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ORIGINAL ARTICLE

The cAMP analogs have potent anti-proliferative effects on medullary thyroid cancer cell lines

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Abstract The oncogenic activation of the rearranged during transfection (RET) proto-oncogene has a main role in the pathogenesis of medullary thyroid cancer (MTC). Several lines of evidence suggest that RET function could be influenced by cyclic AMP (cAMP)-dependent protein kinase A (PKA) activity. We evaluated the in vitro anti-tumor activity of 8-chloroadenosine-3′,5′-cyclic monophosphate (8-Cl-cAMP) and PKA type I-selective cAMP analogs [equimolar combination of the 8-piperidinoadenosine-3′,5′-cyclic monophosphate (8-PIP-cAMP) and 8-hexylaminoadenosine-3′,5′-cyclic monophosphate (8-HA-cAMP) in MTC cell lines (TT and MZ-CRC-1)]. 8-Cl-cAMP and the PKA I-selective cAMP analogs showed a potent anti-proliferative effect in both cell lines. In detail, 8-Cl-cAMP blocked significantly the transition of TT cell population from G_2/M to G_0/G_1 phase and from G_0/G_1 to S phase and of MZ-CRC-1 cells from G_0/G_1 to S phase. Moreover, 8-Cl-cAMP induced apoptosis in both cell lines, as demonstrated by FACS analysis for annexin V-FITC/propidium iodide, the activation of caspase-3 and PARP cleavage. On the other hand, the only effect induced by PKA I-selective cAMP analogs was a delay in G_0/G_1-S and S-G_2/M progression in TT and MZ-CRC-1 cells, respectively. In conclusion, these data demonstrate that cAMP analogs, particularly 8-Cl-cAMP, significantly suppress in vitro MTC proliferation and provide rationale for a potential clinical use of cAMP analogs in the treatment of advanced MTC.

Keywords Medullary thyroid cancer · cAMP analogs · cAMP-dependent protein kinase A (PKA) pathway · Apoptosis · Cell cycle

Introduction

Medullary thyroid carcinoma (MTC) is a neuroendocrine tumor arising from the calcitonin (CT)-producing parafollicular C cells of the thyroid. Surgery is the only curative approach for these patients and curative therapy exists for metastatic disease. Current targeted therapies, such as tyrosine kinase inhibitors that target rearranged during transfection (RET) receptor, are not likely to be curative because of the cytostatic effect of these agents that induce stabilization of cancer in most cases [1, 2].

On these bases, there is an urgent need to develop new anti-tumor drugs for the treatment of advanced MTC [3–5]. Activating mutations of the RET proto-oncogene are implicated in the pathogenesis of several forms of MTC. The RET proto-oncogene encodes a receptor tyrosine kinase able to modulate growth, survival, differentiation, and migration of cells derived from neural crest. Germline mutations of this gene have been detected in almost 100 % of hereditary MTCs, while somatic mutations of RET have been reported in up to 70 % of sporadic forms [6]. RET function could be influenced by the modulation of cyclic

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AMP (cAMP)-dependent protein kinase A (PKA) activity [7, 8]. cAMP is a second messenger that plays a main role in the transduction of several signaling pathways [9]. A major function of cAMP is the activation of PKA that belongs to a family of enzymes playing a key role in the regulation of metabolism and cell proliferation [10]. PKA holoenzymes consist in a heterotetramer of two homodimers represented by two regulatory (R) subunits and two catalytic (C) subunits, resulting in the formation of two isozymes: type I and type II. Type I and type II PKA contain distinct R-subunits, RI and RII, respectively, and each R-subunit has two kinetically different binding domains for cAMP, known as site A and B [11] and four isoforms, RIα, RIβ, RIIα, and RIIβ. cAMP binds R-subunits with the consequent release of the C subunits, which are serine/threonine kinases modulating several cellular functions through the phosphorylation of target molecules. PKA type I and II have different effects on cell proliferation [12]. Although many of the physiologic effects of cAMP can be ascribed to the action of one or more of the PKA isoforms, some of the cAMP-dependent effects appear to be "PKA-independent" [13].

A series of cell permeable cAMP analogs with different specificities for the two binding sites on the R subunit of each PKA isoenzyme have been developed. Most of these compounds showed a potent anti-tumor activity. A site-selective cAMP analog, 8-chloroadenosine-3′,5′-cyclic monophosphate (8-Cl-cAMP), resulted one of the most potent agents and entered phase I/II clinical trials as anti-cancer drug [14]. The inhibition of cell growth by 8-Cl-cAMP is due to the modulation of both PKA type I and type II. However, the relevance of differential modulation of PKA-R subunits during the 8-Cl-cAMP-induced growth inhibition and cytotoxicity is still under debate [15]. Indeed, several studies reported that the anti-tumor activity of 8-Cl-cAMP is also mediated by its metabolite 8-Cl-adenosine and is partially independent of PKA activation and/or alterations of the ratio between type I and type II R subunits [16–22].

Other cAMP analogs with anti-proliferative activity are 8-piperidinoadenosine-3′,5′-cyclic monophosphate (8-PIP-cAMP) and 8-hexylaminoadenosine-3′,5′-cyclic monophosphate (8-HA-cAMP). The use of these compounds in combination allows the selective interaction with both sites A and B of PKA type I [23–26] .

Even if there are several lines of evidence concerning a crosstalk between RET and PKA signaling, the role of cAMP/PKA pathway in the pathogenesis and progression of MTC is not known. Previous studies reported that cAMP inhibits [³H] thymidine incorporation in TT cells [27] and induces differentiation in human MTC cell lines [28]. On the basis of these results, we evaluated the in vitro anti-tumor activity of 8-Cl-cAMP and PKA type I-selective cAMP analogs (equimolar combination of the 8-PIP-cAMP and 8-HA-cAMP) in MTC.

Materials and methods

Drug preparation and cell line cultures

cAMP analogs, 8-Cl-cAMP, 8-HA-cAMP, and 8-PIP-cAMP were provided by Biolog (Basel, Switzerland) and dissolved in 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 which harbor C634W and M918T RET mutations, respectively, were kindly provided by 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 % fetal bovine serum, 2 mM glutamine, and 105 U/l penicillin–streptomycin and maintained in a humidified atmosphere of 5 % CO_2. The cells were grown in 75 cm^2 flasks and passed once every 4–7 days on a 1:2 split.

