Current Review of Small Molecule Ret Kinase Inhibitors

W.J. Zuercher, B.J. Turunen and K.E. Lackey*

GlaxoSmithKline, 5 Moore Drive, Research Triangle Park, North Carolina 27709, USA

Abstract: Aberrant Ret kinase activity has been implicated in multiple carcinomas and disorders. Many strategies have been implemented to identify Ret kinase inhibitors. This review details current efforts to discover novel small molecule Ret kinase inhibitiors. Furthermore, we compile a comprehensive list of Ret kinase inhibitors and describe clinical results of advanced assets with Ret inhibitory activity.

Key Words: Ret kinase, protein tyrosine kinase inhibitors, thyroid cancer.

INTRODUCTION

The protein kinase family of enzymes, comprised of over 500 distinct members, serve to catalyze the phosphorylation of amino acid side chains by the transfer of the γ-phosphate of the ATP-Mg^{2+} complex. Kinases are typically classified into two main subfamilies: Protein Tyrosine Kinases (PTK) and Protein Serine/Threonine Kinases (PSTK), based on the amino acid residue they phosphorylate.

Protein kinases are regulators of a number of vital cell functions including: signal transduction, transcriptional regulation, cell motility, and cell division. Signaling processes are highly regulated, often by complex intermeshed pathways where each kinase will itself be regulated by one or more kinases. Consequently, aberrant or inappropriate protein kinase activity can contribute to disease states. Several oncogenes have been shown to encode protein kinases, suggesting that kinases play a role in oncogenesis [1]. Due to their physiological relevance, variety and ubiquity, protein kinases have become one of the most important and widely studied families of enzymes in biochemical and medical research.

The Ret (**RE**arranged during **T**ransfection) kinase is a receptor protein tyrosine kinase. Ret kinase is expressed in many tissues such as the thymus, salivary gland, spleen, lymph node, testes, and elements of the central and peripheral nervous system [2]. Ret kinase is also expressed in tumors derived from neuroblastomas, pheochromocytomas, and medullary thyroid carcinoma (MTC) [3]. Ret kinase knockout mice exhibit severe developmental kidney anomalies and die within 24 hours after birth due to dysfunctional respiratory control secondary to cervical ganglia development anomalies. These mice also lack enteric neurons and have other nervous system anomalies, suggesting that a functional Ret kinase protein product is required during development [4].

Aberrant Ret kinase expression is associated with multiple endocrine neoplasia (MEN2A and MEN2B), familial medullary thyroid carcinoma (FMT C), papillary thyroid carcinoma (PTC) and Hirschsprung's disease (HSCR). MEN2A is a cancer syndrome resulting from mutation in the extracellular cysteine-rich domain and prompting constitutive activation of the tyrosine kinase. Individuals with this mutation may develop medullary thyroid carcinoma (MTC), parathyroid hyperplasia, and pheochromocytoma. MEN2B is caused by a M918T mutation which changes the tyrosine kinase specificity. MEN2B is similar to MEN2A, but lacks the parathyroid hyperplasia and also leads to development of numerous mucosal ganglia of the lips, tongue, and intestinal tract. Chromosomal rearrangements linking the promoter and N-terminal domains, or unrelated gene(s) to the C-terminus of Ret kinase, result in constitutively activated chimeric forms of the receptor (Ret/PTC) and are thought to be tumor initiating events in PTC [5]. PTC's encompass about 80% of all thyroid carcinomas. Indeed, the strong disease association coupled with the clinical success of kinase inhibition implicate Ret kinase as a valid target for drug intervention.

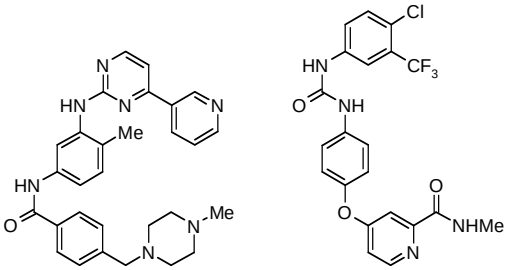


Fig. (1). Imatinib (**1**) and Sorafenib (**2**).

