# 8-Cl-Adenosine Inhibits Proliferation and Causes Apoptosis in B-Lymphocytes via Protein Kinase A-Dependent and Independent Effects: Implications for Treatment of Carney Complex-Associated Tumors

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**Context:** Carney complex, a multiple neoplasia syndrome, characterized primarily by spotty skin pigmentation and a variety of endocrine and other tumors, is caused by mutations in *PRKAR1A*, the gene that codes for the RI$\alpha$ subunit of protein kinase A (PKA). PKA controls cell proliferation in many cell types. The cAMP analogue 8-Cl-adenosine (8-Cl-ADO) is thought to inhibit cancer cell proliferation.

**Objective:** The objective of the study was to study the antiproliferative effects of 8-Cl-ADO on growth and proliferation in B-lymphocytes of Carney complex patients that have PKA defects and to determine whether 8-CL-ADO could be used as a therapeutic agent in the treatment of Carney complex-associated tumors.

**Design:** We used a multiparametric approach (*i.e.* growth and proliferation assays, PKA, and PKA subunit assays, cAMP and $^3$H-cAMP binding assays, and apoptosis assays) to understand the growth and proliferative effects of 8-Cl-ADO on human B-lymphocytes.

**Results:** 8-Cl-ADO inhibited proliferation, mainly through its intracellular transport and metabolism, which induced apoptosis. PKA activity, cAMP levels, and $^3$H-cAMP binding were increased or decreased, respectively, by 8-Cl-ADO, whereas PKA subunit levels were differentially affected. 8-Cl-ADO also inhibited proliferation induced by G protein-coupled receptors for isoproterenol and adenosine, as well as proliferation induced by tyrosine kinase receptors.

**Conclusions:** 8-Cl-ADO in addition to unambiguously inhibiting proliferation and inducing apoptosis in a PKA-independent manner also has PKA-dependent effects that are unmasked by a mutant *PRKAR1A*. Thus, 8-Cl-ADO could serve as a therapeutic agent in patients with Carney complex-related tumors. **(*J Clin Endocrinol Metab* 94: 4061–4069, 2009)**

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Abbreviations: 7AAD, 7-Aminoactinomycin D; ADOHcy, adenosyl-homocysteine; AICAR, 5-aminoimidazole-4-carboxamide 1-$\beta$-D-ribofuranoside; C, catalytic; 8-Cl-ADO, 8-Cl-adenosine; 8-Cl-cAMP, 8-Cl-derivative of cAMP; DIP, dipyridamole; ENT, equilibrate nucleoside transporter; *es*, equilibrate sensitive; GPCR, G protein-coupled receptor; ISO, isoproterenol; LPA, lysophosphatidic acid; mAb, monoclonal antibody; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; mt, mutant; nl, normal; p, phosphorylated; PKA, protein kinase A; PKI, protein kinase inhibitor; R, regulatory; RTK, receptor tyrosine kinase; SFM, serum-free medium.

Carney complex, a multiple neoplasia syndrome, predisposes to a variety of endocrine and other tumors (1), *e.g.* adrenal and pituitary hyperplasia and adenomas, thyroid follicular tumors and gonadal neoplasms (2–4). Carney complex is also associated with spotty skin lesions and nevi; myxomas of the skin, heart, and breast; and psammomatous and melanotic schwannomas (1, 3–5). *PRKAR1A*, a gene located on chromosome 17q22-24, codes for the regulatory (R) I$\alpha$ subunit of the cAMP-dependent protein kinase A (PKA) and is mutated in most Carney complex patients (4). Tumor-specific loss of heterozygosity, within 17q22-24 (6), suggest that RI$\alpha$ is a tumor suppressor gene. Loss of RI$\alpha$, associated with an increase in total PKA activity (7–9), results in tumors in mice (8–10). Effective treatment strategies for many tumors of Carney complex are not available.

The PKA holoenzyme is a heterotetramer consisting of two homodimers of two regulatory subunits (RI-$\alpha$ or -B and RII-$\alpha$ or -B), and two catalytic (C) subunits (C$\alpha$, C$\beta$, or C$\gamma$). PKA is activated by cAMP after the stimulation of G protein-coupled receptors (GPCR) and membrane-located adenyl cyclase. The binding of two cAMP molecules to each of the R subunits (at A and B binding sites) causes PKA activation and the release of two C subunits (11) that phosphorylate downstream targets (12).

PKA modifies the outcome of other signaling pathways (13–15). We previously showed that lysophosphatidic acid (LPA)-activated receptor tyrosine kinase (RTK) induces cell proliferation in B-lymphocytes by interacting with the MAPK cell signaling pathway. These studies also revealed that isoproterenol (ISO) or forskolin-activated PKA interacts with the ERK1/2 cascade of MAPK to increase or decrease proliferation in Carney complex patients and normal subjects by activating B-Raf through Rap-1 or activating c-raf-1, respectively (14–15).

Site-selective analogs of cAMP inhibit growth in many cancer cell types (16–21). Of these analogs, the 8-Cl-derivative of cAMP (8-Cl-cAMP) is thought to be the most potent (18). Our and various other studies in several cancer cell types (16–17, 19–22) show that 8-Cl-cAMP acts through its conversion (by serum phosphodiesterase and 5′nucleotidase) to a dephosphorylated product, 8-Cl-adenosine (8-Cl-ADO) to induce cell cycle phase accumulation and apoptosis (21, 23–25). Other studies suggest that the inhibitory effect of 8-Cl-cAMP involves its binding to RI and RII type PKA isozymes, reducing the RI to RII subunit ratio (18, 26, 27).

