Effects of BP-14, a novel cyclin-dependent kinase inhibitor, on anaplastic thyroid cancer cells

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Abstract. Anaplastic thyroid carcinoma (ATC) is an extremely aggressive human malignancy characterized by a marked degree of invasiveness, absense of features of thyroid differentiation and resistance to current medical treatment. It is well known that ATCs are characterized by deregulation of genes related to cell cycle regulation, i.e., cyclin-dependent kinases (CDKs) and endogenous cyclin-dependent kinase inhibitors (CDKIs). Therefore, in the present study, the effect of a novel exogenous cyclin-dependent kinase inhibitor, BP-14, was investigated in three human ATC cell lines. The ATC-derived cell lines FRO, SW1736 and 8505C were treated with BP-14 alone or in combination with the mTOR inhibitor everolimus. In all ATC cell lines, treatment with BP-14 decreased cell viability and, in two of them, BP-14 modified expression of genes involved in epithelial-mesenchymal transition. Thus, our data indicate that BP-14 is a potential new compound effective against ATC. Combined treatment with BP-14 and the mTOR inhibitor everolimus had a strong synergistic effect on cell viability in all three cell lines, suggesting that the combined used of CDK and mTOR inhibitors may be a useful strategy for ATC treatment.

Introduction

Thyroid cancers are the most common endocrine malignancies, and represent ~1-1.5% of all tumor-related diseases (1). The overall 5-year survival of these tumors is ~85-90%, with the highest mortality rate reported for undifferentiated histotypes. Thyroid cancers are classified as papillary (PTC), follicular (FTC), medullary (MTC) and anaplastic (ATC) carcinomas.

ATC is one of the most aggressive human malignancies. These tumors are poorly defined, fleshy masses with areas of necrosis and hemorrhage and there are no features of thyroid differentiation (2). The mechanisms underlying the development of ATCs are not completely understood. Available therapies for ATCs include chemotherapy, radiotherapy and surgery (3). Nonetheless, patients with ATC have a median survival of 5 months and less than 20% of patients survive 1 year post-diagnosis.

Mutations in genes encoding members of the RAS-MAPK-ERK and PI3K-AKT-mTOR signaling pathways are usually present in well-differentiated tumor components from which most ATCs develop (4-6). These signaling pathways are mainly involved in controlling cell survival, differentiation, proliferation and metabolism (7). Moreover, changes in the activity of these pathways can lead to drug resistance (8), which is a common feature of ATC. In addition, ATCs are characterized by other genetic and epigenetic aberrations, which cause deregulation of genes related to cell cycle regulation and its checkpoints, as well as alteration of chromosome segregation and spindle structure (9).

ATCs show frequent upregulation of cyclin-dependent kinase (CDK) expression, mostly through inactivation of endogenous cyclin-dependent kinase inhibitors (CDKIs) including $p27^{KIP1}$ (10,11). Since CDKs and endogenous CDKIs are frequently deregulated in cancer cells, these have been considered as valid drug targets. A large number of synthetic CDKIs have been tested as anti-proliferative agents in cancer therapy, including roscovitine, one of the first CDKIs produced (12). Inhibitors of the kinase components of oncogenic pathways are also being currently explored as anti-proliferative agents in several human neoplasms, including thyroid cancer (13).

In the present study, we investigated, in three ATC cell lines, the effects of a novel roscovitine derivate, named BP-14, whose efficacy has already been evaluated in hepatocellular carcinoma (12,14). For this purpose, we evaluated cell

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viability, colony-forming capacity and expression of two genes related to epithelial-mesenchymal transition, *CDH1*, a well known marker of epithelial phenotype, and vimentin (*VIM*), a type III intermediate filament (IF) protein that is expressed in mesenchymal cells (15). We, subsequently, studied the effects of a combined treatment using BP-14 and the mTOR inhibitor everolimus (RAD-001) on ATC cells, focusing on cell viability, migration and invasion abilities.

