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Everolimus is an active agent in medullary thyroid cancer: a clinical and *in vitro* study

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Abstract

Everolimus, an mTOR inhibitor, which has been demonstrated to induce anti-tumour effects in different types of neuroendocrine tumours, has never been evaluated in patients with medullary thyroid cancer (MTC). The aim of this study was to evaluate the *in vitro* and *in vivo* effects of everolimus in combination with octreotide in MTC. Two patients with progressive metastatic MTC and high calcitonin levels were treated with everolimus 5–10 mg/day. Both patients were under treatment with octreotide LAR at the study entry. An *in vitro* study was also performed to assess everolimus effects on MTC cell lines (TT and MZ-CRC-1 cells). A tumour response was observed in both patients. Serum calcitonin decreased by 86% in patient 1 and by 42% in patient 2. In TT and MZ-CRC-1 cells, everolimus induced a significant dose-dependent inhibition in cell proliferation. This effect seems to be related to a cell cycle arrest in G_0/G_1 phase in both cell lines and to the induction of cellular senescence in TT cells. Everolimus in combination with octreotide may be active as anti-tumour therapy in patients with progressive metastatic MTC, suggesting to further evaluate this agent in MTC patients in a large prospective study.

Keywords: medullary thyroid cancer • neuroendocrine tumour • calcitonin • somatostatin analogues • everolimus

Introduction

Medullary thyroid cancer (MTC) is a neuroendocrine tumour originating from the C cells within the thyroid gland [1, 2]. Medullary thryroid cancer has been demonstrated to express somatostatin receptors, which are the molecular target of somatostatin analogues [3, 4]. Octreotide and lanreotide, two somatostatin analogues exerting both anti-secretory and anti-tumour activity in different types of neuroendocrine tumours, have shown low efficacy in MTC [5–8]. This is likely explained by the fact that octreotide and lanreotide, the currently available somatostatin analogues, act mainly through the somatostatin receptor subtype 2 which seems not so relevant for obtaining anti-proliferative effects in MTC [9]. Recently, the AKT-mTOR pathway has been demonstrated to be overexpressed in neuroendocrine tumours [10, 11]. In addition, everolimus (RAD001), a novel agent that antagonizes mTOR, has

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been reported to induce encouraging results in terms of neuroendocrine tumour control both *in vitro* [12, 13] and *in vivo* [14, 15]. Everolimus is able to disrupt an important survival pathway of cancer cells thus triggering apoptosis in several tumour cell models [16]. However, alternative mechanisms of programmed cell death have been recently described in papillary thyroid cancer (PTC) cells exposed to everolimus. In fact, it was demonstrated that everolimus can sensitize PTC cells to radiation and chemotherapy through the induction of autophagic cell death [17].

Medullary thyroid cancer may be an optimal model to evaluate the efficacy of everolimus for different reasons: (a) this tumour expresses the specific molecular pathway targeted by everolimus; (b) newly discovered rearranged during transfection (RET) and VEGF inhibitors are now under evaluation in clinical trials and promises to be effective in these patients. However, there is no chemotherapy or biological therapy which is now available to effectively treat patients with metastatic or relapsing MTC; (c) calcitonin and carcinoembryonic antigen (CEA) are highly sensitive and specific markers of MTC progression and both can help to reliably evaluate the response to everolimus; (d) a recent *in vitro* study showed a potent inhibitory effect of everolimus on cell proliferation of MTC cells [18].

Nowadays no data are available concerning the activity of everolimus, either alone or in combination with octreotide, in patients with MTC. The objective of this study was to assess the *in vivo* and *in vitro* efficacy of this drug in patients with progressive metastatic MTC.

Materials and methods

Clinical study

Everolimus was obtained by Novartis Farma S.p.A. (Origgio, Italy) for compassionate treatment in two patients with progressive metastatic MTC, according to Novartis guidelines for compassionate use. Everolimus was started at the dose of 10 mg a day in one patient and 5 mg a day in one other and was lowered to 5 mg a day, 5 mg every other day or discontinued according to toxicity. Both patients were under treatment with octreotide LAR 30 mg a month at the study entry and received everolimus in combination.

Clinical symptom evaluation and calcitonin and CEA measurement were performed monthly. Bone metastases were evaluated by contrast-enhanced MRI of vertebral bodies in patients 1 and 2. Lymph node metastases were evaluated by contrast-enhanced CT scan and colour Doppler ultrasonography in patient 2. Fluoro-18 deoxyglucose positron emission tomography (FDG-PET) was also performed in both patients.

Written informed consent of both patients was obtained. The study was conducted in accordance with the Declaration of Helsinki, adhering to all local regulatory guidelines.

Hormone assays

Serum calcitonin concentrations, evaluated during clinical study, were determined by a commercially available IRMA (Byk Gulden Italia S.p.A., Milan, Italy). The detection limit of the assay was <0.7 pg/ml. The intraassay conduction velocities (CVs) were 6.2% and 2.3% at serum concentrations of 50 and 200 pg/ml, respectively. The interassay CVs were 6.9% and 4.2% at serum calcitonin concentrations of 50 and 200 pg/ml, respectively.

Drug preparation and cell line cultures

Everolimus was kindly provided by Novartis Pharma (Basel, Switzerland), and dissolved in dimethyl sulfoxide (DMSO) to yield a stock solution of 10 mM, which was stored at -20°C. TT and MZ-CRC-1, both human MTC cell lines, were obtained from Prof. Lips (University of Utrecht, The Netherlands). TT and MZ-CRC-1 cells were grown at 37°C in F-12 with Kaighn's Modification medium containing 10% foetal bovine serum, 2 nM glutamine and 10^5 U/l penicillin–streptomycin and maintained in a humidified atmosphere of 5% CO_2. The cells were grown in 75 cm^2 flasks and passaged once every 4–7 days on a 1:2 split.

