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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,

and tumor growth was followed by biweekly tumor diameter measurements using a Vernier caliper. TW was calculated according to the formula: TW (mg) = tumor volume (mm^3) = $d^2D/2$, where d and D are the shortest and the longest diameter, respectively. RPI-1 treatment started when mean TW was about 300 mg (37 days after tumor cell inoculation). For the study on the effect of RPI-1 treatment against oncogene-transformed NIH3T3 cell lines, exponentially growing NIH3T3-^{MEN2A} and NIH3T3$^{H\text{-}RAS}$ cells were injected s.c. (10^5 cells) into the right flank of nude mice. RPI-1 treatment started the same day of cell inoculation. RPI-1 was delivered orally by gavage, at the dose of 150 mg/kg BID for 20 days in TT tumors, and 10 days for the murine tumors. The drug was administered in a volume of 30 ml/kg of body weight. Control mice were treated with vehicle (polisorbate 80:ethanol:distilled water, 20:8:72, v/v/v) in parallel with RPI-1-treated mice. Each control or RPI-1-treated group included 9–11 mice. Tumor growth was followed in each experimental group and growth curves were drawn by plotting mean TW over time. Experimental groups were sacrificed, under slight anesthesia with sevoflurane (Sevorane, by Abbott S.p.A., Campoverde, Italy), by cervical dislocation when mean TW was at a maximum of 2 g.

For histological studies, mice bearing TT tumors of 350–400 mg were treated orally with RPI-1 delivered at the dose of 100 mg/kg BID for 5 days. The day after the last treatment, three mice per group were sacrificed by cervical dislocation. Tumors were excised and fixed. Half of each tumor was fixed in 10% buffered formalin for hematoxylin–eosin staining and TUNEL reaction. The In Situ cell death detection POD kit (Roche Diagnostic GmbH, Mannheim, Germany) was used [22]. The number of apoptotic nuclei and the total number of cells were quantified in non-necrotic areas of each section. The second half of each tumor was fixed in zinc fixative for IHC. Blood vessels were stained with a rat anti-mouse CD31/PECAM-1 monoclonal antibody (kindly supplied by Dr. A. Vecchi, Mario Negri Institute, Milan, Italy), and MVD was quantified as previously described [22].

For statistical analysis, Student's *t*-test (two-tailed) was used to compare TW, MVD, cellularity and number of apoptotic nuclei in control and RPI-1-treated tumors. *P* values lower than 0.05 were considered statistically significant. Analyses were performed with Graph Pad Prism, version 4.0 (Graph Pad Software Inc., San Diego, CA).

2.3. Immunoprecipitation and Western blotting

For the analysis of RET phosphorylation status in TT tumors, mice with a TW of about 300 mg were treated with oral vehicle or RPI-1, 100 mg/kg BID, for 16 days. One hour after the last treatment, two mice per group were sacrificed and the resected tumors snap-frozen in liquid nitrogen. Control and RPI-1-treated tumors were pulverized using the Mikro-Dismembrator II (B. Brown Biotech International, Melsungen, Germany) and resuspended in cold lysis buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 μg/ml pepstatin A, 20 μg/ml chymostatin, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride). Lysates (2 mg/sample) were immunoprecipitated with anti-pTyr antibody as described [17].

For in vitro experiments, floating and adherent cells were collected and whole cell lysates prepared as previously described [17].

Immunoprecipitates or whole tumor/cell lysates were separated on SDS-PAGE followed by Western blotting [17].

2.4. Detection of apoptosis in vitro

Apoptotic DNA fragmentation was detected in cells exposed to vehicle or the indicated concentrations of RPI-1 for 3–7 days, by the TUNEL assay. The In Situ Cell Death Detection Fluorescein Kit (Roche) was used according to the manufacturer's instructions. Briefly, floating and adherent cells were fixed in paraformaldehyde, permeabilized in a solution of 0.1% Triton X-100 in 0.1% sodium citrate, and then incubated in the TUNEL reaction mix for 1 h. After washing, samples were analyzed by a FACScan flow cytometer, and the percentage of apoptosis was calculated on at least 10,000 cells by the CellQuest software (Becton Dickinson, Mountain View, CA).

2.5. ELISA assay for VEGF

TT cells were seeded at 3×10^4 cells/cm^2 in complete medium and cultured for 4 days before serum starvation and concomitant treatment with solvent or 30 μM RPI-1. After 24 and 48 h, the conditioned medium from control or drug-treated cells was harvested and cells were counted. VEGF protein was detected in clarified media by the human VEGF immunoassay kit from Biosource International (Camarillo, CA), according to the manufactures' instructions. Sample analyses were carried out in triplicate. Correction for cell number was not necessary in these experimental conditions. Statistical significance was determined by a two-tailed Student's *t*-test. In parallel experiments, cells treated with solvent or RPI-1 for 48 h were processed for Western blotting with anti-VEGF antibody.