Cell proliferation assay

TT and MZ-CRC-1 cells were plated in 96-well plates at a density of 20×10^3 per well. In the following day, cell culture medium of both cell lines was replaced with medium containing various concentrations (0.1–200 μM) of cAMP analogs (8-Cl-cAMP and the equimolar combination of 8-PIP-cAMP and 8-HA-cAMP) at different times (3 and 6 days).

The plates were then placed in a 37 °C, 5 % CO_2 incubator. After 3 days, medium was refreshed, and cAMP analogs were added again. After 6 days of treatment, cells were harvested for a cell viability assay, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described [29]. All assays were performed in six replicates and were repeated at least three times.

Cell cycle analysis

TT and MZ-CRC-1 cells were plated in duplicates in six-well plates at density of 3×10^5 cell/well. In the following day, cell culture medium of both cell lines was replaced with medium containing 8-Cl-cAMP (5 μM in TT and in MZ-CRC-1) and the equimolar combination of 8-PIP-cAMP/8-HA-cAMP (10 μM in TT and 25 μM in MZ-CRC-1). After 3 days, medium was changed with fresh medium without (control group) or with the drugs. After 6 days, cells were harvested by gentle trypsinization, washed three times with cold PBS (calcium and magnesium free), and collected by centrifugation at $1200 \times g$ for 5 min. The pellets were resuspended and directly stained
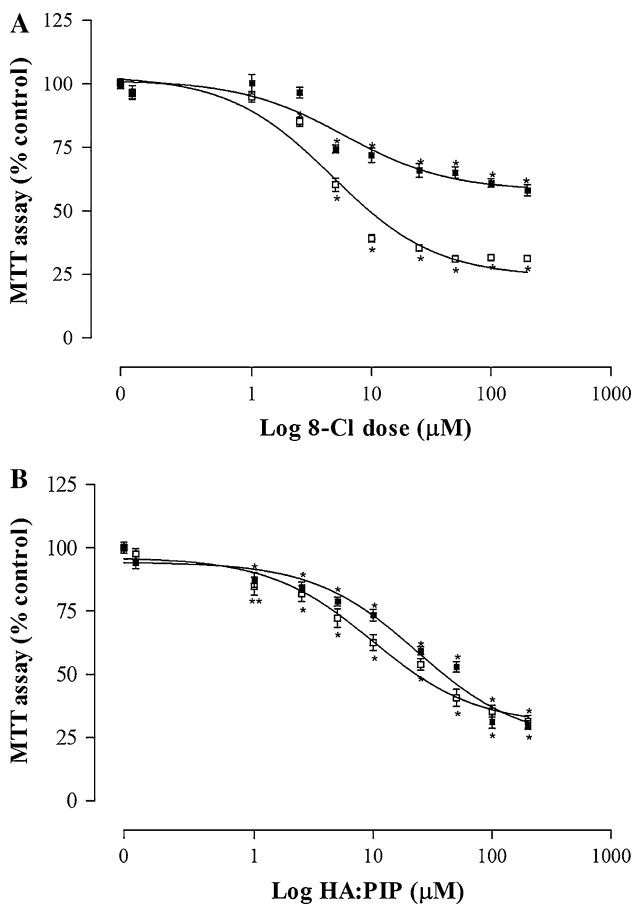
Fig. 1 Effect of 8-chloroadenosine-3′,5′-cyclic monophosphate (8-Cl-cAMP) (a), and PKA type I-selective cAMP analogs (PKA I) (8-PIP-cAMP plus 8-HA cAMP) (b) on cell proliferation in TT (*open square*) and MZ-CRC-1 (*filled square*) cells, as measured by MTT assay. MTC cell lines were incubated for 6 days without or with the drugs at different concentrations. Dose response curves are expressed as non linear regression (curve fit) of log (concentration drug) versus the percentage of control (untreated cells). Values represent the mean and S.E.M. of at least three independent experiments in six replicates. *$p < 0.001$ and **$p < 0.01$ versus control

with propidium iodide (PI) (Sigma-Aldrich, 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 FL2-A with a FACScalibur flow cytometer (Becton Dickinson, Erembodegem, Belgium) using Cell Quest Pro Software. Cell cycle distribution, expressed as percentage of cells in G_0/G_1, S, and G_2/M phases, was determined as previously described [30].

Flow cytometric analysis of apoptosis

The effect of cAMP analogs on apoptosis was analyzed by Annexin V-FITC and PI staining. TT and MZ-CR-1 cells

were plated in duplicates in six-well plates at density of 3×10^5 cell/well and treated with cAMP analogs, as previously described in the section of cell cycle analysis. After 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 $1200 \times g$ for 5 min. The pellets were resuspended in 1X binding buffer (0.1 M HEPES/NaOH, pH 7.4, 1.4 M 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 1X binding buffer was added to each tube. The analysis was performed by FACScalibur on 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 [30].

Western blot analysis

TT and MZ-CRC-1 cells were plated in duplicates in six-well plates at density of 3×10^5 cell/well. In the following day, cell culture medium of both cell lines was replaced with medium containing cAMP analogs as previously described in the section of cell cycle analysis. After 3 days, medium was changed with fresh medium without (control group) or with the drugs. After 6 days, cells were scraped, washed twice in cold PBS, and resuspended in lysis buffer RIPA containing protease and phosphatase inhibitors. Cellular debris was pelleted by centrifugation at $13,000 \times g$ 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 h, and probed with specific antibodies overnight at 4 °C: anti PKA RIa, RIIa, RIIb, anti-extracellular signal-regulated kinase 1/2 (ERK), anti-pERK, anti-caspase-3 and PARP, anti-cleaved caspase, and PARP (Cell Signaling Technology, Beverly, MA, USA) at dilution 1:1000. Blots were detected with ECL-plus kit after incubation with HRP conjugated mouse and rabbit secondary antibodies (dilution 1:5000 for anti-caspase-3 and 1:10,000 for all the other antibodies) and then developed using enhanced chemo-luminescence detection reagents and exposed to X-ray film [16, 31, 32].

