Protein Kinase A-Independent Inhibition of Proliferation and Induction of Apoptosis in Human Thyroid Cancer Cells by 8-Cl-Adenosine

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Purpose: Protein kinase A (PKA) affects cell proliferation in many cell types and is a potential target for cancer treatment. PKA activity is stimulated by cAMP and cAMP analogs. One such substance, 8-Cl-cAMP, and its metabolite 8-Cl-adenosine (8-Cl-ADO) are known inhibitors of cancer cell proliferation; however, their mechanism of action is controversial. We have investigated the antiproliferative effects of 8-Cl-cAMP and 8-CL-ADO on human thyroid cancer cells and determined PKA's involvement.

Experimental Design: We employed proliferation and apoptosis assays and PKA activity and cell cycle analysis to understand the effect of 8-Cl-ADO and 8-Cl-cAMP on human thyroid cancer and HeLa cell lines.

Results: 8-Cl-ADO inhibited proliferation of all cells, an effect that lasted for at least 4 d. Proliferation was also inhibited by 8-Cl-cAMP, but this inhibition was reduced by 3-isobutyl-1-methylxanthine; both drugs stimulated apoptosis, and 3-isobutyl-1-methylxanthine drastically reduced 8-Cl-cAMP-induced cell death. 8-Cl-ADO induced cell accumulation in G1/S or G2/M cell cycle phases and differentially altered PKA activity and subunit levels. PKA stimulation or inhibition and adenosine receptor agonists or antagonists did not significantly affect proliferation.

Conclusions: 8-Cl-ADO and 8-Cl-cAMP inhibit proliferation, induce cell cycle phase accumulation, and stimulate apoptosis in thyroid cancer cells. The effect of 8-Cl-cAMP is likely due to its metabolite 8-Cl-ADO, and PKA does not appear to have direct involvement in the inhibition of proliferation by 8-Cl-ADO. 8-Cl-ADO may be a useful therapeutic agent to be explored in aggressive thyroid cancer. (**J Clin Endocrinol Metab** 93: 1020–1029, 2008)

Thyroid cancer is the most prevalent endocrine malignancy. Although differentiated thyroid cancer has generally good prognosis, a subset of thyroid carcinomas are highly aggressive and respond poorly to available treatment (1–5). New therapies for metastatic, poorly differentiated, and undifferentiated thyroid cancer are needed.

Signals mediated by cAMP and protein kinase A (PKA) have been implicated in the control of cell proliferation in many cell types (6, 7). The PKA holoenzyme consists of a heterotetramer of two homodimers of the regulatory subunits (R-subunits, RIα or RIβ and RIIα or RIIβ) and two catalytic subunits (Cα, Cβ, or Cγ) (8), resulting in the formation of two isozymes, type I (RI) and type II (RII). Binding of cAMP to the regulatory subunits releases the catalytic subunits, which act as serine threonine kinases and

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Abbreviations: 7AAD, 7-Amino-actinomycin D; BrdU, bromodeoxyuridine; 8-Cl-ADO, 8-Cl-adenosine; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IBMX, 3-isobutyl-1-methylxanthine; mAb, monoclonal antibody; MRS1523, 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridinecarboxylate; MTS, 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, salt; NECA, N-ethyl-carbomidoadenosine; PKA, protein kinase A; PKI, protein kinase inhibitor; R-subunits; regulatory subunits; XAC, xanthine amine congener.

phosphorylate target molecules to control a variety of cellular functions (6, 9, 10).

Site-selective cAMP analogs induce growth inhibition in a variety of cancer cell types. Among the cAMP analogs initially tested, the 8-Cl derivative of cAMP (8-Cl-cAMP) was the most potent (11). Although evidence of cellular toxicity was consistently present, conflicting views arose as to the mechanism of its action. One group of studies suggested that the inhibitory effect of 8-Cl-cAMP involves its binding to R-subunits of PKA to modulate both type I and type II PKA isozymes, to reduce the RI to RII subunit ratio (11–13). Other studies provided evidence that 8-Cl-cAMP elicits antiproliferative effects via its extracellular conversion to a dephosphorylated metabolic breakdown product, 8-Cl-adenosine (8-Cl-ADO), by serum phosphodiesterase and 5′-nucleotidase (14–19). The intracellular level of 8-Cl-ADO, however, depends on the activity of two cellular enzymes: adenosine deaminase ($K_m = 17$ μM), which deaminates 8-Cl-ADO to an inactive 8-Cl-inosine, and adenosine kinase ($K_m = 2$ μM), which phosphorylates 8-Cl-ADO to an active 5′,8-chloro-AMP. Higher amounts of 5′,8-chloro-AMP are present in the cell due to the lower K_m of adenosine kinase (20). Some reports suggest that 8-Cl-ADO acts through the inhibition of DNA and/or RNA polymerase (14, 16) or by premature RNA chain termination (21). Finally, others show that the inhibitory effects of both 8-Cl-cAMP (22) and 8-Cl-ADO (23, 24) result in apoptosis; in cells treated with 8-Cl-cAMP, apoptosis is thought to occur only in cells that accumulate in G2/M cell cycle phase (22), whereas cells treated with 8-Cl-ADO are thought to accumulate in the G0/G1 phase without induction of apoptosis (25).

In the present investigation, we studied three human thyroid cancer cell lines: poorly differentiated carcinoma (NPA), follicular carcinoma (WRO), and undifferentiated (anaplastic) cancer (ARO). As a reference cell line, we used HeLa cells. We studied the effect of 8-Cl-cAMP and 8-Cl-ADO on [3H]thymidine incorporation, cell proliferation/metabolism, the cell cycle, and apoptosis. We assessed whether the effect of 8-Cl-cAMP is due to its metabolite 8-Cl-ADO and whether the effect of both drugs is due to an action on A1, A2a and -b, and A3 adenosine surface receptors (17). We also assessed the enzymatic activity of the PKA holoenzyme and the individual PKA subunits in relationship to 8-Cl-ADO. Our data indicated that 8-Cl-cAMP through 8-Cl-ADO inhibited cell proliferation/ metabolism and induced thyroid cell accumulation in the G1/S or G2/M cell cycle phases and apoptosis. There was no apparent involvement of adenosine receptors and no direct involvement of the PKA enzyme and/or R-subunits in the above actions. The data suggest that 8-Cl-ADO (or 8-Cl-cAMP) may be a potential chemotherapeutic agent for the treatment of aggressive thyroid cancer, and its effects will not depend on PKA signaling.

