ZD6474 Suppresses Oncogenic RET Isoforms in a Drosophila Model for Type 2 Multiple Endocrine Neoplasia Syndromes and Papillary Thyroid Carcinoma

Marcos Vidal,1 Samuel Wells,2 Anderson Ryan,3 and Ross Cagan1

1Department of Molecular Biology and Pharmacology, Washington University School of Medicine, Saint Louis, Missouri; 2Duke University Medical Center, Durham, North Carolina; and 3AstraZeneca, Alderley Park, Macclesfield, Cheshire, United Kingdom

Abstract

Patients with hereditary medullary thyroid carcinoma (MTC) associated with multiple endocrine neoplasia (MEN) types 2A and 2B and familial MTC (FMTC) have mutations in the RET proto-oncogene. Approximately 40 percent of patients with papillary thyroid carcinoma (PTC) typically activate the RET receptor tyrosine kinase (RTK). RET kinase inhibitors are likely to be beneficial for patients with hereditary MTC, where currently there is no effective chemotherapy or radiotherapy. Recently, the low molecular weight tyrosine kinase inhibitor ZD6474 was found to block the enzymatic activity of RET-derived oncoproteins in cultured cell lines. We have developed a Drosophila model for MEN2A and MEN2B diseases by targeting oncogenic forms of RET to the developing Drosophila eye. Here we show that, when fed orally, ZD6474 suppressed RET-mediated phenotypes within the context of this in vivo model. Importantly, ZD6474 showed high efficacy and very low toxicity. This compound failed to significantly suppress an activated form of another RTK, the Drosophila epidermal growth factor receptor, nor did it suppress the activity of downstream components of the RET/Ras pathway. Our results support the view that targeting chemokine inhibitors such as ZD6474 to tissues with oncogenic forms of RET is a useful treatment strategy for RET-dependent carcinomas. (Cancer Res 2005; 65(9): 3538-41)

Introduction

The RET receptor tyrosine kinase (RTK) is a 120-kDa transmembrane protein that has been identified as a key regulator of development and a "hotspot" for oncogenic mutations. Mutations that activate RET activity can lead to several cancer syndromes, including multiple endocrine neoplasia type 2A and 2B (MEN2A and MEN2B) and familial medullary thyroid carcinoma (FMTC). MEN2A patients typically contain a mutation that alters one of five cysteines within the extracellular domain of RET, permitting disulfide bonding between receptor pairs and ligand-independent activation (reviewed in ref. 1). The result is a series of oncogenic events, particularly medullary thyroid carcinomas, pheochromocytomas (adrenal medulla tumors), and parathyroid hyperplasia. The more severe MEN2B is usually the result of a methionine-to-threonine substitution at position 918 (M918T) within the tyrosine kinase catalytic domain of RET. MEN2B patients also exhibit MTCs and pheochromocytomas in addition to ganglioneuromas, mucosal neuromas, megacolon, a generalized neural hypertrophy, early defects in bone structure including marfinoid habitus, and possibly other developmental defects (reviewed in ref. 1). Recently, we have created Drosophila models for MEN2A and MEN2B (2). Specifically, we have created three classes of transgenic flies that misexpress Drosophila RET (dRET) isoforms: lines that express wild-type (mimicking FMTC), MEN2A-, and MEN2B-like isoforms. Each dRET isoform was directed to the developing eye to create an easily visible adult phenotype. In this study, we show that oral administration of the kinase inhibitor ZD6474 (3) is effective at suppressing the defects associated with wild-type and oncogenic forms of dRET. Strong phenotypic suppression occurred at doses well below those leading to observed toxicity or lethality. This work represents the first evidence that ZD6474 can be effective in treating RET-related defects in a whole organism.

Materials and Methods

Drosophila stocks. The following previously described stocks were obtained from the Drosophila Bloomington Stock Center: dEGFR[flipout]CyO, sev-rasv12/TM6B, and sev-rasv12/CyO. The generation and detailed phenotypic analysis of GMR-dRET, GMR-dRET695R, and GMR-dRETM918T fly lines will be published elsewhere (2).

