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The discovery of substituted 4-(3-hydroxyanilino)-quinolines as potent RET kinase inhibitors

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Abstract—Substituted 4-(3-hydroxyanilino)-quinoline compounds, initially identified as small-molecule inhibitors of src family kinases, have been evaluated as potential inhibitors of RET kinase. Three compounds, **38**, **31**, and **40**, had K_i's of 3, 25, and 50 nM in an in vitro kinase assay; while a cell based kinase assay showed K_i's of 300, 100, and 45 nM, respectively. These compounds represent potential new leads for the treatment of medullary and papillary thyroid cancer.

RET (Rearranged in Transcription) is a receptor tyrosine kinase expressed in the thyroid gland, elements of the peripheral and central nervous systems, the kidney, and adrenal glands.[1] Gain-of-function mutations in RET can be sporadic or inherited and can lead to certain types of cancer.[2] Inherited point mutations in RET are associated with multiple endocrine neoplasia 2A (MEN2A), MEN2B, and familial medullary thyroid carcinoma (FMTC).[2] Individuals with these cancer syndromes develop various types of tumors, but the most virulent type of tumor, and common to all three syndromes, is medullary thyroid cancer (MTC).[3] A hallmark of MTC is a constitutively activated RET and we hypothesize that it is this RET signaling that renders these tumors generally unresponsive to chemotherapy and radiation through a mechanism of apoptosis inhibition.[3]

Recent reports have examined the ability of well-known kinase inhibitors to inhibit RET activity as an added activity over their intended target.[4] For example, Pyrazolo-pyrimidines, PP1[5] and PP2,[6] ZD6474 (Vandetanib),[7] and BAY 43-9006 (Sorafenib)[8] demonstrated RET kinase inhibition, and all have some drawbacks for use in chemotherapy. In an in vitro RET kinase assay, PP1 and PP2 have reported IC_{50} values of 80 and 100 nM, respectively.[6,9] However, PP1 and PP2 also inhibit numerous other kinases at similar or lower concentrations than their activity in a RET kinase assay.[9] ZD6474, a targeted VEGFR2 inhibitor, has a reported IC_{50} value of 100 nM for RET and is in clinical trials as an anti-angiogenesis agent used to treat metastatic lung, breast, and colon cancer, and more recently to treat MTC.[7] BAY 43-9006 (Sorafenib) was developed as a Raf/VEGFR2 dual kinase inhibitor, but it also inhibits many other kinases, and has recently also been shown to inhibit RET.[8] However, like ZD6474, clinical trials with Sorafenib have shown some significant side effects including hand–foot skin reactions, fatigue, and hypertension.[10]

Based on the evidence of RET kinase inhibition as a viable approach, we believe the design of a small-molecule inhibitor of RET would be an ideal targeted therapeutic agent with the potential for a complementary inhibition profile with reduced side effects. The strategy for identifying a novel RET kinase inhibitor is summarized in Figure 1. We started with a small set of known kinase inhibitors with a broad range of potent activity across a panel of >35 kinase assays. The idea was to use the active compounds identified from this generic set to provide a template to search the GlaxoSmithKline compound collection for more novel, selective, and

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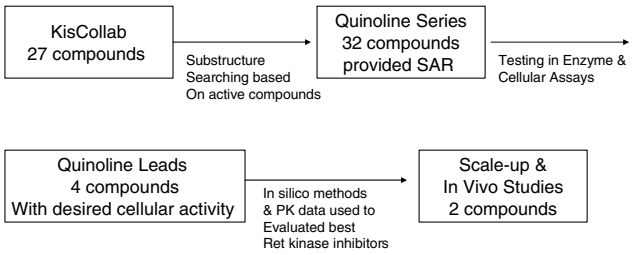


Figure 1. Strategy for rapid identification of a ret kinase inhibitor.

