The Raf-1 pathway: a molecular target for treatment of select neuroendocrine tumors?

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Neuroendocrine (NE) tumors such as medullary thyroid cancer, carcinoid, small cell lung cancer and pheochromocytoma are metastatic in nature, and secrete biogenic amines and hormones. In this review, we will discuss the possibility that activation of the Ras/Raf signaling pathway may be a therapeutic target for patients with select NE tumors. In-vitro activation of Raf-1 in NE tumors either by expression of the ectopic catalytic domain of Raf-1 or by a pharmacologic drug, ZM336372, resulted in growth inhibition. In addition, activation of the Ras/Raf pathway led to a significant reduction in NE markers such as serotonin, chromogranin A and calcitonin. These data support development of Raf-1-activating compounds for treatment of patients with NE tumors of selective subtypes. ***Anti-Cancer Drugs*** **17:139–142 © 2006 Lippincott Williams & Wilkins.**

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Introduction

Ras regulates multiple signaling pathways, of which the best understood is the Ras/Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway. *ras* and *raf* are protooncogenes, and expression of these genes activates signaling pathways, which in turn control cellular growth. Therefore, the Ras/Raf signaling pathway has been recognized as an important process in cancer biology (see reviews [1–5]). Raf was the first identified and is the best-characterized downstream effector kinase of Ras. There are three Raf family members in humans and mice. Despite several findings and new insights on this signaling pathway, the role of Raf in cancer cells remains controversial, yet interesting. In this pathway, extracellular signals mediated by growth factors transmitted through cellular receptors lead to activation of Ras by the confirmation change due to the conversion of GDP to GTP. Ras signaling often leads to activation of Raf, a cytosolic serine/threonine kinase. Raf is the general mediator of Ras and MEK1/2 is the general downstream effector substrate for Raf. Activated Raf then phosphorylates two serine residues of MEK1/2 in the activation loop. Interestingly, MEK acts as a dual specific kinase which can phosphorylate both threonine and tyrosine residues [6]. Then activated MEK phosphorylates downstream ERK1/2. The presence of two ERKs (ERK1 and 2) and MEKs (MEK1 and 2) is interesting, and perhaps surprising, since their functions are similar. However, ERK acts on several substrates ranging from cytoskeletal proteins, phosphatases, kinases to transcription factors [7]. These phosphorylation events lead to activation of transcription factors that play a critical role in tumorigenesis [4]. A schematic diagram of the Ras/Raf pathway is shown in Fig. 1.

Raf gene(s) organization and function

There are three Raf family members in humans and mice: A-Raf, B-Raf and C-Raf (Raf-1) [8–10], and all have the same structural organization (Fig. 2). The N-terminal region contains conserved regions CR1 and CR2, and these regulate the kinase domain (CR3). Overexpression of the kinase domain (CR3) alone elicits oncogenic properties. All three Rafs are able to induce MEK1/2/ERK1/2 activation, but they differ in their strength of activation of ERK. The duration and magnitude of ERK activation have a significant impact on cell cycle event. Even though the structural organization is similar in all three Rafs, they each express a distinct phenotype. Transgenic mice with deletion of B-Raf or C-Raf die *in vitro*. A-Raf-defective transgenic pups are born alive with neurological defects [11]. Transgenic knockout mice experiments suggest that each Raf has its own distinct function even though they have structural similarity. Furthermore, the results of these transgenic mice experiments validate the cell culture results indicating that Raf-1 plays a role in phenotypic differentiation, whereas A-Raf and B-Raf play a specific role, such as

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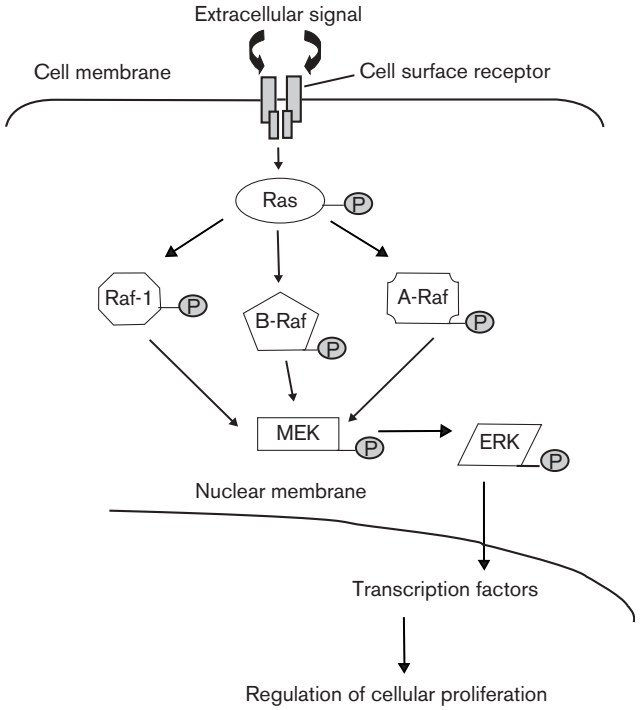
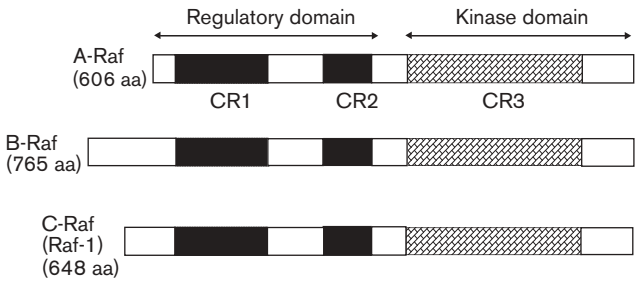
Fig. 1