Cell viability assay

TT and MZ-CRC-1 cells were plated in 96-well plates at a density of 3.5×10^4 per well. The following day, cell culture medium of both cell lines was replaced with medium containing various concentrations of everolimus (range 0.1–50 nM for TT cells and 0.1–100 nM for MZ-CRC-1 cells). The plates were then placed in a 37°C, 5% CO_2 incubator. After 3 days, medium was refreshed and everolimus was added again. After 6 days of treatment cells were harvested for cell viability assay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to analyse the effect of everolimus on cell viability. This assay is based on a colourimetric method in which tetrazolium salt MTT (5 mg/ml in PBS) is added in the plates for 3 hrs to be reduced into coloured formazan compounds by mitochondrial enzymes. It is solubilized with ethanol:DMSO (1:1) solution, and the absorbance of the converted dye is measured at a wavelength of 570 nm with background subtraction at 540 nm, using ELx800 Absorbance Microplate Reader (Bio Tek Instrument, Winooski, VT, USA). All assays were performed in six replicates and were repeated at least three times.

Calcitonin secretion

TT and MZ-CRC-1 cells were plated in 96-well plates at a density of 3.5×10^4 per well. The following day, cell culture medium of both cell lines was replaced with medium containing various concentrations of everolimus (0.5, 1, 2 and 10 nM for TT cells and 1, 10 and 50 nM for MZ-CRC-1). After 3 and 5 days, medium was changed with fresh medium without (control group) or with everolimus. After 6 days conditioned medium was collected to measure calcitonin levels through the CLT-LIAISON kit (DiaSorin Inc., Stillwater, MN, USA), which employs a chemiluminescent immuno assay. The results were obtained by determining the mean value of six replicates, and the experiments were repeated at least three times. The data were corrected for the effect of treatment on cell number.

Cell cycle analysis

TT and MZ-CR-1 cells were plated in duplicates in six-well plates at density of 3×10^5 cell/well. The following day, cell culture medium of both cell lines

was replaced with medium containing everolimus at the concentration of 5 and 50 nM, respectively in TT and MZ-CRC-1 cells. After 3 days, medium was changed with fresh medium without (control group) or with everolimus. After 6 days, cells were harvested by gentle trypsinization, washed three times with cold PBS (calcium and magnesium free), and collected by centrifugation at 1200 *g* for 5 min. The pellets were resuspended in and directly stained with propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) staining solution (50 μg/ml PI, 0.6 μg/ml RNase A and 0.05% Triton X-100 in 0.1% sodium citrate) and incubated at 4°C for 30 min. To evaluate cell-cycle PI, for each tube, 10,000 cells were immediately measured and fluorescence was collected as FL1-A with a FACScalibur flow cytometer (Becton Dickinson, Erembodegem, Belgium) using Cell Quest Pro Software, as previously described [19].

Flow cytometric analysis of apoptosis

The effect of everolimus on apoptosis was analysed by AnnexinV-FITC and PI staining. TT and MZ-CRC-1 (3×10^5 cells/well) were plated in six-well plates and treated with everolimus, as previously described in the section of cell cycle analysis. After 3 and 6 days cells were harvested by gentle trypsinization, washed three times with cold PBS (calcium and magnesium free; Sigma-Aldrich), and collected by centrifugation at 1200 × *g* for 5 min. The pellets were re-suspended in 1× binding buffer (0.1M Hepes/NaOH (pH 7.4), 1.4M NaCl, 25 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Cells (1×10^5 cells) were stained with 5 μl of Annexin V-FITC (BD Pharmingen, San Diego, CA, USA) and 10 μl PI (50 μg/ml in PBS). After 15 min. of incubation at room temperature in dark, 400 μl of 1× binding buffer were added to each tube. The analysis was performed by FACScalibur at 10,000 events for each sample. With the use of CellQuest Pro Software, three subsets of cells, based on intensity of staining with Annexin and PI, were identified: Annexin−/PI− (live cells), Annexin+/PI− (early apoptotic cells), and Annexin+/PI+ (late apoptotic and necrotic cells). Subsequently, the percentage of each population was calculated, as previously described [20].

Western blot analysis

TT and MZ-CRC-1 cells after 3 and 6 days of incubation with everolimus, respectively at the concentration of 5 and 50 nM, were scraped, washed twice in cold PBS and resuspended in lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mM $Na_4P_2O_7$ and 1 mM Na_3VO_4. Cellular debris were pelleted by centrifugation at 13,000 × *g* for 15 min. at 4°C and the supernatant was collected for protein analysis. Cell extracts (30 μg/lane) were resolved on a 10% SDS-PAGE, transferred to nitrocellulose sheets at 100 mA for 1.5 hr, and probed with specific antibodies: anti-caspase 3, anti-caspase 8, anti-caspase 9 and poly(ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Beverly, MA, USA). Blots were then developed using enhanced chemo-luminescence detection reagents and exposed to X-ray film.

Senescence-associated β-galactosidase staining

TT and MZ-CRC-1 cells were plated in six-well plates at a density of 2.5×10^5 per well. The following day, cell culture medium of both cell lines was replaced with medium containing everolimus at a concentration of 5 and 75 nM, respectively, in TT and MZ-CRC-1. After 3 days plates were refreshed and everolimus was added again. After 6 days of treatment, cells were washed with PBS and fixed with 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min. at room temperature. Then, cells were washed twice with PBS and incubated at 37°C (no CO_2) with fresh senescence-associated-β galactosidase stain solution: 1 mg/ml X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM $MgCl_2$ and 30 mM citric acid/phosphate buffer (0.1M citric acid and 0.2M Na_2HPO_4). Staining was evident in 1.5 hr for TT and 3 hrs for MZ-CRC1. β-Galactosidase positive cells were detected and counted by light microscopy. For each determination, in 30 randomly selected fields at 20× magnification β-galactosidase positive and negative cells were counted twice. The number of positive (blue) cells was divided by the total number of counted cells resulting in the percentage of β-galactosidase positive cells. Values of experiments in triplicates were averaged.