Adenosine is transported across the lymphocyte plasma membrane by specialized membrane nucleoside carrier proteins, termed equilibrate nucleoside transporters (ENTs), for subsequent metabolism (28, 29). Two broad types of ENTs exist in mammalian cells; equilibrate bidirectional facilitators and active transport facilitators; the former may be sensitive (*es*) or insensitive to the transport inhibitors nitrobenzylthioinosine and dipyridamole (DIP). However, high concentrations (>100 $\mu$M) of these inhibitors will inhibit both *es* and equilibrate insensitive transporters (29). Adenosine, on intracellular transport by ENT proteins, is metabolized via three main pathways (30–32), two of which lead to apoptosis. It is deaminated to an inactive inosine product (by adenosine deaminase) or phosphorylated by adenosine kinase to AMP, an activator of AMP-activated protein kinase, leading to apoptosis. Furthermore, adenosine is converted to adenosyl-homocysteine (ADOHcy), an inhibitor of S-adenosylmethionine-dependent transmethylation reactions, to inhibit DNA, RNA, and protein methylation; increase p53 levels; and stimulate apoptosis. The effects on AMP-activated protein kinase and ADOHcy can be potentiated by 5-aminoimidazole-4-carboxamide 1-$\beta$-D-ribofuranoside (AICAR) and homocysteine, respectively (33–35). ENTs thus have a profound physiological significance due to their influence on adenosine concentrations available to adenosine receptors (29–31) and for intracellular metabolism (29–35).

Because *PRKAR1A* mutations are present in B-lymphocytes of Carney complex patients (5, 6), we used transformed B lymphoblastoid cell lines from patients with Carney complex and normal subjects to study the effect of 8-CL-ADO on cell growth, proliferation, adenosine transport mechanisms, and apoptosis and the relationship between these parameters and holoenzyme PKA activity and subunit levels, cAMP levels, and cAMP binding. In each case we compared the effect on cell lines with normal (nl) or mutant *PRKAR1A* (mt) from matched subjects. Our data imply that the primary mechanism of inhibition of cell proliferation by 8-Cl-ADO is via ENT-aided intracellular transport and its subsequent metabolism and induction of apoptosis but also include a measurable effect on PKA activity. The data also show that 8-Cl-ADO inhibits cell proliferation in lymphocytes secondarily by altering the function of the PKA pathway and other RTK and GPCR-induced signaling pathways. Because we have demonstrated the effects of 8-Cl-ADO in several cancer cell types, the conclusions from this work may have applications in solid tumors of Carney complex and even in other, non-*PRKAR1A*-mutant systems.

## Materials and Methods

### Materials

HeLa, ARO, WRO, and NPA cancer cell lines were published previously from our laboratory (21). ARO and NPA cells, previously reported as thyroid in origin, have recently been reclassified as colon and melanoma cancer cells, respectively (36).

SW-13 (adrenal cortex carcinoma) and MCF-7 (breast carcinoma) cells were obtained from American Type Tissue Collection (Manassas, VA). Acquisition of remaining materials is described in supplemental methods, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org. All cancer cell lines have been tested for *PRKAR1A* mutations previously and have been found negative (data not shown).

### Lymphocyte cell lines

We previously published the method of lymphocyte cell line establishment and their *PRKAR1A* mutations (14, 15). Peripheral blood samples were collected under a research protocol approved by the Institutional Review Board of the National Institute of Child Health and Human Development, National Institutes of Health (Bethesda, MD), and written informed consent was obtained from each patient. Culture and plating of all cell lines is described in supplemental methods.

### Cell growth and proliferation assays

Cell growth and proliferation was determined by trypan blue and Cell Titer 96 AQ (Promega, Madison, WI) [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)] assays, respectively. Cells, incubated with drugs were counted by hemocytometer using trypan blue viability dye or 96-AQ solution was added (3.5 h, 37 C) before determining absorbance at 490 nm using an ELISA plate reader, respectively.

### Quantitation of apoptosis by flow cytometry

Apoptosis was assessed as reported previously (15). Briefly, cultures were treated with drugs, washed, resuspended in binding buffer, and labeled with 7-Aminoactinomycin D (7AAD)/annexin V. Thirty thousand events were analyzed by flow cytometry. Apoptotic cells were stained by annexin V. Late apoptotic/necrotic cells were stained by both annexin V-fluorescein isothiocyanate and 7AAD.

### PKA activity determinations

PKA activity was measured by modification of a previously described method (37), using [$\gamma$-$^{32}$P]triphosphate, in cultures lysed in a PKA extraction buffer [10 mM Tris (pH 7.5), 0.1 mM dithiothreitol, and 1× protease inhibitor cocktail]. Extracts, exposed to cAMP or cAMP and protein kinase inhibitor (PKI; 15 min, 37 C), were spotted onto phosphocellulose filters. Filters were washed in 0.5% phosphoric acid, air dried, and counts per minute detected in quadruplicate by liquid scintillation. Results were corrected for protein content.

### cAMP assay

cAMP levels were determined using a cAMP-GLO assay kit (Promega) according to the manufacturers' direction. Briefly, cells were lysed, incubated with cAMP GLO reagents, and luminescence measured. Details are given in supplemental methods.

### $^{3}$H-cAMP binding assay

$^{3}$H-cAMP binding was assessed by modification of a previously described method (38). Cell extracts containing $^{3}$H-cAMP were processed and counted for counts per minute as described in detail in supplemental methods.

### Gel electrophoresis

Whole-cell lysates were separated by SDS-PAGE as previously described (14). Proteins, on nitrocellulose membranes, were probed with primary monoclonal antibodies (mAbs), followed by secondary horseradish peroxidase-labeled antibodies against mouse or rabbit IgG. Bands were detected by enhanced chemiluminescence reagent and quantitated by densitometer scanning. Equal sample loading was confirmed when blots were reprobed with $\beta$-actin mAb.