Statistical analysis

All experiments were carried out at least three times and gave comparable results. For statistical analysis, GraphPad
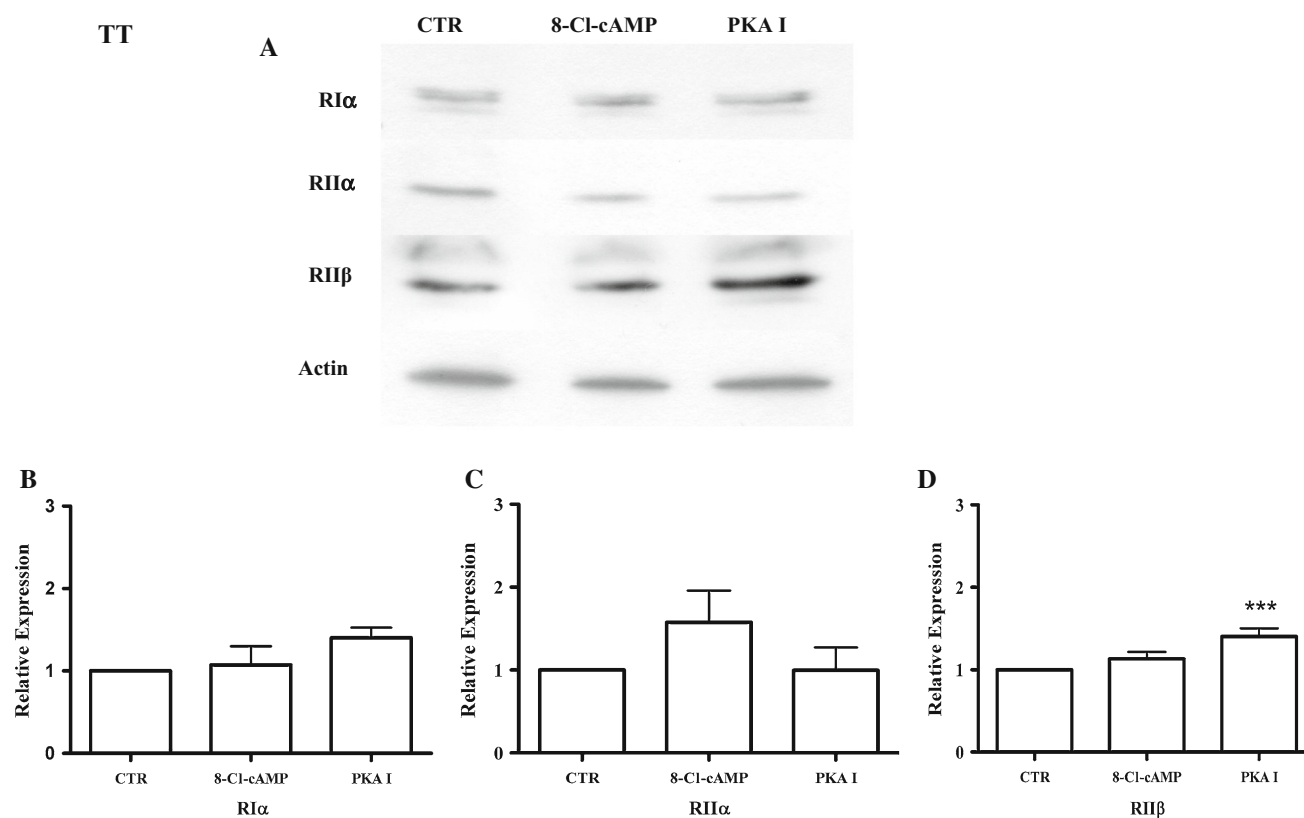
Fig. 2 Expression of type I PKA RIα (**b**) and type II PKA RIIα (**c**) and RIIβ (**d**) after treatment with of 8-chloroadenosine-3′,5′-cyclic monophosphate (8-Cl-cAMP) (5 μM), and PKA type I-selective cAMP analogs (PKA I) (8-PIP-cAMP plus 8-HA-cAMP at the equimolar combination: 10 μM) in TT cell line by Western blotting with specific antibodies, as described in "Materials and methods" section. The expression of house-keeping actin was used as loading control. **a** shows the result of a representative experiment. The intensity of each band associated to PKA was expressed as the ratio between the relative intensities of the bands associated with actin (**b–d**). Values represent the mean and S.E.M. of at least three independent experiments. ***$p < 0.05$ versus untreated control (CTR)

Prism 3.0 (GraphPad Software, San Diego, CA) was used. 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. In all analyses, values of $p < 0.05$ were considered statistically significant. The values reported in figures are the mean ± standard error of the mean (S.E.M.).

Results

cAMP analogs efficiently inhibit MTC cell proliferation

After 6 days of incubation, both 8-Cl-cAMP and PKA type I-selective analogs (8-PIP-cAMP plus 8-HA-cAMP) significantly inhibited the growth of both MTC cell lines in a dose-dependent manner (Fig. 1a, b). Interestingly, the anti-proliferative effects of these compounds were more prominent in TT cells (Fig. 1a: 8-Cl-cAMP: $IC_{50} = 5$ μM, maximal inhibition of proliferation $= -75$ %; b: PKA type

I-selective cAMP analogs: $IC_{50} = 10$ μM, maximal inhibition of proliferation $= -70$ %) compared to MZ-CRC-1 cells (Fig. 1a: 8-Cl-cAMP: $IC_{50} = 5$ μM, maximal inhibition of proliferation $= -42$ %; b: PKA type I-selective cAMP analogs: $IC_{50} = 25$ μM, maximal inhibition of proliferation $= -76$ %). For further experiments, we have selected the IC_{50} concentrations for each cAMP analog (5 μM for 8-Cl-cAMP and 10 μM for PKA type I-selective cAMP analogs in TT cells, 5 μM for 8-Cl-cAMP and 25 μM for PKA type I-selective cAMP analogs in MZ-CRC-1 cells).

