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The Antiproliferative Effect of 8-Chloro-Adenosine, an Active Metabolite of 8-Chloro-Cyclic Adenosine Monophosphate, and Disturbances in Nucleic Acid Synthesis and Cell Cycle Kinetics

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ABSTRACT. 8-Chloro-adenosine, the dephosphorylated metabolite of the antineoplastic agent 8-chloro-cyclic AMP, has been proposed to act on the regulatory subunits of cyclic AMP-dependent protein kinase. 8-Chloro-adenosine has a growth-inhibitory effect, the mechanism of which is unclear. We investigated the effects of 8-chloro-cyclic AMP and 8-chloro-adenosine on nucleic acid synthesis and cell cycle kinetics in two human glioma cell lines. These effects were compared to those of the cyclic AMP analogue 8-(4-chlorophenyl)-thio-cyclic AMP (8-CPTcAMP), which is less susceptible to dephosphorylation. Whereas 8-CPTcAMP almost completely inhibited RNA and DNA synthesis, both 8-chloro-adenosine and 8-chloro-cyclic AMP only partly inhibited synthesis of RNA and DNA at growth-inhibitory concentrations, as demonstrated by using [5-^{3}H]-uridine and [^{14}C]thymidine incorporation. Therefore, the growth-inhibitory effect of 8-chloro-cyclic AMP is not (or not completely) due to activation of cyclic AMP-dependent protein kinase nor to the inhibition of nucleic acid synthesis. Flow cytometric analysis revealed that 8-chloro-cyclic AMP and 8-chloro-adenosine probably block cell cycle progression at the G_2M phase. The effects of 8-chloro-cyclic AMP on nucleic acid synthesis and cell cycle progression were largely prevented by adenosine deaminase, which inactivates 8-chloro-adenosine. This indicates that the effects of 8-chloro-cyclic AMP were at least in part due to its metabolite 8-chloro-adenosine. Incorporation of 8-chloro-adenosine into RNA and DNA might contribute to the disturbance of the cell cycle kinetics and growth-inhibitory effect of 8-chloro-adenosine. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;2: 141–148, 1997.

KEY WORDS. 8-chloro-cyclic AMP; 8-chloro-adenosine; human glioma cells; DNA incorporation; RNA incorporation; cell cycle kinetics

Cyclic AMP is considered to play an important role in cell growth and differentiation [1]. The actions of cAMP are mediated by two isozymes (type I and type II) of PKA. It has been stated that type I PKA is a positive effector of cell proliferation [2, 3]. Conversely, a decrease in the levels of type I PKA and/or an increase in type II PKA levels seems to correlate with differentiation and growth inhibition [4]. Analogues of cAMP that activate PKA are commonly used to induce growth inhibition and differentiation *in vitro* [5–8]. 8ClcAMP is an analogue that has received attention in recent years as a potential antineoplastic agent [9–12]. This compound displays a growth-inhibitory action in a broad range of cancer cells, including human glioma cells, at lower concentrations than other cAMP analogues [13, 14].

An interesting mechanism of action has been proposed to explain why 8ClcAMP is a more potent growth inhibitor than other cAMP analogues [15, 16]. In the inactive state, PKA consists of a complex containing two R and two C subunits. Type I and type II PKA differ in their R subunits, each of which contains two distinct binding sites for cAMP [17]. Binding of cAMP or its analogues to the R subunits results in the release and activation of the C subunits. It has been reported that 8ClcAMP acts as a site-selective cAMP analogue that selectively binds to site B on RII [18]. Incu-

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Abbreviations: ADA, adenosine deaminase; cAMP, cyclic AMP; C, catalytic; 8ClcAMP, 8-chloro-cyclic AMP; 8Cl-adenosine, 8-chloro-adenosine; 8CPTcAMP, 8-(4-chloro-phenyl)-thio-cyclic AMP; PBS, phosphate-buffered saline; PKA, cyclic AMP-dependent protein kinase; R, regulatory; RII, type II R subunit; SRB, sulforhodamine B; TCA, trichloroacetic acid.

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bation with 8ClcAMP (or other cAMP analogues) leads to a decrease in the RI level and an increase in the RII/RI subunit ratio in cancer cells [19, 20]. This increase in the RII/RI subunit ratio may be related to the growth-inhibitory effect of the site-selective analogues, with 8ClcAMP displaying the largest increase in RII/RI ratios [4, 9].