3. Results

3.1. Effects of RPI-1 on RET-C634 mutant-expressing tumors in vivo

We first examined the effects of RPI-1 against the murine tumor model NIH3T3^{MEN2A}, harboring the RET-C634R mutant, in comparison to the effects against the NIH3T3$^{H\text{-}RAS}$ tumor model, in order to confirm the specificity of in vivo activity of RPI-1 against RET-dependent tumor. As shown in Table 1, RPI-1 (150 mg/kg, BID for 10 days) significantly delayed the s.c. growth of NIH3T3^{MEN2A} cells whereas the tumorigenicity of NIH3T3$^{H\text{-}RAS}$ was unaffected. Accordingly, RPI-1 did not affect the in vitro transformed morphology of NIH3T3$^{H\text{-}RAS}$ cells whereas a fully reverted phenotype was induced by the drug in the RET mutant-transformed NIH3T3 cells (Fig. 1).

We previously reported that oral RPI-1 was well tolerated with protracted treatment schedule and active in mice bearing small size s.c. human TT tumor xenografts harboring the RET-C634W mutation [17]. In the present study, at the dose of

Table 1 – Effects of oral RPI-1 treatment against s.c. growth of oncogene-transformed NIH3T3 cells in mice

Tumor	Schedule[a]	Max TWI%[b]	Tox/Tot[c]
NIH3T3^{MEN2A}	2qd × 10	69*	0/8
NIH3T3$^{H\text{-}RAS}$	2qd × 10	0	0/8

[a] Mice received oral RPI-1 at the dose of 150 mg/kg, BID, for 10 consecutive days, starting at day 1 (same day of tumor inoculum).
[b] Maximum tumor weight inhibition percent.
[c] Any death in treated mice occurring before any control mice.
* $P < 0.01$ vs. control mice, by Student's *t*-test.

150 mg/kg, BID, RPI-1 exhibited a substantial efficacy against large tumors (about 300 mg) (Fig. 2). Indeed, at day 67, 10 days after 20 days treatment, the mean TW in the RPI-1-treated mice was significantly smaller than in the vehicle-treated mice (60% TWI, $P < 0.01$). The inhibitory effect was persistent (51% TWI, $P < 0.05$, at day 100) and 2 out of 11 mice were free of tumor at the end of the experiment (day 110). In the first 10 days, regression or growth arrest in 81% (9 out of 11) of treated tumors was achieved whereas 100% of control tumors were growing. Reduction in mice body weight following drug treatment never exceeded 10%, and lethal toxicity was never observed.

To investigate the cellular bases of the antitumor activity achieved by RPI-1 in TT tumor xenografts, the extent of apoptosis and angiogenesis in tumor tissue was assessed. One day after RPI-1 treatment (100 mg/kg, BID for 5 days), tumors were removed and processed for histological analysis. The drug clearly affected angiogenesis, since microvessel density (MVD, CD31$^+$ cells) was significantly decreased in treated compared to control tumors (36% inhibition, $P < 0.0005$, Fig. 3A). By TUNEL reaction analysis, the number of apoptotic cells/field was doubled compared to that of control tumors (210%, $P < 0.0001$, Fig. 3B). These findings may account for the significant loss of cellularity in treated TT tumors (47% inhibition, $P < 0.0001$, Fig. 3C), as detected by histological examination. Moreover, the remaining tumor cells were characterized by shrunken, eosinophilic cytoplasm and pyknotic nuclei or vacuolated cytoplasm, indicating cellular degeneration (Fig. 3D). Necrosis level was comparable in control and treated tumors (not shown).

A biochemical analysis of TT tumor xenografts treated with RPI-1 revealed a strong reduction of tyrosine phosphorylation