Type I PKA regulatory subunits are not affected by cAMP analogs, while PKA type I-selective cAMP analogs cause a slight increase of type II regulatory subunits

We have evaluated the effects of these compounds on the PKA R subunit expression. For this purpose, TT (Fig. 2) and MZ-CRC-1 (Fig. 3) cells were incubated with 8-Cl-cAMP or PKA type I-selective cAMP analogs, and the levels of RIα and RIIα proteins were evaluated by Western
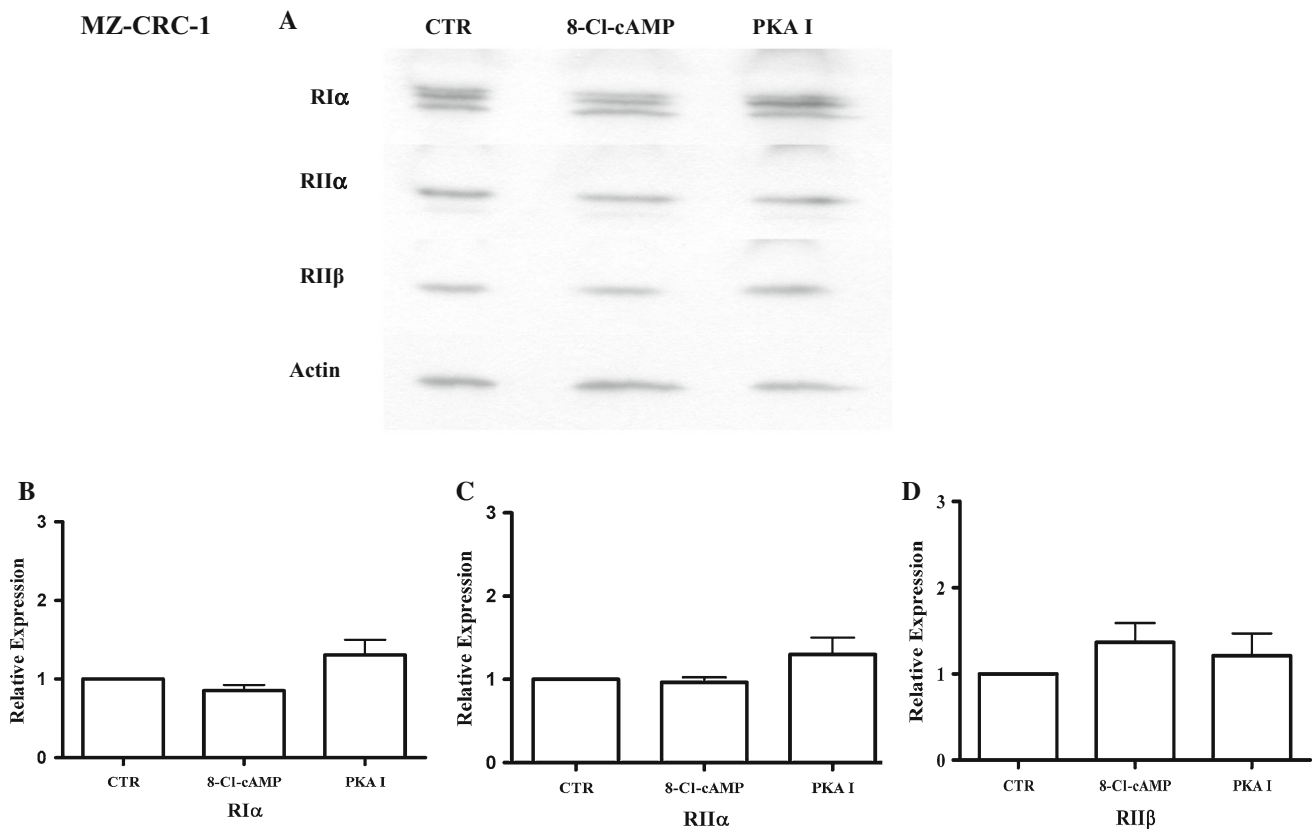
MZ-CRC-1   **A**

Fig. 3 Expression of type I PKA RIα (**b**) and type II PKA RIIα (**c**) and RIIβ (**d**) after treatment with 8-chloroadenosine-3′,5′-cyclic monophosphate (8-Cl-cAMP) (5 μM), and PKA type I-selective cAMP analogs (PKA I) (8-PIP-cAMP plus 8-HA-cAMP at the equimolar combination: 25 μM) in MZ-CRC-1 cell line by Western blotting with specific antibodies, as described in "Materials and methods" section. The expression of house-keeping actin was used as loading control. **a** shows the result of a representative experiment. The intensity of each band associated to PKA was expressed as the ratio between the relative intensities of the bands associated with actin (**b–d**). Values represent the mean and S.E.M. of at least three independent experiments

blot analysis using isoform-specific antibodies. After 6 days of incubation, 8-Cl-cAMP did not modify the expressions of type I regulatory subunits of PKA in both TT and MZ-CRC-1 (Figs. 2, 3) cells, while PKA type I-selective cAMP analogs induced a statistically significant but weak increase of RIIβ subunit of PKA only in TT cells (Fig. 2d).

ERK1–2 activity is reduced by cAMP analogs only in MZ-CRC-1 cells

To study the anti-proliferative mechanism of action of 8-Cl-cAMP and PKA type I-selective cAMP analogs, we examined the effect of these compounds on a key signaling cascade involved in cell growth: the ERK mitogen-activated protein kinase (MAPK) pathway. The exposure of MTC cells to either 8-Cl-cAMP or PKA type I-selective cAMP analogs for 6 days had no effects on the intracellular activation of ERK1–2 (evaluated as phosphorylated isoforms) in TT cells (Fig. 4a, c). On the other hand, we found a significant and strong decrease of ERK activity in MZ-

CRC-1 cells after treatment with cAMP analogs, as demonstrated by the immunodetection of the phosphorylated isoforms of ERK1–2 (Fig. 4b, d).

