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Research Paper

Cl-IB-MECA inhibits human thyroid cancer cell proliferation independently of A3 adenosine receptor activation

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Abbreviations: A3AR, A3 adenosine receptor; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide; NPA, papillary thyroid carcinoma cell line

Key words: A3 Adenosine receptor, Cl-IB-MECA, adenosine receptor antagonist, thyroid cancer, NPA, cell cycle arrest

A3 adenosine receptor (A3AR) agonists have been reported to modulate cellular proliferation. This work was aimed to investigate the expression and the possible implication of A3AR in the human thyroid carcinomas. Normal thyroid tissue samples did not express A3 adenosine receptor, while primary thyroid cancer tissues expressed high level of A3AR, as determined by immunohistochemistry analysis. In human papillary thyroid carcinoma cell line, NPA, at concentrations ≥10 μM, the A3AR-selective agonist 2-chloro-N^6-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide (Cl-IB-MECA) produced inhibition of cell growth, by blocking the G_1 cell cycle phase in a concentration- and time-dependent manner. This effect was well correlated with a reduction of protein expression of cyclins D1 and E2 after 24 hours of Cl-IB-MECA treatment. Moreover Cl-IB-MECA induced dephosphorylation of ERK1/2 in a time- and concentration-dependent manner, which in turn inhibits cell proliferation. The effect of Cl-IB-MECA was not prevented by A3AR antagonists, MRS1191 or MRS1523 or FA385. Furthermore, neither nucleoside transporter inhibitors, Dypiridamole and NBTI, nor the A1, A2A and A2B receptors antagonists were able to block the response to Cl-IB-MECA. Although Cl-IB-MECA has been shown to influence cell death and survival in other systems through an A3AR-mediated mechanism, in NPA cells the growth inhibition induced by micromolar concentrations of Cl-IB-MECA is not related to A3AR activation and hence that its effects on human papillary carcinoma cell line seem to be independent of the presence of this receptor subtype.

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Introduction

It is known that adenosine is a critical modulator of many physiological functions, by binding to specific G-protein coupled receptors, classified as A1, A2A, A2B and A3 receptors.[1,2] Adenosine is able to inhibit growth or induce apoptosis in various tumor cell lines mainly trough activation of A3 adenosine receptor.[3,4] A3AR belongs to the family of the Gi protein-associated cell surface receptors. A3 adenosine receptor expression was detected in several tumor cell lines including astrocytoma, HL-60 leukemia, BF16-F10 and A378 melanoma, human Jurkat T-cell lymphoma and murine pineal tumor cells.[5-7] Interestingly, A3 adenosine receptor is highly expressed in tumor compared to adjacent normal tissue, which may suggest A3AR as a potential target for tumor growth inhibition.[8] N^6-benzyladeno sine-5'-N-methylcarboxamide derivatives, IB-MECA, Cl-IB-MECA and more recently, thio-Cl-IB-MECA were developed as potent and selective A3AR agonists.[9-13]

Thyroid carcinomas are one of the most common neoplasias of the endocrine system[14] and the malignant thyroid carcinoma is one of the most difficult neoplasias to treat. Thyroid nodules can be hyperplastic benign adenomas or malignant lesions, and can be derived from thyroid follicular epithelial cells or C cells.[15] More than 95% of thryroid carcinomas are derived from follicular cells and are usually managed by surgical resection with or without radioactive-iodine ablation. Follicular-cell-derived carcinomas are then divided into well-differentiated, poorly differentiated and undifferentiated types on the basis of histological and clinical parameters. Well-differentiated thyroid carcinoma includes papillary (PTC) and follicular (FTC) types. The differentiated PTCs and FTCs account for most of the malignant tumors, whereas the undifferentiated or anaplastic thyroid carcinoma (UTCs) are higly aggressive but extremely rare.[16] Papillary thyroid carcinoma comprises more than 80% of thyroid epithelial malignancies and the diagnosis of this tumour is based on cytological criteria.[17] However, there is no specific diagnostic tool for the identification of thyroid cancer.[18] Therefore, the identification of new biomarkers are needed to aid in accurate diagnosis and prognosis of most thyroid tumors that can also represent new therapeutic targets.

Given the interest to A3AR agonists as a promising new agents against cancer we have first evaluated the expression of A3 adenosine receptor in human thyroid carcinomas and thus the possible correlation between thyroid differentiation status and A3AR expression degree. We have then characterized the effect of a selective A3AR agonist, Cl-IB-MECA, on papillary carcinoma cell line and the molecular mechanism(s) underlying Cl-IB-MECA-mediated cytotoxicity.

Results

Expression of A3AR in human thyroid tissues. We report for the first time a detailed analysis of A3 adenosine receptor expression in primary thyroid cancer tissues, human specimens from normal thyroid, papillary, follicular and anaplastic thyroid carcinomas, using immunohistochemical analysis with anti-A3 adenosine receptor antibody. The results indicate a negative staining for A3 adenosine receptor in normal thyroid cells (Fig. 1A), whereas all the carcinoma tissues analyzed expressed A3AR protein (Fig. 1B–D). We investigated the relationship between A3AR expression and the various clinico-pathological featureas of thyroid carcinomas. Among them, as shown in Table 1, two of four follicular adenomas expressed a very low level of A3AR protein whereas all the carcinomas except for one case overexpressed it. Furthermore, no significant difference in A3AR overexpression could be established between follicular, papillary and anaplastic carcinomas (Table 1).