The significance of the Ret oncogene has prompted efforts towards the discovery of small molecule Ret inhibitors and previous reviews have described many of the resulting inhibitors [6, 7]. Frequently, Ret inhibitor discovery has been the fortuitous result of broad selectivity screening or cross screening of known kinase templates. Notable examples of

*Address correspondence to this author at the GlaxoSmithKline, 5 Moore Drive, Research Triangle Park, North Carolina 27709;
E-mail: karen.e.lackey@gsk.com

1389-5575/10 $55.00+.00

this includes imatinib (Gleevac, **1**) and sorafenib (Nexavar, **2**) which are used clinically for chronic myeloid leukemia and renal carcinoma, respectively [8, 9]. While both imatinib and sorafenib were originally designed to target a particular kinase, subsequent studies revealed an extended inhibition profile. Provided the additional components to the kinase inhibition profile do not confer toxicity, this strategy can expand the use of existing kinase drugs to smaller patient populations such as thyroid carcinoma. While this approach has been effective in identifying Ret inhibitors, additional means to this end are available, including focused compound set screening and structure-based design or optimization. These latter, more directed approaches may offer advantages such as efficiency, cost effectiveness, and access to different kinase selectivity profiles. Herein we provide a recent review of small molecule Ret kinase inhibitors and describe the strategies employed to identify these compounds. In addition, we review results from clinical studies employing assets with Ret kinase inhibitory activity.

TARGETED Ret KINASE EFFORTS

Lackey and coworkers described focused compound set screening in the identification of Ret inhibitors [10]. First, a screening set was defined comprising 27 templates having activity across a panel of over 35 kinase assays. The activity of the set spanned the entire panel of kinases, but individual compounds provided potent and selective activity towards a small number of kinases. This screening set was evaluated for Ret inhibition, and an active quinoline template then served as the basis for substructure searches of a larger compound collection. Subsequent screening led to the quinoline (Table **1**, compound **3**) which is a potent Ret inhibitor (45 nM in a cell-based kinase assay). Kinase selectivity measurements revealed potent inhibition of c-src, lyn, VEGFR2, and TIE2. The study demonstrated the feasibility and efficiency of screening small, focused compound sets in the discovery of potent and moderately selective Ret inhibitors. This approach is not limited to Ret kinase and should be applicable to the identification of small molecule inhibitors for other kinases, many of which are validated oncology targets.

Structure-based design has been effectively utilized in the development of receptor tyrosine kinase inhibitors [1]. The potential for structure-based Ret inhibitor design stems from the availability of Ret cocrystal structures incorporating the small molecule inhibitors PP1 (PDB code 2IVV) and ZD6474/Zactima (PDB code 2IVU) [11]. Dallavalle, Lanzi, and coworkers employed these crystal structures in docking studies which enabled the activity optimization of RPI-1 to arrive at a structurally-rigidified β-carbolinone (Table 1, compound **4**) with a binding mode distinct from the starting inhibitor [12].

OPPORTUNISTIC Ret KINASE APPROACH

While there are few reported small molecule Ret kinase inhibitors identified via the aforementioned targeted approaches, a number of high affinity Ret inhibitors have been discovered opportunistically by cross screening of compounds known to inhibit other kinases (Tables 2 and 3). This screening has resulted in high affinity Ret inhibitors.

T wo recent examples of potent Ret inhibitors identified from broad screening campaigns evaluating promiscuous kinase templates are the pyrazole benzimidazoles **5** and **6** (Table **2**) [13, 14]. Furthermore, cross screening of compounds **7**-**10** with known kinase targets, revealed potent inhibitory activity *versus* Ret [15-18]. Kinase inhibitor **11** has also been reported to have nanomolar potency although the method of identification was not described [19].

In a recent paper by Ambit researchers, data was collected for kinase activity in their phage-display binding format for 38 advanced (preclinical and be yond) kinase inhibitors across a panel of 317 kinases including Ret and the mutant M918T Ret [20]. We selected nine examples from these data where Ret activity below 500 nM was observed and listed them in Table **3** along with their primary intended targets. Perhaps it is unsurprising that VEGFR family tyrosine kinase inhibitors would not be selective over Ret kinase, given the kinase similarity suggested by the Sugen human kinome branching proximity [21]. Less obvious is the finding that an Aurora or p38 inhibitor would also target Ret kinase activity. For the examples listed in Table **2**, potency values appear nearly equivalent when comparing the inhibition of Ret kinase with the inhibition of the Ret M918T mutant (MEN2B-like). Despite the similarity of the quinazoline