Statistical analysis

The comparative statistical evaluation among groups was first performed by the ANOVA test. When significant differences were found, a comparison between groups was made using the Newman–Keuls test. Student's *t*-test was used to assess differences between two groups. In all analyses, values of $P < 0.05$ were considered statistically significant. Data are reported as mean ± S.E.M.

Case report

Patient 1 (a 60-yr-old male) had a clinical diagnosis of MTC in 1999 on the basis of evidence of cervical enlargement, associated to high serum concentrations of calcitonin (185 pg/ml), thyroid nodules and cervical lymph node enlargement at the neck ultrasonography. An intervention of thyroidectomy plus central and left latero-cervical lymph node dissection was performed and histology confirmed the diagnosis of MTC with lymph node metastases. After surgery, there was persistence of high serum calcitonin concentrations. In 2004, tumour progression occurred with appearance of bone metastases and, subsequently in 2006, with the onset of palpable right latero-cervical lymph node enlargement leading to a second surgery for removal of lymph node metastases. Bone pain was initially controlled by a treatment with zoledronic acid (4 mg a month i.v.) since September 2005. Because of the progressive worsening of clinical symptoms related to bone metastases, a treatment with octreotide LAR 30 mg a month was started in 2006 with poor relief on symptoms. Because an Indium-111-DTPA-Phe1-octreotide Octreoscan (Mallinckrodt Medical, Petten, The Netherlands; 120–200 MBq) was positive in correspondence of vertebral metastases (Krenning score grade 3), three cycles of radio-labelled somatostatin analogue therapy with 90Y-Dotatate were performed in 2006–2007. No significant relief of bone symptoms was observed and a marked progression of vertebral metastases with spinal cord compression and onset of paraplegia and parasthesia occurred in 2008. For this reason, 10 sessions of external radiotherapy for a cumulative radiant dose of 30 Gy were performed in May and June 2008 to treat vertebral metastases of the dorsal tract (D4–D6). However, bone symptoms and signs persisted unmodified and a

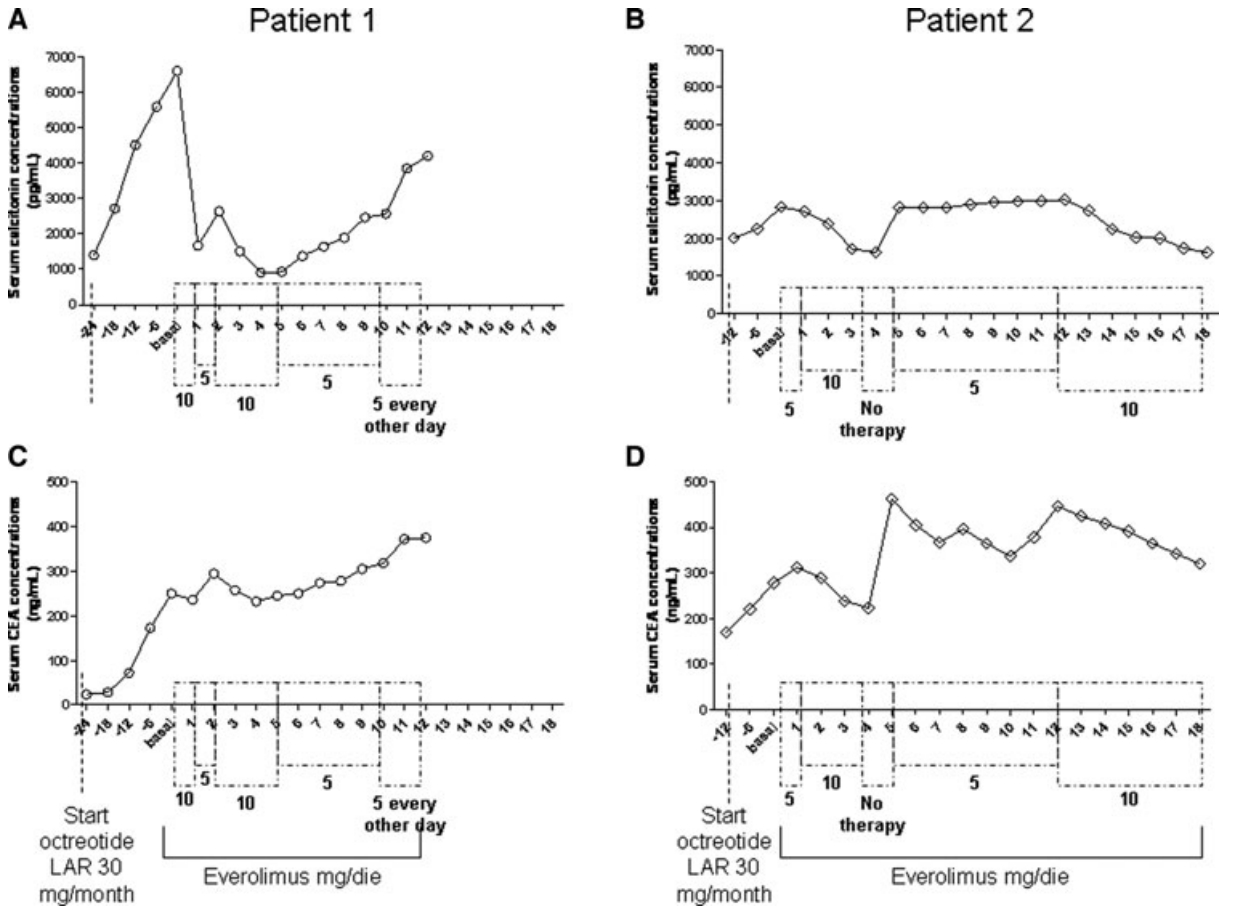

Fig. 1 Calcitonin and CEA serum concentrations in Patient 1 (**A**, **C**) and Patient 2 (**B**, **D**) because the start of the treatment with octreotide LAR 30 mg a month and then during treatment with everolimus at different doses.