### Statistics

Results were analyzed by ANOVA using the PROC Mixed procedure of the Statistical Analysis System as previously described (21). Differences were considered significant at $P < 0.05$. When data were significantly different and sampling done on more than 3 d or with more than three drug concentrations, polynomial response curves of appropriate order were tested for heterogeneity of regression (39, 40) to evaluate treatment effects.

## Results

### 8-CL-ADO inhibits cell growth and proliferation in B-lymphocytes

B-lymphocytes were cultured with 0–30 $\mu$M 8-Cl-ADO for 2–5 d (Fig. 1, A and C), and 4 d (Fig. 1B). 8-Cl-ADO

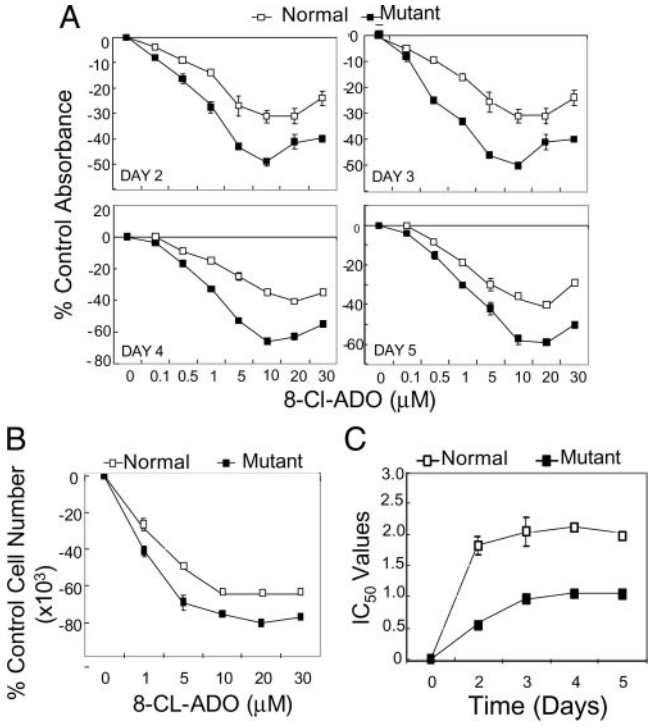


**FIG. 1.** 8-Cl-ADO inhibits cell growth and proliferation in B-lymphocytes. A, 8-Cl-ADO inhibits cell proliferation. Cells were incubated with 0–30 $\mu$M 8-Cl-ADO for 2–5 d and MTS assays performed. One of six experiments is shown. B, 8-CL-ADO inhibits cell growth. Cells were incubated (4 d) with 0–30 $\mu$M 8-Cl-ADO and counted by trypan blue exclusion. C, $IC_{50}$ values for 8-Cl-ADO (from A). Results in A and B were calculated as percent of control absorbance or cell number, respectively (no drugs). Points are mean ± SEM; four to five wells; $P < 0.0001$ (A); mean ± SEM of three experiments; $P < 0.05$ (B); four experiments; $P < 0.0001$ (C); n = 6 nl or mt cell lines.