Materials and methods

Cell lines. The human thyroid cancer cell lines derived from anaplastic thyroid cancer (ATC) used in this study were: FRO (purchased from the European Collection of Cell Cultures, Salisbury, UK), SW1736 (obtained from Cell Lines Service GmbH, Eppelheim, Germany) and 8505C (purchased from Sigma-Aldrich), all harboring a BRAF V600E mutation (16,17). These cell lines were tested for being mycoplasma-free and authenticated by short tandem repeat analysis to be appropriate cell lines of thyroid cancer origin. FRO cells were grown in Dulbecco's modified Eagle's medium (DMEM; EuroClone, Milan, Italy), while SW1736 and 8505C cells were cultivated in Roswell Park Memorial Institute (RPMI)-1640 medium (EuroClone), in a humidified incubator (5% CO_2 in air at 37˚C; Eppendorf AG, Hamburg, Germany). Both media were supplemented with 10% fetal bovine serum (Gibco Invitrogen, Milan, Italy), 2 mM L-glutamine (EuroClone) and 50 mg/ml gentamicin (Gibco Invitrogen). Cultured cells were treated with vehicle (DMSO; Sigma-Aldrich, St. Louis, MO, USA), BP-14 [prepared as described previously (12)] or RAD-001 (everolimus; Novartis, Basel, Switzerland).

Cell viability. To test cell viability, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed, as previously described (18). Briefly, 3,000 cells/well were seeded onto 96-well plates in 200 μl medium. On the following day, the growth medium was replaced with vehicle-treated medium (NT, untreated cultures) or with medium containing different doses of BP-14 (5, 10, 25 and 50 nM) or RAD-001 (25, 50, 100 and 150 nM) alone or in combination, as indicated in Table I. These dose ranges were selected based on previous studies (12,19). The plates were incubated for 0, 24, 48 and 72 h. All experiments were run in quadruplicate, and cell viability was expressed as a percentage relative to the vehicle-treated cells. The percentage of cell viability was used to determine EC_{50} concentrations from dose-response curves, after 72 h of treatment.

Colony-formation assay. The clonogenic activity of the ATC cell lines was evaluated by colony-formation assay. Briefly, the cells were treated with vehicle, BP-14 and RAD-001, alone or in combination for 48 h. FRO, SW1736 and 8505C cells were then seeded in 10-cm plates at a density of 1,000, 500 and 3,000/plate, respectively. Colonies were stained with 0.1% Coomassie Blue solution (Sigma-Aldrich) and counted using Gel Doc (Bio-Rad, Hercules, CA, USA). Data are representative of three independent experiments.

Gene expression assays. Total RNA from the human cell lines, treated either with vehicle, BP-14 at 25 nM or RAD-001 at 100 nM, alone or in combination, was extracted with an RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Total RNA (500 ng) was reverse transcribed to cDNA using random exaprimers and SuperScript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Real-time PCRs were performed using Platinum SYBR Green qPCR SuperMix (Life Technologies) with the ABI Prism 7300 sequence detection systems (Applied Biosystems). The ΔΔCT method, by means of SDS software (Applied Biosystems), was used to calculate mRNA levels. Oligonucleotide primers for *CDH1* (forward, 5'-CAAATCGATGTGGATGTTTCCA-3' and reverse, 5'-CTCGCCCCGTGTGTTAGTTC-3'); *VIM* (forward, 5'-AGCCTCAGGTCATAAACATCATTG-3' and reverse, 5'-AGGTTCTTGGCAGCCACACT-3'); and *β-actin* (forward, 5'-TTGTTACAGGAAGTCCCTTGCC-3' and reverse, 5'-ATGCTATCACCTCCCCTGTGTG-3') were purchased from Sigma-Aldrich.