A schematic representation of the Ras/Raf/MEK/ERK signaling pathway. Binding of extracellular growth factor to the membrane receptor activates Ras by phosphorylation. Activation of Ras leads to a sequential phosphorylation of Raf/MEK/ERK. This event leads to the activation of various transcription factors, which in turn regulates cellular proliferation and apoptosis.

Fig. 2



Schematic representation of *raf* gene organization. The kinase domain or catalytic domain alone can activate the Raf/MEK/ERK pathway. However, the regulatory domain plays a critical role in regulating the expression of the pathway. CR, conserved region

endothelial maintenance and maturation [11]. Although the basic regulatory roles for these Rafs have been elucidated, the reason for the diverse mechanisms is not known.

Role of Raf-1 in growth inhibition in various cancer types

In general, dysregulation of the Ras/Raf pathway leads to tumorigenesis. The overexpression of this pathway may be due to mutation(s) in Ras or Raf. Mutations in this pathway are common among adenocarcinomas of the colon (50%) and pancreas (90%), and in lung and squamous cell cancers (30%) [12]. Due to the activating mutation, the Ras/Raf pathway is highly activated/expressed in these cancer types. However, in neuroendocrine tumors (NE) such as small cell lung cancer (SCLC), medullary thyroid cancer (MTC) and carcinoid cancer the Ras/Raf pathway is not expressed or at least not detected in pathologic specimens [1,13–15]. This suggests that mutation in this pathway is not naturally advantageous [12,16,17]. There is a growing body of literature that suggests that Raf-1 activation may inhibit growth of NE tumors [1,13,18–22]. NE tumors typically metastasize to the liver, and secrete excess biogenic amines and hormones. Due to the secretion of the bioactive hormones, patients with NE tumors often have poor quality of life. Therefore, new therapeutic alternatives are needed. In addition to the secretion of various hormones and biogenic amines, NE tumors express high levels of a transcription factor called human achaete scute homolog-1 (hASH1). Earlier studies have suggested that there is a tight correlation between hASH1 and the NE phenotype. Eliminating hASH1 protein production by antisense treatment led to a decrease in NE marker production [23]. Similarly, in-vivo abolition of hASH1 in transgenic knockout mice resulted in failure to develop pulmonary NE cells and the pubs die at birth [24].

SCLC

Lung cancer is the number one cause of cancer death among both men and women (in 2004 alone there were 160 000 deaths in the USA). In addition, lung cancer accounts for more deaths than prostate, breast and colorectal cancer combined. SCLC accounts for 20% of all lung cancers [25]. Although SCLC is very aggressive, it does response to chemotherapy and radiation. However, the recurrence rate is high even in early-stage treatment. Less than 10% of the patients with SCLC survive for 5 years and for patients with extensive stage cancer the survival is 1–2% due to its aggressiveness and the lack of effective therapies. As mentioned earlier, activation of the Ras/Raf pathway leads to tumorigenicity in many cancer types including pancreatic cancer and prostate cancer. In addition, Ras mutations are common in non-small cell lung cancer (NSCLC) types such as lung adenocarcinomas and squamous cell cancers. In contrast, SCLC cell lines and also SCLC tumor tissue do not have Ras mutations, and, therefore, the Ras/Raf pathway is not overexpressed [13,14]. In a study by Ravi *et al.*, human SCLC cells were transduced with an estrogen-inducible Raf-1 construct. Raf-1 pathway activation in these cells by overexpression of Raf-1 resulted in phenotypic changes and a significant reduction in cellular proliferation [13,14]. Furthermore, it was observed that the reduction in growth is due to the induction of

cyclin-dependent kinase (CDK) inhibitors (CDKIs). However, the mechanism of induction of CDKIs by Raf-1 activation is not clear. One can speculate that there may be several mechanisms by which the CDKIs are induced after Raf-1 activation and perhaps the probable effector/mediator for this is the phosphorylated ERK1/2. Thus, activation of Raf-1 inhibits growth of SCLC cells and suggests that targeting Raf-1 may be a potential treatment strategy for a subset of NE tumors.