Results from other studies suggest that 8ClcAMP does not exert its growth-inhibitory effect through PKA activation [21, 22]. 8ClcAMP can be converted into 8Cl-adenosine in culture medium containing fetal bovine serum in a reaction catalyzed by phosphodiesterases and 5′-nucleotidase(s) [23]. The level of 8Cl-adenosine depends on the activity of two intracellular enzymes: ADA converts 8Cl-adenosine to 8-chloro-inosine, and adenosine kinase phosphorylates 8Cl-adenosine to form 5′-8-chloro-AMP. Adenosine kinase having a lower K_m (2 μM) than ADA (17 μM), intracellularly 8Cl-adenosine will be phosphorylated rather than deaminated [24]. Because 8Cl-inosine has no effect on cell proliferation [22], addition of extracellular ADA inactivates 8Cl-adenosine, making it possible to discriminate between the direct effects of 8ClcAMP and the effects of its metabolite 8Cl-adenosine. In this way, it has been shown that 8Cl-adenosine largely accounts for the growth-inhibitory effect of 8ClcAMP on Chinese hamster ovary cells, Molt-4 lymphoblasts [21], colon cancer cells [25], and human glioma cells [22]. The growth-inhibitory effect of 8Cl-adenosine does not seem to be mediated by classical adenosine A_1- or A_2-receptors [22]. Rather, 8Cl-adenosine exerts its effect intracellularly [20, 21], by a mechanism that is not completely understood. It has been shown that 8Cl-adenosine induces the increase in the RII/RI subunit ratio, an effect previously ascribed to 8ClcAMP [20]. However, this does not necessarily imply that the growth-inhibitory effect of 8Cl-adenosine is due to a shift in type I and type II PKA levels. More insight into the mechanism of action of this purine analogue might prove valuable in the development of new antineoplastic drugs or new therapeutic approaches, even more so now that 8ClcAMP has entered clinical phase I studies.

In an attempt to gain more insight into the mechanism of action of 8Cl-adenosine and 8ClcAMP, we investigated the effects of 8ClcAMP and 8Cl-adenosine on RNA synthesis and DNA synthesis. To examine the role of PKA activation, we compared the effects of 8ClcAMP on cell growth to those of 8CPTcAMP, a cAMP analogue that is much more resistant to hydrolysis by phosphodiesterases than 8ClcAMP [26]. In addition, we investigated the effects of 8ClcAMP, 8Cl-adenosine, and 8CPTcAMP on cell cycle kinetics. Finally, radiolabeled 8Cl-adenosine was used to determine the incorporation of 8Cl-adenosine into RNA and/or DNA, where it might exert its growth-inhibitory effect on human glioma cells.

MATERIALS AND METHODS

Chemicals

8CPTcAMP was obtained from Boehringer Mannheim (Almere, The Netherlands). 8ClcAMP was kindly provided by Dr. Cho-Chung (NCI, Bethesda, MD, U.S.A.). 8Cl-Adenosine was obtained from Biolog Life Science Institute (Bremen, F.R.G.). Tissue culture media and supplements were from Gibco Netherlands BV (Breda, The Netherlands). [5-^{3}H]Uridine (28 Ci/mmol) and [2-^{14}C]thymidine (59.3 mCi/mmol) were from Radiochemical Centre (Amersham, U.K.). [2-^{3}H]8Cl-Adenosine (17 Ci/mmol) was from Moravek Biochemicals, Inc. (Brea, CA, U.S.A.). All other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.).

Cell Culture

The human glioma cell lines WF and D384 have been described previously [27, 28]. They were routinely grown as monolayer cultures (population doubling times: WF, 56 hr; D384, 35 hr) in medium consisting of Ham's F10/Dulbecco's modified Eagle's medium in a 1:1 mixture, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, nonessential amino acids, penicillin (100 IU/mL), and streptomycin (50 μg/mL). Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Maintenance cultures were subcultured weekly, and the culture medium was changed twice a week.

Measurement of RNA and DNA Synthesis

The assays were performed in 96-well filtration plates coated with a hydrophilic PVDF filter with a 0.22-μm pore size (Multiscreen Assay System, Millipore, Bedford, MA, U.S.A.). Three hours after plating of the cells (6×10^4/well, 100 μL/well), drugs were added (final volume 200 μL/well). Cells were incubated for 24 hr, and [2-^{14}C]thymidine (final concentration 2.4 nM, specific activity 59.3 mCi/mmol) or [5-^{3}H]uridine (final concentration 0.9 μM, specific activity 872 mCi/mmol) was present during the final 4 hr of the incubation. Following the incubation, DNA and RNA were precipitated with 8% TCA, and filters were harvested using a Millipore vacuum holder [29, 30]. Incorporation of thymidine and uridine was measured under conditions described previously [31]. Incorporation of the radioisotopes was expressed as pmol/10^6, cells and the drug effects were calculated as a percentage of the incorporation in control wells.