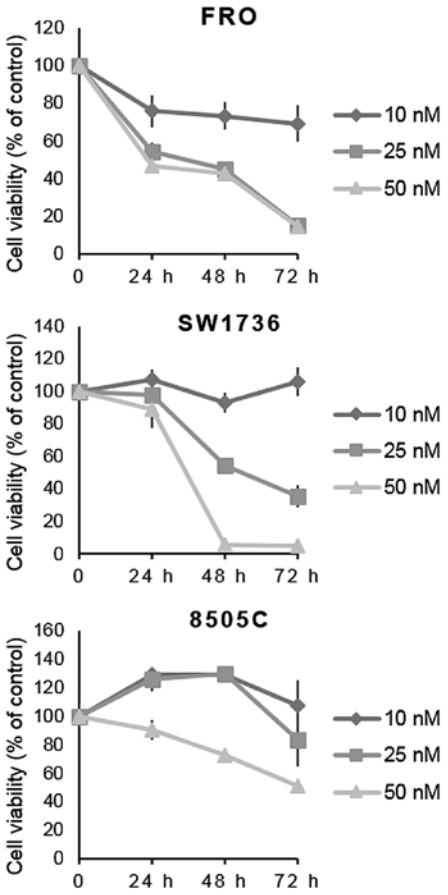

Figure 1. Administration of a CDK inhibitor decreases the viability of anaplastic thyroid carcinoma cell lines. FRO, SW1736 and 8505C cells were exposed to BP-14 at different doses (from 10 to 50 nM). Cell viability was determined by MTT assay after 0, 24, 48 and 72 h and expressed as a percentage of the vehicle-treated cells. All samples were run in quadruplicate.

Combination index (CI) value. Effects of the drug combination used in this study were evaluated using the combination index (CI) equation based on the multiple drug-effect equation of Chou and Talalay (20,21). In all cases where the CI value could be determined, the following diagnostic rule was

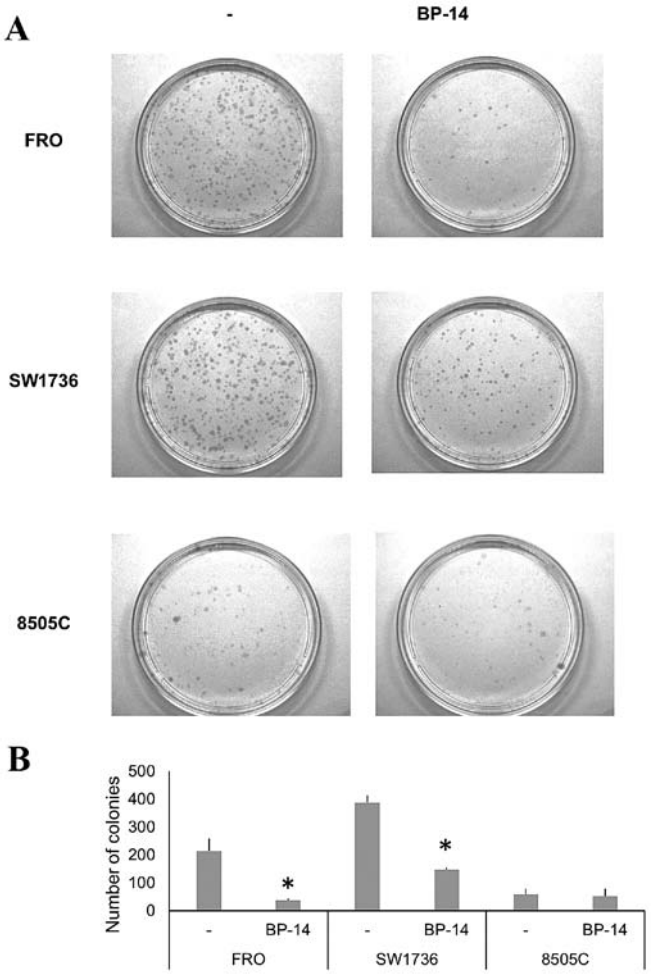

Figure 2. BP-14 treatment decreases the clonogenic ability of anaplastic thyroid carcinoma cell lines. (A) A 48-h treatment with BP-14 at 25 nM significantly decreased colony-formation ability of the FRO and SW1736 cells. (B) Histogram represents the number of colonies per cell line. *P<0.05 by Student's t-test. Data are representative of 3 independent experiments.

