

REVIEW

How thyroid tumors start and why it matters: kinase mutants as targets for solid cancer pharmacotherapy

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Abstract

Treatment of patients with thyroid cancer is usually successful, and most patients are cured of the disease. However, we do not have effective therapies for patients with invasive or metastatic thyroid cancer if the disease is not surgically resectable and does not concentrate radioiodine. Conventional external beam radiotherapy and chemotherapy are of marginal benefit. In other types of cancer, new therapies are being developed that take advantage of our knowledge of cancer pathogenesis to interfere with the activity of specific oncoproteins believed to be important in disease causation. Because these approaches are being considered for thyroid cancer, I will briefly describe in this review examples of recent breakthroughs in medical therapy of certain hematological malignancies and some solid tumors using drugs that work

in this fashion, focusing in particular on compounds that block the enzymatic activity of specific tyrosine kinase oncoproteins. It should be noted, however, that cancers commonly harbor mutations or other disruptions of many genes, each of which could conceivably play a role in disease pathogenesis. This makes the choice of molecular target a difficult and critical decision if these approaches are to succeed. Here I will argue that priority should be given to blocking the function of oncoproteins activated early in tumor development. We have a fairly good understanding of the genetic changes involved in thyroid cancer initiation, and hence these cancers may prove to be particularly well suited for oncoprotein-specific therapies.

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The emerging field of targeted cancer therapies

Targeted cancer therapies attempt to disrupt pathways that are inappropriately activated in cancer cells while leaving normal cells relatively unscathed. Although we still have limited clinical experience with these agents, there has been enough time to draw some useful conclusions. The most notable initial success has been with Gleevec (imatinib) in Philadelphia chromosome/*BCR-ABL* (+) chronic myelogenous leukemia (CML) (Druker *et al.* 2001). The *BCR-ABL* translocation results in expression of a *BCR-ABL* fusion protein, leading to constitutive activation of abl kinase activity and unregulated proliferation and survival of a primitive hematopoietic cell clone. Imatinib is a relatively selective inhibitor of abl kinase, and induces high rates of remission in patients with CML. Imatinib is also effective in patients with gastrointestinal stromal tumors, which are associated with activating

mutations of the tyrosine kinase (TK) receptor *C-KIT* (Heinrich *et al.* 2000, Joensuu *et al.* 2001), and for metastatic dermatofibrosarcoma protuberans, believed to be dependent on the activity of the platelet-derived growth factor receptor kinase. In both cases, imatinib has been shown to have potent inhibitory activity on the activity of these receptor kinases, likely explaining the beneficial effects in patients with these conditions. Another success story in targeted therapies is that of patients with acute promyelocytic leukemia associated with a chromosomal translocation leading to expression of the *PML-RAR α* fusion protein, leading to overexpression of the retinoic acid receptor α . A significant proportion of these patients respond well to treatment with all-*trans* retinoic acid (ATRA) (Fenaux *et al.* 2000). Despite these favorable results, numerous other compounds have failed at early phases of clinical development, dampening some of the initial enthusiasm with targeted therapies

(Katsnelson 2004). Some of the difficulties encountered likely stemmed from inappropriate choice of molecular targets, undesirable side effects or problems with trial design.

Target selection: role of tumor-initiating events

A notable feature of some of the early successes in targeted therapies is that compounds such as imatinib and ATRA inhibit the activity of gene products believed to be early events in tumorigenesis. Thus, *BCR-ABL* and *PML-RAR α* are thought to occur early in leukemogenesis (Brown *et al.* 1997). Their central role in leukemia development is demonstrated by their ability to induce myeloid proliferation when selectively overexpressed in myelocytes of transgenic mice. Recent studies in patients with CML who developed resistance to treatment with the abl kinase inhibitor imatinib showed that many of these had selected and/or acquired tumor clones with point mutations coding for substitutions within the imatinib-binding pocket of BCR-ABL, which interfered with binding and conferred resistance to the antagonist (Hochhaus *et al.* 2002, Shah *et al.* 2002). This illustrates two important points. First, that when placed under selective pressure, CML has potential for recurrence by reactivating the same pathway involved in tumor development (i.e. abl kinase activation) through acquisition of new somatic mutations. Secondly, that the abl kinase is indeed a major driving influence required for survival and expansion of the tumor clone. Because of these considerations, it is possible that oncoproteins implicated in tumor initiation may be particularly well suited as targets for development of inhibitory compounds. This is not to say that interfering with initiation events is all that will be needed, since it may ultimately be necessary to use combination therapies to block multiple pathways. We propose that oncoproteins activated by tumor-initiating mutations are likely to remain essential drivers of tumor expansion even after accumulation of numerous additional genetic changes. This premise, which has major implications for the potential effectiveness of targeted therapies, remains to be proven as a general principle, and in specific tumor types.