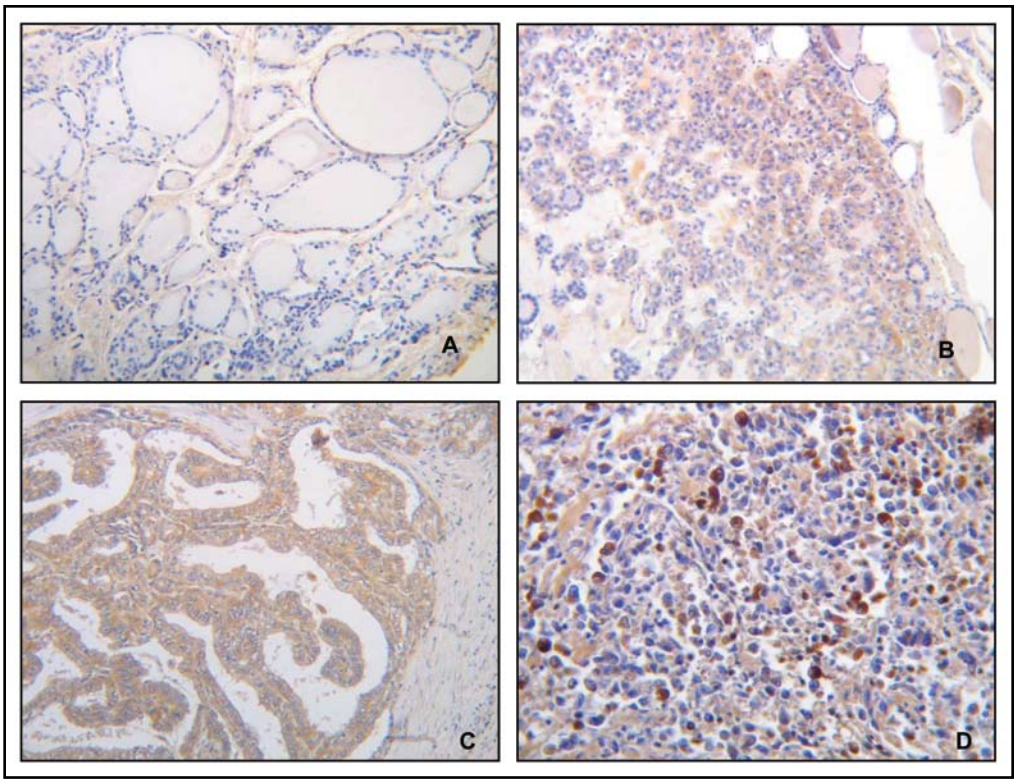

Figure 1. Immunohistochemical analysis of A3AR expression in normal human thyroid and anaplastic human thyroid carcinoma. Localization of A3AR was determined by immunohistochemistry in sections from normal thyroid tissue (A) and three different thyroid carcinomas, follicular (B), papillary (C) and anaplastic (D), respectively.

Table 1 **A3AR localization in normal and pathological human thyroid tissues**

Hystological types of thyroid samples	N° positive cases / n° total cases analyzed by IHC	A3AR staining score		
		1+	2+	3+
Normal thyroid	0/4			
Follicular adenoma	2/4	2		
Papillary carcinoma	7/7	2	2	3
Follicular carcinoma	4/4		1	3
Anaplastic carcinoma	5/5	1	1	3

The percentage of cells with cytoplasmatic staining for A3AR was scored from 0 to 3: 0, no positive cells; 1+, <10% of positive cells; 2+, 11-50% of positive cells; 3+, 76–100% of positive cells.

Cl-IB-MECA inhibits NPA cells growth. The high level of A3 adenosine receptor expression in human thyroid carcinomas prompted us to investigate the effect of a potent and selective agonist of this receptor, Cl-IB-MECA in NPA cell line, derived from a human papillary thyroid carcinoma. NPA cells were treated with increasing concentrations (5–80 μM) of Cl-IB-MECA for 24 h. As shown in Figure 2A Cl-IB-MECA inhibited cell proliferation in a dose-dependent manner, with IC_{50} = 38.29 ± 0.062 μM. Figure 2B shows that Cl-IB-MECA did not induce apoptosis in NPA cells evaluated by flow cytometry analysis and by Western blotting analysis of caspase-3 and -8. These results suggest that this molecule acts by blocking celllar proliferation but it did not induce cell death.

Cl-IB-MECA arrests NPA cells at G_0/G_1 phase of the cell cycle. On the basis of the previous results we analysed the effect of Cl-IB-MECA on cell cycle. The cytometry investigation showed a clear arrest at G_0/G_1 cell cycle phase of thyroid cells treated with Cl-IB-MECA (10–20–40 μM) compared to control cells (Fig. 3A and B) after 24 h of treatment. The accumulation of cells in the G_1 phase was increased by up to 44% ($p < 0.001$) with a corresponding decrease of cells in S phase (down to 63%; $p < 0.001$). These results suggest that Cl-IB-MECA had a primary effect on cell cycle arrest at G_0/G_1 phase.