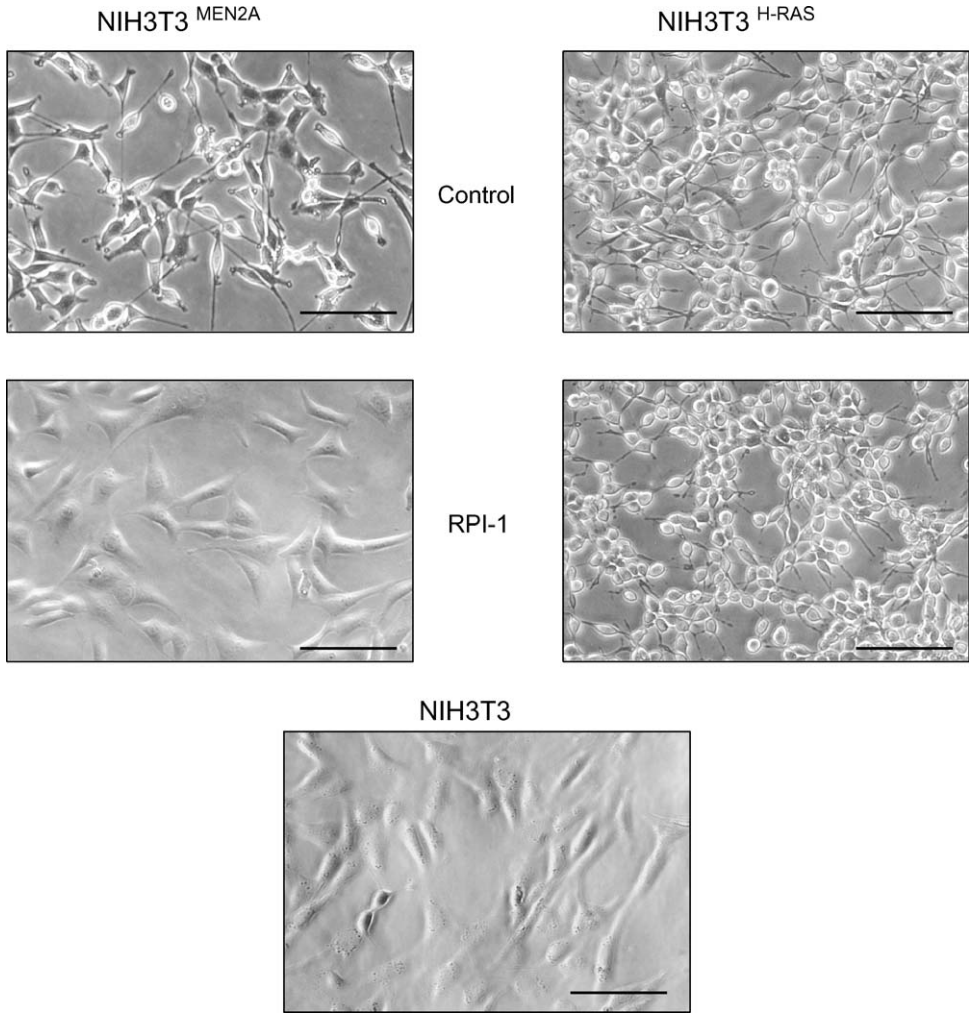


Fig. 1 – Effect of RPI-1 on the transformed morphologic phenotype of murine NIH3T3 fibroblasts transfected with RET-C634R (NIH3T3^{MEN2A}) MEN2A or H-RAS (NIH3T3$^{H\text{-}RAS}$). Cells were exposed to solvent or 10 μM RPI-1 for 24 h and then photographed under a phase-contrast microscope. Parental NIH3T3 cells are shown for comparison. Bar = 200 μm.

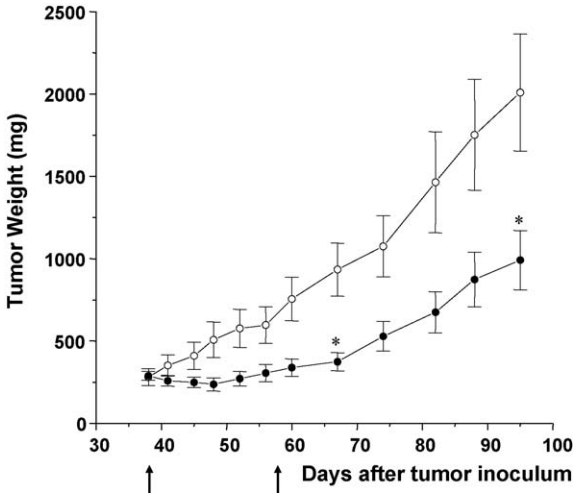


Fig. 2 – Antitumor activity of RPI-1 on large established human TT tumor xenografts. TT cells (2×10^7) were inoculated s.c. in nude mice. When mean tumor weight was approximately 300 mg, mice were treated orally, BID, for 20 days (from day 37 to day 56) with vehicle (○) or 150 mg/kg RPI-1 (●). Each point represents the mean (±S.E.) tumor weight from 9 to 11 mice. Arrows indicate the beginning and the end of RPI-1 treatment. $^{*}P < 0.05$, by the two-tailed Student's t-test.

of Ret immunoprecipitated from RPI-1-treated tumors as compared to vehicle-treated tumors. Analysis of the total tumor lysates showed absence of Ret protein down regulation. Interestingly, in treated tumors a lowered level of VEGF was also observed (Fig. 4).