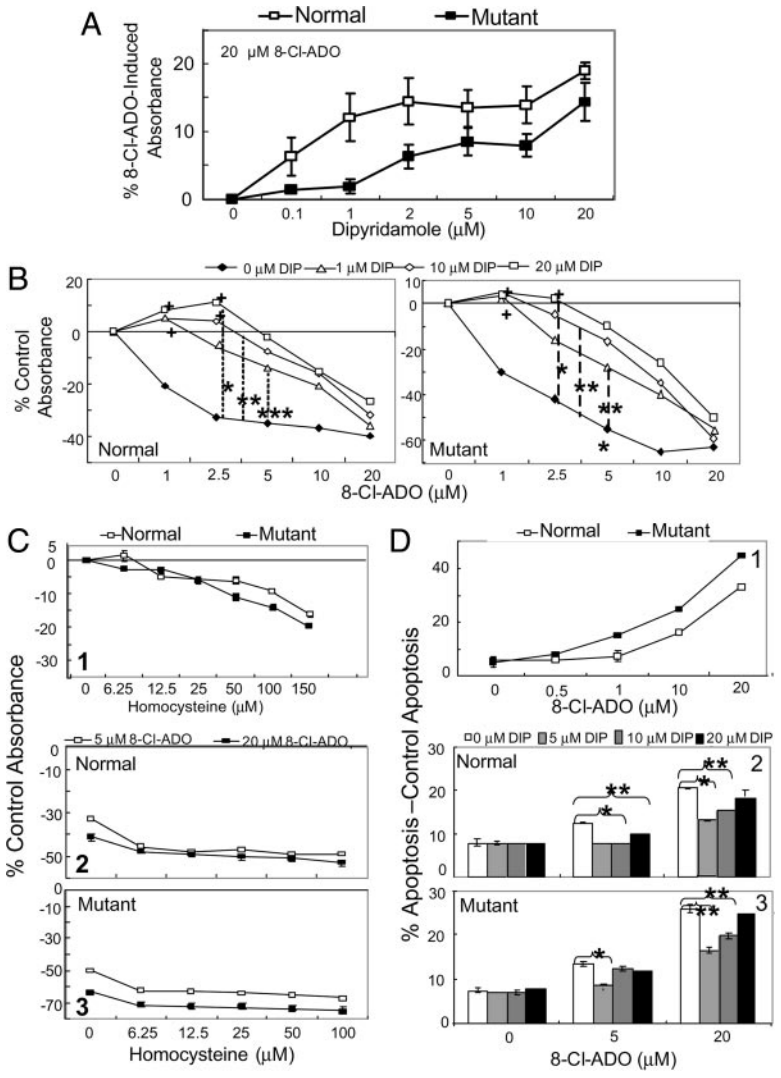


**FIG. 2.** Adenosine nucleoside transporter (ENT) proteins transport 8-Cl-ADO to induce apoptosis. A, DIP reverses 8-Cl-ADO-induced inhibition of proliferation. Cells were incubated with 20 $\mu$M 8-Cl-ADO and increasing DIP concentrations (0–20 $\mu$M). B, Cells were incubated with 8-Cl-ADO alone (♦) or 8-Cl-ADO plus 1 $\mu$M DIP (△), 10 $\mu$M DIP (◇), and 20 $\mu$M DIP (□). C, Homocysteine alone inhibits cell proliferation. The inhibition induced by 8-Cl-ADO is increased by homocysteine. Cells were incubated with homocysteine alone (*panel 1*), homocysteine plus 5 $\mu$M 8-CL-ADO (*panel 2*), or plus 20 $\mu$M 8-CL-ADO (*panel 3*). D, DIP reverses 8-Cl-ADO-induced apoptosis. Cells were incubated with 8-Cl-ADO alone (*panel 1*) or 8-Cl-ADO plus 5, 10, and 20 $\mu$M DIP (*panels 2* and *3*). Cell proliferation and apoptosis were assessed by MTS assay (A–C) and annexin V/7AAD staining (D), respectively. All incubations were for 4 d. Results are representative of two experiments (A and B); three experiments (D); and mean ± SEM of three experiments (C). Results in A are expressed as the percent of the absorbance induced in the presence of 8-Cl-ADO alone (percent 8-CL-ADO induced absorbance) and in B and C as the percent of control absorbance (no drugs). + equals concentration of 8-Cl-ADO and DIP that result in complete reversal of inhibition (B). $P < 0.01$ (A); *, $P < 0.0001$, **, $P < 0.001$, ***, $P < 0.05$ (B); *, $P < 0.01$, **, $P < 0.05$ (D) (n = 4 nl and 4 mt cell lines).

inhibited growth and proliferation in a time- and concentration-dependent manner. Maximal inhibition occurred at d 4 (Fig. 1A) and was greater in cells bearing mt-*PRKAR1A* (mt) *vs.* those with nl genotypes ($P < 0.0001$). $IC_{50}$ values are given (Fig. 1C and supplemental Table S1). No significant inhibition of proliferation was observed on or before d 1 (data not shown). The fourth day time point was used in all subsequent experiments.

## B-lymphocytes transport 8-Cl-ADO by facilitated diffusion to inhibit cell proliferation and induce apoptosis

Because adenosine is transported by facilitated diffusion in lymphocytes via ENT proteins, we determined whether 8-Cl-ADO is transported by facilitated diffusion and metabolized to induce apoptosis in lymphocytes. Cells were incubated with a high 8-Cl-ADO concentration (20 $\mu$M) plus increasing concentrations of the adenosine ENT protein inhibitor, DIP (Fig. 2A). DIP reversed the inhibition by 8-Cl-ADO to a small extent in both cell types (19 and 14%, respectively). However, when cells were incubated with increasing 8-CL-ADO concentrations alone or plus increasing DIP concentrations (0, 1, 10, and 20 $\mu$M, Fig. 2B), a greater reversal of the inhibition by 8-Cl-ADO occurred, with complete reversal mainly at lower 8-Cl-ADO concentrations (see + sign, Fig. 2B). As in Fig. 2A, the reversal was greater in nl cells. These data suggest a competition by 8-Cl-ADO and DIP for ENT proteins and an effect of the mt-*PRKAR1A* on the reversal by DIP.

To determine whether the inhibition by 8-Cl-ADO is affected by stimulants of adenosine metabolism, cells were incubated with AICAR or with homocysteine alone; or with these drugs plus 8-Cl-ADO (AICAR data not shown and Fig. 2C). AICAR alone (to 250 $\mu$M) had no effect on proliferation, and AICAR did not alter the inhibition by 0–20 $\mu$M 8-Cl-ADO (data not shown). Homocysteine alone, however, inhibited proliferation to 20% (Fig. 2C, *panel 1*) with no significant difference between nl and mt cells. Homocysteine increased the inhibition induced by 5 and 20 $\mu$M 8-Cl-ADO to a further 23 and 34% (nl) and 22 and 32% (mt), respectively (*panels 2* and *3*). Inhibition by 8-Cl-ADO plus homocysteine was greater in mt cells. The data suggest that ADOHcy is involved in the inhibition of proliferation.

Because metabolism of ENT-transported adenosine leads to apoptosis in lymphocytes, we determined whether 8-Cl-ADO induces apoptosis in lymphocytes and whether this apoptosis is reversed by DIP. Cells were incubated with increasing concentrations of 8-Cl-ADO alone (Fig. 2D, *panel 1*) or with 8-CL-ADO plus 0 $\mu$M to 20 $\mu$M DIP (*panels 2* and *3*). Apoptosis was induced with 8-Cl-ADO alone, with greater apoptosis ($P < 0.03$) in mt cells, which led to significant levels of necrosis (dot plots, supplemental Fig. S1). Apoptotic values, however, excluded cells that had disintegrated during earlier incubation times. Apoptosis induced by 8-Cl-ADO was relieved by DIP, with a greater effect in mt cells (to 28%; *, $P < 0.01$, **, $P < 0.05$). As in Fig. 2, A and B, 8-Cl-ADO-induced apoptosis was reversed by DIP, with greater reversal in nl cells (to 100%, *, $P < 0.01$, **, $P < 0.05$). These data suggest that 8-Cl-ADO is transported into cells by ENT proteins to stimulate ADOHcy and induce apoptosis and that mt-*PRKAR1A* may interfere with the action of DIP.