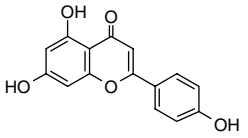
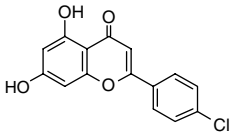
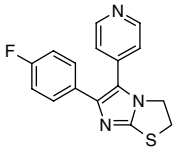
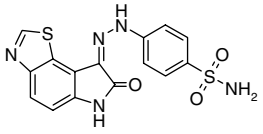
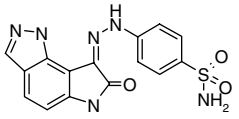
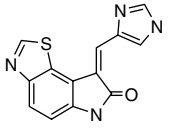
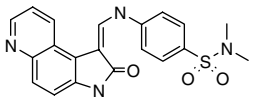
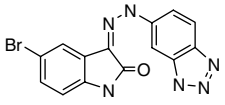
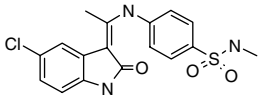
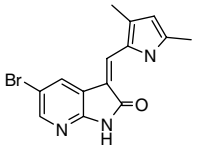
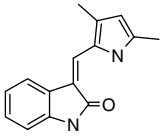
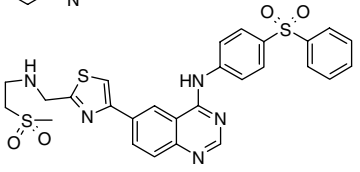
potent RET kinase inhibitors. The next iteration of testing would be done to establish SAR, and the top compounds were selected based on cellular activity. In this report, we will describe the compound selection, synthesis, and SAR that led to the identification of potent RET kinase inhibitors.

The initial set of 27 compounds, named KisCollab shown in Table 1, was chosen based on their potent kinase inhibitory activity. The molecules listed in Table 1 were prepared according to published accounts, where the unreferenced compounds are available commercially.[11–18] Compounds were selected as a set intending to show activity in every kinase in the assay panel of >35 kinases, while individually, providing selective and potent coverage of a few panel members. For example, compound **25** inhibits 2 of 18 kinases tested at a $pIC_{50} > 7.0$. Also compound **13** inhibits 5 of 14 kinases tested at a pIC50 > 7.0, both sets providing mutually exclusive inhibitory coverage of different kinases in the internal assay panel. The KisCollab set was tested at a single concentration in the RET kinase enzyme assay in triplicate to determine active compounds.[19] The data are shown in Table 2 for only the active compounds. This initial screen provided four potential series for further evaluation. Using the substitution patterns on the active compounds and selecting the quinoline as the core scaffold, the GlaxoSmithKline compound collection was searched for more novel and potent RET kinase inhibitors. We chose the quinoline scaffold because there was evidence that we would select drug-like molecules.[20]

Syntheses to generate the compounds in the quinoline series listed in Tables 3–5 are summarized in Schemes 1 and 2. The 6-bromo-4-chloroquinoline starting material shown in Scheme 1 can be obtained from commercial vendors. The introduction of a 6-aryl group is well precedented by using standard Suzuki conditions using 1,4-dioxane and palladium dichloroditriphenyl phosphine on the functionalized core quinoline.[21] The resulting 6-aryl-4-chloroquinoline can then undergo a displacement reaction with a variety of anilines in solvents such as DMSO and acetonitrile to yield the 4-anilino-6-aryl substituted quinolines. 7-Position substituted quinolines were generated using commercially available 4-chloro-7-iodoquinoline using the same methodology as the 6-position analogs.

To synthesize the 6- and 7-position disubstituted compounds, an exemplary synthetic scheme for generating **30** is summarized in Scheme 2. Amine **61** was synthesized

Table 1. KisCollab set

Structure	Compound
	**1**[11]
	**2**[11]
	**3**
	**4**[12]
	**5**[12]
	**6**
	**7**[12]
	**8**[12]
	**9**[12]
	**10**[13]
	**11**[14]
	**12**[15]

(*continued on next page*)

Table 1 (*continued*)