Incorporation of [2-^{3}H]8Cl-adenosine

Glioma cells were cultured in six-well culture plates, and [2-^{3}H]8Cl-adenosine was added at subconfluency in a final concentration of 30 μM (specific activity 42 mCi/mmol). Incorporation of the radioisotope was determined as described previously [29]. In short, following an incubation period of 24 hr, the culture medium was discarded and the

cells trypsinized. Harvested cells were washed with PBS and counted in a hemocytometer. Subsequently, nucleotides were extracted with ice-cold 0.4 M perchloric acid for 15 min. The extract was centrifuged to precipitate DNA and RNA, and the pellet was washed three times with PBS prior to incubation with RNAse A (15 μg in 100 μL Tris-HCl, containing 1 mM EDTA at pH 7.4). After an incubation period of 15 min at room temperature, ribonucleotides were extracted by addition of 30 μL perchloric acid (5 M). The samples were centrifuged to separate RNA (in the supernatant) from DNA (in the pellet). The pellet was washed three times, and DNA was solubilized in Soluene. Radioactivity was measured as described [29]. Incorporation was expressed as pmol/10^6 cells per 24 hr.

Sulforhodamine B (SRB) Assay

Determinations of cell number and proliferation rates were based on a protein-staining method (SRB assay) described previously [32, 33]. For this purpose, cells were inoculated in 96-well culture plates on day 0 (2500 cells/well, 100 μL/well). On day 1, 100 μL of culture medium containing drugs was added to the wells; control wells received standard culture medium. Culture plates were then incubated 1–6 days prior to fixation. Cells were fixed with 50% TCA at 4°C (25 μL/well) for 1 hr. TCA-fixed cells were washed with distilled water and stained at room temperature for 15 min with 0.4% (w/v) SRB dissolved in 1% acetic acid (50 μL/well). Subsequently, the wells were rinsed four times with 1% acetic acid to remove unbound stain. The culture plates were air-dried, and protein-bound SRB was solubilized by adding 150 μL of 10 mM unbuffered Tris base per well. Absorbance was measured at 540 nm on a Titertek Multiscan reader. There was a linear relationship between cell number and absorbance in both control and drug-treated cells.

Growth in treated cultures was expressed as a percentage of the increase in absorbance of control cultures between days 2 and day 7, using the equation

$$\% \text{ growth} = \frac{A(cAMP7) - A(c2) \times 100}{A(c7) - A(c2)}$$

where A(c2) is the mean absorbance in six control wells determined on day 2, A(c7) is the mean absorbance in six control wells determined on day 7, and A(cAMP7) is the absorbance determined on day 7 in wells containing drugs. Concentrations that cause 50% inhibition of the maximal growth (IC_{50}) were calculated from concentration-response curves with the help of NFIT software (University of Texas, Galveston, TX, U.S.A.).

Flow Cytometry Analysis

Glioma cells cultured in 25-cm^2 flasks were harvested by gentle trypsinization. Harvested cells were centrifuged, and the cell pellet was resuspended in 900 μL Tris buffer containing 30 μg/mL trypsin. Following 10 min incubation at room temperature, 750 μL Tris buffer containing trypsin inhibitor (0.5 mg/mL) and RNAse A (0.1 mg/mL) was added to the lysate. Subsequently, nuclei were stained with the DNA-specific fluorochrome 4,6-diamino-2-phenyl indole. Trout erythrocytes served as the DNA ploidy standard. DNA histograms were generated on a CA II flow cytometer (Partec, Munster, F.R.G.) using DPAC software. The percentage of cells in each cycle phase was calculated using Multicycle software (Partec, Munster, F.R.G.).