further tumour progression at skeletal level was observed at the MRI evaluation. In December 2008, everolimus was started at the dose of 10 mg a day per os under compassionate use.

Patient 2 (a 74-yr-old male) had a clinical diagnosis of MTC in 1974. Medullary thyroid cancer was revealed by appearance of cervical enlargement, associated to thyroid nodules at the neck ultrasonography and high serum concentration of calcitonin (215 pg/ml). A total thyroidectomy was performed and the diagnosis of MTC was confirmed at the pathological examination. After surgery, serum calcitonin levels were still positive and progressively increasing. Two other operations were performed in 2001 and 2007 to remove lymph node metastases detected at the right and left latero-cervical compartments. Post-operative follow-up revealed persistence of high serum calcitonin concentrations and evidence of cervical and mediastinal lymph node metastases and bone metastases associated to clinical symptoms (diarrhoea, flushing and bone pain).

In March 2008, a treatment with octreotide LAR 30 mg a month was started resulting in mild improvement of diarrhoea and flushing but progressive worsening of bone pain from vertebral metastases. A treatment with zoledronic acid (4 mg a month i.v.) was started in October 2008 and discontinued in December 2008 because of insurgence of severe gastro-intestinal symptoms at each administration. In February 2009, everolimus was started at the dose of 5 mg a day per os under compassionate use.

Results

In vivo anti-proliferative and anti-secretive effects of everolimus

Patient 1 had a rapid serum calcitonin decrease 1 month after the start of everolimus, then the levels moderately raised again at 2 month follow-up, after 2 weeks of drug discontinuation for haematological toxicity (grade 3 thrombocytopenia and grade

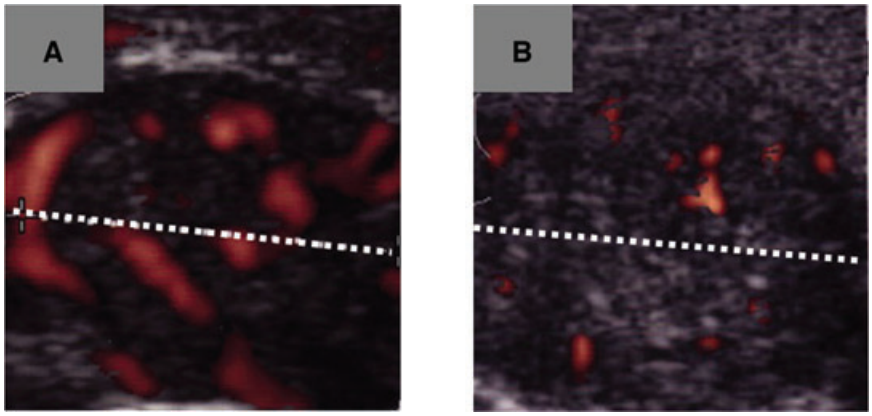

Fig. 2 Colour Doppler ultrasonography imaging of a laterocervical lymph node metastasis before (**A**) and after 3 months of treatment with everolimus 10 mg a day (**B**).

2 anaemia) and resumption of everolimus treatment at a dose of 5 mg a day for 2 other weeks (Fig. 1A and C). Because the third month of treatment, everolimus was given at the dose of 10 mg a day resulting in a progressive decrease of calcitonin until the 5-month follow-up. Since month 6, the dose was lowered to 5 mg a day for 2 months and then to 5 mg every other day because of grade 2 thrombocytopenia and grade 3 abdominal toxicity, including nausea, diarrhoea and abdominal pain. This change in the dose of everolimus resulted in a mild and progressive increase of calcitonin concentration. Serum CEA variations after treatment paralled at a lower extent CT variations along the follow-up.

At the morphological evaluation, skeletal MRI was substantially unchanged after 6 months of treatment with everolimus; however, a mild decrease was reported for vertebral lesions of the lumbar tract. Neurological symptoms were consistent with paraplegia and incapacity of bladder empting with necessity of urinary catheterization at the beginning of everolimus treatment. Both paraplegia and bladder dysfunction markedly improved 1 month after treatment with partial recovery of walking and bladder function. This condition was maintained for 6 months. After everolimus dose decrease and then discontinuation, paraplegia and bladder dysfunction relapsed and progressively worsened along the follow-up.

Everolimus was withdrawn by the patient himself in December 2009 after 1 year of treatment due to a further tumour progression at skeletal level with involvement of all vertebral bodies and skull and consequent worsening of neurological symptoms.

