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Apoptotic cell death induction and angiogenesis inhibition in large established medullary thyroid carcinoma xenografts by Ret inhibitor RPI-1

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Abbreviations:
BID, bis in day
GDNF, glial cell line-derived neurotrophic factor
IHC, immunohistochemistry
MEN-2, multiple endocrine neoplasia 2
MTC, medullary thyroid carcinoma
MVD, microvessel density
PTC, papillary thyroid carcinoma
TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick end labeling
TK, tyrosine kinase
TW, tumor weight
TWI, tumor weight inhibition

ABSTRACT

Recent evidence indicates that the success of molecular targeted therapies may depend on the identification of drug targets which are essential for the survival of subsets of tumors. RET oncogenes that have been implicated in the development of thyroid carcinomas are emerging as potential therapeutic targets. In the present study, we investigated the efficacy and the cellular bases of antitumor activity of the indolinone Ret tyrosine kinase inhibitor RPI-1 against large established s.c. TT tumor xenograft, a human medullary thyroid carcinoma (MTC) harboring oncogenic MEN-2A-type RET mutation. Oral treatment with RPI-1 caused growth arrest or regression in 81% treated tumors. Following treatment suspension, tumor inhibition was maintained (51%, $P < 0.05$, 100 days) and cures were achieved in 2/11 mice. In treated tumors, Ret was tyrosine dephosphorylated. Moreover, compared to control tumors, a significant increase in apoptotic cells (210%, $P < 0.0001$), loss of cellularity (47%, $P < 0.0001$) and reduction of microvessel density (36%, $P < 0.0005$) were detected. In vivo effects of RPI-1 were reflected in activation of BAD, cleavage of caspases, apoptotic DNA fragmentation and inhibition of VEGF production observed in in vitro RPI-1-treated TT cells. These findings thus indicate that RPI-1 antitumor effect on the MTC was characterized by apoptosis induction and angiogenesis inhibition. The results, consistent with a dependence on RET oncogene activation for maintenance and survival of MEN2A-type MTC, provide further preclinical rationale for a pharmacological RET-targeted intervention in thyroid cancer.

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1. Introduction

Several alterations in oncogenes and tumor suppressor genes have been implicated in the pathogenesis and progression of thyroid cancers [1]. Gain-of-function mutations of the RET protooncogene are associated with MTC and PTC [2,3]. MTC, which arises from the calcitonin-producing C cells of the thyroid gland [4], often occurs as a sporadic malignancy, although in about 25% of cases it occurs as part of the inherited MEN-2 syndromes MEN-2A, MEN-2B, and familial MTC.

Recently, it was assessed that activating germline point mutations in the RET protooncogene play a causative role in the pathogenesis of the MEN-2 syndromes [5]. Moreover, somatic RET mutations are found in 40–50% of sporadic MTC [3]. PTC, which arises from the follicular thyroid cells, is the most common thyroid malignancy. Somatic rearrangements of the RET genes generating chimeric oncogenes designated as RET/PTC are present in 5–30% of sporadic and in 60–70% of radiation-associated PTC [6]. As a consequence of such molecular alterations, Ret receptor tyrosine kinase (TK) is frequently aberrantly activated in thyroid carcinoma.

Activation of Ret by ligand binding or oncogene transformation triggers a complex network of signaling cascades which, in general, include pathways mediated by Ras/RAF/ERK, PI3K/Akt, JNK, p38 and PLCγ [3]. Through activation of these pathways, Ret has been reported to control cell survival, differentiation, proliferation, migration and other cellular functions in a cell specific way. Recent studies suggest that RET oncogenes can maintain the neoplastic phenotype by controlling proliferation and survival of thyroid cancer cells [7–9]. Such biological effects of RET oncogenes, along with their early activation in tumor development [10,3] and their ability to drive the development of thyroid cancers in transgenic mice [11], suggest a pivotal role in thyroid cancer.

The Ret receptor TK represents an attractive target for specific therapeutic approaches, mainly considering that current chemotherapy has a marginal role in patients with MTC or PTC, and the prognosis of inoperable or metastatic patients remains poor [4,12]. Preclinical studies aimed to the abrogation of RET oncogenic potential, including gene therapy with dominant-negative RET mutants, selective inactivation of RET RNA by ribozymes, or inactivation of Ret activity by small molecule TK inhibitors, have shown that the inhibition of RET function is sufficient to revert the neoplastic behavior (reviewed in [10,13,14]).