## Other cancer cell types transport 8-CL-ADO by facilitated diffusion

We previously showed (21) that 8-Cl-ADO inhibits cell proliferation in HeLa, NPA, ARO, and WRO cells (see $IC_{50}$ values, supplemental Table S1). To determine whether these cells and other cancer cells transport 8-CL-ADO, we obtained $IC_{50}$ values for 8-Cl-ADO in MCF-7 and SW-13 cells (data not shown and supplemental Fig. S2), using MTS assays. $IC_{50}$ values (supplemental Table S1) indicate that 8-Cl-ADO inhibits cell proliferation at comparable levels in a variety of cell types. When cells were incubated with DIP alone, no effect on proliferation occurred. However, when cells were incubated with DIP (to 20 $\mu$M) plus 10 and 20 $\mu$M 8-Cl-ADO (Fig. 3), a complete reversal of the inhibition occurred in all cell types (except in SW-13), with the greatest reversal in NPA cells. The reversal was greater at a lower 8-Cl-ADO concentration (10 $\mu$M; $P < 0.05$–$0.0001$). Together the data suggest a competition for ENT proteins by 8-Cl-ADO and DIP in other cell types.

## 8-Cl-ADO alters PKA activity, PKA subunit levels, cAMP levels, and $^3$H-cAMP binding in B-lymphocytes

Basal PKA activity was higher in mt cells (Fig. 4A, $P < 0.01$) and was increased by 8-Cl-ADO (Fig. 4, *panel 1*). After exposure to cAMP (Fig. 4, *panel 2*) or cAMP plus the PKA-specific inhibitor, PKI (Fig. 4, *panel 3*), PKA activity was increased by 44 and 48% (nl and mt cells, respectively) or decreased (95–98%), respectively. 8-Cl-ADO altered PKA subunit levels, with a reduction in RI$\alpha$, a small decrease in RII$\beta$, and a large increase in RII$\alpha$ and C$\alpha$ (Fig. 4B) as well as a reduction in the ratio of RI/RII (supplemental Fig. S3). cAMP levels were also assayed in extracts of cells grown in media with and without 10% serum [serum-free medium (SFM)] plus 8-Cl-ADO (Fig. 4C). All extracts showed increased cAMP levels (*, $P < 0.0001$; **, $P < 0.01$), but the increase was greatly enhanced in SFM, possibly due to a lack of cAMP breakdown by serum phosphodiesterase and 5′ nucleotidase. 8-Cl-ADO inhibited $^3$H-cAMP binding (Fig. 4D) but only when cells were previously incubated (4 d) with 8-Cl-ADO and not when extracts were simultaneously incubated with 8-Cl-ADO and $^3$H-cAMP (data not shown). The data suggest that 8-Cl-ADO affects PKA activity via a change in levels of PKA subunits, cAMP, and $^3$H-cAMP binding.

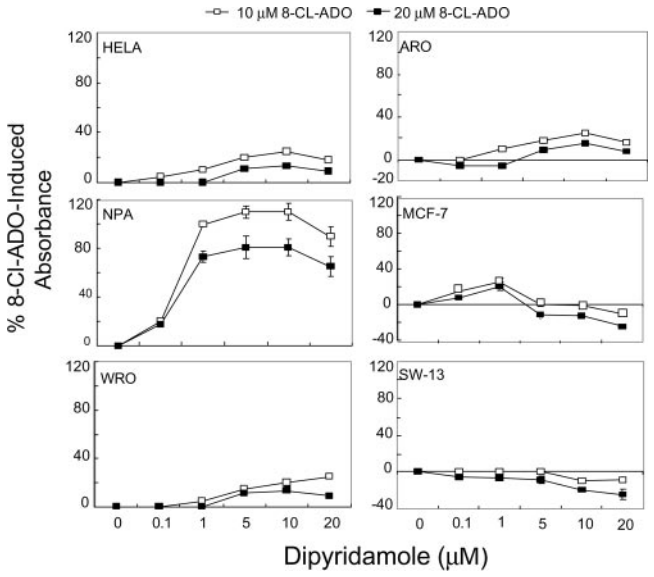


**FIG. 3.** Adenosine nucleoside transporter (ENT) proteins transport 8-Cl-ADO to inhibit proliferation in other cancer cell types. Cells were incubated (4 d) with increasing DIP concentrations and 10 or 20 $\mu$M 8-Cl-ADO and MTS assays performed. Results are expressed as the percent of 8-Cl-ADO-induced absorbance (no DIP) and are mean ± SEM of three experiments (n = 4 nl or 4 mt cell lines).

## 8-Cl-ADO decreases ISO-induced cell proliferation and phosphorylated (p) ERK1/2 in B-lymphocytes

When cell proliferation was stimulated (mt) or inhibited (nl) with the GPCR agonist ISO, 8-Cl-ADO (20 $\mu$M) decreased or had no significant effect, respectively, on these responses (Fig. 5A). Likewise, 8-Cl-ADO (to 30 $\mu$M) inhibited RTK-induced proliferation as stimulated by LPA (data not shown). 8-Cl-ADO also inhibited levels of pERK1/2 (Fig. 5B). 8-Cl-ADO, however, had a small and nonspecific inhibitory effect on A1, A2a, and A2b adenosine receptors (supplemental Fig. S4, A and B) but no effect on A3 receptors (data not shown). Together, these data indicate that 8-Cl-ADO inhibits proliferation induced by different GPCRs and proliferation induced by other receptor types (*e.g.* RTK).