Tumor-initiation events in thyroid cancer: the *RET/PTC* oncogenes

Thyroid cancers stand out among solid tumors because many of the tumor-initiating genetic events have been identified. Notable among them are the *RET/PTC* oncogenes, which are believed to play a causative role in the pathogenesis of a significant proportion of papillary carcinomas of the thyroid (PTC), in particular those arising after radiation exposure, and in pediatric cancers. Chromosomal rearrangements linking the promoter and N-terminal domains of unrelated gene/s to the C-terminal

fragment of *RET* result in the aberrant production of a chimeric form of the receptor in thyroid cells that is constitutively active (Santoro *et al.* 2002). Several forms have been identified that differ according to the 5' partner gene involved in the rearrangement, with *RET/PTC1* and *RET/PTC3* being the most common. *RET/PTC1* is formed by a paracentric inversion of the long arm of chromosome 10 leading to fusion of *RET* with a gene named *H4/D10S170*. *RET/PTC3* is also a result of an intrachromosomal rearrangement and is formed by fusion with the *RFG/ELE1* gene. Multiple lines of evidence point to *RET/PTC* as one of the key first steps in thyroid cancer pathogenesis (Fig. 1): (i) Thyroid-specific overexpression of either *RET/PTC1* (Jhiang *et al.* 1996, Santoro *et al.* 1996) or *RET/PTC3* (Powell *et al.* 1998) in transgenic mice leads to development of tumors with histological features consistent with papillary thyroid carcinoma, indicating that these oncoproteins can recreate the disease in an animal model. (ii) There is a high prevalence of *RET/PTC* expression in occult or microscopic PTC (Viglietto *et al.* 1995, Sugg *et al.* 1998, Corvi *et al.* 2001), pointing to the activation of this oncogene at early stages of tumor development. (iii) Exposure of cell lines (Ito *et al.* 1993) and fetal thyroid explants (Mizuno *et al.* 1997) to ionizing radiation results in expression of *RET/PTC* within hours, supporting a direct role for radiation in the illegitimate recombination of *RET*. (iv) The breakpoints in the *RET* and *ELE1/RFG* genes resulting in the *RET/PTC3* rearrangements of radiation-induced pediatric thyroid cancers from Chernobyl are consistent with direct double-strand DNA break resulting in illegitimate reciprocal recombination (Nikiforov *et al.* 1999). Moreover, the *H4* and *RET* genes, although lying at a considerable linear distance from each other within chromosome 10, are spatially juxtaposed during interphase in thyroid cells and presumably present a target for simultaneous double-strand breaks in each gene after ionizing radiation, thus giving rise to the *RET/PTC1* rearrangement (Nikiforova *et al.* 2000). These data provide evidence that ionizing radiation, the major risk factor for development of papillary thyroid cancer, can directly induce *RET* recombination events, and link environmental events to tumor initiation through this genetic pathway.