RESULTS

DNA and RNA Synthesis

[^{14}C]Thymidine and [5-^{3}H]uridine incorporation were used to determine the effects of 8Cl-adenosine and 8ClcAMP on the synthesis of DNA and RNA, respectively. Tables 1 and 2 show the effects of 8Cl-adenosine and 8ClcAMP on the nucleic acid synthesis in two human glioma cell lines (WF and D384). The incorporation of thymidine and uridine were inhibited concentration-dependently by both 8Cl-adenosine and 8ClcAMP. In WF glioma cells, the inhibi-

TABLE 1. Effect of 8Cl-adenosine (% of control) on growth and on DNA and RNA synthesis in human glioma cell lines D384 and WF

	Thymidine incorporation		Uridine incorporation		Proliferation	
[8Cl-Adenosine]	D384	WF	D384	WF	D384	WF
3 μM	102 ± 5	92 ± 4	74 ± 3*	86 ± 4	19 ± 2	49 ± 7
10 μM	86 ± 8	84 ± 8	53 ± 4	64 ± 9	1 ± 1	22 ± 3
30 μM	63 ± 8†	53 ± 1‡	40 ± 3‡	56 ± 5	3 ± 3‡	10 ± 4‡
3 μM + ADA	106 ± 1	86 ± 3	97 ± 2	N.D.	N.D.	N.D.
30 μM + ADA	109 ± 3	87 ± 2	103 ± 4	N.D.	91 ± 2	86 ± 1
ADA (2 U/mL)	108 ± 6	89 ± 3	105 ± 1	N.D.	93 ± 1	81 ± 5

D384 and WF glioma cells were incubated with 8Cl-adenosine and/or ADA for 24 hr to determine their effects on DNA and RNA synthesis. [2-^{14}C]Thymidine and [5-^{3}H]uridine were present during the final 4 hr of the incubation period, after which the incorporation of the radiolabel was determined. [2-^{14}C]Thymidine and [5-^{3}H]uridine incorporation is expressed as a percentage of the respective control value. SRB assays were performed at the beginning and the end of 6 days' incubation with 8Cl-adenosine and/or ADA to determine their effects on glioma cell proliferation. Proliferation is expressed as a percentage of cell growth in untreated control groups. Values are the mean ± SEM of three independent determinations. Statistical differences were determined by Student's *t*-test. N.D., not determined. There were no statistically significant differences between groups treated with either ADA alone or ADA + 8Cl-adenosine.

*Different from 3 μM + ADA, $P < 0.01$.

†Different from 30 μM + ADA, $P < 0.02$.

‡Different from 30 μM + ADA, $P < 0.001$.

TABLE 2. Effect of 8ClcAMP (% of control) on growth and on DNA and RNA synthesis in human glioma cell lines D384 and WF

	Thymidine incorporation		Uridine incorporation		Proliferation	
[8ClcAMP]	D384	WF	D384	WF	D384	WF
1 μM	N.D.	N.D.	96 ± 4	95 ± 12	99 ± 7	95 ± 5
10 μM	106 ± 1	90 ± 3	73 ± 4*	83 ± 7	45 ± 3	49 ± 5*
30 μM	99 ± 3	74 ± 5	N.D.	N.D.	17 ± 6	25 ± 1
100 μM	83 ± 3†	68 ± 5	59 ± 4†	60 ± 5	9 ± 4‡	11 ± 2‡
10 μM + ADA	106 ± 3	89 ± 1	96 ± 3	N.D.	N.D.	84 ± 6
100 μM + ADA	103 ± 3	75 ± 5	85 ± 4§	N.D.	60 ± 10	51 ± 3‖
ADA 2 U/mL	108 ± 6	89 ± 3	105 ± 1	N.D.	82 ± 9	77 ± 6

D384 and WF glioma cells were incubated with 8ClcAMP and/or DA for 24 hr to determine their effects on DNA and RNA synthesis. [2-^{14}C]Thymidine and [5-^{3}H]uridine were present during the final 4 hr of the incubation period, after which the incorporation of the radiolabel was determined. [2-^{14}C]Thymidine and [5-^{3}H]uridine incorporation are expressed as a percentage of the respective control values. SRB assays were performed at the beginning and the end of 6 days' incubation with 8ClcAMP and/or ADA to determine their effects on glioma cell proliferation. Proliferation is expressed as a percentage of cell growth in untreated control groups. Values are the mean ± SEM of three independent determinations. Statistical differences were determined by Student's *t*-test. N.D., not determined.