The block of cell cycle induced by Cl-IB-MECA well correlated with a reduced expression of cyclins D1 and E2 compared with control cells (Fig. 4A and B).

We further investigated the signal transduction pathway by which Cl-IB-MECA may inhibit thyroid cell proliferation by analysing the changes in ERK1/2 phosphorylation. Figure 5A and B shows a Western blot experiment of pERK1/2 after treatment of NPA cells with Cl-IB-MECA at different time points and different concentrations, respectively. The ERK1/2 phosphorylation reduction was

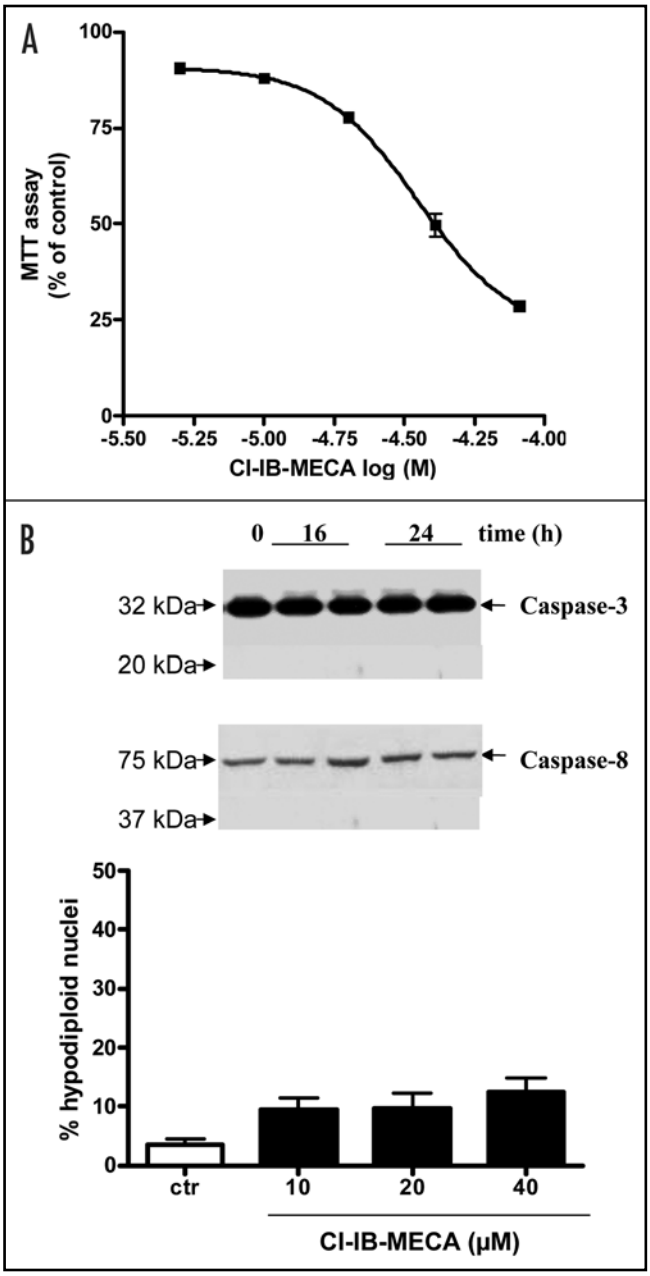

Figure 2. Effect of Cl-IB-MECA on human NPA cells. (A) cytotoxic effect measured by MTT analysis. NPA cells were treated with Cl-IB-MECA at the indicated concentrations for 24 hours. The results are expressed as percentage of the OD relative to untreated cells assumed as 100 % of cell viability. Values indicate the mean ± SEM in triplicate tests. (B) apoptosis of NPA cells after treatment with Cl-IB-MECA analysed by flow cytometry and expressed as percentage of hypodiploid nuclei (n = 3). Representative blots show caspase-3 and caspase-8 expression in NPA cells treated with 40 μM Cl-IB-MECA at the indicated times.

rapid (5 min) and prolonged for 2 h after Cl-IB-MECA treatment, reaching the maximum value at the concentration of 40 μM.

Inhibition of NPA cell proliferation by Cl-IB-MECA is not mediated through A3 adenosine receptor. To verify whether the effect of Cl-IB-MECA in NPA cells was due to activation of A3 adenosine receptor we tested two human A3AR-selective antagonists, MRS1523 and FA385 for their ability to reverse the Cl-IB-MECA effect on NPA proliferation. As shown in Figure 6A MRS1523 (0.03–10 μM) and FA385 (0.5–5 μM) did not counteract Cl-IB-MECA inhibitory effect on growth of NPA cells at 24 h incubation. Similar results were obtained with another A3 adenosine receptor antagonist, MRS 1191 (data not shown). Furthermore, MRS1523 and FA385 did not affect the ERK1/2 phosphorylation reduction induced by Cl-IB-MECA after short period of incubation (15 min) (Fig. 6B). Thus, the effect of Cl-IB-MECA in NPA cells appears not to be mediated through A3 adenosine receptor activation.