Table 1. Ret Inhibitors Identified *via* Targeted Approach

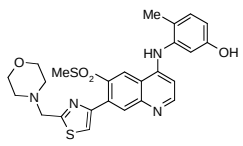
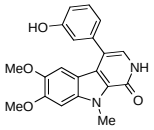
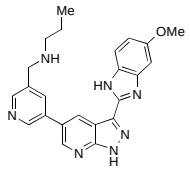
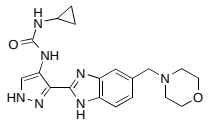
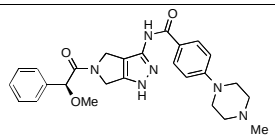
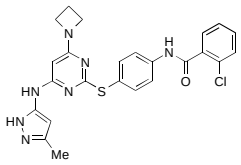
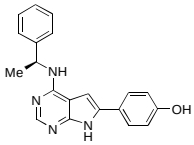
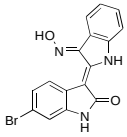
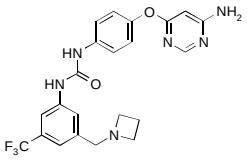
Compound	Structure	Ret IC_{50}	ID Method
3		35 nM	Focused screening
4		90 nM	Structure based design

Table 2. Ret Inhibitors Identified *via* Opportunistic Approach

Compound	Structure	Ret IC_{50}	ID Method
5		36 nM	Broad screening
6		< 100 nM	Broad screening
7 PHA-739358		31 nM	Cross screening of Aurora inhibitor
8		8 nM	Cross screening of Aurora inhibitor
9 PKI-166		4 nM	Cross screening of EGFR inhibitor
10		50 nM	Cross screening of GSK/CDK inhibitor
11		80 nM	Unknown

structures and potencies of the EGFR TK inhibitors, gefinitib and CI1033 showed no Ret kinase inhibition detected at the 10 μM concentration tested.

Clinical Stage Assets with Ret Kinase Inhibition Activity

Several advanced clinical kinase assets were specifically evaluated for their Ret-mediated activity in cancer. The structures of the compounds with their reported Ret kinase activity are shown in Table **3**. All of these compounds potently inhibit a member of the VEGFR family of protein kinases.

SU5416 (**21**) was originally designed to target VEGFR tyrosine kinase and is presently used in clinical trials for VEGFR, c-kit, and FLT-3 receptors TKs [22]. Mologni and co-workers reported that SU5416 blocks Ret kinase activity and affects cell proliferation as expected [23]. According to this work, the *in vitro* Ret kinase activity of SU5416 using an ELISA-based kinase activity assay afforded an IC_{50} value of 170 nM in sharp contract to the fivefold higher value reported by Kim and co-workers [24]. Inhibition of Ret V840M could not be detected up to the maximum concentration tested of 30 μM. Experiments were done with varying

Table 3. Late Stage Assets with High Affinity Ret Inhibition Identified by Ambit Screening

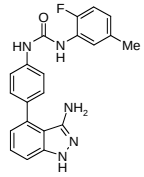
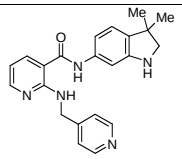
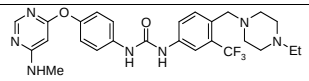
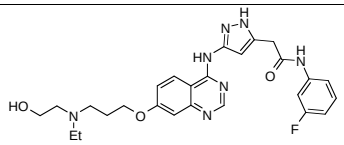
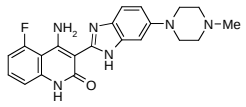
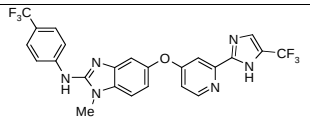
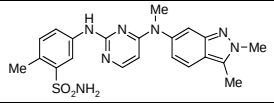
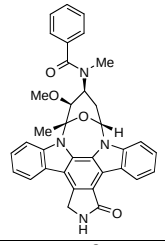
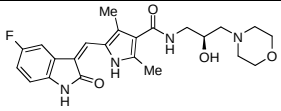
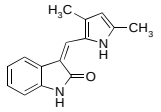
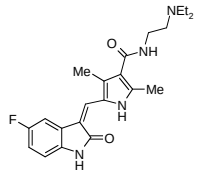
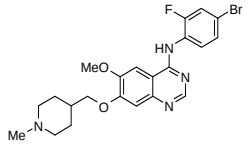
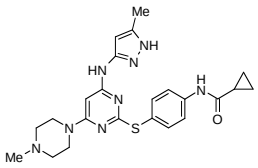
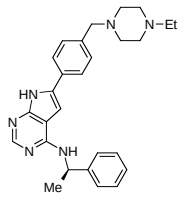
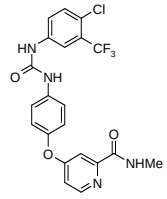
Compound	Structure	Primary Target(s)	Status	Ret IC_{50} (nM)	Ret M918T IC_{50} (nM)
12 ABT-869		FLT3, CSF1R, VEGFR2	I	100	120
13 AMG-706		VEGFR2, FLT1/4, KIT	III	14	20
14 AST-487		FLT3, KIT	Preclin	17	4.5
15 AZD-1152HQPA		AURKB	I	80	120
16 CHIR-258		FLT3, FGFR3	I	71	47
17 CHIR-265		BRAF, VEGFR2	I	150	72
18 GW786034		VEGFR2, FLT1/4	III	310	270
19 PKC-412		FLT3, KIT	III	350	130
20 SU-14813		VEGFR2, FLT1/3, PDGFRB, KIT	II	37	24