Patient 2 received initially everolimus at the dose of 5 mg a day because of a mild chronic anaemia (haemoglobin: 10.5 g/dl) suggesting not to administrate the full dose of 10 mg a day. As the drug was clinically and biochemically well-tolerated, since month 2 the dose was increased to 10 mg a day. This change in the dose of everolimus resulted in a marked decrease of serum calcitonin, which was substantially unchanged after the first month of treatment with 5 mg a day (Fig. 1B and D). Serum CEA levels were even increased under everolimus 5 mg a day as compared to baseline and mildly decreased after increasing the dose to 10 mg. Then, because of insurgence of grade 2 anaemia and grade 3 neutropenia with fever and need of antibiotic therapy, everolimus was discontinued until recovery to grade 1 haematological toxicity. Since month 6, everolimus was restarted to 5 mg a day resulting in stable calcitonin concentrations and variable CEA concentrations with initial decrease and subsequent regrowth. Since month 13, everolimus was increased to 10 mg a day resulting in a progressive decrease of calcitonin and CEA concentrations. The treatment with everolimus was still ongoing at the last follow-up at the same dose with good tolerance. After the beginning of everolimus, no diarrhoea and flushing were complained by the patient while bone pain was quite well-controlled only requiring short-time treatment with oral analgesics.

At the skeletal MRI, vertebral lesions were substantially unchanged after 6 months of treatment with everolimus, then increased in number at 12-month follow-up but not further progressed at 18-month follow-up. At the multislice CT scan, mediastinal lymph node metastases were unchanged in size along the follow-up; however, a large area of necrosis was evident within the biggest lesion at pre-vascular level since the first CT evaluation 3 months after the beginning of everolimus treatment. At the colour Doppler ultrasonography, a dramatic change of tumour vascularization was observed in the laterocervical lymph node metastases (Fig. 2), with a mild decrease in the left laterocervical lymph node metastasis after treatment as compared to baseline (15 mm *versus* 22 mm, respectively).

In vitro anti-proliferative and anti-secretory effects of everolimus

Everolimus significantly suppressed the growth of TT cells [median inhibition concentration (IC_{50}) 0.33 nM, maximal inhibition of proliferation: −48%] in a dose-dependent manner after 6 days of incubation (Fig. 3A). The anti-proliferative effect of everolimus was less prominent in MZ-CRC-1 cell line, as shown by the higher IC_{50} (2.3 nM) and lower maximal inhibition of proliferation (−22.5%) compared to TT cells (Fig. 3B). Incubation of TT cells (Fig. 3C) and MZ-CRC-1 (Fig. 3D) with everolimus for 6 days did not significantly affect calcitonin secretion.

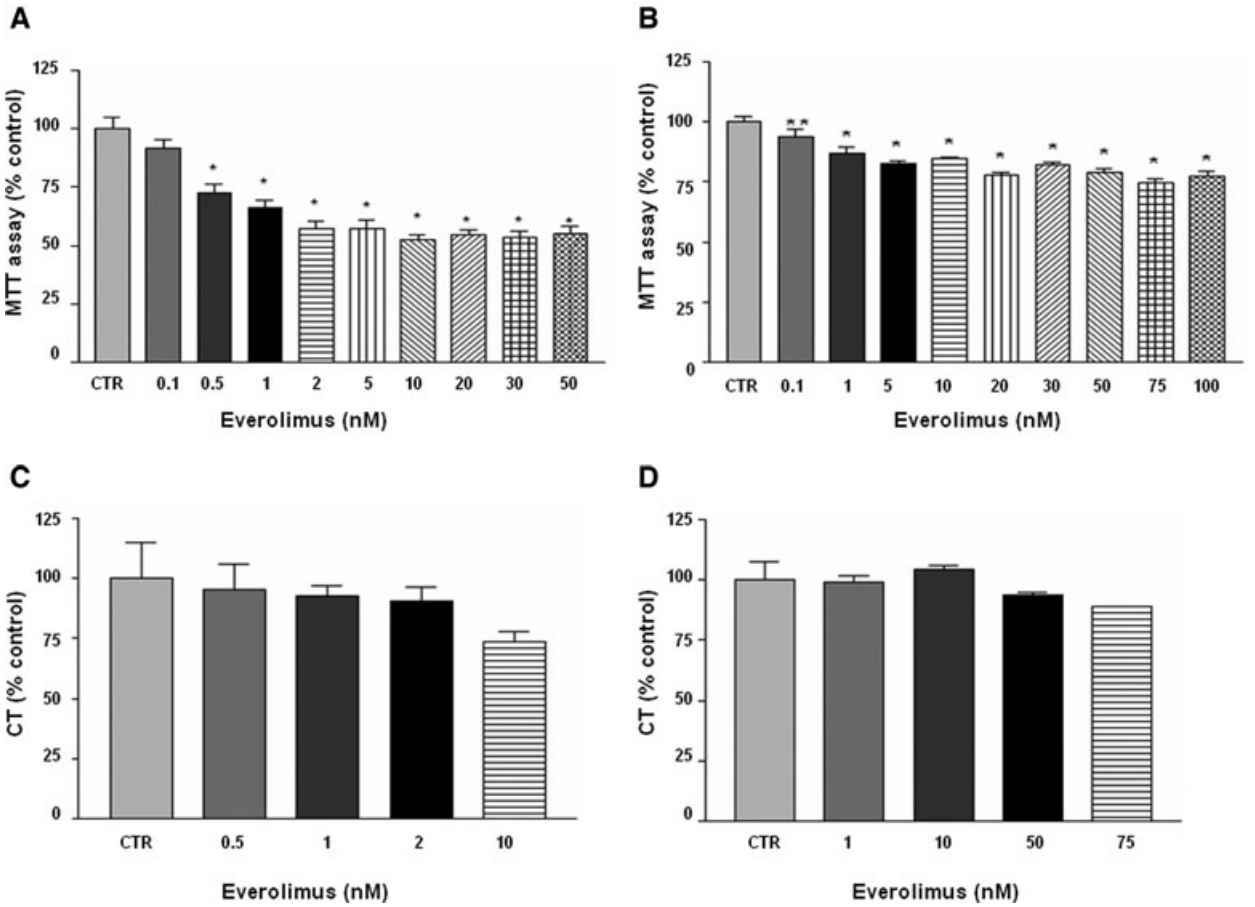

Fig. 3 Effects of 6 days of incubation with or without everolimus on cell proliferation (**A**, **B**) and CT secretion (**C**, **D**) in TT (**A**, **C**) and MZ-CRC-1 (**B**, **D**). Values are expressed as the percentage of control (untreated cells) and represent the mean ± S.E.M. of at least three independent experiments in six replicates. $^{*}P < 0.001$ and $^{**}P < 0.05$ *versus* control.

