_{50}). Compounds were dissolved to a stock concentration of 10 mM in dimethyl sulfoxide (DMSO) and evaluated at six concentrations each in quadruplicate. The final concentration of DMSO in the assay was 1%. IC_{50} values were derived by non-linear regression analysis.