Selective antagonists of adenosine receptor subtypes in Cl-IB-MECA-induced response in NPA cells. The adenosine exerts many of its affects by activation of four specific cell surface receptors, designed A1, A2A, A2B and A3.[22] In radio-receptor binding studies[6,23-25] it has been reported that the affinity of Cl-IB-MECA for A3 adenosine receptor is in the nanomolar range. However, we observed the inhibition of NPA cells proliferation at concentrations ≥10 μM. To test whether the inhibition of NPA cells proliferation induced by Cl-IB-MECA was not due to a non specific action of this molecule on any of adenosine receptors, we evaluated the effect of Cl-IB-MECA in combination with DPCPX (an A1 receptor antagonist), ZM 241385 (a selective A2A receptor antagonist) or PSB 1115 (a selective A2B receptor antagonist) on NPA cell proliferation and on ERK1/2 phosphorylation. As shown in Figure 7A and B, the selective antagonists of adenosine receptor subtypes were not able to block the Cl-IB-MECA effect on cell proliferation or to prevent the ERK1/2 dephosphorylation, respectively. It is important to note that the selective receptors antagonists used in this work had no effect on cell proliferation or ERK 1/2 phosphorylation when used alone or all together (data not shown). These data strongly suggest that adenosine receptor-mediated signals are not directly responsible of Cl-IB-MECA-induced NPA cell cycle arrest.

Nucleoside transporters inhibitors in Cl-IB-MECA-induced response in NPA cells. The observations that adenosine receptors antagonists did not inhibit cell proliferation arrest induced by Cl-IB-MECA led us to hypothesize that Cl-IB-MECA is transported into NPA cells to act intracellularly. We further analysed this possibility for Cl-IB-MECA in our experimental conditions by using an adenosine uptake inhibitor, NBTI or the adenosine transport inhibitor, dipyridamole. Neither NBTI (10 μM) nor dipyridamole (10 μM) prevent the Cl-IB-MECA effects on NPA cells (Fig. 8). NBTI itself or dipyridamole had no effect on cell proliferation (data not shown). However, we cannot rule out the possibility that Cl-IB-MECA might enter the cells by a nucleoside transporter-independent mechanism. Therefore, further studies are needed to understand the exact mechanism(s) underlying the observed effects of Cl-IB-MECA.

Discussion

In the present study we have investigated the expression of adenosine receptor A3 in the human thyroid cancer tissues and cell lines. The results obtained from the immunohistochemical analysis demonstrated for the first time, to our knowledge, the existence of A3 adenosine receptor in human thyroid carcinomas. The A3 adenosine receptor expression level was higher in thyroid carcinomas than in normal tissues. In anaplastic (undifferentiated) carcinoma, A3AR expression was high with no significant difference from papillary or follicular carcinoma. Furthermore the expression of A3AR was not correlated to a differentiation degree of carcinoma cells. However

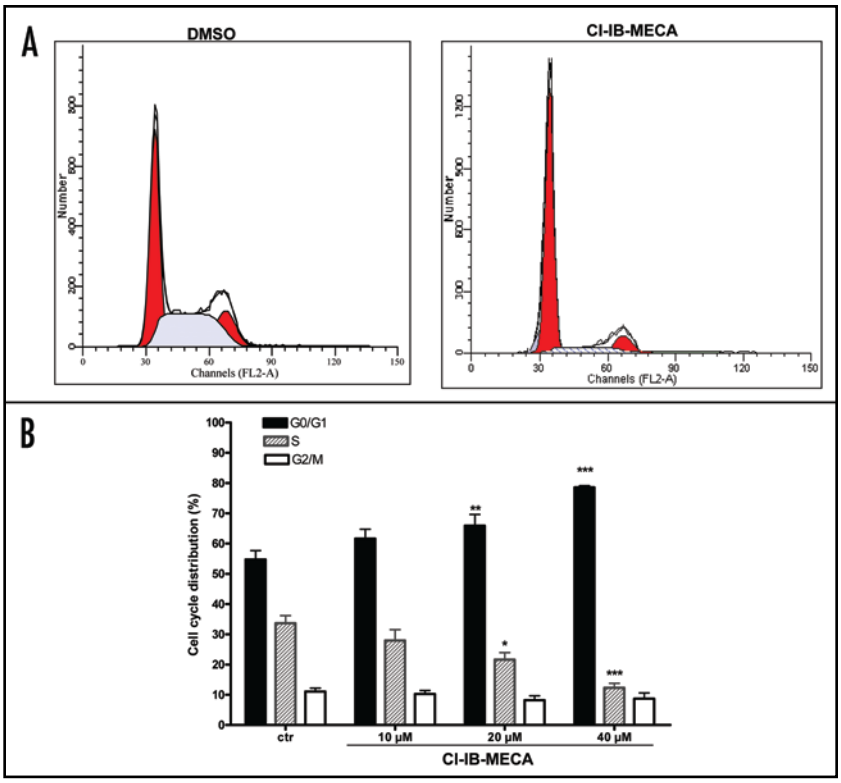

Figure 3. Cell cycle analysis of NPA cells treated with Cl-IB-MECA for 24 h. A, representative flow cytometric analysis of cell cycle. *DMSO panel*, NPA cells treated for 24 h with Cl-IB-MECA vehicle. *Cl-IB-MECA panel*, NPA cells treated for 24 h with 40 μM Cl-IB-MECA. B, quantitative analysis of cells in G_0/G_1, S, and G_2/M phases treated with the indicated concentrations of Cl-IB-MECA. Data are expressed as mean ± SEM percentage of cell cycle distribution, *P<0.05, **P<0.01 and ***P<0.001 vs control, n=5.