Materials and Methods

Cell lines and other materials

Thyroid cancer cell lines have been extensively described elsewhere (26, 27). HeLa cells were from American Type Tissue Collection (Rockville, MD); RPMI 1640 media, AIM V media, fetal bovine serum (FBS;

GIBCO/Invitrogen, Carlsbad, CA), and anti-bromodeoxyuridine (anti-BrdU)-fluorescein isothiocyanate (FITC) monoclonal antibody (mAb) from Invitrogen (Carlsbad, CA); FBS from Hyclone (Logan, UT); forskolin, isoproterenol, 8-Cl-cAMP, 8-Br-cAMP, 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridinecarboxylate (MRS1523), N-ethyl-carbomidoadenosine (NECA), adenosine, xanthine amine congener (XAC), 3-isobutyl-1-methylxanthine (IBMX), and LY294002 from SigmaAldrich (St. Louis, MO); 8-Cl-adenosine from the NIH/NCI (Bethesda, MD); protein kinase inhibitor (PKI) and Cell Titer 96 AQ assay 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, salt (MTS) assay] from Promega (Madison WI); annexin V-FITC, 7-amino-actinomycin D (7AAD) from BD Pharmingen (San Diego, CA); PKA assay kit and [γ^{-32}P]triphosphate from Amersham Pharmacia (Piscataway, NJ); [3H]thymidine from MP Biomedicals (Solon, OH); and FACScalibur flow cytometer and Cell Quest software from Becton Dickinson (San Jose, CA).

Cell culture and plating

Cells were grown in RPMI 1640 media with 1% L-glutamine, 10% FBS, and 0.05% gentamicin. Population doubling times were as follows: ARO, 24 h; WRO, 36 h; HeLa, 77 h; and NPA, 79 h. Cells were plated as follows: in 24-well plates (2×10^4 cells/ml) or in 96-well plates (1 to 2.5×10^3 cells/well) for [3H]thymidine and MTS assays, respectively; in six-well plates (3×10^4 cells/ml) for apoptosis experiments; in 175-cm² flasks (1×10^6 cells/ml) for PKA assays and gel electrophoresis; and in 75-cm² flasks (2×10^5 cells/10 ml) for cell cycle analysis. In all experiments, cells were allowed to attach overnight before the addition of drugs.

Cell proliferation

Cell proliferation was determined by [3H]thymidine incorporation or MTS assay. Cells, incubated with media alone or with media, drugs, and 2 μCi/well [3H]thymidine, were washed with PBS (pH 7.4), and 10% TCA (23 C) added. Proteins were solubilized with 0.5 N NaOH and [3H]thymidine detected by scintillation count. For MTS assays, cells were incubated with drugs, and Cell Titer 96 AQ solution was added (37 C) before determining the absorbance at 490 nm.

Quantitation of apoptosis

Apoptosis was quantified as reported previously (10) and is the percentage of cells that excluded 7AAD and exteriorized membrane phosphatidylserine, detected by the binding of annexin V-FITC (28). Briefly, cultures treated with drugs were washed, resuspended in binding buffer, labeled with 7AAD and annexin V-FITC, and incubated, and a minimum of 20,000 events were analyzed by flow cytometry (Cell Quest software). Apoptotic cells were stained by annexin V-FITC; late apoptotic/necrotic cells were stained by both annexin V-FITC and 7AAD.

Cell cycle analysis

Cell cycle analysis was performed as previously described (10). Briefly, cells were synchronized in low-serum medium and released from synchronization with 10% FBS/RPMI. BrdU was added 1 h before each time point. After 1 h, cells were collected, washed, and resuspended in PBS followed by ethanol. Samples were centrifuged and washed with HCl/Triton X-100, resuspended in H₂O, and boiled. PBS/Triton X-100 was added followed by centrifugation. Anti-BrdU-FITC mAb was added, and samples were kept in the dark. Propidium iodide was added, and a minimum of 20,000 events were analyzed by flow cytometer using CellQuest software.

Gel electrophoresis

Briefly, proteins in whole-cell lysates were separated by SDS-PAGE on 10% Tris-glycine gels (7). Proteins, transferred to nitrocellulose membranes, were probed with primary antibodies to PKA R-subunit proteins, followed by secondary antibodies against mouse or rabbit IgG. Bands
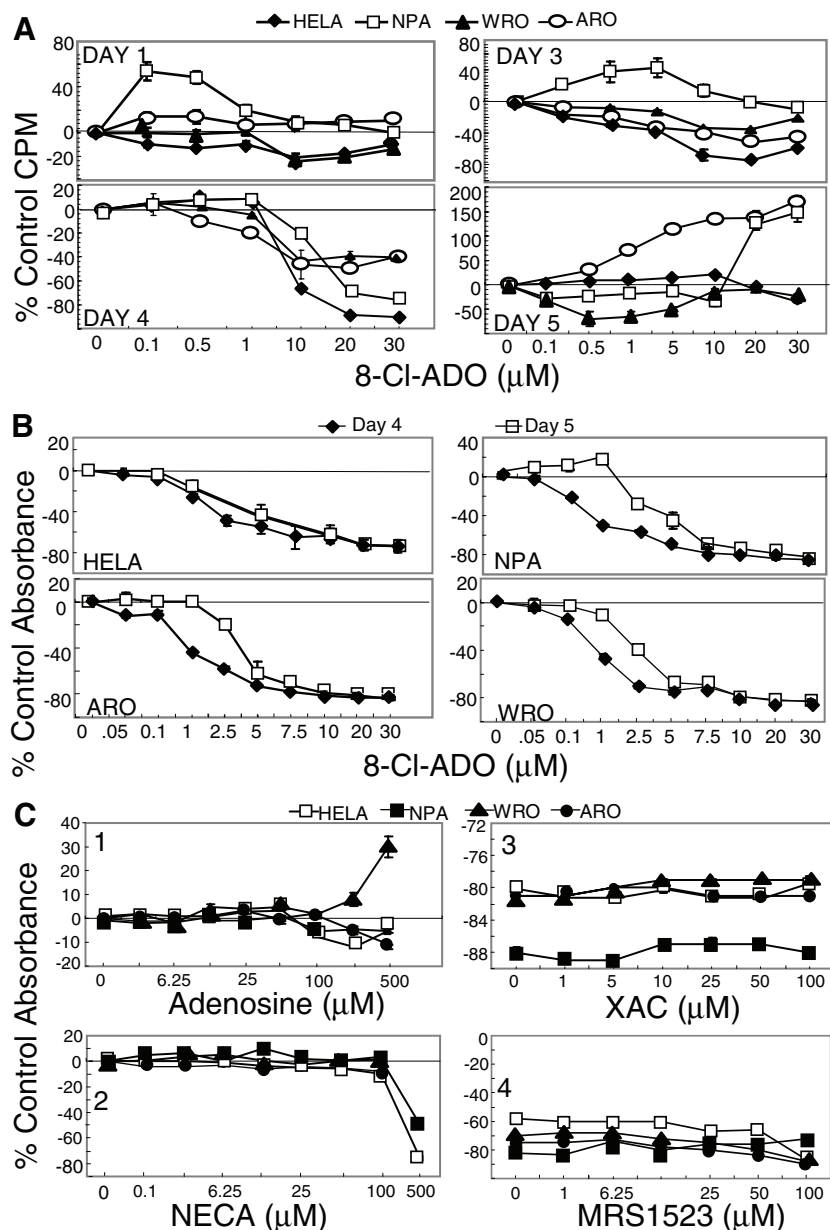
FIG. 1. 8-Cl-ADO inhibits [³H]thymidine uptake and cell metabolism in thyroid cancer cells and in HeLa cells, with no effect on adenosine receptors. A, 8-Cl-ADO inhibits [³H]thymidine uptake in a time- and concentration-dependent manner. Cells in 24-well culture plates were incubated for 1–5 d with increasing 8-Cl-ADO concentrations (0–30 μM), in the presence of 2 μCi [³H]thymidine per well. Counts per minute (CPM) were determined by scintillation count, and values were calculated as percent of control counts per minute. One representative experiment is shown. B, Cells in 96-well culture plates were incubated for 4 and 5 d with 0–30 μM 8-Cl-ADO. C, Cells in 96-well culture plates were incubated for 4 d with adenosine (0–500 μM, *panel 1*), NECA (0–500 μM, *panel 2*), XAC (0–100 μM) plus 20 μM 8-Cl-ADO or 8-Cl-ADO alone (*panel 3*), and MRS1523 (0–100 μM) plus 20 μM 8-Cl-ADO, or 8-Cl-ADO alone (*panel 4*). In B and C, Cell Titer 96 AQ solution was added 3.5 h before plates were read using an ELISA reader at 460 nm. Results in B and C are percentage of control absorbance. In C (*panels 3* and *4*), zero represents 20 μM 8-Cl-ADO alone. *Points* are mean ± SEM of four wells (A), mean ± SEM of four experiments (B), and mean ± SEM of three experiments (C); *P* < 0.0001 (B).