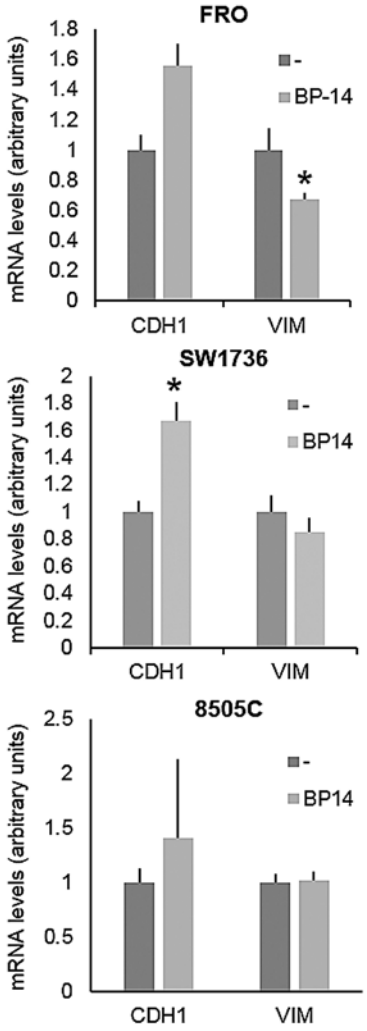

Figure 3. Expression levels of the *CDH1* and *VIM* genes in ATC cell lines after BP-14 treatment. FRO, SW1736 and 8505C cells were treated either with BP-14 at 25 nM or vehicle for 72 h, and *CDH1* and *VIM* mRNA expression was evaluated by qPCR. All samples were run in triplicate. *P<0.05 by Student's t-test. Data are representative of 3 independent experiments.

applied: CI<1 indicates synergism, CI=1 indicates an additive effect and CI>1 indicates antagonism. The analysis was carried out using CompuSyn software (ComboSyn Inc., Paramus, NJ, USA).

Statistical analysis. Cell viability, colony-forming capacity and evaluation of mRNA levels were expressed as means ± SD, and significances were analyzed with the Student's t-test, performed with GraphPad Software for Science (San Diego, CA, USA).

Results

In a first set of experiments, we evaluated the biological effects of BP-14, a novel roscovitine derivate, in three human anaplastic thyroid cancer-derived cell lines (FRO, SW1736 and 8505C), in a time course of treatments with different doses of BP-14. As shown in Fig. 1, incubation with different doses of BP-14 significantly reduced FRO and SW1736 cell viability, at different time-points, while it affected 8505C cell viability only at high doses.

Based on the obtained data, in further experiments, we decided to use the median effective dose of 25 nM for a 48-h treatment, i.e., dose and time required to achieve 50% of the theoretical maximal effect in the FRO and SW1736 cell lines.

When focusing on clonogenic activity, a 48-h treatment with BP-14 at 25 nM reduced the number of colonies in the FRO and SW1736 cells when compared to the number of colonies in the control cells. In particular, as shown in Fig. 2, we detected a 5.5- and 3-fold reduction in the FRO and SW1736 cells, respectively. The 8505C cell line displayed low colony formation efficiency that was not affected by BP-14.

To further investigate the effects of BP-14 on aggressiveness parameters, we analyzed the expression of various genes involved in EMT and, in particular, we focused on *CDH1* and vimentin (*VIM*). A significant increase in *CDH1* gene expression was noted in the FRO and SW1736 cells (FRO, P=0.005; SW1736, P=0.002), while 8505C cells showed no variation in *CDH1* expression levels. *VIM* expression levels were significantly decreased only in FRO cells (P=0.007, Fig. 3).

Table I. Combination index (CI) data for the combined treatment of BP-14 and RAD-001 in ATC cell lines.

	BP-14 dose (nM)	RAD-001 dose (nM)	CI in FRO	CI in SW1736	CI in 8505C
Point 1	5.0	25.0	0.30202	1.097E8	18596.3
Point 2	5.0	50.0	2.12135	0.39369	0.05979
Point 3	5.0	100.0	3.73150	0.50684	0.52049
Point 4	5.0	150.0	5.34166	0.94557	0.05011
Point 5	10.0	25.0	0.31318	0.69159	305.585
Point 6	10.0	50.0	0.50569	0.33884	0.12378
Point 7	10.0	100.0	4.24271	0.47682	6.17151
Point 8	10.0	150.0	0.26274	0.91167	0.10012
Point 9	25.0	25.0	0.55719	15.6815	0.22520
Point 10	25.0	50.0	0.52671	0.36697	0.24538
Point 11	25.0	100.0	0.63991	0.55161	0.24814
Point 12	25.0	150.0	0.54472	0.98968	0.34295
Point 13	50.0	25.0	1.00195	3.44322	0.43611
Point 14	50.0	50.0	0.98965	0.35021	0.46149
Point 15	50.0	100.0	1.04564	0.54217	0.50288
Point 16	50.0	150.0	0.96844	0.97197	0.41918