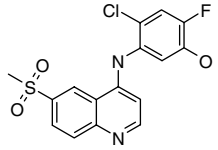
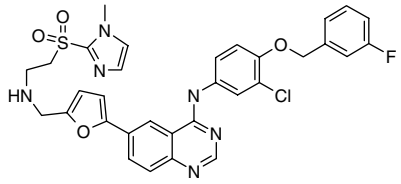
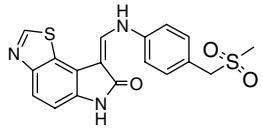
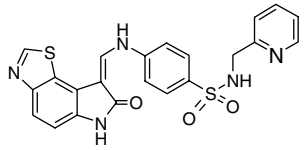
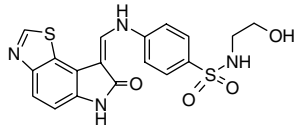
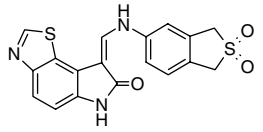
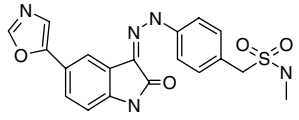
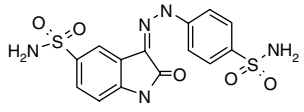
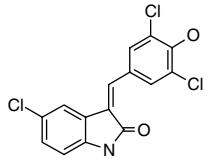
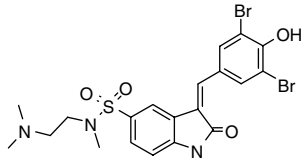
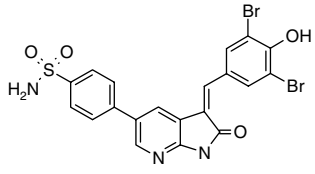
Structure	Compound
	**13**[16]
	**14**[15]
	**15**[12]
	**16**[12]
	**17**[12]
	**18**[12]
	**19**[12]
	**20**[12]
	**21**[17]
	**22**[17]
	**23**[13]

Table 1 (*continued*)

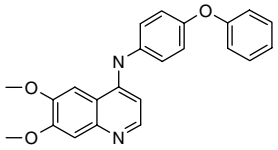
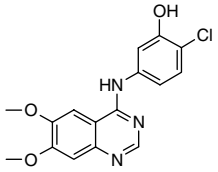
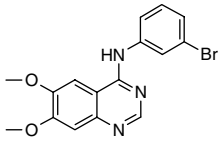
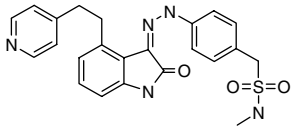
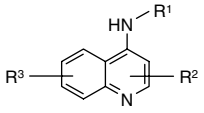
Structure	Compound
	**24**[18]
	**25**[18]
	**26**
	**27**[12]

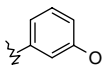
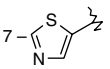
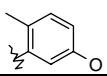
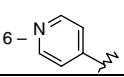
Table 2. Compounds with activity <1500 cpm tested at 5000 nM[a]

Compound	cpm
6	950 (±142)
7	1208 (±130)
15	1312 (±41)
18	428 (±23)
23	355 (±44)
25	298 (±7)
13	460 (±55)

[a] Values of counts per minute (cpm) are means of three experiments, standard deviation is given in parentheses.[19]

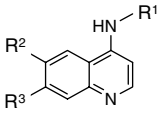
Table 3. Trisubstituted 4-anilinoquinoline

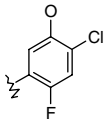
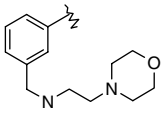
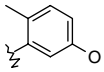
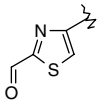
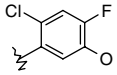
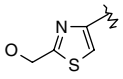
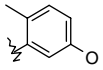
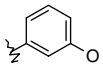
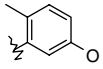
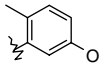
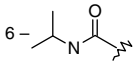
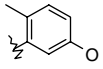
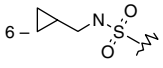
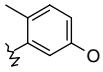
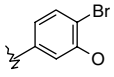
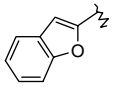


Compound	R^1	R^2	R^3	IC_{50} (nM)
28		3-Cl		100
29		2-CF_3		>1000

from commercially available 2-bromo-1-fluoro-4-nitrobenzene. The fluoro substituent of the nitrobenzene was displaced using sodium sulfoxide followed by reduction of the nitro group using iron/acetic acid. The key intermediate, 6-methylsulfonyl-4-chloro-7-bromoquinoline, was synthesized using well-established procedures from the functionalized aniline.[22–24] Anilines can be

Table 4. 6- or 7-substituted 4-anilinoquinoline



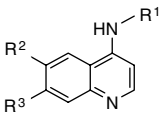
Compound	R^1	R^2	R^3	IC_{50} (nM)
30		H		75
31		H		25
32		H		25
33		H		75
34		H		6
35			H	80
36			H	95
37			H	90
38			H	3
39		H		85

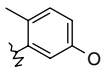
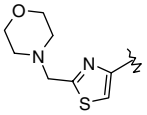
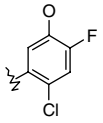
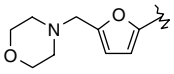
introduced in the 4-position with the aforementioned displacement reaction, followed by a Pd coupling reaction to install the heterocycle in the 7-position. A variety of amines can be used to probe the SAR by generating a diverse set via a reductive amination step.