ZD6474 administration. The required amount of ZD6474 was added directly to standard fly medium and sonicated extensively until homogenized. The fly medium was filtered first to remove large particles. Embryos (n = 15-20) of the appropriate genotype were loaded onto 0.2 mL of medium.

Scanning electron microscopy. Adult flies were fixed in 95% ethanol, dried with a critical point drier, sputter-coated, and visualized using a Hitachi S-2600H scanning electron microscope.

Results and Discussion

Drosophila RET directs abnormal eye development. The Drosophila retr locus encodes a transmembrane protein that is 53% identical to human RET in its kinase domain; the extracellular domain of dRET shows limited conservation in its primary sequence to RET but shares overall structural similarity including a cysteine-rich region (4, 5). The MEN2A-associated mutation was mimicked by replacing a cysteine at position 695 with an arginine (C695R); this site is analogous to human...
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RET 634, a site mutated in approximately half of analyzed MEN2A patients. To mimic the MEN2B mutation, a methionine-to-threonine point mutation was engineered into a full-length dRET cDNA at codon 1007 (analogous to position 918 within human RET subdomain VIII). The altered dRET isoforms (in addition to a wild-type, unaltered isoform) were each cloned behind the eye-specific GMR promoter (6) and stable transgenic fly lines were established by standard methods to generate GMR-dRET, GMR-dRETC695R, and GMR-dRETm1007T flies that express the wild-type, C695R, or M1007T dRET isoforms, respectively, specifically in their developing eyes. Cultured Drosophila can survive without functional eyes, and targeting these isoforms permitted us to study these dominant dRET isoforms in stable, viable lines. Targeting either isoform to the developing eye leads to overproliferation, alterations in cell fate differentiation, and ectopic cell death, giving rise to "rough" adult eyes consisting of fused, poorly patterned lenses and a reduction in the eyes' overall size (Figs. 1B and 2A; data not shown; ref. 2); these eye phenotypes are due to activation of a broad palate of signaling pathways, including Ras, c-Jun NH2-terminal kinase, Src, etc. (2). Many of these pathways have been connected to human RET signaling, further indicating that these transgenic fly lines are functionally useful MEN2 models.

**ZD6474 suppresses Drosophila RET–mediated defects.** The anilinoquinazoline compound ZD6474 was originally identified as a chemical inhibitor of the VEGFR2 RTK, with additional activity against epidermal growth factor receptor (EGFR; e.g., refs. 7–9). Recently, ZD6474 has been found to also inhibit RET signaling in two human papillary thyroid carcinoma (PTC) cell lines and RET/PTC-transformed fibroblasts injected into nude mice (3). To explore the ability of ZD6474 to alleviate the disruptive effects of oncogenic RET isoforms within an epithelium in situ, transgenic flies were fed differing concentrations of ZD6474 by mixing the compound into their medium. When fed with ZD6474, GMR-dRET animals were rescued in a dose-dependent manner: low concentrations of ZD6474 in the flies' medium led to a partial rescue of GMR-dRET, GMR-dRETC695R, and GMR-dRETm1007T flies (Figs. 1C–D and 2B; and data not shown). In all three lines, higher doses led to transgenic eyes with eyes that were phenotypically indistinguishable from wild-type animals (Fig. 2C; data not shown).

At concentrations effective for rescue of the eye phenotype, ZD6474 did not affect viability or fertility of the animals. The only effect observed was a delay in development; pupariation time was delayed 4 days on average. This effect may reflect effects by ZD6474 on tissue growth or more indirect effects such as affecting the willingness of the animals to consume food. RTK signaling is required for development of a broad spectrum of tissues and cell types; the fact that ZD6474-treated animals are overall healthy and fertile argues against the prospect that the compound affects the full spectrum of normal RTK signaling necessary for development and homeostasis.