Mapping of signaling pathways used by *RET/PTC* to induce thyroid cell transformation provides clues for discovery of new thyroid oncogene

The *RET/PTC* rearrangements result in illegitimate expression of chimeric proteins consisting of an N-terminal fragment donated by one of the heterologous gene partners, fused to the intracellular TK domain of *RET*. The fusion proteins dimerize in a ligand-independent manner due to motifs present in the N-terminal domains. This results in

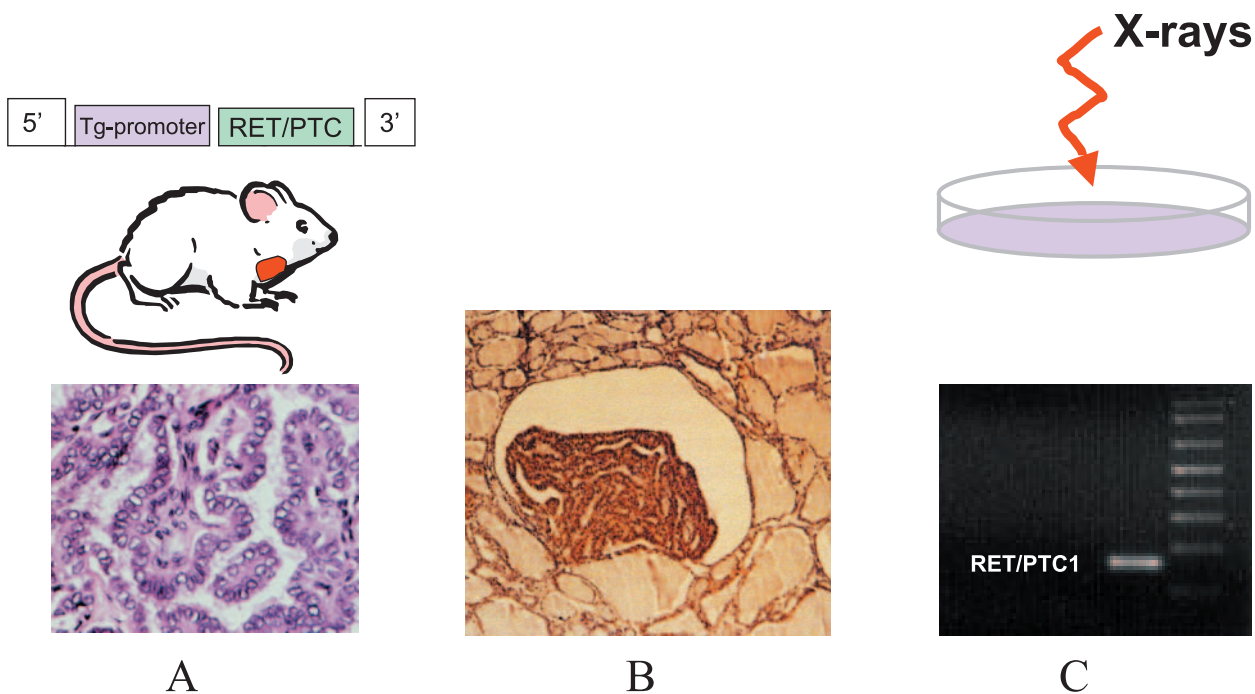


Figure 1 *RET/PTC* rearrangements are involved in papillary thyroid carcinoma initiation. Several lines of evidence support the concept that *RET/PTC* is an early genetic event predisposing to development of papillary thyroid cancer. (A) Thyroid-specific expression of *RET/PTC1* (Jhiang *et al.* 1996, Santoro *et al.* 1996) or *RET/PTC3* (Powell *et al.* 1998) in transgenic mice result in development of tumors with cellular features resembling papillary thyroid carcinoma. (B) *RET/PTC* expression is detected in human thyroid micropapillary carcinomas (Viglietto *et al.* 1995, Sugg *et al.* 1998, Corvi *et al.* 2001). (C) External irradiation of human thyroid fetal explants and human carcinoma cell lines results in detectable *RET/PTC* rearrangements after short time intervals (Ito *et al.* 1993). Elements of this Figure are reproduced with permission from Powell *et al.* (1998) and Corvi *et al.* (2001).