3.2. *Effects of RPI-1 on VEGF production and induction of apoptosis in vitro*

To better investigate the reduction of VEGF level observed in the TT tumor xenogratfs, we assessed, by a specific ELISA test, the release of VEGF protein in the culture medium derived from TT cells exposed to solvent or RPI-1 (Fig. 5). A time-dependent increase in the growth factor secretion was observed in the control cell supernatant, between 24 and 48 h (66.18 ± 0.99 pg/ml versus 219.14 ± 17.41 pg/ml). The VEGF content in the conditioned medium of TT cells exposed to RPI-1 for 24 h was reduced as compared to the control (44% inhibition, $P = 0.04$, Fig. 5). After 48 h, the drug treatment prevented the increase in secreted VEGF and a further reduction over control value was observed (63% inhibition, $P = 0.03$). At this time, a reduced level of VEGF was also detected in lysates from treated cells by Western blotting (inset in Fig. 5).

Having established the occurrence of apoptosis in TT tumor xenografts following RPI-1 treatment (Fig. 3B), we assessed whether apoptosis induction could be a direct effect of the

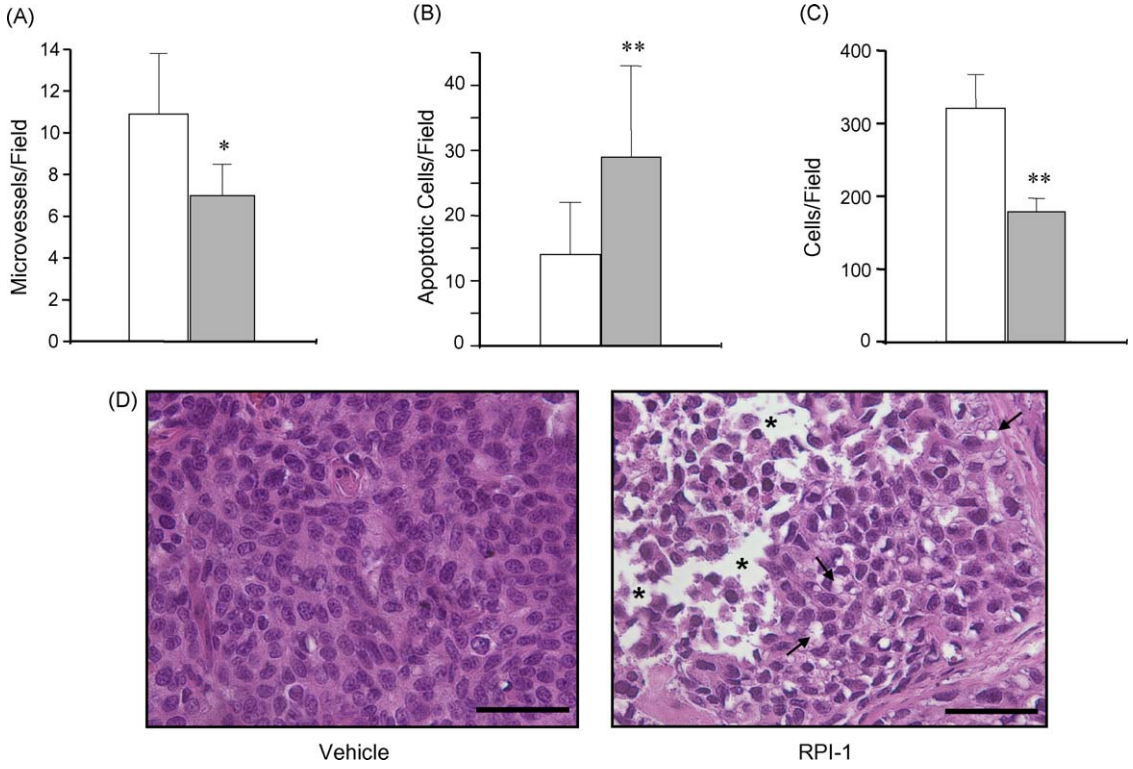


Fig. 3 – Effects of RPI-1 on angiogenesis of TT tumor xenografts and in vivo apoptosis induction. Mice bearing established TT tumors (about 350–400 mg) were treated orally, BID, for 5 days with: vehicle (white columns) or RPI-1, 100 mg/kg (gray columns). One day after the last treatment, tumors were surgically removed and processed for histology. (A) Microvessel density ($CD31^+$ cells), by IHC analysis. (B) Number of apoptotic cells, by TUNEL assay. (C) Total number of cells, by HE staining. All analyses were performed in six fields per section at a 400× magnification. Mean values/field are reported. Bars represent S.D. (D) Representative tumor sections of mice treated with vehicle or RPI-1. Stars show areas of loss of cellularity; arrows show vacuolated cells. HE staining. Bar = 100 μm. $^{*}P < 0.0005$, $^{**}P < 0.0001$ vs. control tumors, by Student's t-test.