were detected by ECL reagent and quantitated by densitometer scanning (Molecular Dynamics, Sunnyvale, CA). Equal sample loading was confirmed, and arbitrary values were calculated when blots were stripped and reprobed with β-actin mAb.

PKA activity determinations

PKA activity was measured as described previously (29), using [γ-³²P]triphosphate, in cultures that were lysed in a PKA extraction

buffer [2.5 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol]. Extracts were exposed to cAMP or cAMP and PKI before determination of PKA activity. All determinations were performed twice for each sample and corrected for protein content.

Statistics

Results were analyzed by ANOVA using the PROC Mixed procedure of the Statistical Analysis System (SAS Institute, Cary, NC). When more than two groups were compared, the PDIFF procedure (SAS Institute) was used to compare differences between treatment means. Differences were considered significant at *P* < 0.05.

Results

8-Cl-ADO inhibits cell proliferation

Cells were cultured with 8-Cl-ADO and [³H]thymidine (Fig. 1A) or with 8-Cl-ADO alone in cell metabolism studies (Fig. 1B). 8-Cl-ADO inhibited [³H]thymidine incorporation in all cells in a time and concentration-dependent manner. Cell numbers used are based on cell-titration experiments. A maximal inhibitory effect in the MTS assay was observed in a 4-d incubation period (data not shown). Under these conditions, optimal inhibition occurred on d 4 (to 90%, Fig. 1A; to 82%, Fig. 1B). Curves were biphasic (Fig. 1A and 0–7.5 μM 8-Cl-ADO, Fig. 1B) and inhibition was decreased on d 5. Curves (Fig. 1B) were statistically different for thyroid cells (*P* < 0.0001) only. High levels of inhibition occurred with both assays, with a reversal of the inhibition on d 5. Cells (Fig. 1C) were incubated (4 d) with the A1, A2, and A3 adenosine receptor agonist adenosine; the A1, A2, adenosine receptor agonist NECA; the A1, A2a, and A2b adenosine receptor antagonist XAC; or the A3 adenosine receptor antagonist MRS1523 (*panels 1–4*, respectively) and 20 μM 8-Cl-ADO (*panels 3* and *4*). The adenosine receptor agonists (except NECA at a toxic concentration, 500 μm) did not inhibit cell metabolism, and no reversal of the inhibition by 8-Cl-ADO occurred with the adenosine receptor antagonists. These data exclude the possibility that 8-Cl-ADO's effect is by an effect on adenosine receptors.

8-Cl-ADO undergoes intracellular metabolism

We preincubated 8-Cl-ADO in complete media (to 7 d; 37 C) without cells, followed by incubation with cells (4 d in the same solution; Fig. 2A). No significant effect on the inhibition of cell
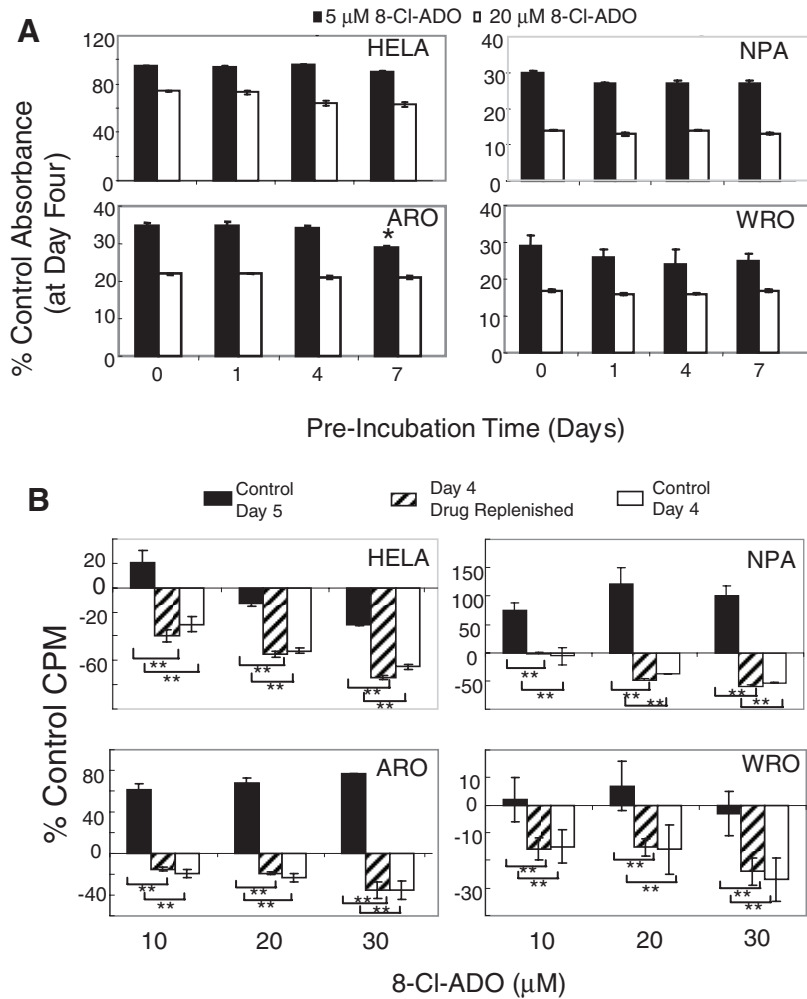
FIG. 2. Loss of inhibition with 8-Cl-ADO with prolonged incubation time: intracellular drug metabolism. A, 8-Cl-ADO (5 and 20 μM) was preincubated (minus cells) for 0–7 d at 37 C before its addition to cells for an additional 4 d; B, cells in 24-well culture plates were incubated with 8-Cl-ADO (20 μM) and [^{3}H]thymidine (2 μCi per well) for 4 and 5 d. In some cultures, 8-Cl-ADO was replenished on d 4 and cultures assayed on d 5. Levels of intranuclear counts per minute (CPM) were determined by scintillation count, and values expressed as percent of control counts per minute. In A, Cell Titer 96 AQ solution was added 3.5 h before plates were read using an ELISA plate reader. Results are expressed as percentage of control absorbance. Points are mean ± SD of two experiments (A) and mean ± SEM of three experiments (B); *, $P = 0.032$ (A); **, $P < 0.0001$ (B).