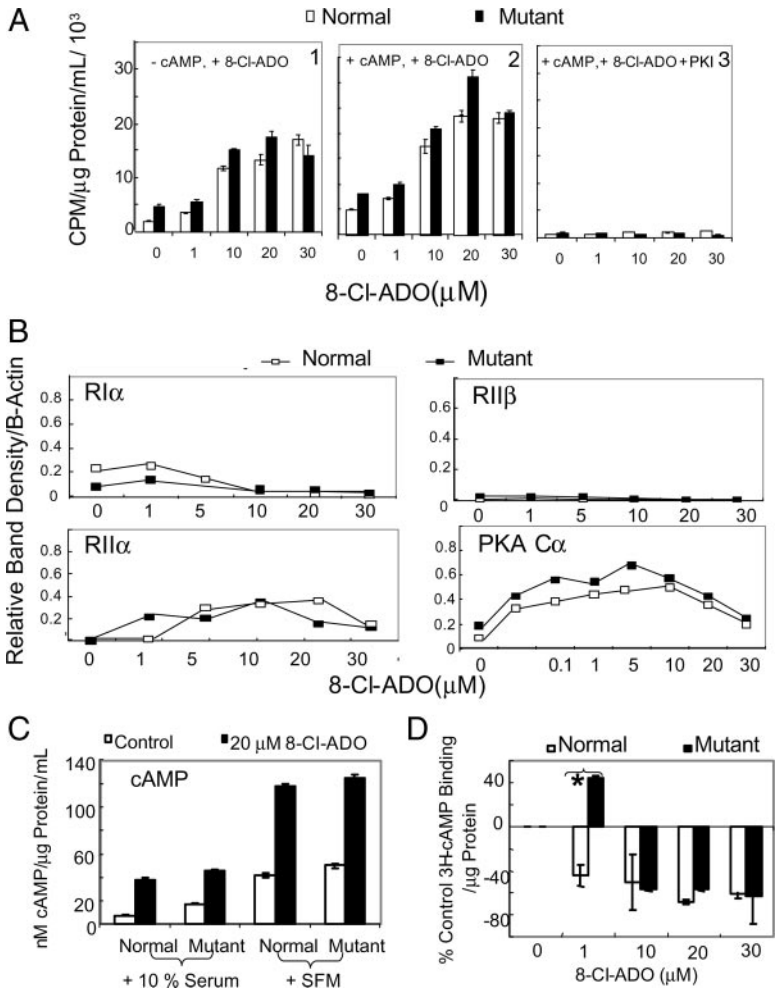


**FIG. 4.** 8-Cl-ADO alters PKA activity, PKA subunit levels, and cAMP and $^3$H-cAMP binding in B-lymphocytes. A, Cells were incubated with 8-Cl-ADO. Cell extracts were exposed to cAMP (*panel 2*) or cAMP plus PKI (*panel 3*). PKA activity was determined. B, 8-Cl-ADO alters PKA subunit levels. Cells were incubated with 8-Cl-ADO; lysed; and immunoblot assays performed using RI$\alpha$, RII$\alpha$, RII$\beta$, and C$\alpha$ PKA subunit mAbs. C, 8-CL-ADO stimulates cAMP. Cells were incubated with 8-Cl-ADO in media containing 10% fetal bovine serum or SFM and cAMP assayed using a cAMP-GLO kit. D, 8-Cl-ADO inhibits $^3$H-cAMP binding. Cells were incubated with 8-Cl-ADO. Cell supernatants, exposed to $^3$H-cAMP, were counted for counts per minute in triplicate by liquid scintillation. All incubations in intact cells were for 4 d. Results are representative of three experiments each (A–D). Points are mean ± SEM of quadruplicate samples (A) [n = 6 nl or mt cell lines (A and B) and 3 nl or mt cell lines (C and D)]. *, $P <$ 0.0001; **, $P < 0.01$ (C); *, $P < 0.0001$ (D).

## Discussion

In the present investigation, we determined whether 8-Cl-ADO inhibits cell proliferation in human Epstein-Barr virus-transformed B-lymphocytes from Carney complex patients and normal subjects and whether the PKA cell signaling pathway is directly involved in this inhibition. We also explored other mechanisms of the inhibition: we showed that ENT proteins transport 8-Cl-ADO into the cell by facilitated diffusion to induce apoptosis and affects GPCR- and RTK receptor-induced proliferation. Although PKA is increased by 8-Cl-ADO, it is apparent that this effect may be overlooked because other direct effects of 8-Cl-ADO and its metabolism are more significant in terms of inhibition of proliferation and induction of apoptosis.

We previously showed (14) that basal growth rates in mt lymphocytes surpassed that of nl cells by 2- to 3-fold. In this study, to eliminate differences in basal growth, where necessary, results were calculated as percent of control. The data show that 8-Cl-ADO inhibits proliferation in a time- and concentration-dependent manner, and inhibition was 2-fold greater in mt cells (Fig. 1). $IC_{50}$ values (Fig 1C and supplemental Table S1) confirmed these data. The level of inhibition was comparable with that in other cell types (21).