Effects of everolimus on the cell-cycle

After 6 days of incubation, everolimus (5 nM) induced a significant accumulation of TT cells in G_0/G_1 phase (+13%, $P = 0.05$; Fig. 4A) and decreased the fraction of cells in S phase (−35%, $P < 0.001$; Fig. 4B) compared with the control. Similarly, everolimus (75 nM) increased the percentage of MZ-CRC-1 cells in G_0/G_1 phase (+18%, $P < 0.05$; Fig. 4C) and decreased the fraction of cells in S phase (−40%, $P < 0.001$; Fig. 4D).

Effects of everolimus on apoptosis

In TT and MZ-CRC-1 cells, the effects on the apoptosis of 6 days of treatment with everolimus, respectively at the concentrations of 5 and 75 nM, were examined by flow cytometry with Annexin V and propidium iodide. No significant change in the percentage of early and late apoptotic cells was observed during incubation with everolimus compared to control in both cell lines. To exclude any effect of everolimus on apoptosis, we investigated the activation of caspase-3, caspase-8, caspase-9 and PARP. After 6 days of incubation everolimus did not modify the expression of cleaved caspases and PARP in both cells compared to untreated cells. Figure 5 shows the effects of everolimus on apoptosis in MZ-CRC-1 after 6 days of incubation with everolimus, similar results were observed in TT cells and after 3 days of incubation in both cell lines (data not shown).

Effects of everolimus on senescence

To evaluate the effects of mTOR inhibitors on cellular senescence, TT and MZ-CRC-1 cells were treated with everolimus at the concentrations of 5 and 75 nM, respectively. As shown in Figure 6A, analysis of senescence based on cellular β-galactosidase activity revealed that the percentage of senescent TT cells to be doubled after everolimus treatment compared to the control (14.6 ± 3.3% and 7.2 ± 1.3%, respectively, $P < 0.001$). Although in MZ-CRC-1

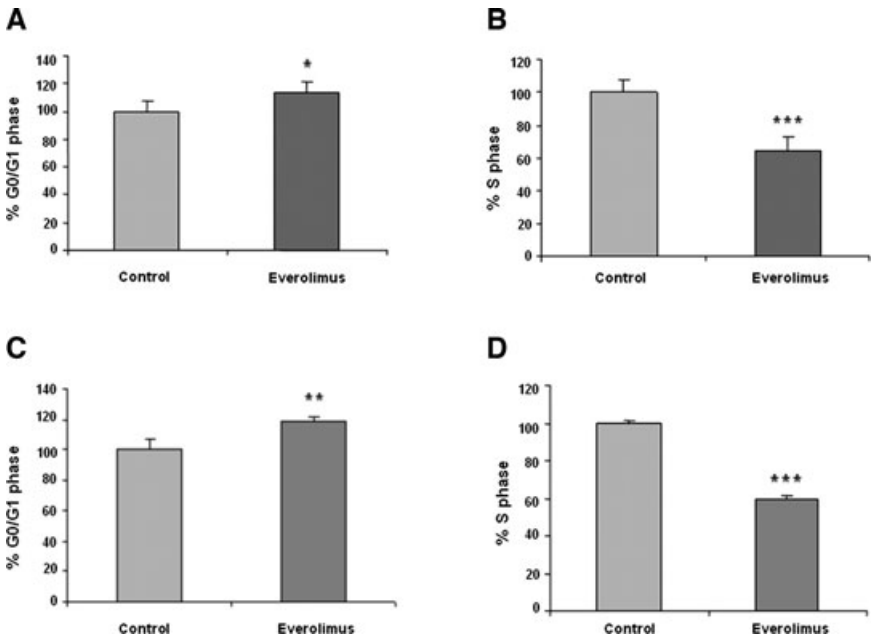

Fig. 4 Cell cycle analysis of TT (**A**, **B**) and MZ-CRC-1 (**C**, **D**) cells after 6 days of incubation with everolimus, respectively at the concentrations of 5 and 75 nM. Data are expressed as mean ± S.E.M. of the percentage of cells in the G_0/G_1 (**A**, **C**) and S (**B**, **D**) phase of three independent performed experiments, as compared with untreated control cells. Control values have been set to 100%. $*P = 0.05$, $**P < 0.05$ and $***P < 0.001$ *versus* untreated control.