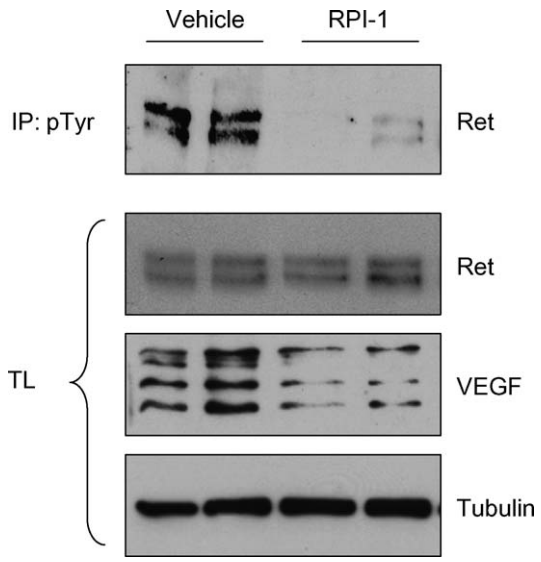


Fig. 4 – Inhibition of Ret phosphorylation and reduction of VEGF levels in TT tumor xenografts by RPI-1 treatment. Mice bearing established TT tumors (about 300 mg) were treated orally, BID, for 16 days with vehicle or RPI-1, 100 mg/kg. One hour after the last treatment, two tumors per group were resected and pTyr-containing proteins immunoprecipitated (IP) from tumor lysates. Immunoprecipitates were subjected to Western blot analysis for Ret. Sample of the respective total tumor lysates (TL) were directly blotted for Ret, VEGF or tubulin as indicated.