Table 4. Clinical Stage Assets with Ret Kinase Inhibition Activity

Compound	Structure	Primary Target(s)	Ret IC_{50} (nM)
21 SU5416		VEGFR	170

(Table 4). Contd…..

Compound	Structure	Primary Target(s)	Ret IC_{50} (nM)
22 Sunitinib Sutent		VEGFR, PDGFR	224
23 Vandetanib		VEGFR2	NA
24 VX-680/ MK-0457		Aurora A/B	43
25 AEE788		EGFR, VEGFR	740
2 Sorafenib		VEGFR2, BRAF	13

SU5416 (21)

levels of ATP to show that SU5416 was competitive with ATP. The homology docking model of the Ret TK domain was built as the active form using the phosphorylated active insulin receptor kinase domain crystal structure. The model does support the observation that SU5416 binds in the ATP binding site and when the gatekeeper residue is mutated, SU5416 would be expected to clash with MET-804. To further support the biochemical evidence of Ret inhibition, the authors evaluated SU5416 in cellular assays and showed an IC_{50} value of 5 μM for inhibiting Ret/PTC2 phosphorylation which was in good agreement with an IC_{50} value of 7.9 μM in a Ba/PTC stably transfected line. As with SU11248 discussed below, studies to demonstrate the reversion of transformation of NIH3T3 cells expressing Ret/PTC after treatment with SU5416 showed the expected Ret mediated effect albeit at 10 μM. (note: in the Kim paper the max concentration tested of 5416 was 1 μM which explains why they did not see phosphorylation of STAT3 or reversion of the transformed state of the cells with SU5416 in their studies) Finally, the same mechanism based correlation between Ret inhibition and efficacy in TPC-1 cells (IC_{50} 2.7 μM) was shown using 10 μM of SU5416 and measuring important downstream markers of the Ret signaling pathway. The authors conclude that the inhibition of proliferation is due to a G1 arrest, and not apoptosis. *In vivo* modulation of Ret autophosphorylation was measured in a mouse xenograft model using NIH-PTC cells. SU5416 (50 mg/kg, i.p.) was given to the animals after tumors were established in the flank, and biopsies taken after 6 hrs post treatment confirmed inhibition of Ret autophosphorylation with no effect on protein levels. Despite the favorable pharmacokinetic properties and other kinase inhibition activities of SU5416 in the clinical setting, the authors suggest that it is a good lead structure for new, more selective Ret inhibitors.

SU11248 (22, Sunitinib, Sutent)

SU11248 (**22**) was originally discovered and developed as an orally available VEGFR and PDGFR tyrosine kinase inhibitor [25]. It was found highly effective in many pre-

clinical models of efficacy and mechanism of action studies for these and other primary targets. More recently, SU11248 was evaluated for its potential usefulness in Ret/PTC-positive thyroid cancers [26]. As evaluated in an *in vitro* Ret/PTC3 assay measuring the inhibition of phosphorylation of a peptide substrate, SU11248 was reported to have an IC_{50} value of 224 nM. Using a transiently transfected NIH3T3 cell system, SU11248 was confirmed to have intracellular inhibition of Ret/PTC autophosphorylation plus blocked transactivation of STAT3 where activation has been linked with cell transformation. Since Ret/PTC transfection leads to enhanced phosphorylation of p42/44 MAPK, SU11248 was evaluated and found to decrease phosphorylation levels at the two concentrations tested, 0.5 and 1.0 μM. These data were supported by observing the reversion of morphological changes after treating NIH3T3 fibroblasts transformed by Ret/PTC expression with SU11248. No such effect was observed when Ras-transformed lines were used, suggesting that SU11248 does not operate by inhibiting downstream signaling events. Finally, these effects were correlated to a dose-dependent inhibition of TPC-1 (endogenous Ret/PTC1 expression) cell proliferation.