the A3AR overexpression on carcinomas compared to benign adenoma or normal tissue may suggest a role of A3AR in malignant transformation.

Cl-IB-MECA is a synthetic nucleoside recently developed with high selectivity and affinity to A3AR with antitumor activity.[4,5,11,12] On this line, we examined the anti-proliferative activity of Cl-IB-MECA against human papillary carcinoma cell line NPA and investigated the possible mechanism(s) of action. We found that Cl-IB-MECA in a μM range of concentrations inhibited the proliferation of NPA cells by induction of cell cycle arrest at G_1 phase. Cell cycle arrest at G1 phase well correlated to the reduction of cyclin D1 and cyclin E2 protein expression. We also found that Cl-IB-MECA is able to inhibit ERK1/2 phosphorylation, necessary for cell proliferation, in a time- and concentration-dependent manner, as previously demonstrated in other cell lines.[26] In addition, in NPA cells Cl-IB-MECA at the concentrations used in this work did not induce apoptosis. Although the reported role of A3 adenosine receptors in cancer modulation, the present results with selective A3AR antagonists, MRS1191, MRS1523 and FA385, clearly show that Cl-IB-MECA does not interact with A3 receptor and hence that its effects on human NPA cells are independent of the presence of this receptor subtype. Moreover, the effects of Cl-IB-MECA in NPA cells are not mediated by A1, A2A, or A2B receptors, either. In fact, our results show that none of the adenosine receptors antagonists DPCPX, ZM241385, PSB1115 highly selective for A1, A2A or A2B receptors, respectively, were able to reverse the effects of Cl-IB-MECA on cell proliferation and on ERK1/2 phosphorylation. Most convincingly, there was absolutely no reduction in the accumulation of cells in G_1 phase or ERK1/2 dephosphorylation induced by Cl-IB-MECA when a combination of antagonists against all four subtypes was used (data not shown). We also tested the possibility of an intracellular action of Cl-IB-MECA, as a second messenger, after being transported into the cells. The nucleoside transport inhibitors, NBTI or dipyridamole, used at a final concentration of 10 μM, which is an excess of the IC_{50} values for inhibition of nucleoside transporters[27] failed to inhibit or reduce the effects of Cl-IB-MECA in a significant manner. However, we cannot rule out the possibility that Cl-IB-MECA might operate directly with some intracellular targets, after being transported into the cell by a nucleoside transporter-independent mechanism.

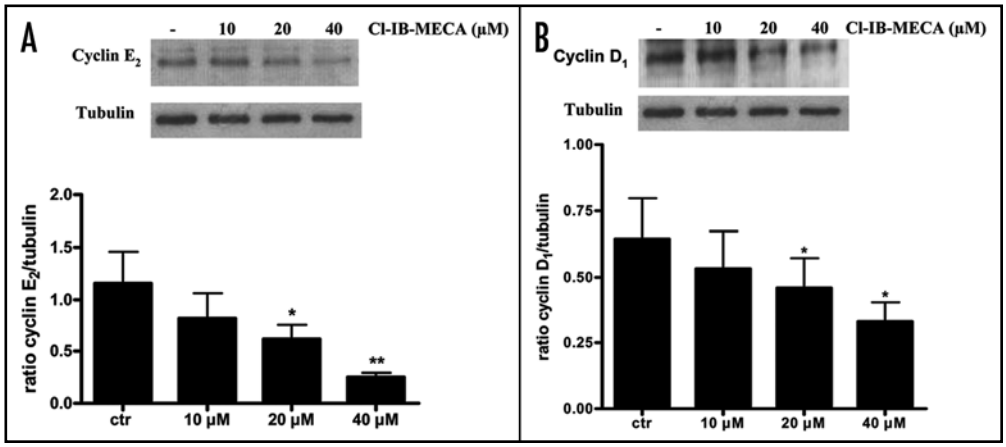

Figure 4. Analysis of cyclin E2 (A) and cyclin D1 (B) protein expression after 24 h in Cl-IB-MECA-treated NPA cells performed by Western blots. Data are expressed as mean ± SEM of ratio with tubulin. *p < 0.05 and **p < 0.01 vs control, n = 3 for each experiment.