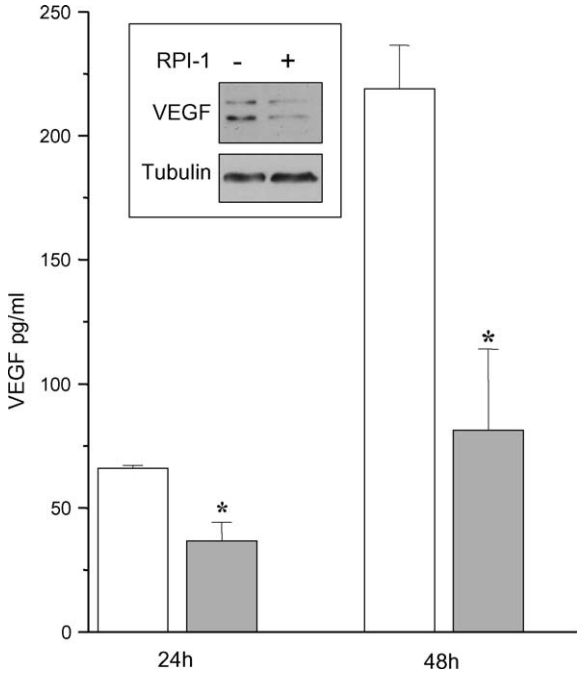


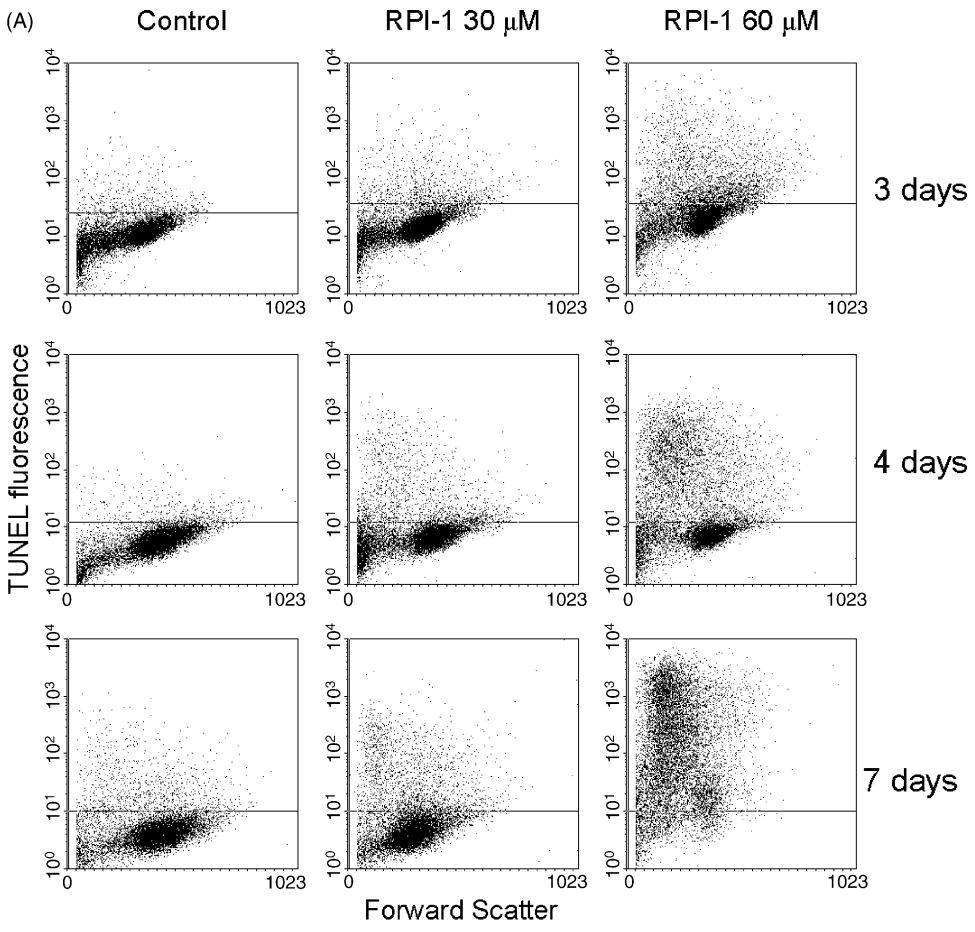
Fig. 5 – Effect of RPI-1 on secreted and intracellular VEGF in TT cell culture. Cells were serum starved and exposed to solvent (white columns) or 30 μM RPI-1 (gray columns) for 24 or 48 h before harvesting the conditioned medium. VEGF protein in the medium was detected by ELISA assay. Data represent the mean values ± S.E. of three replicates. *$P < 0.05$ by student's t-test. Inset: cells treated for 48 h with solvent or RPI-1 were processed for Western blotting with anti-VEGF antibody. Anti-tubulin blot shows the protein loading. One experiment representative of two is shown.

drug on the MTC cells. TT cells were exposed in vitro to RPI-1 for prolonged periods (3, 4 and 7 days) to mimic the protracted treatment of the in vivo studies. As detected by the TUNEL reaction, the extent of apoptotic DNA damage increased in a time- and dose-dependent manner (Fig. 6A). Indeed, after a week, the control cell population presented about 14% of TUNEL positivity, which was increased by nearly two-fold (26%) and six-fold (91%) in cells exposed to 30 or 60 μM RPI-1, respectively (Fig. 6B).

We next examined in TT cell cultures the effects of long lasting RPI-1 treatment on cellular pathways regulating survival and apoptosis. As shown in Fig. 7A, a 4-day treatment with RPI-1 persistently inhibited Ret-pTyr1062 docking site phosphorylation and Ret protein expression, as well as the activation of Ras/ERK and PI3K/AKT downstream pathways, as indicated by the disappearance of activating phosphorylations of AKT and ERKs. We further examined the activation status of the pro-apoptotic protein BAD. Phosphorylation on serine 112 and serine 136, which depends on the Ras/ERKs and PI3K/AKT pathways, respectively, is known to inactivate BAD function [23,24]. Both serines were indeed dephosphorylated in RPI-1-treated cells (Fig. 7B), thus suggesting that the treatment was able to promote activation of the pro-apoptotic factor in TT cells. To further explore the pathways involved in apoptotic cell death induction by RPI-1, we examined caspase activation [25]. Involvement of the initiator caspase-9 in treated cells was suggested by the down-regulation of its proform. In addition, cleavage of caspase-3 and PARP, a caspase-substrate, confirmed the activation of caspase-dependent apoptosis and suggested the involvement of the intrinsic apoptotic pathway (Fig. 7B). On the contrary, caspase-8, an effector of the extrinsic apoptotic pathway, was not activated by RPI-1 treatment (not shown).