†Different from 100 μM + ADA, $P < 0.01$.
*Different from 10 μM + ADA, $P < 0.01$.
§Different from ADA alone, $P < 0.01$.
‡Different from 100 μM + ADA, $P < 0.001$.
‖Different from ADA alone, $P < 0.02$.

tion of DNA synthesis was comparable to that of RNA synthesis, whereas in D384 cells, the incorporation of uridine was more strongly inhibited than that of thymidine. Surprisingly, at growth-inhibitory concentrations (i.e. concentrations inducing 90% or more inhibition of the glioma cell proliferation, determined by SRB assay) the incorporation of thymidine was only partly inhibited by 8ClcAMP (100 μM) and 8Cl-adenosine (30 μM). Addition of ADA (2 U/ml), which inactivates 8Cl-adenosine by deamination to 8Cl-inosine, prevented the reduction of the nucleic acid synthesis induced by 8Cl-adenosine or 8ClcAMP (Tables 1, 2). However, in cultures of D384 cells treated with 8ClcAMP (100 μM) + ADA, the incorporation of uridine was still significantly lower than in cultures treated with ADA alone. Determination of uridine incorporation in WF cells in the presence of ADA failed to provide reproducible results.

Because 8CPTcAMP is more suitable than 8ClcAMP for studying the effects of PKA activation, we compared its effect with that of 8ClcAMP (Fig. 1). 8CPTcAMP (1 mM) almost completely blocked both DNA and RNA synthesis (the remaining [^{14}C]thymidine and [5-^{3}H]uridine incorporation was 3% and 7% of the respective control values). 8CPTcAMP was much more potent than 8ClcAMP in inhibiting nucleic acid synthesis. In contrast, 8ClcAMP inhibited glioma cell proliferation more potently than did 8CPTcAMP, which caused approximately 100% growth inhibition only at a concentration of 1 mM (data not shown).

Incorporation of 8Cl-adenosine

Incorporation of 8Cl-adenosine into either RNA or DNA may contribute to its growth-inhibitory effect. Therefore, D384 and WWF glioma cells were incubated with [2-^{3}H]8Cl-adenosine for 24 hr. Radioactivity was detected in both RNA and DNA from either cell line (Fig. 2). Despite the difference in population doubling times, there was little difference between WF and D384 cells in the amount of [2-^{3}H]8Cl-adenosine incorporated into DNA. Incorporation of [2-^{3}H]8Cl-adenosine into RNA was higher in D384 cells than in WF cells. Considering the rate of nucleic

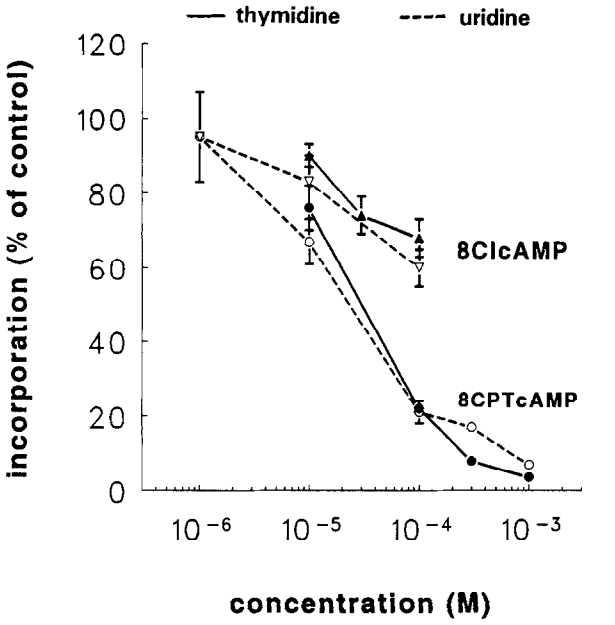

FIG. 1. 8CPTcAMP inhibited thymidine (-●-) and uridine (-○-) incorporation more strongly than did 8ClcAMP (-▲-, thymidine; -▽-, uridine incorporation). WF cells were incubated with 8ClcAMP or 8CPTcAMP for 24 hr. [^{14}C]Thymidine or [5-^{3}H]uridine was present during the final 4 hr of the incubation period, after which the incorporation of radiolabeled in DNA or RNA, respectively, was determined. Values are the mean ± SEM of three independent determinations.