RPI-1 is an orally available indolinone-based synthetic molecule, which was initially described as an inhibitor of the product of RET/PTC-1, the most frequent RET oncogene found in sporadic PTC [6,15,16]. We have recently reported that RPI-1 is very effective in the control of growth of TT tumor xenograft, a human MTC harboring the C634W MEN-2A-type mutation of RET. According to such an effect, RPI-1 was able to inhibit TK activity, expression and signaling of Ret in TT cells [17].

The present study was designed to further characterize in vitro and in vivo the cellular bases of RPI-1 antitumor activity. The results indicate that oral treatment with the Ret inhibitor achieved an outstanding growth inhibition with the occurrence of tumor regressions even in large established TT tumor xenografts. The antitumor effect of RPI-1 was associated with induction of apoptotic tumor cell death and angiogenesis inhibition.

2. Materials and methods

2.1. Reagents and cell culture

The synthesis and chemical structure of RPI-1 (1,3-dihydro-5,6-dimethoxy-3-[(4-hydrophenyl)methylene]1-H-indol-2-one) have been reported [15]. For the antitumor activity studies, RPI-1 was dissolved in polisorbate 80 (20% of final volume). The solution was stirred and maintained at 4 °C. Just before use, it was diluted by slowly adding a cold solution of 10% ethanol in distilled water under stirring on ice. The final RPI-1 concentration was 5.0 mg/ml (maximum concentration allowed). For in vitro experiments, an RPI-1 stock solution was prepared in 100% DMSO and diluted in culture medium for use (final concentration: 0.5% (v/v) DMSO).

The human TT cell line was derived from a metastatic MTC harboring a MEN-2A-type RET mutation, specifically a Cys-to-Trp substitution at codon 634 (C634W) [18,19]. TT cells were maintained in Ham's F12 medium (BioWhittaker, Vervier, Belgium) supplemented with 15% fetal bovine serum (Life Technologies, Gaithersburg, MD) at 37 °C in a 5% CO_2 atmosphere. NIH3T3^{MEN2A} and NIH3T3$^{H\text{-}RAS}$ cells are murine fibroblasts transformed by transfection with the C634R mutant RET or H-RAS oncogenes, respectively [20]. They were cultured in Dulbecco's modified Eagle medium (DMEM, Biowhittaker) supplemented with 5% calf serum (Colorado Serum Company, Denver, CO). The parental cell line NIH3T3 was maintained in DMEM supplemented with 10% calf serum. All murine cells were incubated at 37 °C in a 10% CO_2 atmosphere.

The following polyclonal rabbit antibodies were used: anti-Ret H-300, anti-pRet (Tyr1062), anti-pBAD (Ser136) and anti-caspase-9 p10 (H-83) and anti-VEGF (147) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-pAKT (Ser473), anti-p-p44/42 MAP kinase (Thr202/Tyr204), anti-pBAD (Ser112), anti-cleaved caspase-3 (D175), and anti-cleaved PARP (D214) from Cell Signaling Technology (Beverly, MA); anti-MAP kinase1/2 (ERK 1/2 CT) and anti-PARP from Upstate Biotechnology (Lake Placid, NY).

The following mouse monoclonal antibodies were used: anti-phosphotyrosine (p-Tyr), clone 4G10 (Upstate Biotechnology); anti-PKBα/Akt and anti-BAD from Transduction Laboratories (Lexington, KY); anti-β-tubulin from Sigma Chemical Company (St. Louis, MO).

2.2. In vivo studies

All in vivo experiments were performed using 8–11-week-old female athymic nude CD-1 mice (Charles River, Calco, Italy). Mice were maintained in laminar flow rooms with constant temperature and humidity. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori (Milan, Italy), according to the United Kingdom Coordinating Committee on Cancer Research Guidelines [21].

Exponentially growing TT cells were injected s.c. (2×10^7 cells) into the right flank of mice. TT cells were injected on day 0,