Taken together, our findings indicate that Cl-IB-MECA might affect the thyroid carcinoma cells proliferation by a mechanism independent of classical adenosine receptors. Previous studies have shown that A3AR agonists, IB-MECA or Cl-IB-MECA, at micromolar concentrations, inhibited growth of different cell lines through mechanism not related to A3 adenosine receptor signalling. Kim and coworkers (2002) have shown that Cl-IB-MECA induced apoptosis in two leukemic cell lines through an A3AR-indipendent mechanism.[28] In addition, micromolar concentrations of the A3AR-selective agonist, IB-MECA, completely inhibited growth of the human breast cancer cell line MCF-7 which do not express appreciable

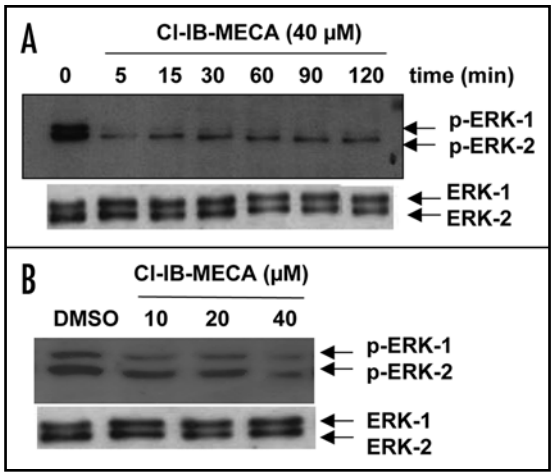

Figure 5. Time- and dose-dependent effects of Cl-IB-MECA on phosphorylation of ERK1/2. (A) NPA cells were stimulated with 40μM Cl-IB-MECA for the indicated times. (B) dose-response effect of Cl-IB-MECA in cells stimulated for 15 minutes before determination of ERK1/2 phosphorylation. Blots shown are representative of at least three independent experiments.

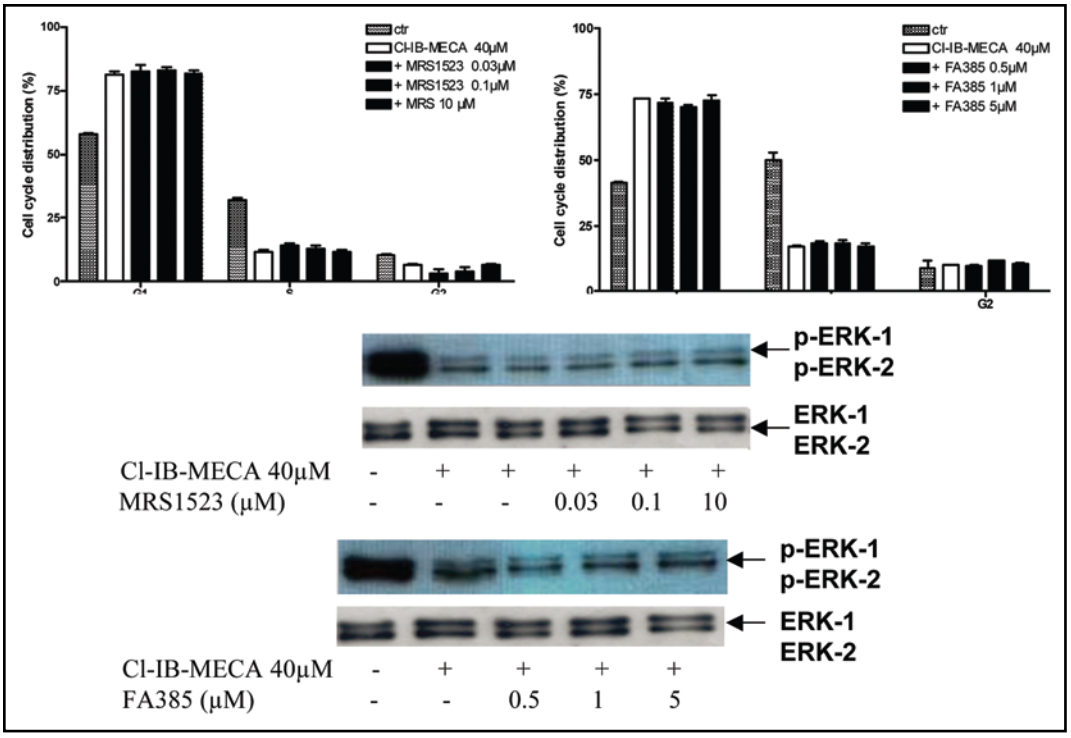

Figure 6. Effects of the A3AR antagonists MRS1523 and FA385 on Cl-IB-MECA-induced cell cycle arrest and ERK1/2 dephosphorylation. NPA cells were treated with different concentrations of A3 receptor antagonists, MRS1523 or FA385, 30 minutes before incubation with 40 μM Cl-IB-MECA. Cells cycle distribution were determined by flow cytometry after 24 h of stimulation (A). ERK1/2 phosphorylation was determined after 15 minutes of Cl-IB-MECA stimulation by Western blot analysis (B). Data are expressed as mean ± SEM percentage of cells cycle distribution, n = 3. The immunoblots show one representative experiment.

levels of A3AR mRNA. In this cells overexpression of A3 adenosine receptor did not increase the sensitivity to IB-MECA. Furthermore, the growth inhibition of MCF-7 was not abolished by A3AR antagonist.[29] Previous reports have also highlighted that adenosine-induced apoptosis in El-4 thymoma cells was mediated through a nonclassical adenosine receptor, as selective adenosine-receptors antagonists failed to attenuate the adenosine-induced cell death.[30] Other studies have proposed a novel low affinity receptor for adenosine, classified as "atypical A2a and A3 adenosine receptors".[31,32]

In conclusion, A3 adenosine receptor has been identified for the first time in thyroid carcinoma tissues. Even though the A3AR has been identified in these cells, the anti-proliferative effects of Cl-IB-MECA appears not to be mediated by this receptor. This results may lead to Cl-IB-MECA or its analogues as drugs to treat thyroid cancers. However, the precise mechanism of this molecule needs to be further investigated.