constitutive activation of the TK function of RET, autophosphorylation at selected tyrosine residues, and initiation of intracellular signaling by engagement with effectors through specific tyrosine-phosphorylated domains of the receptor. Three RET protein variants (RET9, RET43 and RET51) have been shown to be generated by alternative splicing. They have identical primary structures until amino acid 1063, followed by unique C-terminal sequences (Myers *et al.* 1995). Three sites of tyrosine phosphorylation are common to all these variants, and have been shown to function as docking sites for signaling molecules. pY905 mediates the recruitment of the SH2 domain-containing proteins Grb7 and Grb10. Phospholipase C γ associates with RET via pY1015 (Borrello *et al.* 1996), and Shc and Frs2 interact with pY1062 (Arighi *et al.* 1997, De Vita *et al.* 2000, Melillo *et al.* 2001). Regardless of its phosphorylation state, Y1062 also interacts with the Enigma protein, which targets RET/PTC isoforms to the inner surface of the plasma membrane (Durick *et al.* 1998). Several investigators have explored the contribution of signaling effector pathways activated via Y1062 of RET on cell growth and transformation, primarily in NIH3T3 cells and in the rat pheochromocytoma cell line PC12 (Asai *et al.* 1996, De Vita *et al.*

2000, Segouffin-Cariou & Billaud 2000). In these cells, RET-Y1062, acting via either PI3K or MAPK, is required for the effects of RET on cell transformation, survival and migration. In thyroid follicular cells, RET/PTC requires Y1062 (for clarity, amino acid numbering corresponds to that of wild-type RET) to activate Shc-Ras-Raf-Mek-Erk, and this pathway is in turn required for RET/PTC-dependent stimulation of DNA synthesis (Knauf *et al.* 2003).

Buckwalter *et al.* (2002) investigated the contribution of these signaling pathways to RET/PTC1-induced thyroid tumor formation *in vivo* by characterizing transgenic mice expressing thyroid-targeted RET/PTC1 mutants with phenylalanine substitutions at either Y905, Y1015 or Y1062. Tumor formation was significantly decreased in all of the mutants, but in particular by RET/PTC1 Y905F. This points to significant contributions mediated by all of these pathways to RET/PTC-induced thyroid cell transformation. The interpretation of these experiments is complicated by the fact that RET/PTC expression in thyroid cells causes primary hypothyroidism through impaired expression of many of the specialized proteins required for thyroid hormonogenesis, and the degree of hypothyroidism and of the consequent thyrotropin