Effects of cAMP analogs on cell cycle distribution of MTC cells

Cell cycle phase distribution was evaluated by FACS analysis in TT and MZ-CRC-1 cells treated with 8-Cl-cAMP and PKA type I-selective cAMP analogs (Fig. 5a–f). After 6 days of incubation, we observed that 8-Cl-cAMP significantly decreased the percentage of TT cells both in G_0/G_1 (-23 %; $p < 0.05$ compared to the untreated cells) and in S phase (-70 %; $p < 0.001$ compared to the untreated cells). PKA type I-selective cAMP analogs significantly increased the population of TT cells in G_0/G_1 phase ($+22$ %, $p < 0.05$ vs. control) and decreased the cell number in both S (-34 %, $p < 0.001$ compared to the untreated cells) and in G_2/M phase (-12 %, $p < 0.05$ vs. control) (Fig. 5a–c). In MZ-CRC-1 cells, 8-Cl-cAMP induced a significant decrease of S phase (-36 %, $p < 0.01$
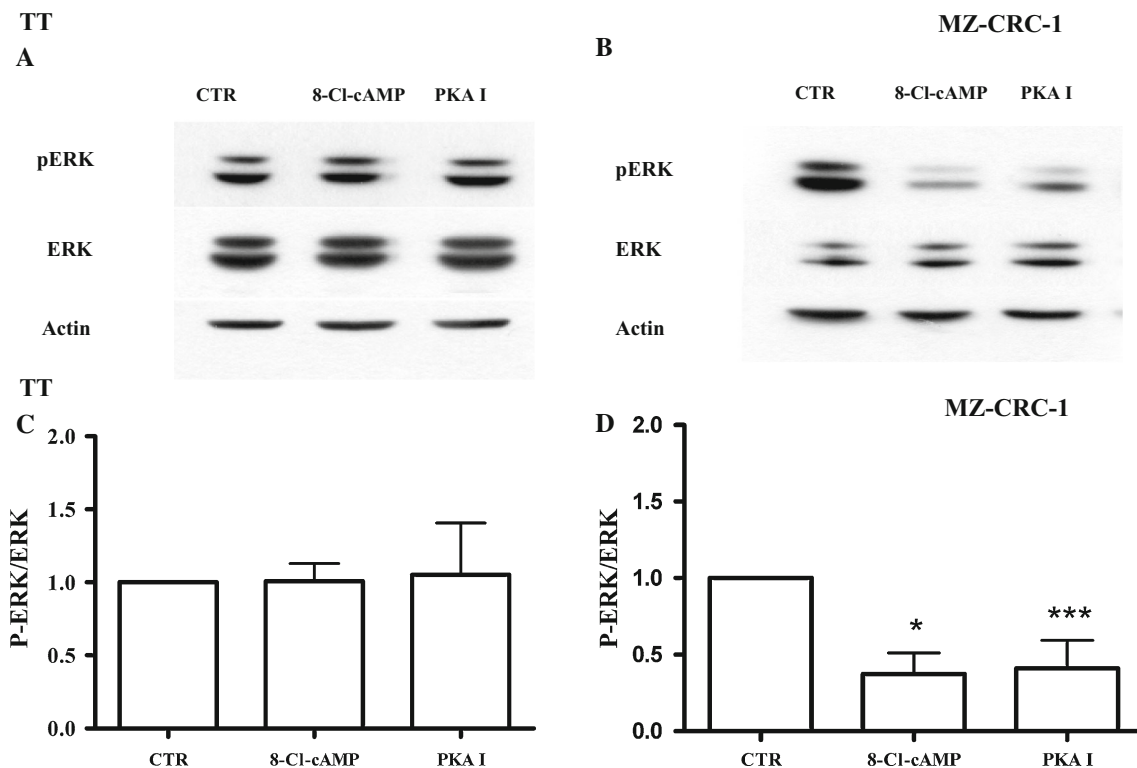
Fig. 4 Expression of pERK and ERK after treatment with 8-chloroadenosine-3′,5′-cyclic monophosphate (8-Cl-cAMP) (5 μM in TT and in MZ-CRC-1), and PKA type I-selective cAMP analogs (PKA I) (8-PIP-cAMP plus 8-HA-cAMP at the equimolar combination: 10 μM in TT and 25 μM in MZ-CRC-1) by Western blotting with specific antibodies, as described in "Materials and methods" section. **a**, **b** show the result of a representative experiment in TT and MZ-

CRC-1 cell lines, respectively. Densitometric analyses of the bands associated to the pERK and ERK in TT (**c**) and MZ-CRC-1 (**d**) cell lines are expressed as the ratio between pERK and ERK relative intensities of the bands. The expression of house-keeping actin was used as loading control. Values represent the mean and S.E.M. of at least three independent experiments. *$p < 0.001$, ***$p < 0.05$ versus untreated control (CTR)

vs. control), whereas there were no significant changes of the other phases of the cell cycle. After the treatment with PKA type I-selective cAMP analogs, we observed a decrease of MZCRC-1 cell number in G_2/M phase of the cell cycle (-22 %, $p < 0.01$ vs. control) (Fig. 5d–f).

8-Cl-cAMP but not PKA type I-selective cAMP analogs induces apoptosis in MTC cells

We evaluated the effects of cAMP analogs on both early and late apoptosis by flow cytometry with Annexin V and PI after 6 days of treatment (Fig. 6a–d). 8-Cl-cAMP strongly increased the percentage of TT cells in both late ($+273$ % compared to untreated cell; $p < 0.05$) and early apoptosis phases ($+811$ % compared to untreated cells; $p < 0.01$). On the other hand, a lower but statistically significant increase of late apoptosis was observed after incubation with 8-Cl-cAMP ($+64$ % vs. control; $p < 0.01$) in MZ-CRC-1 cells. No significant changes in the percentage of both early and late apoptotic cells were recorded after incubation of both TT and MZ-CRC-1 cells with PKA type I-selective cAMP analogs.

These data were confirmed by evaluating the effects of cAMP analogs on key enzymes involved in the apoptotic cascade, such as poly(ADP-ribose) polymerase (PARP) and caspase-3 (Fig. 7). In detail, after 6 days of treatment, 8-Cl-cAMP significantly increased PARP degradation, as shown by a higher amount of cleavage products if compared to untreated TT and MZ-CRC-1 control cells. Similarly, the activity of caspase-3 increased after 6 days of incubation with 8-Cl-cAMP in both MTC cell lines. No significant changes in caspase-3 activity and PARP cleavage were observed after the exposure of both cell lines to PKA type I-selective cAMP analogs.