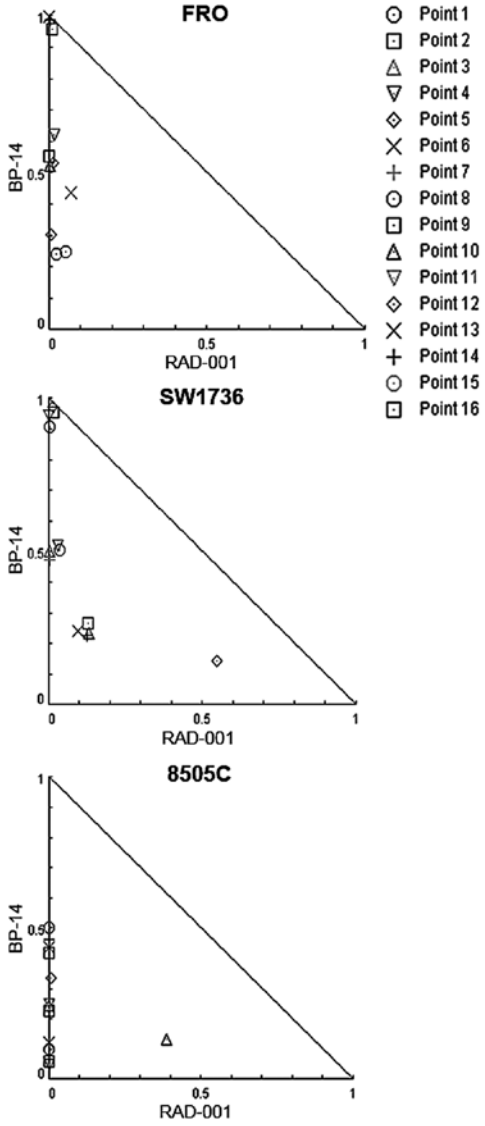

Figure 4. Synergistic effect of the combined treatment of a CDK inhibitor and an mTOR inhibitor in ATC cell lines. Normalized isobolograms representing FRO, SW1736 and 8505C cell viability after a 48-h treatment with different doses of BP-14 (from 5 to 50 nM) and RAD-001 (from 25 to 150 nM).

Since a promising approach to cancer therapy is the combined use of different drugs, we evaluated whether BP-14 interacts synergistically with other compounds already used in cancer treatment, such as everolimus (RAD-001). For this purpose, we evaluated the effect of a combined treatment of BP14 and RAD-001 by measuring the CI values, according to the Chou and Talalay equation (20,21). The two-drug combination elicited a strong decrease in cell viability, compared to the untreated cells (CI values were <1) (Table I and Figs. 4 and 5). Our results revealed that BP-14 and RAD-001 exhibited a synergistic effect in decreasing cell proliferation at a quite high range of doses in all three ATC cell lines.

Then, we evaluated changes in EMT-related processes, i.e. colony formation as well as *CDH1* and *VIM* gene expression, after a combined treatment with BP-14 at 25 nM and RAD-001 at 100 nM. A 48-h BP-14 treatment significantly reduced colony formation in the FRO (P=0.0002) and SW1736 (P=0.004) cells (comparison of Fig. 2 and 6A). The treatment with RAD-001 at 100 nM reduced the clonogenic ability only in the SW1736 cells (P=0.027). In contrast, the combined treatment significantly reduced the clonogenic capacity of the FRO (P=0.0007) and SW1736 (P=0.0002) cells. The 8505C cells displayed, again, no benefit after either BP-14 or RAD-001 treatment, alone or in combination.

Following analysis of the expression of EMT-related genes, treatment with BP-14 at 25 nM increased the *CDH1* mRNA level in the FRO (P=0.005) and SW1736 (P=0.002) cells and reduced the *VIM* mRNA level only in the FRO cells (P=0.007) (comparison of Fig. 3 and 6B). Treatment with RAD-001 at 100 nM modestly modified *CDH1* (FRO, P=0.006; SW1736, P=0.0047; 8505C, P=0.02) and *VIM* (FRO, P=ns; SW1736, P=0.007; 8505C, P=ns) mRNA levels in all three cell lines. The synergistic treatment with BP-14 and RAD-001 greatly increased the *CDH1* mRNA level in the 8505C (P=0.0003), so far considered 'unresponsive' to single agent treatment.