Carcinoid tumors

Carcinoid tumors are the most common type of NE tumors frequently found in the gastrointestinal (GI) system. Although this type of cancer is rare (1–5:100 000), the incidence is very high due to its well-differentiated nature and slow-growing properties. However, it frequently metastasizes to the liver and is second to colorectal carcinoma as the most common source of isolated hepatic metastases [26–28]. Due to its metastatic nature, patients with carcinoid cancers have a less than 30% 5-year survival probability [4,9]. Due to the high rate of metastasis, curative surgical resection for carcinoid is uncommon, emphasizing the need for development of novel therapies. Other forms of therapy including chemoembolization, radiofrequency ablation, cryoablation, chemotherapy and liver transplantation have had limited efficacy [29–36]. In addition, patients with carcinoid tumors often have debilitating symptoms, such as uncontrollable diarrhea, flushing and skin rashes due to the hypersecretion of biogenic amines and hormones. Therefore, new therapeutic options are needed. We have previously reported the construction of an estrogen-inducible Raf-1 in the human pancreatic carcinoid tumor cells, BON. Activation of Raf-1 by the addition of estradiol in the medium led to a significant reduction in levels of NE hormones such as serotonin and chromogranin A [15,37]. Therefore, Raf-1 activation could be a potential palliative treatment for patients with GI carcinoids.

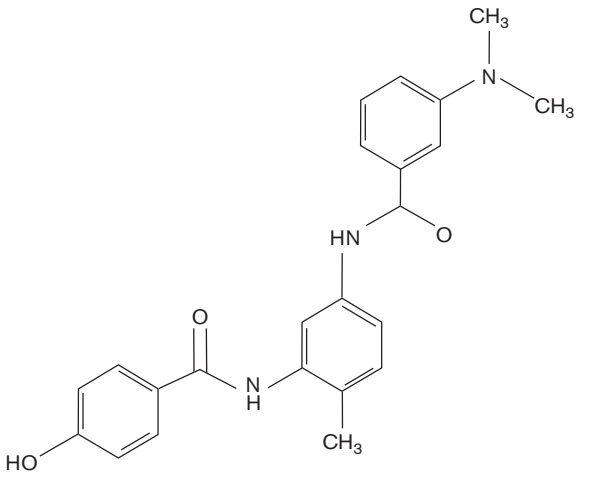
MTC

MTC, which accounts for 3–5% of all thyroid cancers, is also a NE tumor and originates from the C cells of the thyroid [1,38,39]. MTC tends to be a slow-growing tumor, but maintains the differentiation features of the parental C cells. Similar to carcinoid disease, the primary therapy for patients with MTC is surgical resection. However, MTC also frequently metastasizes, especially to the liver and regional lymph nodes, precluding patients from a curative resection. Earlier we reported that hASH1, a neural transcription factor, is highly expressed in MTC, but not in normal tissues, and may be important for tumor proliferation [1,19,39]. It is speculated that decreasing hASH1 levels may inhibit MTC tumor growth [19,21,40]. Recently, we and others have reported that Raf-1 activation in the MTC-TT cell line results in growth suppression as well as a reduction in NE hormones (such as calcitonin and serotonin), hASH1 and levels of the RET protooncogene [21,40]. Furthermore, it has been shown that growth inhibition by Raf-1 activation in MTC-TT cells induces an autocrine/paracrine protein, leukemia-inhibitory factor (LIF), and this alone could mediate differentiation and cell growth inhibition [21]. This finding is interesting because Raf-1 activation not only activates its own Raf-1/MEK/ERK pathway, but also crosstalk with other pathway(s), which in turn could possibly regulate growth.

ZM336372: a Raf activator in NE tumors

These results suggest that Raf-1 activation may be a possible therapeutic target in certain NE cancers. Other than gene therapy, applications to deliver activated Raf-1 to tumor cells are limited. Therefore, we explored the possibility of pharmacologically activating Raf-1. The compound ZM336372 (Fig. 3) was originally identified as a small-molecule inhibitor of Raf-1 [41]. Although the drug inhibited Raf-1 activation in in-vitro kinase experiments, it surprisingly showed a paradoxical response of more than 100-fold Raf-1 activation in cell culture experiments [41,42]. The explanation authors provided for the activation of Raf-1 is that the drug possibly inhibits/blocks the negative feedback loop of Raf-1 regulation. However, to date the mechanism of the negative regulation of the Raf-1 pathway activation is unclear. When we treated NE tumor cell lines such as pancreatic carcinoid and pulmonary carcinoid cancer cell lines with ZM336372, there was significant reduction in growth and hormone production [22]. Furthermore, we have shown the phosphorylation of Raf-1 and ERK1/2 by ZM336372. It is possible that activation of the Raf-1 pathway is required at least in carcinoid tumor types for the anti-tumor proliferation effect. Taken together, the

Fig. 3



Chemical structure of ZM336372.

results of overexpression of ectopic Raf-1 and activation of endogenous Raf-1 by ZM336372 in carcinoid cells suggests that Raf-1 could be a potential target for novel therapeutic anti-cancer strategies. Therefore, further research is warranted for identifying more compounds to activate Raf-1. These compounds could be tested for growth inhibition in these types of cancer.

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