metabolism by preincubated 8-Cl-ADO was observed in all cell types, except ARO (-14%, 5 μM, 7 d; $P = 0.032$). Thus, the reduced inhibition on d 5 (Fig. 1, A and B) is not due to extracellular drug metabolism. Cells were incubated with 8-Cl-ADO for 4 and 5 d (control d 4 and 5), or the drug was replenished on d 4 (d 4 drug replenished) and [^{3}H]thymidine incorporation determined on d 5 (Fig. 2B). In all cells, decreased inhibition occurred on control d 5 ($P < 0.0001$) compared with control d 4. In cultures where drug was replenished on d 4 and assayed on d 5, the inhibition of control d 4 was maintained. These data suggest an intracellular mechanism of drug metabolism.

8-Cl-cAMP is metabolized to 8-Cl-ADO

Cells were incubated (4 d) with 8-Cl-ADO, 8-Cl-cAMP, and 8-Cl-cAMP plus the phosphodiesterase inhibitor IBMX (50 μM) in complete media containing Hyclone serum and 8-Cl-cAMP in AIM V serum-free media (Fig. 3A). Although for 8-Cl-ADO,

IC$_{50}$ values were greater in [^{3}H]thymidine than in MTS assays, in MTS assays, 8-Cl-cAMP inhibited cell metabolism at IC$_{50}$ values similar to that of 8-Cl-ADO (see IC$_{50}$ values, Fig. 3B). However, the inhibition by 8-Cl-cAMP was greatly reduced by IBMX (to 62%, $P < 0.0001$–0.01) and further reduced in serum-free media (to 75%, $P < 0.0001$; Fig. 3A). No inhibition occurred with IBMX alone, and IBMX did not alter the inhibition by 8-Cl-ADO (data not shown). The level of inhibition by 8-Cl-cAMP also depended on the source of FBS (e.g. Hyclone vs. GIBCO), with media containing Hyclone serum producing the greatest inhibition by 8-Cl-cAMP (Fig. 3C). Accordingly, IBMX showed a decrease in the inhibition (20–72%, HeLa to ARO cells, respectively) by 8-Cl-cAMP (25 μM; Fig. 3D). A sharp decline was observed in the recovery from inhibition, likely due to toxicity at high IBMX concentrations (400–800 μM). Therefore, the data suggest that 1) 8-Cl-cAMP is metabolized to 8-Cl-ADO and 2) the inhibitory effect of 8-Cl-cAMP is due to its by-product 8-Cl-ADO.

8-Cl-ADO and 8-Cl-cAMP induce apoptosis

Cells were incubated (4 d) with 8-Cl-ADO, 8-Cl-cAMP, or 8-Cl-cAMP plus 50 μM IBMX (Fig. 4, A–C, respectively) in media containing Hyclone serum. Both 8-Cl-ADO and 8-Cl-cAMP induced apoptosis in all cell types in a concentration-dependent manner. Apoptosis induced by 8-Cl-cAMP was reduced (to 65%; $P = 0.004$ to <0.001) by IBMX. In cells treated with 8-Cl-ADO or 8-Cl-cAMP (Fig. 4, A and B, respectively), apoptosis led to significantly high levels of necrosis (Fig. 4, A and B, *upper panels*). Detectable total cell death (early apoptosis plus necrosis) was high (to 57%) in all cell types. Thus, values given for apoptosis exclude apoptotic cell death in cells that had disintegrated during earlier incubation times. Total cell death, however, was similar to levels of inhibition seen in cell metabolism and [^{3}H]thymidine studies (Fig. 1, A and B) on d 4.