Discussion

The search for new target therapies for ATC is urgently needed. In fact, this neoplasm, although a rare histotype of thyroid cancer, is characterized by an extremely poor prognosis (22). The complete loss of differentiation makes it unresponsive to radioiodine treatment (23), and current treatments, based on a combination of surgery, chemotherapy and external radiotherapy, are not effective. The discovery of molecular alterations occurring in such tumors, has permitted the selection of a series of novel agents able to act against such molecular targets. For an initial screening of these novel potential drugs, several human ATC cell lines, which carry the

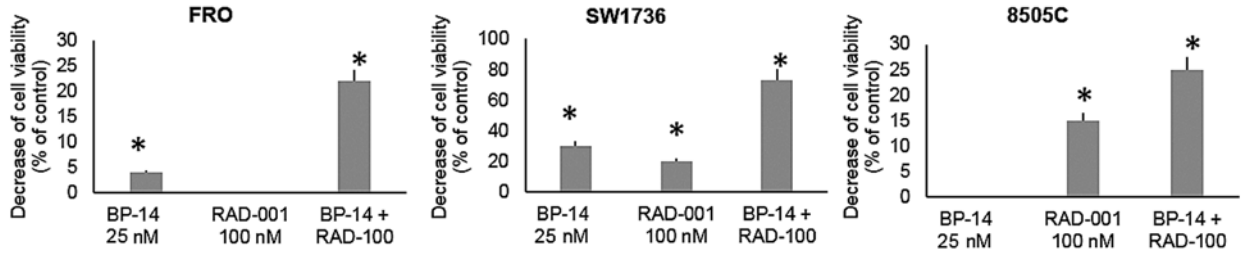

Figure 5. Effect of the combined treatment of BP-14 at 25 nM and RAD-001 at 100 nM on ATC cell viability. Cells were treated for 48 h with BP-14 at 25 nM and RAD-001 at 100 nM, alone or in combination. Bars indicate the percentage of viable cells vs. control (vehicle-treated cells). *P<0.0001 by Student's t-test. Data are representative of 3 independent experiments.