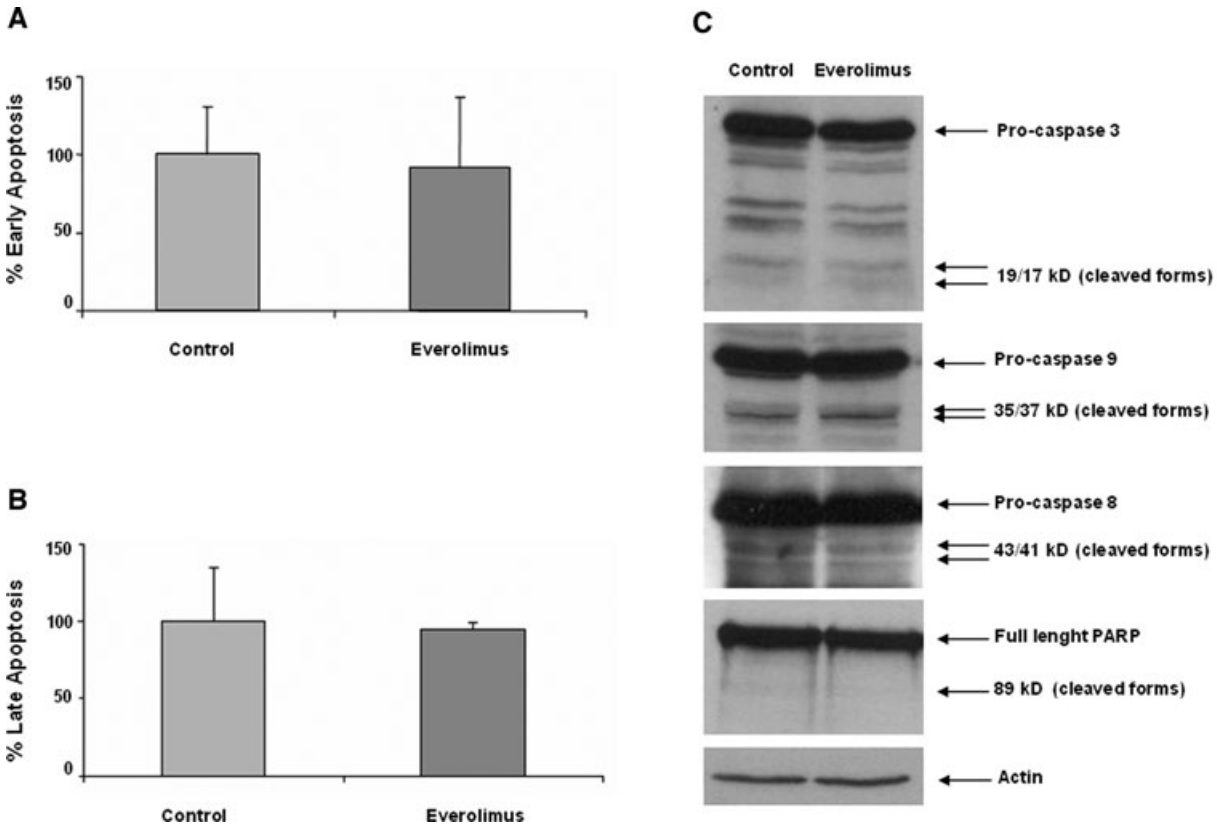

Fig. 5 Effect of everolimus on apoptosis in human MZ-CRC-1 cell line. MZ-CRC-1 cells were incubated with 75 nM of everolimus for 6 days. Subsequently, the proportions of cells in early (**A**) and late (**B**) apoptosis were analysed by flow cytometry. Values are demonstrated as average and S.E.M. of three independent performed experiments. During this experiment total cell lysates were analysed by Western blot to detect the cleavage of caspases 3, 8, 9 and PARP (**C**). Actin was used as control for equal lading.

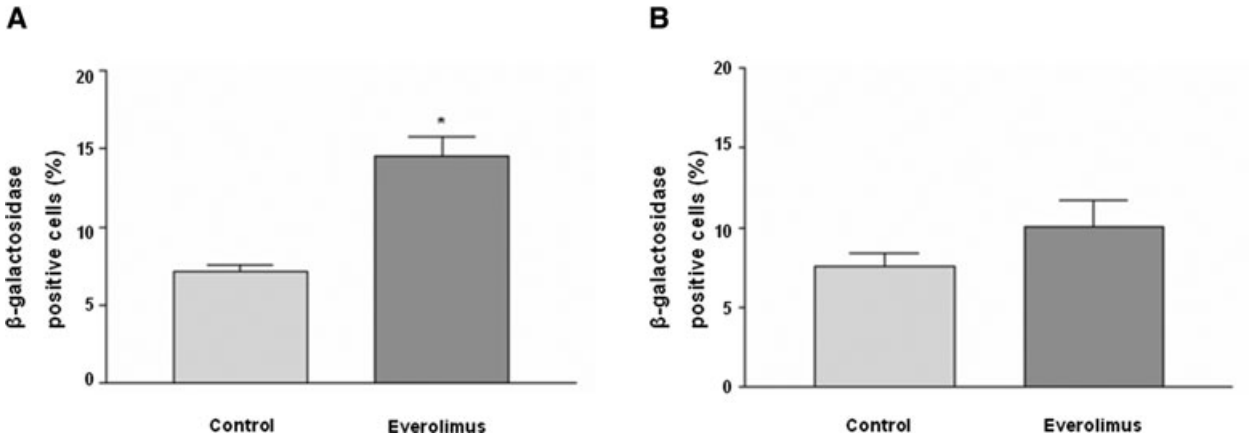

Fig. 6 Effect of everolimus on cellular senescence. TT (**A**) and MZ-CRC-1 (**B**) cells were treated with everolimus for 6 days at the concentrations of 5 and 75 nM, respectively, and then were stained for β-galactosidase. The results were reported as the percentage of β-galactosidase positive cells. The experiments were repeated at least three times. $*P < 0.001$.

there was no significant increase of senescent cells after everolimus treatment (Fig. 6B).