8-Cl-ADO alters cell proliferation independently of the PKA signaling pathway

Basal levels of PKA activity (Fig. 5A) were similar in all cell lines (~40 cpm/μg protein/ml). After exposure to cAMP, PKA activity was significantly increased in WRO and ARO cells (20 and 40%, respectively; $P = 0.0005$ to <0.0001) only at the high 8-Cl-ADO concentration (30 μM) and in ARO cells (10%; $P < 0.0001$) at the lower 8-Cl-ADO concentration (15 μM). However, PKA activity was decreased by 85 and 40% in HeLa cells at 15 and 30 μM, respectively ($P < 0.0001$). No effect was seen at 15 μM in NPA or
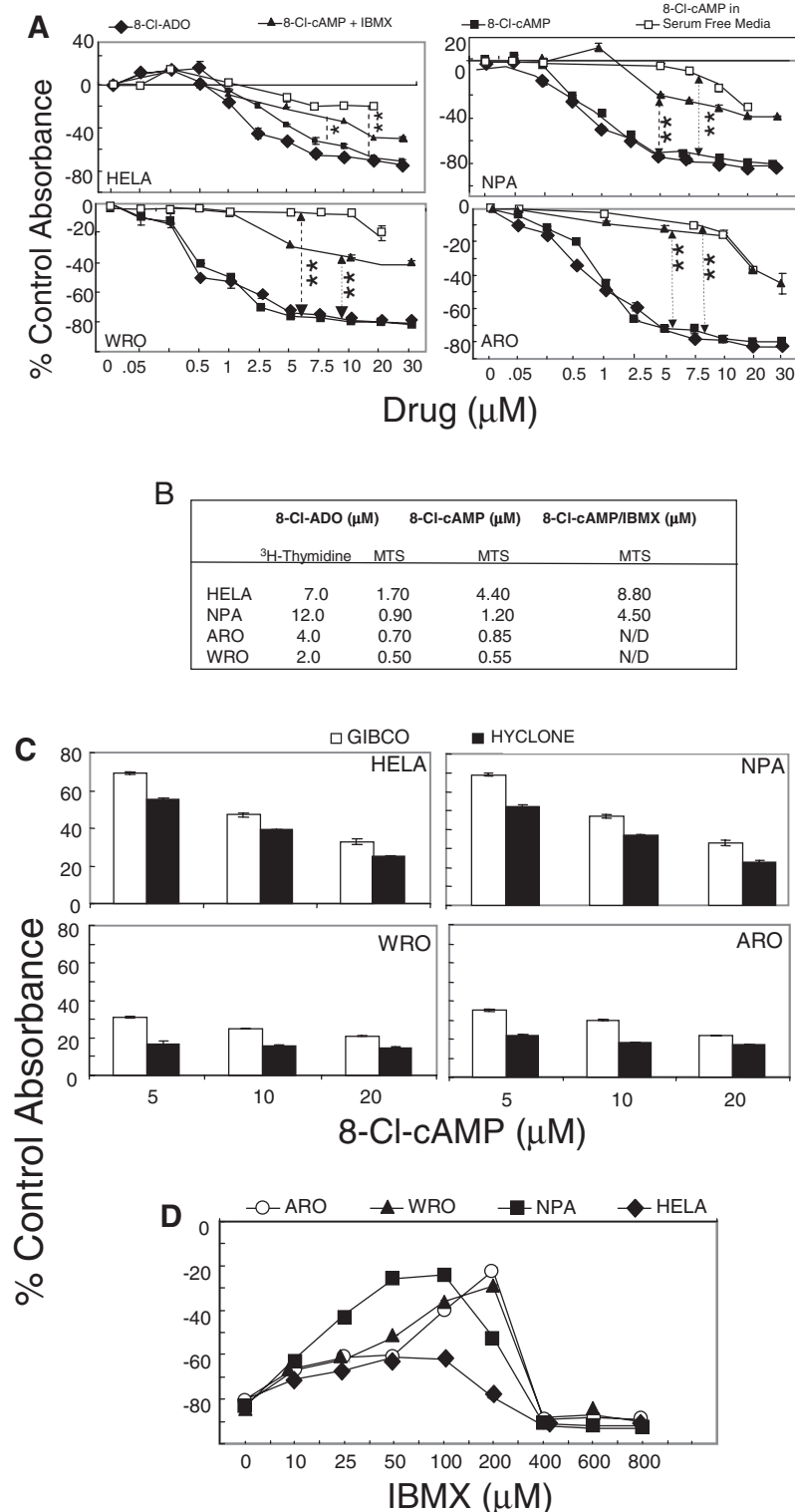
FIG. 3. Metabolism of 8-Cl-cAMP to 8-Cl-ADO: effect of IBMX and serum-free media on extracellular drug metabolism. A, Cells in 96-well culture plates were incubated with increasing concentrations (0–30 μM) of 8-Cl-ADO, 8-Cl-cAMP, or 8-Cl-cAMP plus IBMX (50 μM) in media containing 10% FBS (Hyclone). Cells were also incubated with 8-Cl-cAMP in serum-free medium. All cultures were incubated for 4 d. B, List of IC$_{50}$ values from [^{3}H]thymidine and MTS assays. C, Cells in 96-well culture plates were incubated (4 d) with increasing concentrations of 8-Cl-cAMP in media containing serum from GIBCO/Invitrogen or from Hyclone. D, Cells in 96-well culture plates were incubated (4 d) with increasing concentrations of IBMX (0–800 μM) and/or 8-Cl-cAMP (25 μM). In A, C, and D, Cell Titer 96 AQ solution was added for 3.5 h before plates were read using an ELISA plate reader at 460 nm. Results are expressed as percentage of control absorbance and are mean ± SEM of six experiments (A) and mean ± SD of two experiments (C and D). *, $P = 0.01$; **, $P < 0.0001$ (A).

WRO cells. Thus, there was no consistent effect of 8-Cl-ADO on HeLa or thyroid cells. After exposure to cAMP and 8-Cl-ADO, PKA activity was reduced to basal levels (minus cAMP) by the PKA-specific inhibitor PKI. Although PKA R-subunit levels were altered by 8-Cl-ADO, they were differentially affected (Fig. 5B) with no reduction in the ratio of RI- to RII-type subunits (Fig. 5C). The data indicate a differential effect of 8-Cl-ADO on PKA activity in HeLa *vs.* thyroid cells with a small stimulatory effect in thyroid cells and a much greater inhibition in HeLa cells. Because the PKB/Akt cell signaling pathway also promotes cell proliferation in cancer cells (30), we determined whether the PKA and the PKB/Akt signaling pathways were involved in the inhibition of cell proliferation/metabolism by 8-Cl-ADO. Cultures were incubated (4 d) with PKA pathway stimulants [8-Br-cAMP, forskolin (FSK), and isoproterenol (ISO), Fig. 5D] and the PKA and PKB pathway inhibitors H89 (50 nM) and LY294002 (30 μM), respectively (Fig. 5E). PKA stimulants had little or no effect on cell proliferation/metabolism (Fig. 5D). Although in ARO cells a small inhibition occurred with 8-Br-cAMP (to 35%; $P < 0.0001$), it was 2.6-fold less than that exerted by 8-Cl-ADO in the same cell line. LY294002 inhibited cell metabolism by 50–70% ($P < 0.0001$), whereas H89 inhibited metabolism only in NPA cells (to 17%; $P = 0.01$). This suggests a small stimulatory effect of 8-Cl-ADO on PKA activity in thyroid cells but, in general, no direct involvement of the PKA cell signaling pathway in cell proliferation/metabolism. Instead, the data confirm that the PKB/Akt cell signaling pathway is involved in cell proliferation/metabolism (30).

Accumulation of cells in G1/S or G2/M phases of the cell cycle

Cell cycle analysis of synchronized cells treated with 8-Cl-ADO (20 μM, 72 h), indicated that NPA cells accumulate in the G2/M phase of the cell cycle, whereas WRO and ARO cells accumulate in G1/S. No phase accumulation was observed with HeLa cells (Fig. 6).