All compounds tested with a substituent in the 2-position of the 4-anilinoquinoline series showed no activity at the concentrations tested. The 3-position substituents retained activity, but did not offer any advantages in binding properties. Two representative examples are shown in Table 3. In an era of emphasis on ligand efficiency,[25] we opted to keep 2- and 3-position as unsubstituted in our pursuit of a RET kinase inhibitor.

Generally speaking, there appeared to be considerable tolerance for substitution in the 6- and 7-position. Based on experience with other kinase inhibitor drug discovery programs, we believed we could use these as attachment points to examine improved cellular activity.[26] Table 4 includes representative examples of the active quinoline compounds tested where either the 6- or 7-position has a substituent. All compounds tested with 7-position substitution contain the key 3-phenol pharmacophore in the 4-anilino substituent. Additional substitution on the 4-anilino portion is well tolerated as seen in examples **30**, **31**, and **32**. Compounds **33** (IC_{50} value 75 nM) and **32** (IC_{50} value 6 nM) differ by a single methyl substituent in the 2-position of the 4-anilino portion of

Table 5. 4-Anilino-6,7-disubstituted quinoline

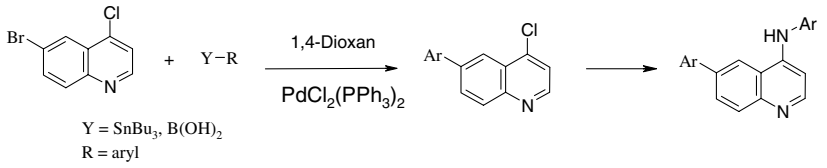


Compound	R^1	R^2	R^3	IC_{50} (nM)
40				34
41		Cl		>1000

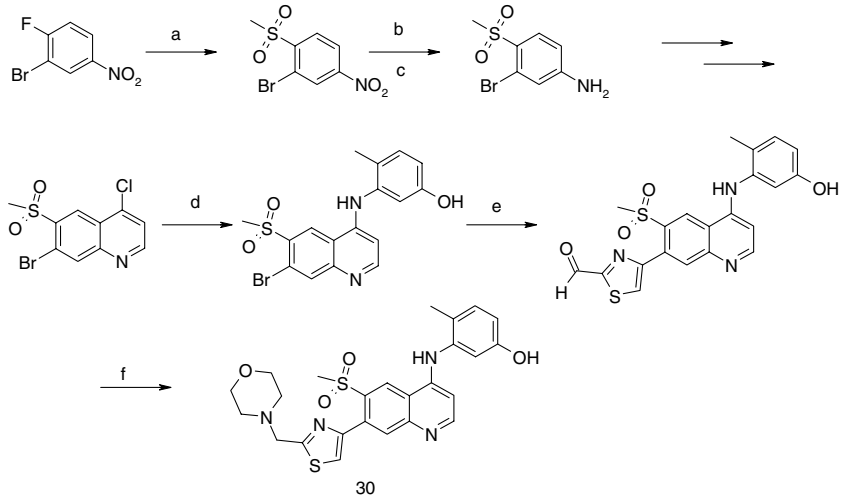
the molecule and show a log difference in potency. Changes in the 6-position, **35**, **36**, and **37**, show no significant difference in potency, all with IC_{50} determinations in the range of ~85 nM. However, the methyl sulfone in the 6-position, as in **38**, results in a log increase in potency compared to compounds **35**, **36**, and **37**.

Finally, Table 5 summarizes examples of compounds where both the 6- and 7-position are simultaneously substituted. While disubstitution of this ring is tolerated, it appears that the methyl sulfone confers a specific added potency advantage when compared with the halogen substituent.