The authors suggest several advantages for SU11248 over other agents with potent Ret kinase activity. The pharmacokinetic properties measured in Phase I clinical trials suggest the appropriate plasma levels (~250 nM) can be achieved after oral dosing with low toxicity [27]. While the anti-angiogenic properties may confer an efficacy advantage for the patients, most of the clinical kinase inhibitors with Ret kinase activity also have anti-angiogenic biological targets in their inhibition profile.

ZD6474 (23, Vandetanib)

ZD6474 was originally developed as a small molecule inhibitor of VEGFR2 tyrosine kinase, but it also effectively inhibits Ret and EGFR tyrosine kinases [28-30]. Since ZD6474 is an inhibitor of many mutant forms of Ret, with the exception of V804M, Knowles and co-workers studied the protein-ligand interactions of the Ret kinase domain in wild type and mutant crystal structures to devise strategies to overcome resistance [11]. They provided a structural rationale for the observed wild type and mutant Ret inhibition profile of ZD6474 and defined models which might be utilized for the design of new inhibitors.

A research team developed a Drosophila model for MEN2A and MEN2B diseases and demonstrated a Ret-mediated efficacious effect by treatment with ZD6474 [31]. They demonstrated that ZD6474 did not have any effects through EGFR or downstream components of the ras pathway. Three drosophila Ret (dRet) isoforms were created to mimic wild type, MEN2A-like and MEN2B-like Ret to form three transgenic flies with characterized visible eye defects. Treatment of the whole organism easily detected effective blocking of Ret-mediated activities while rapidly assessing toxicity. The work showed that flies fed with ZD6474 had effective restoration of the eye in a Ret-driven system, but higher expression levels created by different gene insertion points led to a diminished rescue response. It is very important to note that ZD6474 treatment did not affect the viability of the drosophila model.

Clinical trial information has been reported for medullary thyroid carcinoma for two doses, 300 mg/day, which is the maximum tolerated dose, and 100 mg/day [32]. Interim reported results showed partial responses were observed at the high dose (3 out of 15 patients), and additional results are pending.

VX-680/MK-0457 (24)

VX-680 was originally designed and developed as an anti-mitotic agent targeting Aurora A/B kinase with mechanistically consistent effects on mitotic histone H3 phosphorylation, the downstream substrates of both Aurora A and Aurora B [33]. VX-680 is a very potent Aurora kinase inhibitor with K_i values between 0.6-18 nM for Aurora A, B, and C with activity on flt3 and abl kinases observed as well [34]. Consequently, VX-680 was found to be a potent, cell permeable inhibitor of the Aurora kinases toxic to human cancer cells with anti-tumor activity in multiple xenograft models. Based on these pre-clinical results, VX-680 was progressed to human clinical trials where encouraging efficacy was observed for CML (chronic myeloid leukemia) and ALL (acute lymphocytic leukemia) [35]. More recently, a single compound patent application published claiming VX-680 as an agent to be used in the treatment of Ret mediated diseases with an IC_{50} value of 43 nM [36]. Given the promising results in the clinic for other components of VX-680's kinase profile, the consequences of its Ret inhibition are eagerly anticipated.

AEE788 (25)

Originally developed as an EGFR and VEGFR tyrosine kinase inhibitor, and an optimized derivative of PKI-166, AEE788 demonstrated mechanism consistent efficacy in preclinical antitumor and antiangiogenic models driven by these families of cancer targets [37]. The researchers showed a correlation between plasma/tissue drug levels, inhibition of target phosphorylation and blocking tumor growth. In an *in vitro* panel of kinase assays, activity was reported to be 0.74 μM for R*et al*ong with the following kinases with IC_{50} values below 1 μM: ErbB family, VEGFR family, PDGFR-β, c-abl, c-src, c-kit, and c-fms. A very interesting functional interaction occurs between EGFR and Ret in Ret/PTC-induced cell proliferation. Activated Ret/PTC occurs by constitutive dimerization via the intracellular components, and lacks the extracellular and transmembrane domains. The authors showed a compelling link between ligand-induced EGFR activation resulting in phosphorylation of Ret [38]. PKI-166 had no measurable Ret inhibition, but could inhibit Ret/PTC mediated cell growth through the EGFR TK inhibition. AEE788 inhibited the Ret-induced growth well below the moderate IC_{50} value, presumably due to the combined effects of EGFR and Ret kinase inhibition. AEE788 was more effective at modulating Ret-induced cell growth than PKI-166, suggesting that the dual EGFR/Ret activity conferred some advantage. These data provide evidence for designing multiple kinase inhibition profiles into drugs for more effective clinical agents.