Because adenosine, a structural analog of 8-Cl-ADO, is transported across the lymphocyte plasma membrane by ENT proteins and metabolized intracellularly to induce apoptosis (28, 29, 33), we explored the possibility that 8-Cl-ADO is also transported into and metabolized in lymphocytes to induce apoptosis. Experiments with the ENT protein inhibitor DIP and a high concentration of 8-Cl-ADO (20 $\mu$M) showed a modest reversal of the inhibition by 8-Cl-ADO (Fig. 2A). This effect was enhanced with increasing 8-Cl-ADO and DIP concentrations (Fig. 2B). At lower 8-Cl-ADO concentrations, the inhibition was completely reversed (see + sign, Fig. 2B), indicating a competition between 8-Cl-ADO and DIP for the ENT protein and suggesting that 8-Cl-ADO is transported into lymphocytes by facilitated diffusion.

We determined the effect of 8-CL-ADO on components of the adenosine metabolic pathway. AICAR, alone or with 8-Cl-ADO (data not shown), did not effect cell proliferation. However, homocysteine alone inhibited proliferation (Fig. 2C, *panel 1*), and inhibition by 8-Cl-ADO was increased when homocysteine was present (Fig. 2C, *panels 2* and *3*). Our data also show that 8-Cl-ADO may increase levels of p53, another component of adenosine metabolism (supplemental Fig. S5). These data suggest that ADOHcy is involved in the inhibition.

Because adenosine metabolism results in apoptosis (28, 29, 33), we determined whether 8-Cl-ADO induces apoptosis in lymphocytes and whether this apoptosis is reversed by

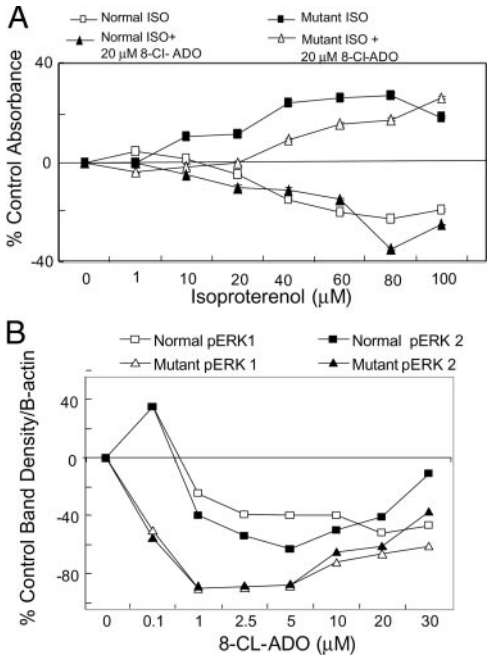


**FIG. 5.** 8-Cl-ADO inhibits ISO-induced proliferation and pERK1/2 in B-lymphocytes. A, 8-Cl-ADO inhibits ISO-induced alteration of cell proliferation. Cells were incubated with ISO (4 d), with and without 8-Cl-ADO and proliferation determined by MTS assays. B, 8-Cl-ADO inhibits pERK1/2. Cells were incubated with 8-Cl-ADO (4 d), lysed, and immunoblot assays performed. Results are mean ± SEM of three experiments (A) and one representative experiment (B) (n = 3 nl or mt cell lines).

DIP. Apoptosis was induced by 8-Cl-ADO, was greater in mt cells (Fig. 2D, *panel 1*; $P < 0.03$), and was reversed by DIP (Fig. 2D, *panels 2* and *3*; *, $P < 0.05$ to **, $P < 0.01$). As in proliferation studies (Fig. 2, A and B), the effect was dependent on concentrations of both drugs and indicated a competition by 8-Cl-ADO and DIP for the ENT protein. Reversal of 8-Cl-ADO-induced inhibition and apoptosis by DIP (Fig. 2, A, B, and D) was less apparent in mt cells and may indicate an alteration or decrease in ENT proteins or a decreased response to DIP by mt cells, reflecting an effect of mt-*PRKAR1A* on ENT activity. Together, these data suggest that 8-Cl-ADO is transported in and metabolized to ADOHcy to induce apoptosis in lymphocytes.

We next examined PKA's involvement in the inhibition by 8-Cl-ADO. 8-Cl-ADO increased basal and cAMP-induced PKA activity (Fig. 4A) and altered PKA subunit levels (Fig. 4B), with an overall reduction in the RI/RII subunit ratio (supplemental Fig. S3), whereas C$\alpha$ levels were 2-fold that of RII. These changes have been reported by others for 8-Cl-cAMP (18, 26, 27).

We determined the manner in which 8-Cl-ADO increases PKA activity: cell extracts, previously incubated (4 d) with 8-Cl-ADO, showed increased cAMP levels (Fig. 4C). This increase was more evident in the absence of serum in the medium, suggesting an involvement of phosphodiesterase in the maintenance of cAMP levels. 8-Cl-ADO also inhibited $^3$H-cAMP binding (Fig. 4D) but did not effect binding when the 4-d incubation period was bypassed and extracts were incubated (50 min) simultaneously with 8-Cl-ADO and $^3$H-cAMP (data not shown). These data imply that inhibition of $^3$H-cAMP binding by 8-Cl-ADO requires preexposure to 8-Cl-ADO and suggests a time-required down-regulation of cAMP binding.