4. Discussion

We previously showed that prolonged oral treatment with the RET inhibitor RPI-1 induced complete regressions of small TT human MTC xenografts carrying a MEN-2A-type RET mutation [17]. In the present study, we provide direct evidence of Ret target inhibition in vivo since Ret was found dephosphorylated in TT tumors from treated mice. Moreover, drug treatment was able to delay the s.c. growth of murine NIH3T3 cells transformed by a RET-MEN2A-type mutant whereas the tumorigenicity of the same cells transformed by the H-RAS oncogene was unaffected. Since the efficacy of pharmacological agents may be critically dependent on tumor size, the study on large tumors was performed to further explore the therapeutic potential of the Ret inhibitor. The present results confirm the antitumor activity of drug treatment against large established TT tumor xenografts, in terms of growth delay and



(B)

Percentages of apoptotic cells

days	Control	RPI-1 30 μM	RPI-1 60 μM
3	4.6 ± 2.8	6.3 ± 2.8	33.7 ± 3.0
4	5.4 ± 2.9	14.6 ± 1.9	46.9 ± 5.4
7	14.5 ±4.0	25.9 ± 5.7	91.1 ± 3.9

Fig. 6 – Apoptosis induction in TT cells by RPI-1 treatment. Cells were incubated in the presence of solvent (control) or RPI-1, 30 or 60 μM, for 3, 4 or 7 days. (A) Apoptosis was assessed by FACS analysis on TUNEL-stained cells. A representative experiment is reported. (B) Percentages of apoptotic cells. Values are the mean ± S.D. from at least three independent experiments.

regression of tumor burden evident in most of mice under treatment. A complete regression was found in two animals, with no evidence of disease at the end of experiment, about two months after treatment end.

The antitumor effect of RPI-1 was reflected in an increased apoptosis associated with loss of cellularity and reduced MVD. Our data suggest that the apoptotic response of TT tumors is a direct effect of RPI-1 on tumor cells. In fact, apoptosis was also detected in TT cells in vitro after a prolonged drug exposure in a dose- and time-dependent manner. Furthermore, the drug-induced pro-caspase cleavage indicated activation of the caspase-dependent apoptotic machinery involving the intrinsic mitochondrial pathway [25].

Several lines of evidence implicate Ret in the control of programmed cell death. A pro-survival role of Ret is well established in the development of the mammalian enteric nervous system in which Ret mediates the survival function of the GDNF family of ligands [26]. Mice expressing null mutations in either RET or GDNF die shortly after birth and lack enteric neurons throughout the digestive tract [27,28]. Accordingly, in the absence of a functional Ret receptor, enteric neuron precursors in the foregut of embryos were shown to undergo apoptotic cell death [29]. The prosurvival function of Ret is kinase-dependent and is mainly mediated through the PI3K-AKT pathway. Indeed, in a neuroectodermic cell line, AKT activation by Ret was shown to be essential to

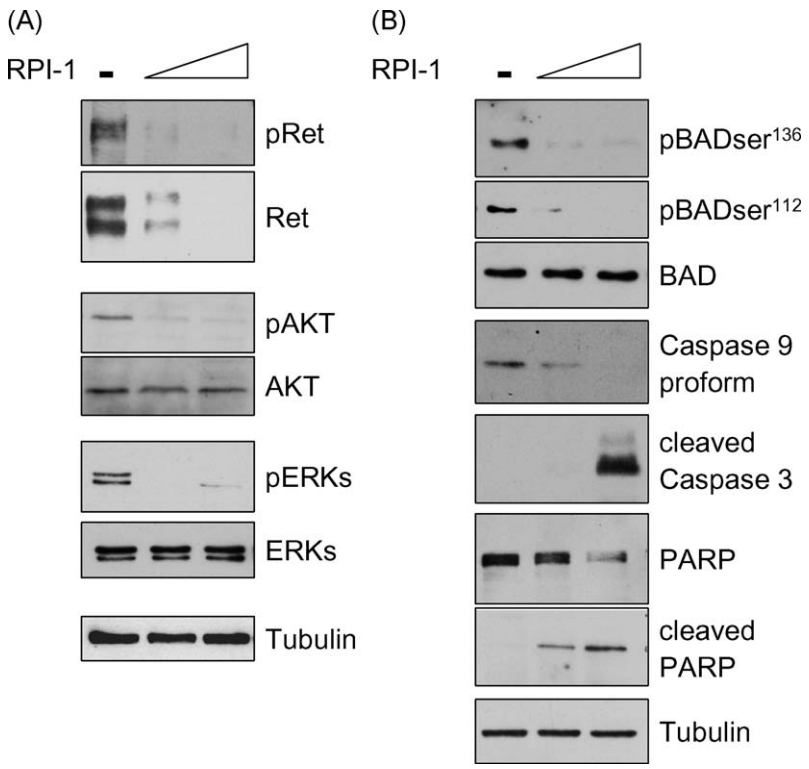


Fig. 7 – Effects of RPI-1 on apoptosis-regulating pathways in TT cells. Cells were continuously exposed to solvent (▬) or RPI-1 (30 or 60 μM), for 4 days. Whole cell lysates were analyzed by Western blotting with the indicated antibodies. (A) Inhibition of Ret phosphorylation (Tyr1062) and expression, and inhibition of activating phosphorylations of AKT (Ser437) and ERKs (Thr202/Tyr204). (B) Dephosphorylation of BAD on Ser136 and Ser112 and cleavage of caspase-9 proform, caspase-3 and PARP. The blots probed with phospho-specific antibodies were re-probed with antibodies directed against the respective proteins after stripping. Blots anti-tubulin are shown as a control of protein loading.