Sorafenib (2)

While sorafenib was originally designed as a Raf inhibitor, [39] it is actually a more potent inhibitor of Ret kinase

activity (IC_{50} 5.9 nM) and the Ret V804M mutant often associated with resistance to small molecules (IC_{50} 7.9 nM) [40]. The *in vitro* studies were done using catalytically active kinase domain and measuring the inhibition of phorphorylation of an immobilized Ret-derived peptide substrate. The values are in close agreement with the Ambit values shown in Table **3**. Plaza-Menacho and co-workers proposed a ligand-protein interaction in the ATP binding site with an inactive DGF^{out} conformation determined by correlation with Gleevec/c-abl, sorafenib/b-Raf, and the unphosphorylated state of Ret. The authors offer a plausible advantage of sorafenib Ret kinase inhibition which is equipotent on oncogenic Ret and Ret^{V804M} over that observed for ZD6474, which is inactive in cells with the V804M mutations, based on the interactions with the gate keeper residue often associated with developing resistance [41]. Tumor cell lines with wild type or a mutant Ret (MTC-TT, TPC1, MZ-CRC-1 and TGW-1) were used to assess the Ret mediated signal inhibition of sorafenib (both references). For consistency, HEK293 cells were transfected with the same Ret forms, and in all cases (tumor and transfected lines) the Ret activity was inhibited by 90 minute treatment with sorafenib in a concentration range between 15 and 150 nM and was accompanied by a decrease in ERK1/2 phosphorylation. It is important to note that experiments were done to show that the inhibition of downstream ERK1/2 phosphorylation was from Ret inhibition and not via the Raf kinase inhibitory activity. Furthermore, after exposures of 48 hours, sorafenib inhibited cell proliferation through a signal inhibition mechanism and reduced Ret protein levels via internalization from the plasma membrane for lysosomal degradation.

A response was obtained in a spontaneous, advanced MTC patient with sorafenib in combination with tipifarnib, a farnesyltransferase inhibitor (FTI) [42]. The farnesyltransferase enzyme is important for ras mediated signaling and inhibition alone has not proven as broadly effective in clinical trials despite very elegant preclinical studies showing mechanism based efficacy [43, 44]. Sorafenib (twice daily, 28 d) plus tipifarnib (twice daily, 21 d) were administered to the patient after surgical resections and failed chemotherapy. Tumor shrinkage was confirmed by Response Evaluation Criteria in Solid Tumors (RECIST) criteria to be 36% by 8 weeks and 46% by 10 months. The two markers that were used and tracked with the imaging of tumor reduction were carcinoembryonic antigen (CEA) and calcitonin. Tumor tissue resected prior to sorafenib/tipifarnib treatment was analyzed for Ret mutations and a unique 6-bp deletion mutation (TGTGCG) in Exon 11 was discovered. The authors hypothesize that this mutation would result in a change in the extracellular cysteine-rich region, potentially resulting in constitutive activation of the Ret kinase. It is also possible that the RAS/MAPK pathway is required for Ret-induced transformation, and inhibiting multiple points in the pathway is a significantly more effective way to block the signal in solid tumors [45].

CONCLUSION

Clinical trial data provide evidence that Ret inhibition could have therapeutic value for thyroid cancer. These data come from using drug candidates that were designed for a different kinase target but included Ret inhibition in the overall profile. This approach is a straightforward means to expand the therapeutic potential of small molecule kinase inhibitors. However, despite these promising results, the full therapeutic potential of Ret inhibition has yet to be realized. In working toward this end, it is expected that Ret *directed* inhibitors will have significant utility. Several approaches are available for the identification of additional small molecule inhibitors targeting Ret kinase, including focused screening, structure-guided optimization and structure-based de novo design of new templates.

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