Discussion

Approximately 50% of the patients with MTC has persistent or relapsing disease after surgery. Although MTC is a slowly growing tumour, more than half of patients with advanced MTC will not survive beyond 10 years [1, 2]. Considering that surgery is the only curative approach for these patients and no effective therapy exists for metastatic disease, a novel agent, with a potential anti-secretive and anti-proliferative activity in MTC, may represent a consistent therapeutic advance. A new clinical scenario is now emerging because of the availability of different compounds, which target specific molecular pathways. In the field of hereditary MTC, interesting data have been recently reported by using vandetanib, a selective oral inhibitor of RET, vascular endothelial growth factor receptor, and epidermal growth factor receptor signalling [21]. On the basis of investigator assessments, 20% of patients (*i.e.* six of 30 patients) experienced a confirmed partial response (median duration of response at data cut-off, 10.2 months). An additional 53% of patients (*i.e.* 16 of 30 patients) experienced stable disease at ≥24 weeks, which yielded a disease control rate of 73% (*i.e.* 22 of 30 patients). The authors concluded that vandetanib may provide an effective therapeutic option in patients with advanced hereditary MTC.

Because MTC belongs to the family of neuroendocrine tumours, it has been also investigated for the expression of somatostatin receptors, the key molecules allowing somatostatin analogues to exert their anti-tumour activity [3, 4]. However, no anti-tumour effects have been observed in MTC patients treated with octreotide and lanreotide, the actually available somatostatin analogues [5–8]. AKT-mTOR pathway has been demonstrated to be overexpressed in neuroendocrine tumours as well as everolimus, a mTOR inhibitor, was demonstrated to be effective in neuroendocrine tumour cell lines [10, 13]. In a recent *in vitro* study on MTC, everolimus significantly inhibited cell viability in a dose- and time-dependent manner. It diminished phosphorylation of mTOR and p70S6K in both TT cells and cultured human MTCs, induced cell cycle arrest in the G_0/G_1 phase in TT cells, but had no effect on apoptosis [18]. Interestingly, another study highlighted that the combination of everolimus with octreotide/pasireotide was significantly more anti-proliferative than either agent alone in neuroendocrine tumour cell lines. The dose-dependent increase of Akt phosphorylation observed with everolimus was completely inhibited by the combination therapy [13]. Furthermore, in patients with digestive neuroendocrine tumours, the combination therapy everolimus/octreotide was reported to have important effects on tumour proliferation, resulting in objective tumour response and stabilization in about 20% and 70% of cases, respectively [14].

In this study, everolimus showed to induce significant anti-proliferative effects in both TT and MZ-CRC-1 MTC cell lines. This anti-tumour activity seems to be mediated by the induction of cell cycle arrest in G_0/G_1 phase, but not of apoptosis. Beyond the apoptosis it is emerging recently that senescence could be a potential endpoint for tumour therapy [22]. Cellular senescence is an irreversible growth arrest, preferentially in G_1 phase, that limits the lifespan of mammalian cells and prevents unlimited cell proliferation protecting against the development and progression of cancer. Senescent cells are unable to divide, penetrate the surrounding tissues and metastasize [23]. In humans, the role of mTOR in longevity and cellular senescence is complex [24, 25]. It has been suggested that activation of p53 induces cell cycle arrest, after which its ability to exercise senescence or quiescence is dependent on its interaction with the mTOR pathway [26]. On the other hand, Ota *et al.* recently described that everolimus induces senescence involving down-regulation of Sirtuin 1 in human endothelial cells

[27]. In our study we showed that everolimus induced senescence exclusively in TT cells, as shown by the significant increase in β-galactosidase positive cells during incubation with mTOR inhibitor. This could partially explain the better anti-proliferative activity of everolimus in TT cells compared to MZ-CRC-1. However, we cannot exclude that additional indirect mechanisms (effects on growth factors production, inhibition of angiogenesis, *etc.*) are involved in the anti-tumour activity of everolimus in MTC. Moreover, because we have exposed MTC cells to everolimus for a long incubation time, we cannot exclude that secondary necrosis occurred aside senescence. However, our findings suggest a relevant, even if not necessarily unique, role of senescence in the biological effects observed in our experimental conditions.

Finally, everolimus in combination with octreotide was evaluated for the first time in the treatment of patients with MTC. In this preliminary observation, calcitonin hypersecretion was significantly decreased in both patients under study, while CEA decrease paralleled calcitonin variations but at a lower extent. Since everolimus did not result in a significant inhibition of calcitonin secretion on MTC cell lines, the decrease of serum calcitonin concentrations observed during treatment with everolimus may be explained by a potent anti-tumour activity of this agent. In fact, a tumour response was observed in both patients, as expressed by indirect signs of inhibition of bone metastases, such as decrease of nerve compression and improvement of paraplegia in patient 1, tumour necrosis and decrease in tumour size and vascularization of lymph node metastases in patient 2.

A relevant point is that all the anti-proliferative effects were observed when everolimus was given at the full dose of 10 mg a day and not when a lower dose was administered. On the other hand, the continuous administration of everolimus 10 mg a day resulted in grades 2–3 side effects which required dose reduction. Perhaps a helpful option could be to start the treatment with lower dose of everolimus, less active on tumour growth but being able to induce a gradual patient adaptation to the drug toxicity, and then to switch to the full dose to obtain prolonged anti-proliferative effects. In this study, patient 2 experienced more gradual but more durable anti-secretive and anti-proliferative effects than patient 1 and this may in part reflect the different modality of administration of everolimus. It would be also crucial to introduce this treatment in a early stage of disease when patient conditions are less compromised by tumour spread. The clinical results of this report are only preliminary and need to be confirmed by clinical trials on a larger series of patients that we are beginning to enrol in an ongoing clinical trial in our institutions.

In conclusions, this study reports for the first time that everolimus in combination with octreotide may be effective as anti-tumour therapy in patients with progressive and metastatic MTC, suggesting to perform further larger studies to confirm these potential effects.

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Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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