Alteration of PKA activity (as suggested for 8-Cl-cAMP) (18, 26, 27) may be a result of 8-Cl-ADO's ability to change the site selectivity of cAMP toward R-subunits. We show that 8-Cl-ADO increases PKA activity and levels of RII$\alpha$ and C$\alpha$ but decreases RI$\alpha$ and RII$\beta$, resulting in a decreased ratio of RI/RII (Fig. 4 and supplemental Fig. S3). These data suggest (supplemental Fig. S6) that 8-Cl-ADO competes with cAMP and preferentially binds to RII$\alpha$, causing degradation of RI$\alpha$, decreases in $^3$H-cAMP binding, increase in free cAMP, and stimulation of PKA activity. Because R- and C-subunits are maintained in a 1:1 ratio in the cell, RII$\alpha$ may recruit C$\alpha$ from the degraded RI$\alpha$ resulting in an increase in C$\alpha$. RII$\alpha$ may stimulate nuclear cAMP response element for the production of more RII$\alpha$ (18), increasing RII$\alpha$ subunits within the cell. However, the ability of C$\alpha$ to

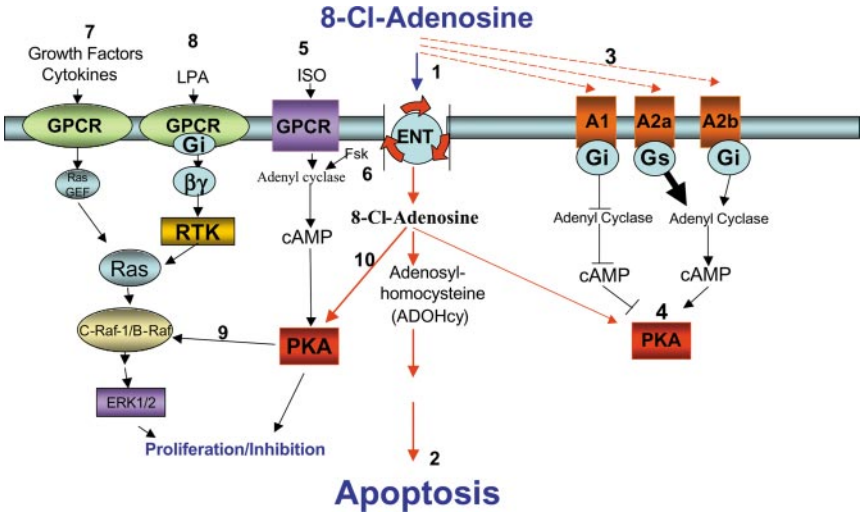


**FIG. 6.** Mechanism of the inhibition by 8-Cl-ADO in B-lymphocytes. 1, 8-Cl-ADO is transported intracellularly by an *es*-type ENT protein and metabolized to ADOHcy to (2) induce apoptosis. 3), Extracellular 8-Cl-ADO alters the action of A1, A2a, and A2b adenosine receptors that (4) activate or inhibit PKA. 5), PKA is stimulated extracellularly at GPCRs by ISO or intracellularly at (6) adenyl cyclase by forskolin (Fsk). 7), Growth factors or cytokines activate GPCRs to stimulate the ERK1/2 cascade of the MAPK signaling pathway. 8) LPA stimulates GPCRs that interact with RTK that activate ERK1/2. 9) PKA interacts with c-Raf-1 or B-Raf of the ERK1/2 MAPK pathway to stimulate or inhibit cell proliferation. 10, 8-Cl-ADO alters the activity of PKA.

induce proliferation may be counteracted by the ability of 8-Cl-ADO to induce cell cycle arrest and apoptosis (21).

8-Cl-ADO transport also occurs in other cell types, seen by proliferation studies in HeLa, NPA, WRO, and ARO cell lines. Proliferation was inhibited at $IC_{50}$ values similar to those reported previously (21) (supplemental Table S1). DIP completely reversed inhibition in all cell types tested except SW-13, in which inhibition was partially decreased (see Fig. 3 and supplemental Fig. S2). SW-13 cells may be only partly sensitive to DIP at these concentrations. Because ENT protein inhibitors can show biphasic effects, higher concentrations of DIP (>100 $\mu$M) or other transport inhibitors (*e.g.* dilazep or nitrobenzylthioinosine) (29) should be tested in these cells. 8-Cl-ADO transport by lymphocytes and other cancer cells may indicate a general mechanism of action for the effect of 8-Cl-ADO on proliferation.

8-Cl-ADO may also affect GPCR- and RTK receptor-induced proliferation. GPCRs may be affected extracellularly (adenosine receptors; see supplemental Fig. S4B) and intracellularly (ISO, Fig. 5) at the level of PKA to alter MAPK, whereas RTK-induced proliferation may be affected intracellularly at the level of MAPK/PKA (14, 15). The possibility exists that differences in the degree of inhibition in lymphocytes may be due to differences in the function of nl- and mt-*PRKAR1A*.

In conclusion, we present a hypothesis (Fig. 6) for the mechanism of action of 8-Cl-ADO on cell proliferation in lymphocytes, based on this work, our former studies (21), and those of others (16–19, 26, 27). The inhibition by 8-CL-ADO may occur due to: 1) stimulation of an *es* type ENT protein that transports 8-Cl-ADO intracellularly for metabolism to ADOHcy to induce apoptosis; 2), intracellular 8-Cl-ADO may stimulate holoenzyme PKA activity. However, the ability of transported 8-Cl-ADO to inhibit proliferation via an effect on PKA activity would depend on the cell's ability to use the PKA signaling pathway to induce proliferation (21); 3) PKA activity may be increased by 8-Cl-ADO's ability to selectively bind to R-type PKA subunits; 4) 8-Cl-ADO may inhibit GPCR- and RTK-induced proliferation; and 5) ENT proteins may transport 8-Cl-ADO in other cell types to inhibit proliferation.

Finally, because the effects of 8-Cl-ADO on mt *vs.* nl lymphocytes were not diametrically opposed and varied only by degree, these differences may reflect the activity of mt-*PRKAR1A* (14, 15). After establishing pharmacokinetics & pharmacodynamics, 8-Cl-ADO may be a potent therapeutic tool for use in the treatment of tumors of Carney complex.

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