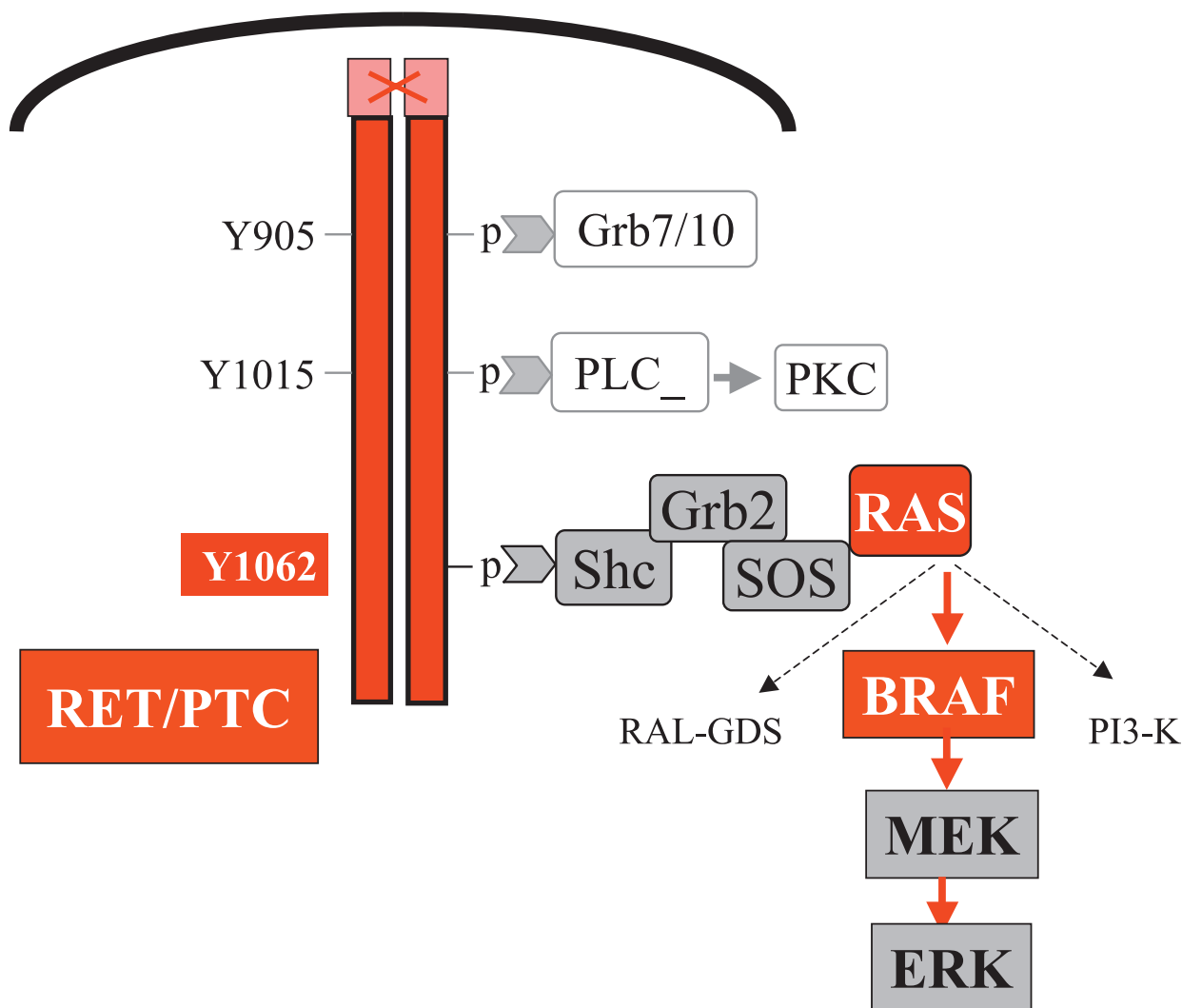


Figure 2 Mutations of three genes that signal in tandem implicate constitutive activation of the Ras-RAF-MAP kinase pathway in PTC pathogenesis. Several *in vitro* studies point to a critical role of RET Y1062, signaling via Ras and Raf, in cell transformation and thyroid cell dedifferentiation. Recent analysis of human papillary thyroid cancers demonstrate the presence of mutually exclusive mutations of either *RET/PTC*, *RAS* or *BRAF*, with little overlap between them, providing compelling genetic evidence for the critical role of this pathway in pathogenesis of these cancers.

elevation may have varied in severity between the mice expressing the different RET/PTC mutants. Nevertheless, this study indicates that none of these RET/PTC tyrosine residues alone is absolutely required for tumor formation, and all appear to contribute to some extent to the ultimate phenotype.

By contrast, *in vitro* data in thyroid cells point to an absolute requirement of Y1062 for RET/PTC-induced dedifferentiation, as determined by decreased expression of thyroid-specific gene products such as the sodium iodide symporter, thyroglobulin or PAX-8. RET/PTC-mediated dedifferentiation requires activation of Shc-RAS-RAF-MAP kinase (Knauf *et al.* 2003), thus providing a good

rationale to explore the contribution of mutations of other effectors in this pathway to thyroid cancer pathogenesis (Fig. 2).

B-RAF: the most prevalent thyroid oncogene

There are three isoforms of the serine-threonine kinase Raf in mammalian cells: A-Raf, B-Raf, and C-Raf or Raf1. C-Raf is expressed ubiquitously, whereas B-Raf is expressed at higher levels in hemopoietic cells, neurons and testis (Daum *et al.* 1994). B-Raf is also the predominant isoform in thyroid follicular cells (L Zhang, N