A model of **40** bound into the ATP binding pocket of RET kinase is shown in Figure 2 and compares favorably with the structures published by Phillip P. Knowles and co-workers.[27] The sulfone appears to be within hydrogen bonding distance of the backbone NH and side chain hydroxyl of Ser811 as well as the side chain of Arg878. A sulfonamide at the analogous position significantly increased the potency of CDK2 inhibitors, so it is reasonable to conclude that this interaction is important in boosting affinity.[12] The meta-substituted 4-aniline group likely interacts with the side chains of Asp892 and Lys758 as observed in other quinoline structures.[28] Potency is often achieved by kinase inhibitors that bind in the ATP binding pocket by interacting with the side chain located on the first turn of the helix at the solvent interface. In RET, this residue is a glycine, making the potential interactions minimal. Longer substituents, like the morpholinomethylthiazole, at the 7-position could potentially interact with Glu818, while shorter groups, like the thiophene carbaldehyde, may interact directly with the backbone of the hinge. However, since these positions are very solvent exposed, it is difficult to predict the strength of these types of interactions. There are no cysteine or serine residues close to the aldehyde that could form a covalent bond (Ser411 is ~4 to 5 Å). Substitutions in the 2-position are not accommodated since the quinoline ring packs tightly against the back of the pocket. Small substituents in



Scheme 1. Reagents and conditions for 4,6-substituted quinolines.



Scheme 2. Reagents and conditions: (a) $MeSO_2Na$, DMA; (b) Fe/ACOH; (c) Na_2CO_3/H_2O; (d) 3-amino-4-methylphenol, AcCN, Δ; (e) 4-(tributylstannanyl)-1,3-thiazole-2-carbaldehyde, $Pd(PPh_3)_2Cl_2$, Ag_2O, dioxane; (f) Morpholine, Mol. Sieves, AcOH, $NaBH(AcO)_3$, $NaHCO_3$.

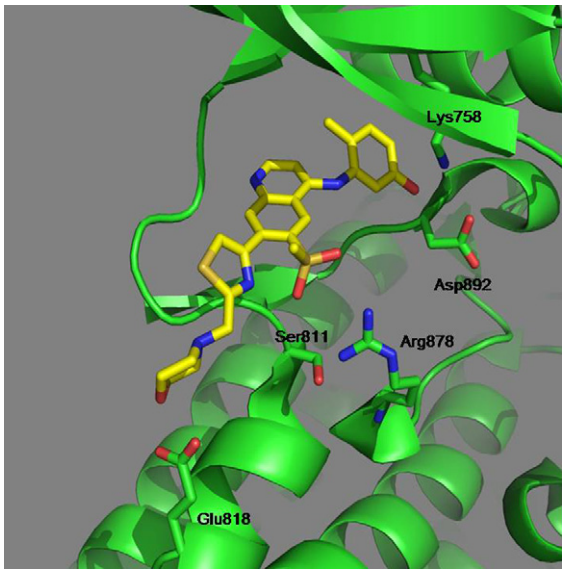


Figure 2. Model of 40 bound in the active site of RET based on the protein coordinates of PDB2IVV. The figure was generated with PYMOL.

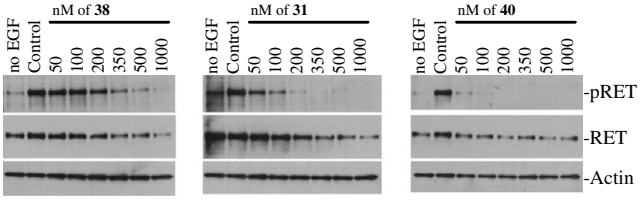


Figure 3. Western blot showing dose–response of RET inhibition by compounds.