Discussion

Although MTC is a slowly growing tumor, after the discovery of distant metastases, the mean overall survival of the patients is around 25 % at 5 years and 10 % at 10 years, respectively. Surgery is the only curative approach for these patients. Treatment with tyrosine kinase inhibitors is currently used in patients with advanced and
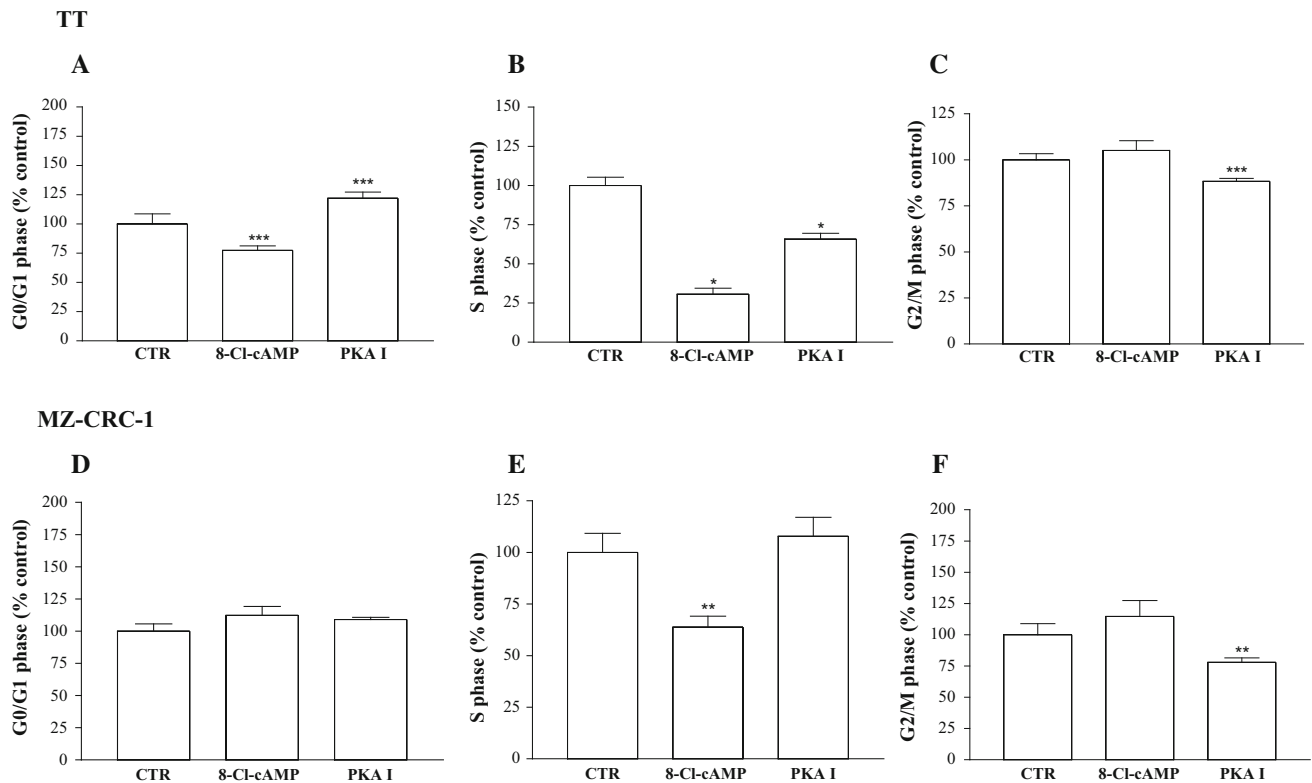
Fig. 5 Cell cycle analysis of TT (**a–c**) and MZ-CRC-1 (**d–f**) cells after 6 days of incubation with or without 8-chloroadenosine-3′,5′-cyclic monophosphate (8-Cl-cAMP) (5 μM in TT and in MZ-CRC-1), and PKA type I-selective cAMP analogs (PKA I) (8-PIP-cAMP plus 8-HA-cAMP at the equimolar combination: 10 μM in TT and 25 μM in MZ-CRC-1), evaluated by FACS after labeling with PI, as described in "Materials and methods" section. Data are expressed as mean ± S.E.M. of the percentage of cells in the different phases of the cell cycle of three independent experiments, as compared with untreated control cells (CTR). Control values have been set to 100 % *$p < 0.001$ and **$p < 0.01$, ***$p < 0.05$ versus CTR

progressive MTC, significantly increasing progression free survival but with a low complete response rate. Indeed, the activation of several tumor escape pathways is thought to be the cause of these nonsustained tumor responses [33].

cAMP signaling represents an interesting target for the selective induction of growth inhibition and apoptosis in cancer cells through the PKA-dependent or independent pathways. 8-Cl-cAMP is a potent cAMP analog that is under investigation as a potential therapeutic agent for its anti-proliferative effect in preclinical studies. Phase I clinical trials in patients with solid tumors revealed its safety and clinical activity [34, 35], while a Phase II pilot study evaluation of 8 Cl-cAMP in the treatment of relapsed or refractory multiple myeloma has been completed (ClinicalTrials: NCT00004902). It has been reported that 8-Cl-cAMP shows a potent growth inhibitory effect and has a reverse-transforming activity in cancer cells [36]. Its anti-tumor activity is exerted via different mechanisms (1) by its metabolite 8-Cl-adenosine, acting via AMP-activated protein kinase (AMPK), inducing p38 MAPK phosphorylation and inhibition of mammalian target of rapamycin complex (mTORC) 1, leading to the induction of autophagy [37]; (2) by suppression of c-myc and c-ras

oncogene expression, inducing changes in cell morphology [38]; (3) through increasing the RI to RII ratio and reducing ERK phosphorylation; (4) by increasing apoptotic process through caspase-3/7 activation [16], Bcl-2 inactivation [39], and through cell cycle cell arrest [40].