Mitsutake, J A Knauf & J A Fagin, unpublished observations). Although all Raf isoforms activate MEK phosphorylation, they are differentially activated by oncogenic Ras. In addition, B-Raf has higher affinity for MEK1 and MEK2 and is more efficient in phosphorylating MEKs than other Raf isoforms (Peyssonnaud & Eychene 2001). *BRAF* somatic mutations were recently reported in a high proportion of benign nevi (Pollock *et al.* 2003) and malignant melanomas (Davies *et al.* 2002), and in a smaller subset of colorectal and ovarian cancers (Davies *et al.* 2002). A total of 98% of the mutations in melanomas resulted from thymine-to-adenine transversions at nucleotide position 1796, resulting in a valine-to-glutamate substitution at residue 600 (V600E), formerly designated as V599E. Recent resolution of the crystal structure of the wild-type and B-Raf V600E kinase domains helps understand the mechanisms of mutational activation of the protein (Wan *et al.* 2004). B-Raf exhibits the characteristic bilobar structure of protein kinases. In its inactive conformation, B-Raf residues G596-V600 in the activation loop form hydrophobic interactions with residues G464-V471 in the ATP-binding site (P loop), resulting in a structure that is not aligned for binding to ATP or substrate. Oncogenic mutations in the activation loop or the P loop disrupt their interaction and destabilize the inactive conformation. Most, but not all of known oncogenic B-Raf substitutions allow the formation of new interactions that fold the kinase into a catalytically competent structure (Dhillon & Kolch 2004). Paradoxically, some of the oncogenic Braf mutants impair *in vitro* kinase activity (Wan *et al.* 2004). Despite this, these low-activity kinase Braf mutants induce ERK phosphorylation, which is due to activation of Raf1, presumably by heterodimerization (Wan *et al.* 2004).

The *BRAF*^{T1796A} mutation is the most common genetic change in PTC, and present in about 36–69% of cases (Cohen *et al.* 2003, Fukushima *et al.* 2003, Kimura *et al.* 2003, Namba *et al.* 2003, Nikiforova *et al.* 2003, Soares *et al.* 2003, Xu *et al.* 2003, Trovisco *et al.* 2004). *BRAF*^{T1796A} mutations are unique to PTC, and not found in any other form of well-differentiated follicular neoplasm arising from the same cell type. There is practically no overlap between PTC with *RET/PTC*, *BRAF* or *RAS* mutations, which altogether are found in about 70% of cases (Kimura *et al.* 2003, Soares *et al.* 2003). The lack of concordance for these mutations provides compelling genetic evidence for the requirement of this signaling system for transformation to PTC (Fig. 2). As these signaling proteins function along the same pathway in thyroid cells, this represents a unique paradigm of human tumorigenesis through mutation of three signaling effectors lying in tandem (Fig. 2). *BRAF* mutations can occur early in development of PTC, based on evidence that they are present in microscopic PTC (Nikiforova *et al.* 2003). Moreover, PTC with *BRAF* mutations have more aggressive properties, present more often with extrathyroidal

invasion and at a more advanced clinical stage. The tall-cell variant papillary thyroid cancers, widely regarded as more aggressive, have a particularly high prevalence of *BRAF* mutations (Nikiforova *et al.* 2003). Undifferentiated or anaplastic carcinomas arising from preexisting papillary thyroid cancers have a significant prevalence of *BRAF* mutations, whereas those arising from preexisting follicular carcinoma do not (Namba *et al.* 2003, Nikiforova *et al.* 2003). These data indicate that *BRAF* mutations may be an alternative tumor-initiating event in papillary thyroid cancer, and that tumors with this genotype carry a less favorable prognosis. The role of oncogenic Braf as a tumor-initiating event has been confirmed in mice with targeted expression of BRAFV600E in thyroid cells. These animals develop papillary thyroid cancers with high penetrance early in life, and progress to dedifferentiation, capsular and microvascular invasion, confirming many of the features found in the human tumors (J A Knauf, N Mitsutake, L Zhang, Y E Nikiforov & J A Fagin, unpublished observations).

Small molecule kinase inhibitors in thyroid cancer

Protein kinases are involved in transmitting intracellular signals that eventuate in all biological properties of cancer cells, including growth, survival, motility, invasion and metastasis. It is no surprise that these signaling effectors have been considered prime targets for interference by anticancer therapies. This approach, initially greeted with skepticism because of concerns of lack of specificity due to common catalytic mechanisms and structural similarity between kinases, has now shown clinical value with the emergence of the abl kinase inhibitor imatinib. Most of the protein kinase antagonists in development are directed towards the ATP-binding site. However, there are other potential approaches to inhibit specific kinases, by interfering with expression of the kinase, folding of the mature protein or interaction with substrates (Dancey & Sausville 2003). Among solid cancers, thyroid carcinomas represent a particularly promising paradigm for targeted therapy because some of the key oncogenic events are activating mutations of genes coding for TKs, and these occur early in cancer development.