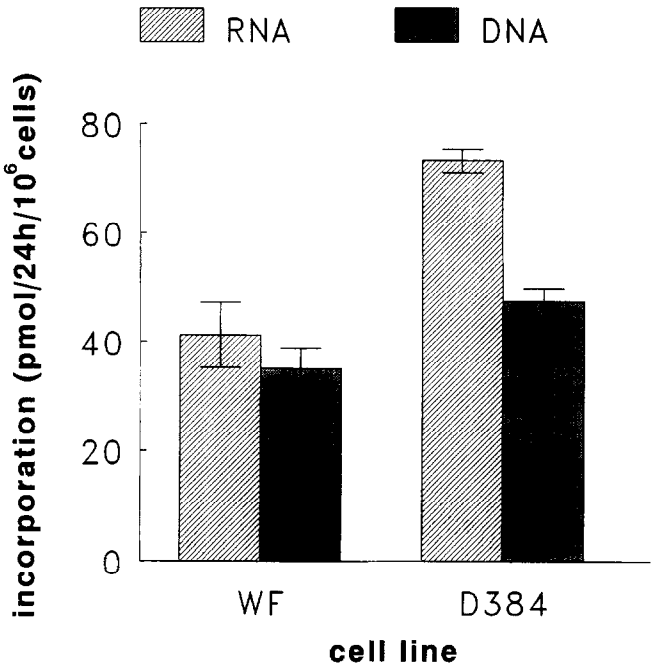

FIG. 2. Incorporation of [^{3}H]8Cl-adenosine into RNA (hatched bars) or DNA (shaded bars) of WF and D384 cells. [^{3}H]8Cl-Adenosine (30 μM) was present for 24 hr in subconfluent cultures. Values are the mean ± SEM of three independent determinations.

acid synthesis, relatively more [2-^{3}H]8Cl-adenosine was incorporated into RNA than into DNA in both cell lines: in experiments performed in the presence of 30 μM 8Cl-adenosine, the amount of [2-^{3}H]8Cl-adenosine incorporated into DNA was 5–8% of the [^{14}C]thymidine incorporation into DNA, expressed as pmol/10^6 cells/24 hr; incorporation of [^{3}H]8Cl-adenosine into RNA was 22% and 46% of the amount of [5-^{3}H]uridine incorporated into the RNA of D384 and WF cells, respectively.

Flow Cytometry Analysis

Initially, we studied the effect of only 8ClcAMP on WF and D384 cells. Subsequently, D384 glioma cells were used to investigate the effects of 8Cl-adenosine, 8ClcAMP, and 8CPTcAMP on the cell cycle kinetics in more detail (Table 3). In most experiments, these compounds were used at growth-inhibitory concentrations. Incubation of D384 cells with 1 mM 8CPTcAMP for 6 days did not significantly affect cell cycle distribution (Fig. 3; Table 3)—no more than 8ClcAMP or 8Cl-adenosine did at their IC_{50} (data not shown). The percentages of cells in S and G_2M phases were only slightly decreased in cultures treated with 8CPTcAMP, compared with control cultures (Table 3). In contrast, at growth-inhibitory concentrations, both 8ClcAMP and 8Cl-adenosine caused considerable shifts in the cell cycle distribution: the percentage of cells in the G_0/G_1 phase was decreased, whereas the percentage of cells in the S and G_2M phases was increased (Table 3). In addition, the peak of cells in the G_2M phase was considerably broadened (Fig. 3D, H). Addition of ADA to cultures incubated with 8ClcAMP largely prevented these changes in cell cycle distribution. The shift in cell cycle distribution of WF cells was less conspicuous, but here, too, an increase in the percentage of cells in the S-phase was observed after treatment with 8ClcAMP (Table 3).

TABLE 3. Effects of 8ClcAMP and 8CPTcAMP on the cell cycle distribution of WF and D384 glioma cells

Cell line	Drug	Cell cycle distribution		
		% G_0/G_1	% S	% G_2/M
WF	Control	79.2	9.2	11.6
	8ClcAMP (100 μM)	69.2	18.3	12.5
D384	Control	72.2	19.4	8.4
	8CPTcAMP (1 mM)	77.0	13.3	9.7
	8ClcAMP (100 μM)	27.5	16.9	55.6
	8ClcAMP (100 μM) + ADA (2 U/mL)	63.0	28.4	8.5
	8Cl-Adenosine (30 μM)	33.8	43.0	23.3

WF and D384 glioma cells were incubated with drugs or drug vehicle (control) for 6 days. Subsequently, the cell cycle distribution was determined by flow cytometry. Values represent the fraction of cells in a given phase of the cell cycle and are derived from a representative experiment. Repeated experiments gave similar results.

Cultures were incubated with 8ClcAMP (100 μM) or 8Cl-adenosine (30 μM) for several periods to investigate at what point in time the shifts in the cell cycle distribution occur. At 24 hr, 8Cl-adenosine induced an increase in the number of cells in both the S phase and G_2M phase (Fig. 3B). At 48 hr, the percentage of cells in the G_1 phase was decreased in favour of the percentage of cells in the S and G_2M phases (Fig. 3C). Between 48 hr and 6 days, the most prominent change in the cell cycle distribution was the continued increase in the percentage of cells in the S and G_2M phases. In addition, there was an accumulation of cells with a DNA content between diploidy and tetraploidy (Fig. 3D). In cultures incubated with 8ClcAMP, we found similar shifts in the cell cycle distribution, although these shifts occurred more slowly than in cultures treated with 8Cl-adenosine (Fig. 3F–H).