We have recently demonstrated that cAMP analogs selective for PKA type I, such as 8-PIP-cAMP plus 8-HA-cAMP, inhibited the growth of a wide range of human carcinoma cell lines including BRAF-positive carcinoma cell lines (ARO and NPA) [16, 41]. cAMP-dependent pathway interacts with a variety of intracellular signaling pathways modulating cell growth [42] including Ca^{2+}-mediated [43] and cytokine-dependent pathways [44]. The latter acts via Janus kinase/signal transducers and activators of transcription (JAK/STAT) [45] and Ras/Raf/ERK inhibition MAPK [46] that is also activated by RET. Moreover, several reports demonstrated a direct interaction between RET and PKA. The RET proto-oncogene encodes a receptor tyrosine kinase that is essential for the development of cells derived from neural crest through the interaction with the glial cell line-derived neurotrophic factor (GDNF) protein family. In the neural crest cells, the increase of cAMP level, through the activation of PKA,
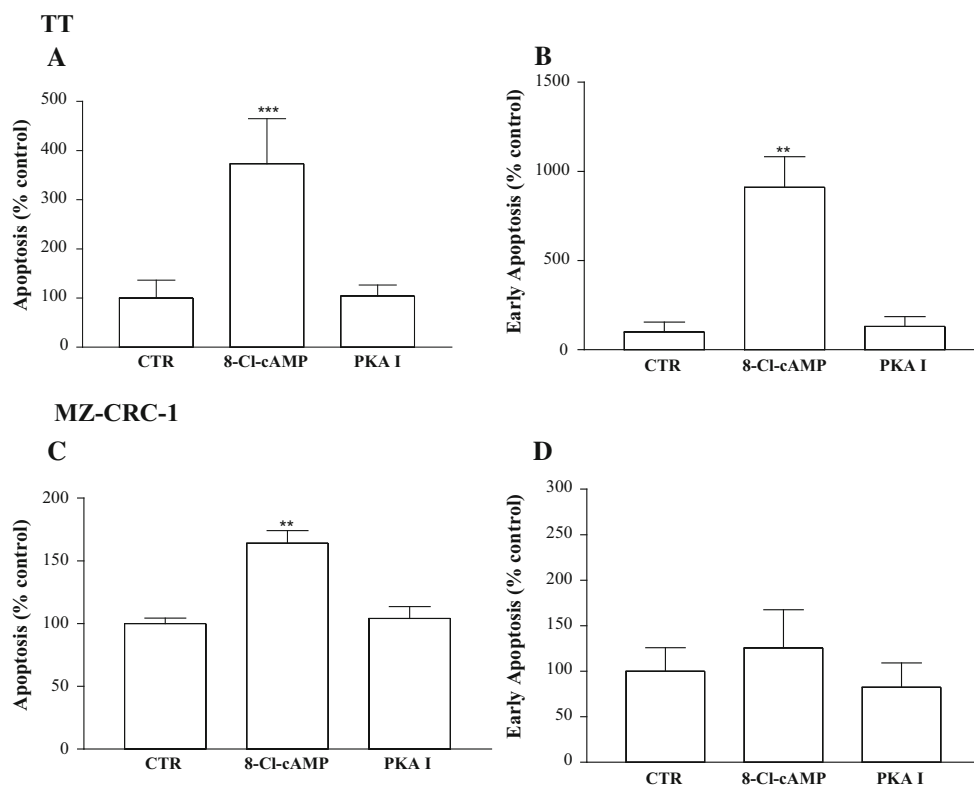
Fig. 6 Effect 8-chloroadenosine-3′,5′-cyclic monophosphate (8-Cl-cAMP) (5 μM in TT and in MZ-CRC-1), and PKA type I-selective cAMP analogs (PKA I) (8-PIP-cAMP plus 8-HA-cAMP at the equimolar combination: 10 μM in TT and 25 μM in MZ-CRC-1) on cell death in TT (**a, b**) and MZ-CRC-1 (**c, d**) cell lines after 6 days of treatment. The proportions of cells in apoptosis were evaluated by FACS analysis after double labeling with PI and Annexin V, as described in "Materials and methods" section and showed as *bars* in the graphs. Values are expressed as the percentage of control (untreated cells) and represent the mean and S.E.M. of at least three independent experiments. **p < 0.01, ***p < 0.05 versus control (CTR)

inhibited lamellipodia formation, migration, and neurite outgrowth by counteracting RET activation [47]. This suggests the possibility of a new strategy to block RET oncogenic activity through cAMP analogs in tumors arising from neural crest cells, such as MTC [8, 48, 49].

In the present manuscript, we have evaluated in vitro the anti-tumor activity of cAMP analogs in MTC cells. 8-Cl-cAMP and the combination of PKA I-selective cAMP analogs induced a significant anti-proliferative effect in both TT and MZ-CRC-1 cell lines. This anti-tumor activity was higher in TT cells, especially after treatment with 8-Cl-cAMP, and it appeared to be mediated by the inhibition of mitogenic ERK-dependent pathway in MZ-CRC-1 cell line. In addition, this effect appeared to be independent from changes in the expression of type I PKA subunits that were not mainly affected by treatment with either PKA I-selective cAMP analogs or 8-Cl-cAMP in both cell lines. Only PKA I-selective cAMP analogs slightly increased PKA RIIβ expression in TT cells.

In our experimental model, the anti-tumour activity of the cAMP analogs appeared to be due to cell cycle modulation. Several reports have demonstrated that cAMP analogs can disrupt different phases of the cell cycle in a cell-dependent manner. 8-Cl-cAMP induced an accumulation in the G_0/G_1 phase and a decrease in the proportion of cells in the S phase [20] in human point-mutated c-Ha-ras, and the c-erbB-2 proto-oncogenes transformed MCF-10A cells and a G_2/M block in HL-60 leukaemic cells [46]. It has also been demonstrated that PKA I-selective cAMP analogs induced a slight increase in the number of cells in G_0/G_1 in ARO cells [50]. Our data showed that cAMP analogs induced a perturbation in cell cycle through different mechanisms: 8-Cl-cAMP appeared to significantly block the transition of the TT cell population from G_2/M to G_0/G_1 phase and from G_0/G_1 to S phase. Similar effects were recorded in MZ-CRC-1 cells, where an inhibition of the transition from G_0/G_1 to S phase was observed. On the other hand, PKA type I-selective cAMP analogs induced a delay in G_0/G_1-S and S-G_2/M progression in TT and MZ-CRC-1 cells, respectively.