RET is a logical target for selective inhibition in both medullary and papillary thyroid cancers. Several groups have published preclinical studies with compounds showing inhibitory effects on RET kinase activity at low nanomolar concentrations, and impairment of cell growth *in vitro* and in mouse xenografts (Carlomagno *et al.* 2002a,b, 2003, Lanzi *et al.* 2003, Strock *et al.* 2003) (Table 1). At least one of these compounds, ZD6474, originally developed as an anti-angiogenic agent through its inhibition of the vascular endothelial growth factor receptor KDR (Hartman *et al.* 2002), is now entering clinical trials for patients with medullary thyroid cancer.

Table 1 Selected RET and Raf small molecule kinase inhibitors

	Agent	Structure	IC50	Reference
Target RET	RPI-1	2-Indolinone	20–40 μ M	Lanzi <i>et al.</i> (2000)
	PP1	Pyrazolopyrimidine	80 nM	Carlomagno <i>et al.</i> (2002b)
	PP2	Pyrazolopyrimidine	100 nM	Carlomagno <i>et al.</i> (2003)
	ZD6474 ¹	Quinazoline	100 nM	Carlomagno <i>et al.</i> (2002a)
	CEP-701 ²	Indolocarbazole	<100 nM	Strock <i>et al.</i> (2003)
	CEP-751	Indolocarbazole	<100 nM	Strock <i>et al.</i> (2003)
RAF	BAY43-9006 ³	Bis-aryl urea	12 nM (Raf1)	Lyons <i>et al.</i> (2001)
	L-779,450 ⁴	Triarylimidazole	1 μ M	Shelton <i>et al.</i> (2003)

¹Astra Zeneca; ²Cephalon; ³Bayer/Onyx; ⁴Merck.

B-Raf represents an attractive target for treatment of papillary thyroid cancers because of its possible role in tumor initiation, its high prevalence, and its association with tumors presenting at an advanced stage. The compound BAY43–9006 is a potent and effective Raf inhibitor *in vitro* and in mouse xenografts, and is presently in clinical trials for other forms of cancer (Karasarides *et al.* 2004). Other Raf inhibitors, in the form of small molecule kinase inhibitors as well as antisense oligonucleotides, are also in development (Dancey & Sausville 2003). In due course it is likely that at least one of these compounds or others with similar properties will be tested in patients with advanced papillary thyroid cancer. It should be noted that other signaling pathways and molecular targets (Braga–Basaria *et al.* 2004), although not directly activated through genetic mutations, may prove to be crucial for thyroid cancer progression and thus appropriate for targeted inhibition.

Conclusions

We are finally seeing the emergence of new approaches for development of thyroid cancer therapies, and there is reason for optimism that we may soon have new options for our patients. A note of caution is still in order. The activity of the oncogenic kinases may no longer be required once the tumors have progressed to highly malignant cancers and accumulated numerous other genetic changes. Some have suggested that at least some thyroid cancers may not be entirely clonal for the mutated kinase, raising the prospect of resistance to therapy. The latter can also take place through somatic development of new mutations within the respective kinase genes, rendering the oncoproteins resistant to the inhibitory compounds. Certain mutations of *BCR-ABL*, including some resulting in substitutions within the imanitib–contact sites in the ATP-binding pocket, have been associated with imanitib resistance and CML recurrence (Hochhaus *et al.* 2002, Shah *et al.* 2002). In the case of RET, an oncogenic point mutation in codon 804 present in some

patients with MEN2 is associated with resistance to many of the known RET antagonists (Carlomagno *et al.* 2004). Finally, inhibition of kinase activity may induce cytostatic effects rather than cell death, and/or require co-administration of other therapies for beneficial effects to manifest.

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