Discussion

Our study addressed the questions of 1) whether or not 8-Cl-ADO has growth-inhibitory effects on human thyroid cancer and HeLa cells and 2) whether the inhibitory
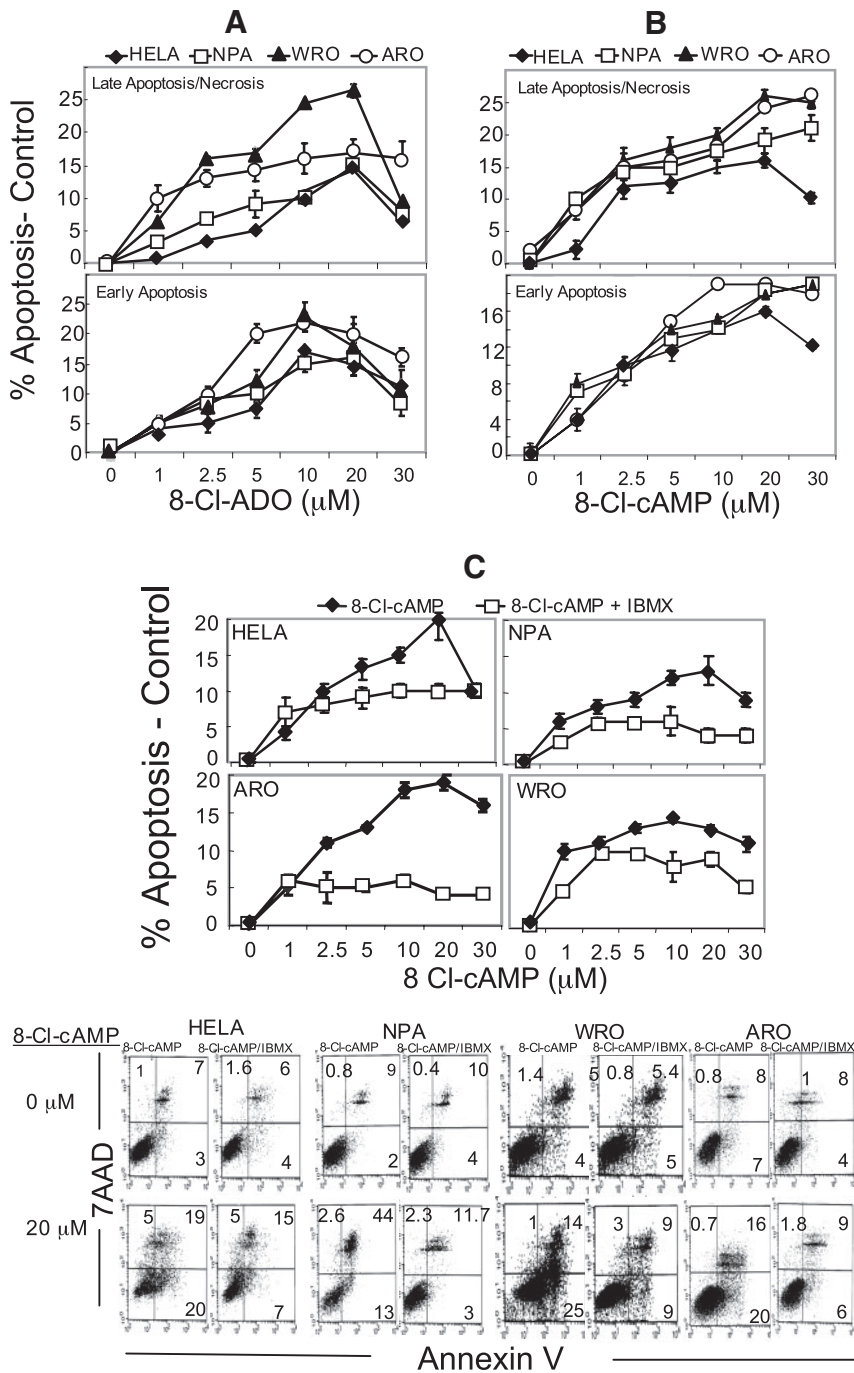
FIG. 4. 8-Cl-cAMP and 8-Cl-ADO stimulate apoptosis in HeLa cells and in thyroid cancer cells. IBMX inhibits 8-Cl-cAMP-induced apoptosis. Cells were incubated with increasing concentrations of 8-Cl-ADO (A), 8-Cl-cAMP (B), or 8-Cl-cAMP plus (50 μM) IBMX (C). Apoptosis was assessed by annexin V-FITC/7AAD assay staining and measured by FACScalibur flow cytometry. *Points* are mean ± SEM of three experiments (A), four experiments (B), and three experiments (C), and results are expressed as percentage of apoptosis (minus apoptosis in untreated cells). *Dot plots* in C are of one representative experiment. P = 0.004 to <0.0001 (C).

IC_{50} values determined by [3H]thymidine assay were about 10-fold higher than those obtained by MTS assay (Fig. 3B), possibly due to the difference in sensitivity of the two assays (34).

The inhibition by 8-Cl-ADO also decreased with time (on d 5 of incubation) (Fig. 1, A and B). By preincubating 8-Cl-ADO (37 C, up to 7 d without cells), followed by incubation with cells and the same preincubated drug, or by replenishing the drug on d 4, we determined whether the decrease in inhibition was due to intracellular (Fig. 2B) and/or extracellular (Fig. 2A) drug metabolism, respectively. We found no difference in the inhibitory effect with the preincubated drug, and the decrease in inhibition on d 5 could be eliminated by replenishing the drug on d 4. This suggests that 8-Cl-ADO is not metabolized by the media at 37 C but may be degraded within the cell. As shown by others (17, 19, 35), the possibility exists that by d 5, intracellular adenosine deaminase may convert the active 8-Cl-ADO to an inactive product, 8-Cl-inosine, thereby reducing inhibition.

We addressed the ongoing controversy as to whether or not 8-Cl-cAMP acts as a pro-drug and is metabolized by cellular or serum phosphodiesterase and 5′-nucleotidase (18, 19, 31, 35) to the highly potent 8-Cl-ADO. We first found that 8-Cl-cAMP inhibits cell proliferation in all cell lines and that the inhibition is comparable to that of 8-Cl-ADO when Hyclone instead of GIBCO/Invitrogen serum (Fig. 3, A–C) was used. The inhibition by 8-Cl-cAMP was greatly reduced by IBMX (Fig. 3, A and D) and further reduced in serum-free media in thyroid cells. There was, however, less reduction in inhibition in HeLa cells with IBMX and serum-free media. These studies and those of others (15–18, 33, 35, 36) suggest that 8-Cl-cAMP is metabolized to 8-Cl-ADO by serum phosphodiesterase (and possibly 5′-nucleotidase) and that the source of the serum plays a large part in this conversion. These data may explain the large difference in IC_{50} values for 8-Cl-ADO and 8-Cl-cAMP that has also been reported by others (17, 31).