Materials and Methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA solution (1x) and antibiotic solution (penicillin and streptomycin), phosphate-buffered saline (PBS) were from Cambrex Biosciences. 3-(4,5-Dimethylthiazol2--yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide, Triton X-100, sodium citrate, formamide, MRS 1191, MRS 1523, NBTI and mouse-monoclonal anti-tubulin primary antibody were purchased from Sigma (Milan, Italy). Rabbit polyclonal anti-A3AR, anti-caspase-3 and anti-caspase-8 primary antibodies and horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG were from Santa Cruz Biotechnology (DBA; Milan, Italy). Rabbit polyclonal anti-p-ERK1/2, anti-ERK1/2, anti-cyclin D1, anti-cyclin E2 primary antibodies were from Cell Signaling Technology (Celbio; Milan, Italy). Biotinylated goat anti-rabbit IgG was from Vectostain ABC kits (Vector Laboratories). Cl-IB-MECA, DPCPX, PSB 1115, ZM 241385 and dipyridamole were from Tocris Bioscience (Tocris Cookson Ltd., UK). ECL reagent was from Amersham Pharmacia Biotech, UK. FA 385 was synthesized and kindly provided by Dr. Vittoria Colotta.[19]

Cell culture. NPA cells, a human papillary thyroid carcinoma cell line[20] were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 units/ml streptomycin and 2 mmol/L glutamine. At the onset of each experiment, cells were placed in fresh medium and then cultured in the presence of Cl-IB-MECA (10–20–40 μM) or other reagents followed by further analyses.

Immunohistochemical analysis. Immunohistochemical analysis was performed as previously described.[21] Briefly, speciemens from normal and pathological human thyroid were isolated, rinsed with

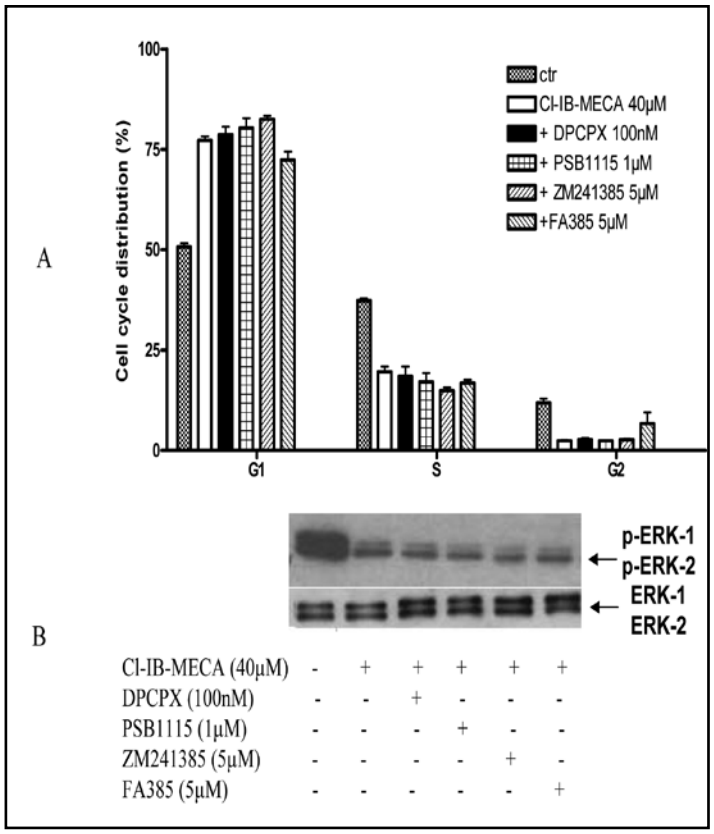

Figure 7. Effect of DPCPX (100 nM), PSB1115 (1 μM), ZM241385 (5 μM) and FA385 (5 μM) A1, A2A, A2B, A3 adenosine receptor antagonists, respectively on Cl-IB-MECA-induced cell cycle arrest and ERK1/2 dephosphorylation. NPA cells were treated with different adenosine receptor antagonists for 30 minutes before Cl-IB-MECA stimulation (40μM). Cell cycle analysis was determined after 24 h of Cl-IB-MECA treatment (A) (n = 3). ERK1/2 phosphorylation was analysed by Western blot after 15 minutes of stimulation with Cl-IB-MECA. Blot shown is representative of at least three independent experiments.