counteract the apoptotic signal on mitochondria, and Ret-mediated activation of ERK was found to contribute to the ability of GDNF to prevent the cleavage of pro-caspase-3 [30]. The data reported in the present study indicate that apoptosis occurs in human MTC cells following in vitro and in vivo treatment with a Ret inhibitor, thus supporting a pro-survival function of oncogenic RET in this tumor type. Indeed, in TT cells cultured in the continuous presence of RPI-1, AKT and ERK were both maintained inactive (dephosphorylated). Such an effect was associated with dephosphorylation of Ret-Tyr1062, consistent with the pTyr1062 docking site requirement for activation of PI3K/AKT and ERK-dependent proliferation/survival downstream pathways [31]. AKT inhibition may have different outcomes that result in apoptosis [32]. Removal of the inhibitory phosphorylation of pro-caspase-9 by AKT may allow the cytochrome *c*-induced proteolytic processing of this initiator caspase [33]. Accordingly, pro-caspase-9 cleavage was detected in RPI-1-treated cells. Furthermore, the pro-apoptotic protein BAD dephosphorylation at serine 136 and 112 was consistent with the drug-induced inactivation of AKT and ERKs. In such a dephosphorylated state, BAD is able to inhibit anti-apoptotic proteins of the Bcl-2 family by direct binding, thereby promoting the intrinsic apoptotic pathway [23,24]. Our data thus support a mechanism of apoptosis induction by RPI-1 through the inhibition of Ret-sustained survival pathways.

In spite of evidence of in vivo pharmacodynamic activity of RPI-1, such an effect was not apparently associated with a reduction in Ret receptor expression, in contrast to that observed in the TT cell line and in other Ret-expressing cell lines treated in vitro [17]. Further studies are needed to elucidate this discrepancy, which could be related to different exposure or time course in the two systems.

An additional finding in this study was the significant reduction of MVD in TT tumors from mice receiving RPI-1 treatment. Although a direct inhibitory effect on endothelial cells cannot be ruled out, an indirect antiangiogenic effect could be related to inhibition of the oncogene-dependent angiogenic phenotype in light of the marked inhibitory effect of RPI-1 in VEGF expression/secretion by the TT tumor cells. Such property is of particular relevance considering that VEGF secretion has been found constitutively activated in some thyroid cancers including MTCs [34]. Even the loss of cellularity observed in treated tumors might be at least in part the consequence of tumor hypoxia. Studies are underway to elucidate the bases of such antiangiogenic activity, which could contribute to the drug activity.

Other small molecule TK inhibitors of different classes have been reported to inhibit Ret kinase activity [15–17,20,35–38]. Evidence of in vivo activity was reported with indolocarbazole derivatives (CEP-751 and its prodrug), which delayed the growth of TT tumor xenografts by s.c. administration [35].

The pyrazolopyrimidine PP1 [36] and the anilinoquinazoline ZD6474 [37] were shown to inhibit the tumorigenicity of RET/PTC3-transformed NIH3T3 cells in mice by s.c. and i.p. administration. Together with our results obtained with the orally delivered RPI-1, these studies indicate that pharmacological inhibition of RET oncoproteins is feasible and may be an effective strategy in the treatment of RET-driven thyroid carcinomas.

Whereas it was generally believed that the antitumor effect of agents that target growth-factor signaling pathways would be manifested as a cytostatic effect, recent evidence indicates that inhibition of protein kinases "pivotal" in neoplastic pathophysiology can effectively lead to cell death and decrease in neoplastic mass, as already reported with clinical TK inhibitors [39]. In a few studies with Ret inhibitors, the occurrence of some degree of sub-G1 DNA [37] or nuclear condensation [35] in treated thyroid carcinoma cells suggests apoptosis induction in vitro. The present study documents that oral RPI-1 treatment is able to induce tumor cell death, resulting in tumor tissue destruction and tumor regression. Although our pharmacological approach does not exclude the existence of additional targets in MTC cells, the effects of RPI-1 are in keeping with similar results obtained when a selective disruption of RET signaling was achieved by a dominant-negative RET mutant [8].

In conclusion, our results support the possibility to abrogate the "pivotal" function of RET oncogenes in the control of proliferation and survival of MTC by a pharmacological approach with an orally available drug. Such findings might have therapeutic implications for a targeted intervention and possibly rational combination treatments designed to promote selective apoptotic cell death of RET-dependent tumors.

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