One objective of our study was to examine the mechanisms of inhibition of cell proliferation by 8-Cl-ADO and 8-Cl-cAMP. We measured apoptosis (Fig. 4) in 8-Cl-ADO- and 8-Cl-cAMP-treated cells using the annexin V/7AAD assay (37). Treatment with 8-Cl-ADO (Fig. 4A) and 8-Cl-cAMP (Fig. 4B) for 4 d resulted in apoptosis in all cell lines. The rank order of apoptosis (data not shown) was similar to the order
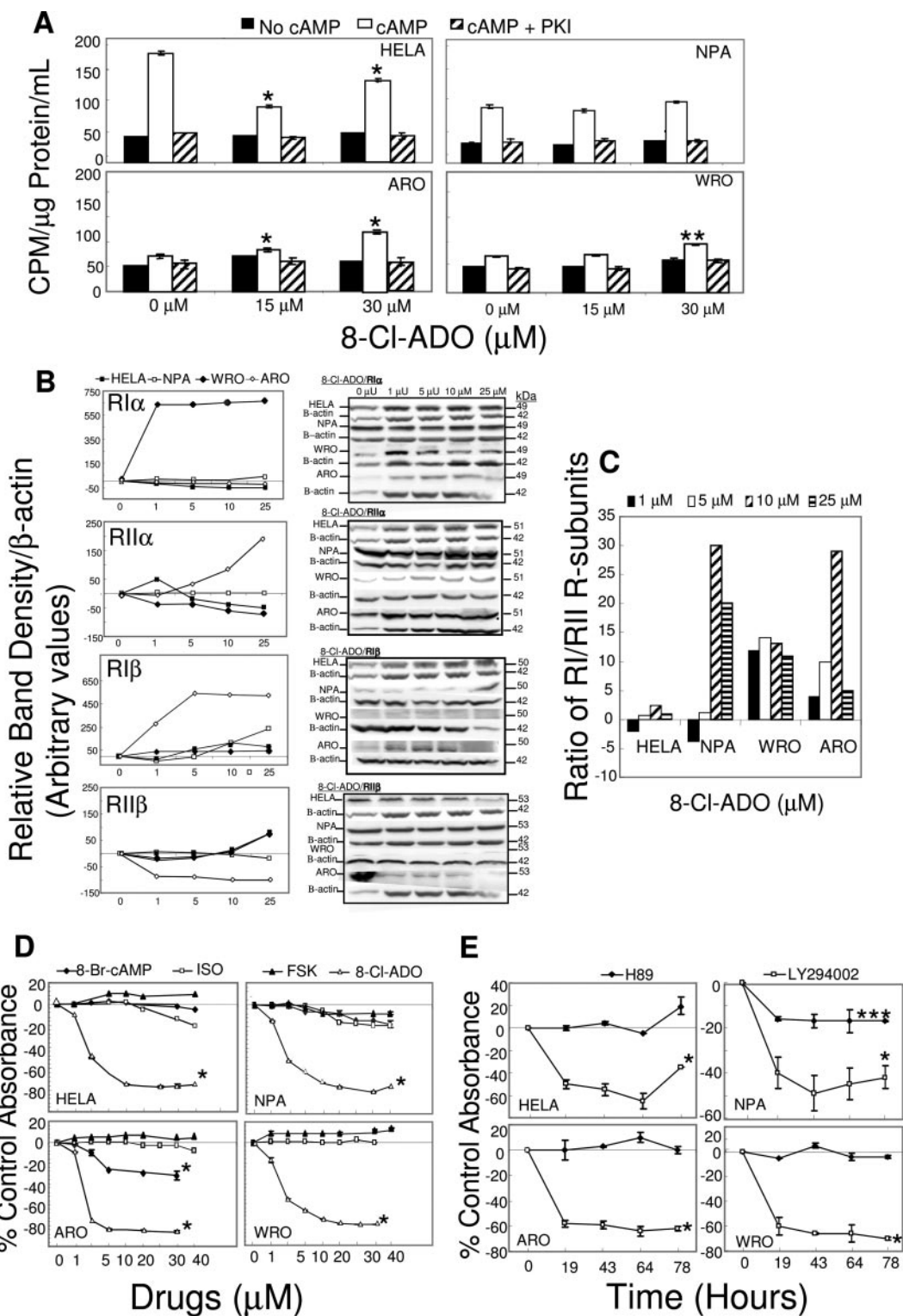
effects of the parent compound, 8-Cl-cAMP, are due to its metabolic by-product, 8-Cl-ADO. We also examined possible mechanisms of action of 8-Cl-ADO on cell growth. We first showed that 8-Cl-ADO inhibited cell proliferation/metabolism and [3H]thymidine incorporation in all thyroid and HeLa cell lines (Fig. 1). IC_{50} values obtained by MTS assay were comparable to those reported for other cancer cell lines (19, 31–33). However,

FIG. 5. Differential effect of 8-Cl-ADO on PKA activity in thyroid cancer cells and in HeLa cells: no significant effect of PKA pathway stimulants or the PKA activity inhibitor H89 on cell metabolism/proliferation. A, Cells in 175-cm² flasks were incubated with 8-Cl-ADO (15 and 30 μM) for 4 d, and cell extracts were exposed to cAMP or to cAMP plus the PKA-specific inhibitor PKI. PKA activity levels were then determined. Results are expressed as cpm [γ³²P]dATP per microgram protein per milliliter and are mean ± SEM of three experiments. B, Cells in 175-cm² flasks were incubated with 8-Cl-ADO (0–25 μM) for 4 d and lysed, and gel electrophoresis and immunoblot assay were performed using antibodies to PKA R-subunits. C, Ratio of PKA R-subunits RIα plus RIβ/ RIIα plus RIIβ from B above. D, Cells in 96-well culture plates were incubated for 4 d with increasing concentrations (0–40 μM) of the PKA pathway stimulants 8-Br-cAMP, isoproterenol (ISO), and forskolin (FSK), or with 8-Cl-ADO. E, cells were incubated with the PKA pathway inhibitor, H89 (50 nM) or with the PKB (Akt)-pathway inhibitor LY294002 (30 μM) for 0–78 h. Cell titer 96 AQ solution was added to cultures in D and E for 3.5 h, before plates were read using an ELISA plate reader at 460 nm. *Points* in B are mean ± SEM of three experiments, expressed as percentage of control band density/B-actin (arbitrary values). Bands are representative of one experiment. Results are expressed as percentage of control absorbance ± SEM of three experiments (D) or mean ± SD of two experiments (E). *, $P < 0.0001$ (A, D, and E); **, $P = 0.0005$ (A); ***, $P = 0.01$ (E) compared with baseline.
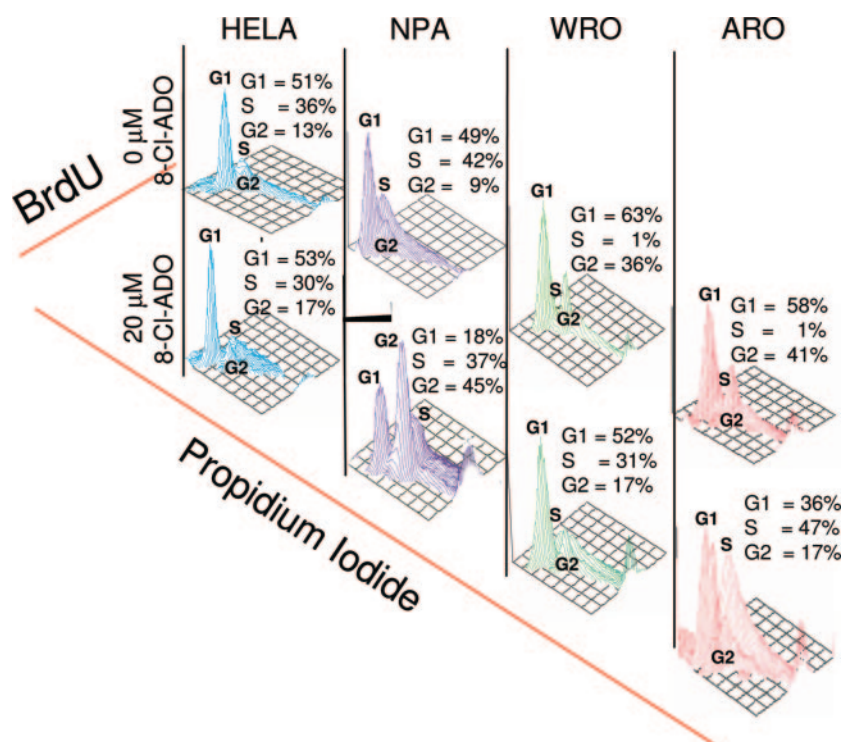
FIG. 6. 8-Cl-ADO induces the accumulation of cells in G1/S and G2/M phases of the cell cycle. Cells, synchronized (72 h) in low-serum (0.1%) media were incubated for 3 d with or without 8-Cl-ADO (20 μM) in complete medium. Cells were pulse labeled with BrdU, followed by the addition of anti-BrdU-FITC mAb and propidium iodide. Cell cycle analysis was determined by FACScalibur flow cytometry, using Cell Quest software. On the y-axis is the percentage of newly formed DNA (BrdU) content as indicated by anti-BrdU-FITC mAb in each cell cycle phase on the x-axis is propidium iodide. Three-dimensional plots are from one representative experiment.