The lowest concentration that showed significant rescue of the eye phenotype was 0.08 mmol/L; flies showed a detectable decrease in viability at concentrations at and above 2.5 mmol/L. Whereas we do not know the precise concentration of drug that entered each fly's system, if the amount is linear to the concentration present in the food, these results would indicate a therapeutic index in the range of 30-fold. We did not observe differences in the ability of ZD6474 to rescue different dRET isoforms, as long as the initial phenotypes were comparable. Different insertion sites for each of the transgenes lead to differing severity of the GMR-dRET, GMR-dRETC695R, and GMR-dRETm1007T phenotypes (2); as anticipated, lines with the strongest phenotypes were less sensitive to the rescuing effects of ZD6474 than lines with moderate or weak phenotypes (data not shown). That is, ZD6474 was not able to show clear rescue at all levels of dRET isoform expression. Importantly, the GMR promoter is an artificial promoter construct that contains multimerized glass enhancer elements (6) and represents one of the strongest promoters known in Drosophila.

**ZD6474 is less effective with Drosophila isoforms of epidermal growth factor receptor, Ras, and Raf.** Two major downstream targets of RET/dRET signaling are Ras and Raf.
Targeting an activated form of Drosophila Ras1 (sev-ras\textsuperscript{v12}) or Raf (sev-raf\textsuperscript{torso9}) to the eye leads to a roughened eye that is reminiscent of GMR-dRET eyes (Fig. 3C; data not shown; refs. 10, 11). Interestingly, the ZD6474 compound did not rescue the phenotypes resulting from the expression of either isoform (Fig. 3D; data not shown). This indicates that either ZD6474 is suppressing dRET activity that is independent of Ras pathway signaling or, more likely, the compound acts upstream of dRas1 and dRaf. These results provide in vivo evidence to support previous reports that ZD6474 acts directly on the receptor and suggests that any inhibition of downstream cytoplasmic kinases linked to Ras signaling is likely to be minor.

ZD6474 has been previously shown to inhibit mammalian EGFR in vitro, although more poorly than RET (3, 9). dEGFREllipse\textsuperscript{v507} is an activated form of the Drosophila EGFR orthologue dEGFR/DER/Tor/Flb (12). It yields a mildly rough eye due to a mutation in its kinase domain that is readily modified by mutations in other loci (e.g., refs. 13, 14). Surprisingly, ZD6474 failed to modify the dEGFREllipse\textsuperscript{v507} phenotype (compare Fig. 3A and B). This suggests that, in Drosophila, ZD6474 shows stronger in situ preference for dRET than dEGFR, further indicating that the compound does not inhibit all RTKs with similar efficacy.

ZD6474 is a candidate for drug therapy. Our results indicate that ZD6474 can act as an in vivo inhibitor of the RET signaling pathway. In addition, two lines of evidence suggest that the compound shows at least limited specificity. First, the compound did not affect viability of the fly models. Drosophila requires the activity of a broad spectrum of RTKs in addition to the Ras signal transduction pathway for viability. Second, ZD6474 did not significantly affect the activity of activated forms of either an alternate RTK (dEGFR) or either of two downstream components of the Ras pathway (Ras1 or Raf). The strong suppression of RET-dependent phenotypes that we observed in Drosophila supports the view that targeting chemical kinase inhibitors such as ZD6474 to tissues with oncogenic forms of RET may offer a useful treatment strategy for RET-dependent carcinomas.

To date, pathologies that result from mutations in RET have proven relatively refractory to drug intervention. Recently, some success has been achieved by targeting tyrosine kinases with kinase inhibitors that target inactive forms of the kinases. For example, imatinib mesylate (Gleevec) has proven successful in the treatment of chronic myelogenous leukemia patients that contain the Bcr-Abl chimeric kinase (e.g., ref. 15), and is
currently being tested in a widening number of kinase-associated diseases (e.g., refs. 16–18). Cetuximab (Erbitux) and gefitinib (Iressa), antagonists of EGFR isofoms, have shown efficacy for patients with metastatic colorectal cancer and non–small cell lung cancer, respectively (19–21). These compounds have shown the somewhat surprising observation that kinase inhibitors can target oncogenic forms of protein kinases with relatively low toxic side effects. We do not know how successful simple model systems such as Drosophila will be for predicting efficacy of compounds during the treatment of human diseases, but the remarkable advances gained from studying, e.g., signal transduction pathways in Drosophila suggests optimism. This work represents one of the first published accounts of testing a drug in Drosophila that is strongly considered for use in a specific human disease. Such trials would prove an important first test of the utility of using flies to screen directly for human therapeutics.

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References
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