DISCUSSION

8Cl-Adenosine is a potent inhibitor of human glioma cell growth *in vitro*. This compound is an active metabolite of 8ClcAMP, which in recent years has received increasing attention as a potential antineoplastic agent. To reveal its site of action, we have investigated the effects of 8Cl-adenosine on the synthesis of nucleic acids. Both 8ClcAMP and 8Cl-adenosine only partly inhibited the synthesis of DNA and RNA at concentrations that almost completely inhibited proliferation. In contrast, at cytostatic concentrations, 8CPTcAMP inhibited the synthesis of nucleic acids nearly 100%. This is in good agreement with the observation that an elevation in the cAMP concentration in he-

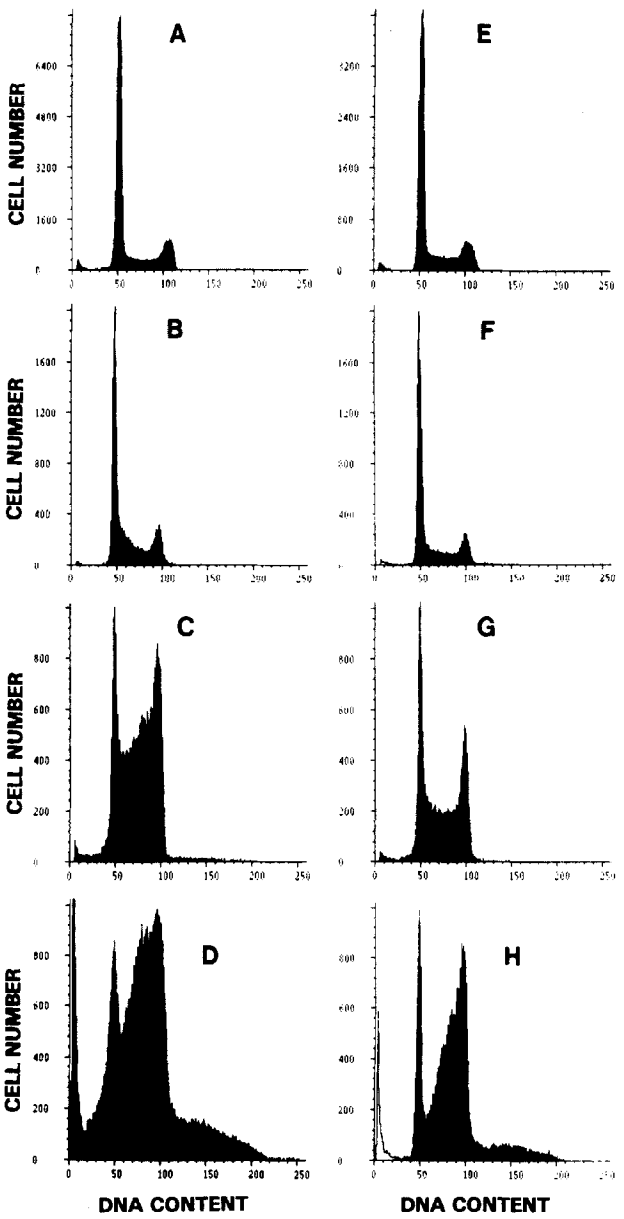

FIG. 3. DNA histograms of D384 cells treated with 30 μM 8Cl-adenosine (B, C, D), 1 mM 8CPTcAMP (E), or 100 μM 8ClcAMP (F, G, H). (A) DNA histogram of untreated cells in the log phase of growth. D384 cells were incubated with drugs for 24 hr (B, F), 48 hr (C, G) or 6 days (D, E, H). Repeated experiments gave similar results.

patocytes blocks the cell cycle progression at a point immediately before the G_1/S border [34].