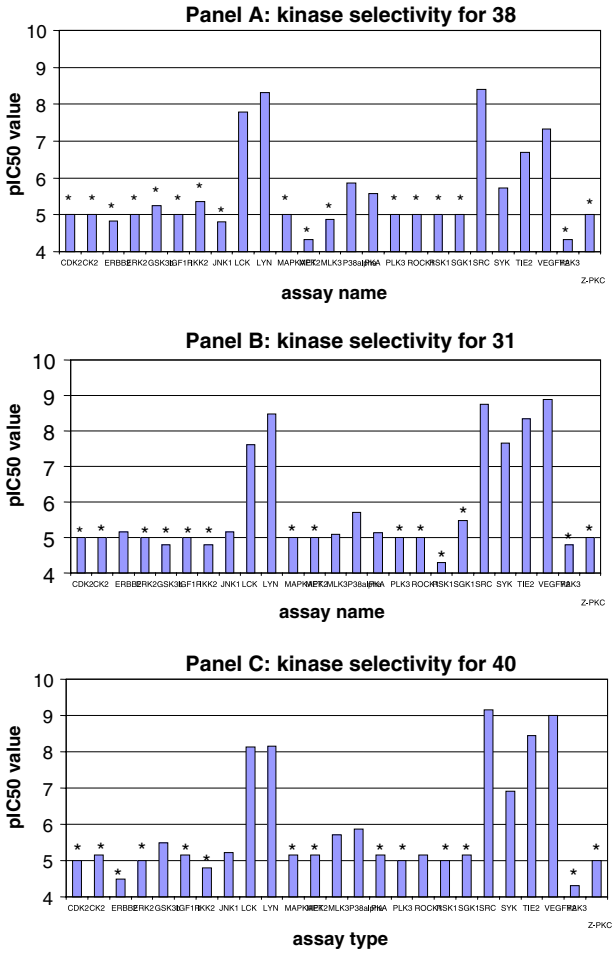


Figure 4. Selectivity profile of representative assays for 38 (panel A), 31 (panel B) and 40 (panel C). *Denotes maximum concentration tested not pIC50 value determined.

the 3-position are tolerated due to the presence of the small gatekeeper residue, valine.

Three compounds (**31**, **38**, **40**) were selected for further evaluation in a ret kinase cellular assay. The data are shown in Figure 3 as a Western blot with increasing concentration of each inhibitor and using antibody detection.[29] The assay uses SK-N-MC cells that express a chimeric EGFR/RET protein (EGFR is extracellular, RET is intracellular). The cells are incubated for 1 h with compound before activating RET by adding 100 ng/mL of EGF for 15 min. The actin levels were used to demonstrate equivalent loading of total protein. The phosphorylated levels of a chimeric EGFR-RET protein were measured to determine the effectiveness of each RET kinase inhibitor's ability to block a signaling event. In a dose responsive manner, each of these three compounds demonstrated potent activity with approximate ED_{50} values in the 45–300 nM range. The protein levels (RET) were compared with the inhibition of phosphorylation (pRET) to be certain that only the signaling was inhibited, as opposed to cytotoxicity or other mechanism that might reduce the phosphorylation levels. It is noteworthy that these compounds were also effective in cellular systems that contained the MEN2A and MEN2B mutations. TT cells are human MTC where RET kinase is constitutively activated by the MEN2A mutation, whereas MZ-CRC-1 cells are human MTC where RET kinase is constitutively activated by the MEN2B mutation. Western blots of TT and MZ-CRC-1 cell lysates treated with 0.5 μM kinase inhibitor for increasing amounts of time from 30 min to 24 h are included in the Supplementary data. The data demonstrate the inhibition of mutant RET phosphorylation in both cell lines compared to a non-treatment control (Supplementary data).

Another important factor to examine was the overall kinase inhibition profile for these effective ret kinase inhibitors. A summary bar graph of a representative set of kinase assays is shown in Figure 4. The inhibition pattern across the different kinase families suggests that these three compounds are quite similar. The other targets inhibited include c-src,[30] lyn, [31] VEGFR2,[32] and TIE2[32] which are also potentially important anticancer targets.

We used a strategy to identify a ret kinase inhibitor that included a small kinase probe set of 27 compounds created by selecting compounds whose inhibition profiles covered a panel of >35 kinases. The seven active compounds found from screening this generic set allowed

us to rapidly identify the quinoline series with the appropriate substitutions that confer potent RET kinase inhibition. Several compounds, **31**, **38**, and **40**, demonstrated potent inhibition of intracellular autophosphorylation in a ligand stimulated chimeric assay system. Finally, these compounds had similar kinase inhibition profiles that included important anticancer targets such as c-src, VEGFR2, TIE2, and lyn. They will be evaluated in more extensive models of efficacy and mechanistic information in tumors driven by RET activity.[33]

Acknowledgments

Kinase selectivity data were supplied by the Molecular Discovery Chemistry, GlaxoSmithKline enzyme screening department. Kinase inhibition data for Table 1 are included in Supplementary data.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.07.104.

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