Another potential mechanism involved in the anti-tumor activity of cAMP analogs is the activation of apoptosis. Several reports showed that cAMP analogs induced apoptosis in cancer cells [40, 51]. In addition, it was reported
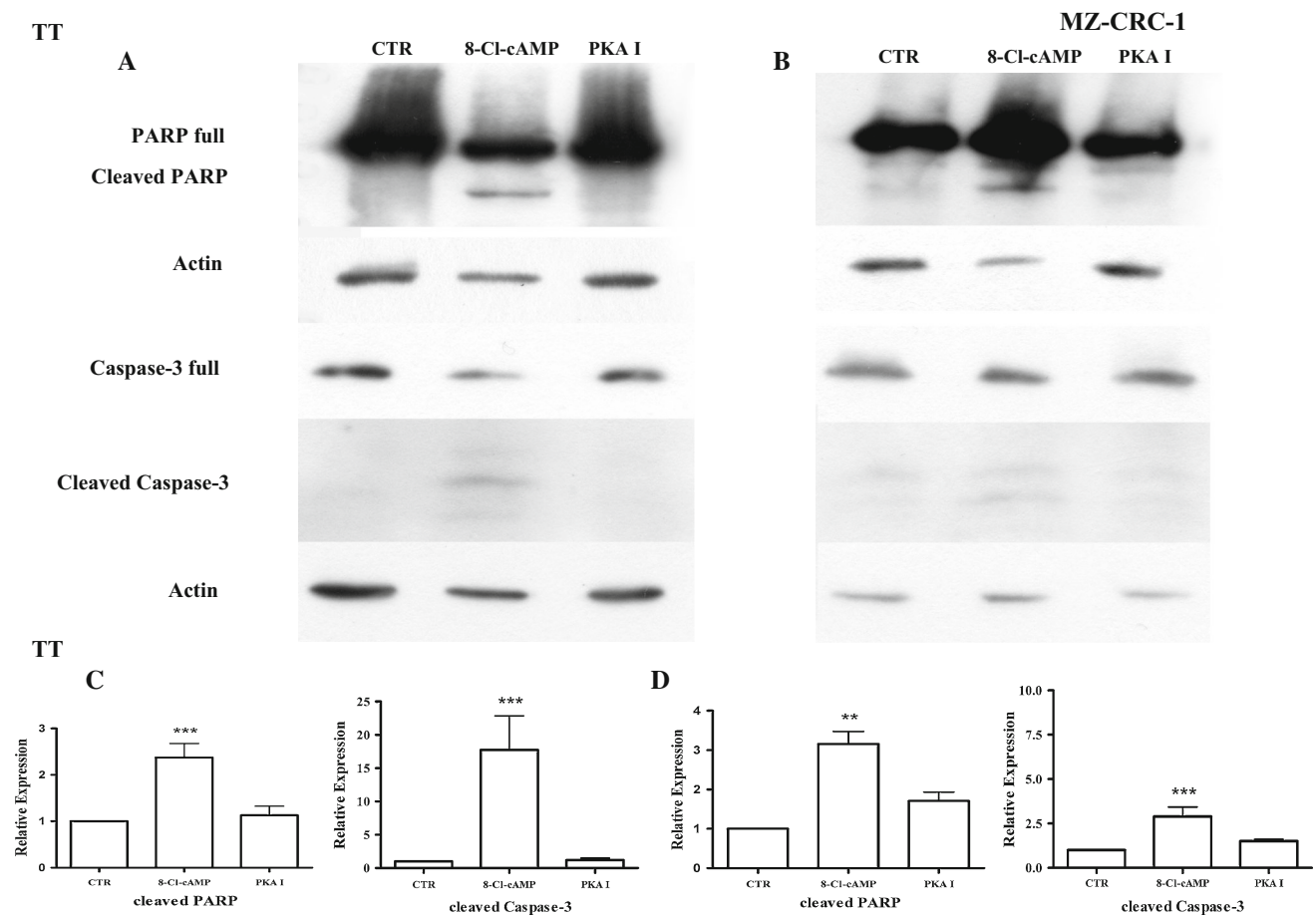
Fig. 7 Expression of the total and cleaved PARP and caspase-3 cells after treatment with 8-chloroadenosine-3′,5′-cyclic monophosphate (8-Cl-cAMP) (5 μM in TT and in MZ-CRC-1), and PKA type I-selective cAMP analogs (PKA I) (8-PIP-cAMP plus 8-HA-cAMP at the equimolar combination: 10 μM in TT and 25 μM in MZ-CRC-1) by Western blotting with specific antibodies, as described in "Materials and methods" section. **a**, **b** show the result of a representative experiment in TT and MZ-CRC-1 cell lines, respectively. Densitometric analyses of the bands associated to the cleaved PARP and cleaved caspase-3 in TT (**c**) and MZ-CRC-1 (**d**) cell lines are expressed as the ratio between the relative intensities of the bands associated with actin. Expression of the house-keeping protein actin was used as loading control. Values represent the mean and S.E.M. of at least three independent experiments. $*p < 0.001$ and $**p < 0.01$, $***p < 0.05$ versus untreated control (CTR)

that the activation of the cAMP-dependent pathway can potentiate the apoptotic effects of several cytokines with potential anti-cancer activity, such as IFN-α or leptin [52, 53]. On this light, we have analyzed the effects of cAMP analogs on apoptosis in MTC cell lines. The family of caspases mediates proteolytic cleavages in the early process of apoptosis [54, 55]. Caspase-3 represents the central effector caspase in mammalian cells and is finally activated by either intrinsic or extrinsic apoptotic pathways [56]. Several proteins which are cleaved during apoptosis have been shown to be specific targets for caspase-3 including PARP [57] in the late phase of apoptosis [58, 59]. The latter is an enzyme involved in DNA repair, replication, differentiation, transcription, and activation of cellular protection mechanisms against DNA damage [60]. In the present study, only 8-Cl-cAMP induced the apoptosis,

evaluated by flow cytometry, in both TT and MZ-CRC-1 cells. The induction of apoptosis by 8-Cl-cAMP was confirmed by the activation of caspase-3 and PARP cleavage in both cell lines. Interestingly, the pro-apoptotic activity of 8-Cl-cAMP appeared to be PKA type I-independent, since PKA I-selective cAMP analogs were not able to induce apoptosis in both cell lines. In addition, the potent pro-apoptotic activity observed after 8-Cl-cAMP incubation and not after PKA I-selective cAMP analogs could explain the stronger anti-proliferative effects of 8-Cl-cAMP than that of 8-PIP-cAMP plus 8-HA-cAMP in MTC cells.

In conclusion, our results provide for the first time experimental evidence that 8-Cl-cAMP and the PKA I-selective cAMP analogs have a potent anti-proliferative effect in MTC cell lines, supporting a potential future application of cAMP analogs in the treatment of this disease.

In particular, 8-Cl-cAMP appeared to have a potent anti-tumor activity mediated by cell cycle arrest and induction of apoptosis. On these bases, cAMP signaling cascades seem to play a critical role in the control of MTC cell growth. Future preclinical and clinical studies are mandatory to confirm the efficacy and safety of 8-Cl-cAMP in MTC and to evaluate the anti-tumoral activity of cAMP analogs in combination with RET tyrosine kinase inhibitors to maximally suppress the activity of signaling pathways that contribute to MTC tumorigenesis and progression.

Conflict of interest No potential conflict of interest relevant to this article was reported.

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