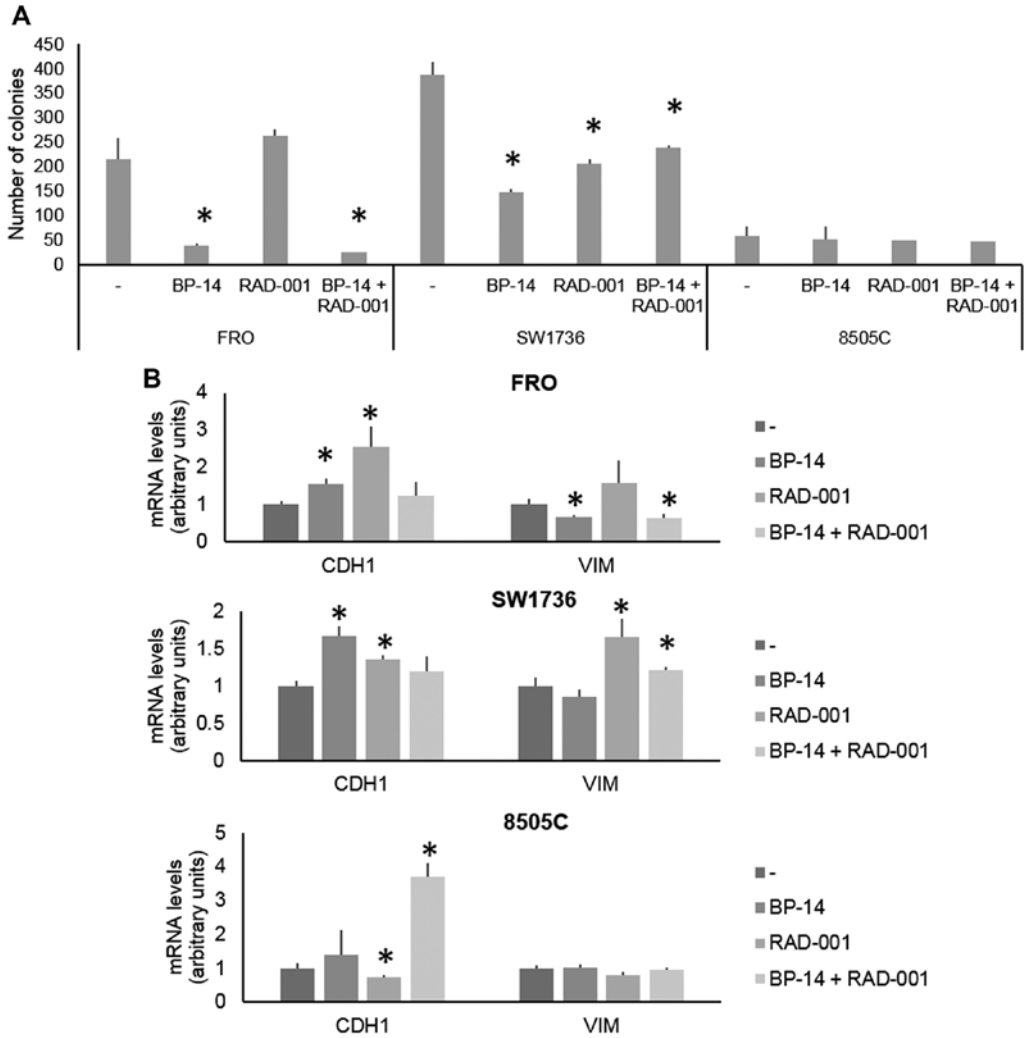

Figure 6. Synergy of a combined treatment of BP-14 and RAD-001 on EMT-related phenomena in anaplastic thyroid carcinoma cell lines. (A) A 48-h combined treatment with BP-14 at 25 nM and RAD-001 at 100 nM significantly decreased the colony-formation ability of FRO and SW1736 cells. (B) FRO, SW1736 and 8505C cells were treated either with BP-14 at 25 nM or RAD-001 at 100 nM, alone or in combination, for 72 h and *CDH1* and *VIM* mRNA expression was evaluated by qPCR. All samples were run in triplicate. *P<0.05 by Student's t-test. Data are representative of 3 independent experiments.

same genetic and epigenetic alterations of human neoplasias, have been largely exploited.

Among the inhibitors affecting major oncogenic pathways, the most attractive are those targeting the kinases involved in cell cycle regulation (24). Since data indicate that CDKs and CDKIs play a role in thyroid tumorigenesis (25-27), we focused on the efficacy of a novel compound, BP-14, which is able to antagonize CDK1/2/5/7 and CDK9 (14). Apart from '*in vitro*' effects, using cultured cell lines (12,14), BP-14 has been previously investigated '*in vivo*' using xenografts derived from hepatoma cell lines and chemically induced liver cancer (14). By these approaches, it has been demonstrated that, at the dose of 1 mg/kg, BP-14 significantly reduces tumor growth without any side effects. We showed that BP-14 affected the viability of three different ATC cell lines at very similar concentrations to those active in hepatoma cells (14): 25 nM for FRO and

SW1736 cells and 50 nM for 8505C cells. Therefore, considering data obtained '*in vitro*' and '*in vivo*' with hepatoma cells, our findings suggest that BP-14 could be used '*in vivo*' for ATC treatment.

A major finding in our resarch was the demonstration of synergy between BP-14 and the mTOR inhibitor everolimus. In fact, in all three ATC cell lines analyzed, the combined use of BP-14 and everolimus significantly decreased cell viability to a greater extent than using the two compounds alone. The simultaneous use of compounds targeting distinct signaling pathways appears to be a very efficient anticancer strategy (28). Accordingly, previous research has demonstrated '*in vivo*' the synergy between mTOR and MAPK inhibitors (29). Since the combination of distinct molecular drugs appears to be quite effective in ATC (30), our data would indicate a novel strategy for treatment of this type of tumor.

Acknowledgements

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