of the IC$_{50}$ values (Fig. 3B) for both drugs as determined by the MTS assay. Both drugs caused rapid late apoptosis/necrosis (Fig. 4, A and B, *upper panels*). Early apoptosis was decreased at the higher 8-Cl-ADO and 8-Cl-cAMP concentration (30 μM). This may be accounted for by a population of cells that underwent cell death earlier in the incubation. These cells would disintegrate by the fourth day and would not be assessed by the assay. Because IBMX decreased 8-Cl-cAMP-induced antiproliferation (Fig. 3, A and D), we determined whether IBMX also reduces 8-Cl-cAMP-induced apoptosis. 8-Cl-cAMP-induced apoptosis (Fig. 4C) was reduced with IBMX, as was late apoptosis/necrosis (data not shown). These data show that both drugs induce apoptosis and confirm that 8-Cl-cAMP, acting as a pro-drug, is converted to 8-Cl-ADO to induce an inhibition of proliferation and apoptosis.

Next, we examined the involvement of the PKA cell signaling pathway in the inhibition by 8-Cl-ADO. Because 2-Cl-adenosine activates adenosine receptors that stimulate adenyl cyclase and PKA activity to affect cell proliferation in some cell lines (17, 38), the possibility exists that the inhibition by 8-Cl-ADO is due to its action as an adenosine receptor agonist, similar to 2-Cl-adenosine. However, in our studies, incubations with adenosine receptor agonists and antagonists with and without 8-Cl-ADO (Fig. 1C) showed no effect on proliferation/metabolism. The effect of 8-Cl-ADO on PKA activity (Fig. 5A) was also examined. PKA activity was altered in a differential manner by 8-Cl-ADO, with decreased PKA activity in HeLa cells and a small increase in

activity in thyroid cells, mostly at the high 8-Cl-ADO concentration (30 μM). 8-Cl-ADO did not affect basal PKA activity or PKA activity determined in the presence of the PKA inhibitor PKI. 8-Cl-ADO did differentially alter levels of PKA R-subunits (Fig. 5B). These changes, however, were contrary to those reported previously (12, 13, 39), with no reduction in the ratio of RI to RII subunits (Fig. 5C).

Known stimulants of PKA activity (Fig. 5D) at 8-Cl-ADO inhibitory concentrations showed little or no inhibition of cell metabolism, compared with that with 8-Cl-ADO. Likewise, the PKA pathway inhibitor H89 had no or only a small inhibitory effect (to 17%, only in NPA cells), whereas inhibition was greatly induced (to 70%) by the PKB/Akt pathway inhibitor, LY294002, as seen by others with 8-Cl-cAMP (40). These data, and the fact that 8-Cl-ADO has no effect on adenosine receptors (Fig. 1C) suggested that although in thyroid cells, there is a small stimulation of PKA activity at high 8-Cl-ADO concentrations and a differential effect on R-subunit levels, PKA most likely does not mediate changes in growth directly but may act indirectly by modulating the activity of factors that can affect tumor cell growth, as surmised by other investigators (31). The PKA cell signaling pathway and the activity of the PKA holoenzyme are in all probability not directly involved in the inhibition by 8-Cl-ADO. These studies point to an effect of the drugs on the PKB/Akt cell signaling pathway (40); more studies, however, are required to determine the mechanism of action of 8-Cl-ADO on signaling by PKB/Akt.

As in other studies (22, 25), our results show that 8-Cl-ADO-induced inhibition of thyroid cell metabolism is related to cell cycle arrest. Initially (Fig. 1A), we showed that the incorporation of [³H]thymidine on d 5 is increased by 8-Cl-ADO, whereas in MTS assays, the inhibition of proliferation (at the higher 8-Cl-ADO concentrations) on d 5 remains the same as on d 4 (Fig. 1B). These data suggest that cells treated with high 8-Cl-ADO concentrations retain the ability to incorporate [³H]thymidine (Fig. 1A) but are unable to divide (Fig. 1B). Indeed, cell cycle analysis of 8-Cl-ADO-treated cells shows that by 3 d of incubation, NPA cells accumulate in G2/M phase of the cell cycle, whereas WRO and ARO cells accumulate in G1/S phase (Fig. 6), indicating cell cycle arrest by the drug. Our results with NPA cells show cell cycle arrest in G2/M and confirm the findings of others (22). However, our results in all cell types are contrary to those of others who show cell accumulation in G0/G1, with no induction of apoptosis (25). These results may be due to differences in cell type, methods used, intracellular drug metabolism, and cell cycle transit time. Also, although we have shown cell accumulation in G1/S and G2/M cell cycle phases, as well as apoptosis with

8-Cl-ADO, the existing data do not determine whether the cells that accumulate in G1/S or in G2/M are the same cell population that undergoes apoptosis.

In summary, our data indicate that proliferation of HeLa and three widely used thyroid cancer cell lines can be inhibited by 8-Cl-ADO and 8-Cl-cAMP. We showed that 1) inhibition of proliferation by 8-Cl-cAMP is via the 8-Cl-cAMP metabolic breakdown product 8-Cl-ADO, which may also undergo intracellular degradation over time; 2) cells accumulate in G1/S or G2/M of the cell cycle and undergo apoptosis, suggesting that 8-Cl-ADO may cause cell cycle arrest, although HeLa cells also undergo apoptosis without an apparent phase accumulation; and 3) PKA activity and the PKA R-subunits have no direct effects on 8-Cl-cAMP or 8-Cl-ADO actions. Because we found subtle differences in the effect of 8-Cl-ADO on the four cell types tested, generalizations cannot be made for the effect of 8-Cl-ADO on all cancer cell types; thus, a thorough determination of inhibition of proliferation and mechanism of action should be established for each cancer cell type. Finally, these data may indicate that for aggressive thyroid cancer, 8-Cl-ADO may be a useful, if not powerful, drug.

Acknowledgments

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