PBS, fixed in 4% buffered neutral formalin, and embedded in paraffin. 5-6 μm thick paraffin sections were then deparaffinized and placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min and then washed in PBS before immunoperoxidase staining. Slides were then incubated overnight at 4°C in a humidified chamber with antibody anti-A3AR diluted 1:100 in PBS and subsequently incubated, first with biotinylated goat anti-rabbit IgG for 20 min and then with premixed reagent ABC (Vector Laboratories) for 20min. The immunostaining was performed by incubating slides in diaminobenzidine (Dako) solution containig 0.06 mM diaminobenzidine and 2 mM hydrogen peroxide in 0.05% PBS (pH 7.6) for 5 min, and after chromogen development, slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips using a permanent mounting medium (Permount).

MTT assay. NPA cells (1 x 10^5 cells/ml in 96-well culture plates) were incubated for 24 hours with different concentrations of Cl-IB-MECA. After treatment, MTT solution (5mg/ml in PBS) was added to each well. After three hours incubation lysis buffer (200g/L SDS, 50% Formamide, pH4.7) was added to each well to dissolve formazan. The absorbance was measured at 620 nm in a microplate reader. The results were expressed as percentage relative to untreated control and the IC_{50} value was calculated using nonlinear regression analysis.

Flow Cytometry Analysis. NPA cells (1 x 10^6 cells/ml in 12-well culture plates) were incubated for 24 h in the presence or absence of Cl-IB-MECA. The cells were then washed with PBS and suspended by trypsinization. Cells were centrifugated at 2000 x g for 10 min, and incubated with a staining solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 mg/ml propidium iodide at 4°C for 30 min in the dark. Samples were analyzed by Becton Dickinson FACScan flow cytometer. The cell cycle distribution, expressed as percentage of cells in the G_0/G_1, S, and G_2/M phases, was calculated by using ModFit LT 3.0 program. The apoptotic cells are expressed as percentage of hypodiploid nuclei.

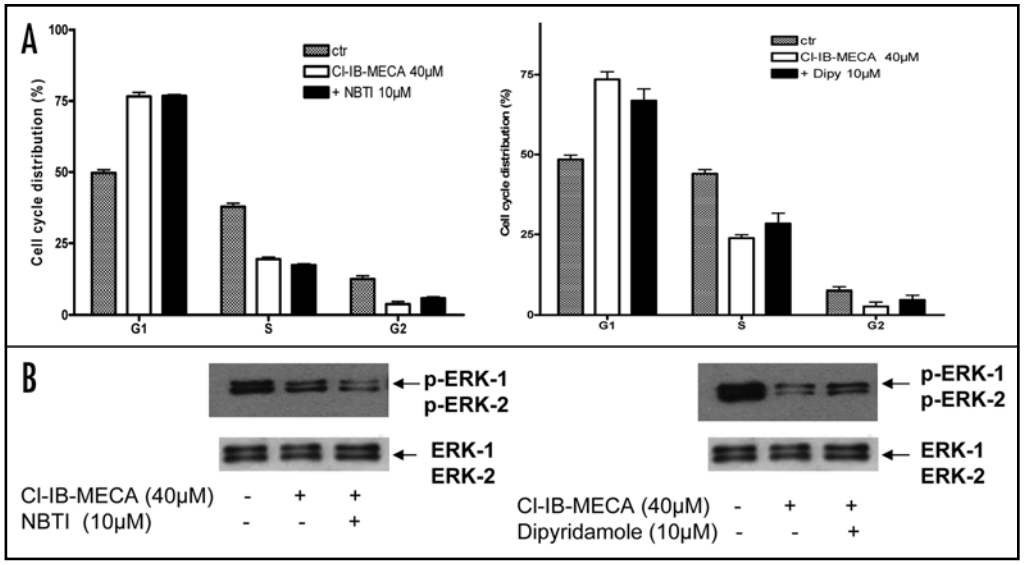

Figure 8. Effects of the transporters inhibitors, NBTI (10μM) and dipyridamole (10 μM) on cell cycle phases and on ERK1/2 dephosphorylation. NPA cells were treated with NBTI or dipyridamole 15 minutes before Cl-IB-MECA stimulation (40 μM). Cell cycle distribution was determined after 24 h of Cl-IB-MECA treatment (A) (n = 3). ERK1/2 phosphorylation was determined after 15 minutes of treatment (B).

Western blotting analysis. Cells were plated at a density 5 x 10^6/ml in normal culture conditions and incubated with or without Cl-IB-MECA at different times. 40 μg of total whole-cell protein per lane were separated through 10% denaturing-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in TBS-Tween 20 (0.1% v/v) and then incubated overnight at 4°C with the primary antibodies. Following incubation with a horseradish peroxidase-conjugated secondary antibodies signals were detected with ECL reagent. Each filter was reprobed with mouse monoclonal anti-tubulin antibody. Immunoreactive bands were quantified by densitometry.

Statistical analysis. Results are expressed as mean ± SEM. The one-way ANOVA test was used for statistical analysis followed by Bonferroni's Multiple Comparison test. A value of $p < 0.05$ was considered statistically significant.

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