DNA synthesis, as observed in glioma cell cultures exposed to 8ClcAMP, is not necessarily accompanied by an increase in cell number. Using flow cytometric analysis, we have demonstrated that both 8ClcAMP and 8Cl-adenosine, but not 8CPTcAMP, blocked progression in the cell cycle at the G_2M phase, which explains the difference in the effects of 8CPTcAMP and 8ClcAMP (or its metabolite) on DNA synthesis. The fact that 8ClcAMP increased the percentage of WF cells in the S phase, but not in the G_2M phase, may be related to the relatively high population doubling time of this cell line (56 hr, versus 35 hr in the D384 cell line). We observed that an increase in the percentage of D384 cells in the S phase occurred before the cells accumulated in the G_2M phase. It is conceivable that retardation of the cell cycle progression by 8ClcAMP or 8Cl-adenosine prevented the WF cells accumulated in the S phase from reaching the G_2M phase within 6 days (i.e. before the flow cytometry was performed).

The differences between the effects of 8CPTcAMP and 8ClcAMP on nucleic acid synthesis in human glioma cells suggest that 8ClcAMP did not inhibit glioma cell growth through PKA activation. Moreover, we observed a similarity between the effects of 8ClcAMP and 8Cl-adenosine on nucleic acid synthesis and cell cycle kinetics. These observations, however, do not provide conclusive evidence for the hypothesis that 8Cl-adenosine accounts for the effects induced by 8ClcAMP. Therefore, in a number of experiments ADA was added to the culture medium. ADA catalyzes the conversion of 8Cl-adenosine into 8Cl-inosine, which does not affect cell proliferation [22, 35]. Because ADA inactivates 8Cl-adenosine without affecting the concentration of 8ClcAMP [22], differences between the effects of 8ClcAMP + ADA and of 8ClcAMP alone are most likely due to the presence of 8Cl-adenosine.

Although the results of most experiments indicate that the effects of 8ClcAMP are largely due to its metabolite 8Cl-adenosine, it should be noted that the effect of 8ClcAMP (100 μM) on RNA synthesis in D384 cells was not completely prevented by ADA. This result may indicate that 8ClcAMP inhibited the synthesis of RNA partly through its active metabolite, 8Cl-adenosine, and partly through PKA activation. Another explanation may be that the concentration of ADA (2 U/mL) was not high enough to convert all 8Cl-adenosine present in the culture medium into 8Cl-inosine. Higher concentrations of ADA have not been used, however, because ADA itself also inhibits glioma cell growth [22].

There has been substantial debate on the question of whether the effects of 8ClcAMP on cell growth are mediated by its metabolite, 8Cl-adenosine, or by interference with type I and type II PKA [23, 36]. Tagliaferri and coworkers found that 8ClcAMP, used at its IC_{50} did not affect cell cycle progression in a colon cancer cell line, whereas 8Cl-adenosine (at its IC_{50}) increased the number of cells in the G_1 phase [19]. This seemed to suggest that 8Cl-adenosine and 8ClcAMP do not share the same mechanism of action. However, we failed to observe disturbances of the cell cycle distribution when 8ClcAMP and 8Cl-adenosine were used at their IC_{50}. The results from our present study indicate that the growth-inhibitory effect of 8ClcAMP and 8Cl-adenosine on human glioma cells is not due to an accumulation of cells in the G_1 phase, but rather that it is related to a block in the G_2M phase. Using normal and neoplastic mouse lung epithelial cell lines, Lange-Carter and coworkers recently showed that changes elicited by 8ClcAMP in the proportion of cells in the G_1, S, and G_2M

phases were similar to those induced by 8Cl-adenosine [20]. However, not all the lung cell lines behaved in the same way with respect to the cell cycle progression after exposure to 8ClcAMP or 8Cl-adenosine. Thus, species differences as well as differences between the various tumor cell lines may account for discrepancies between our results and those of others. By adding ADA, we were able to demonstrate that the shifts in the cell cycle distribution of human glioma cells induced by 8ClcAMP can be largely attributed to 8Cl-adenosine.

Radiolabeled 8Cl-adenosine was incorporated into both DNA and RNA. Although the incorporation of 8Cl-adenosine into RNA was higher in D384 cells than in WF cells, the rate of RNA synthesis was lower in the latter cell line. Because adenosine forms base pairs with thymidine and uridine in DNA and RNA, respectively, the amount of thymidine and uridine incorporated should equal the amount of adenosine incorporated. Thus, the incorporation of [2-^{3}H]8Cl-adenosine, expressed as a percentage of thymidine or uridine incorporation, indicates to what extent adenosine is replaced by 8Cl-adenosine. From these data, it appears that relatively more [2-^{3}H]8Cl-adenosine was incorporated into the RNA of WF cells than into the RNA of D384 cells. Whether incorporation into RNA and/or DNA contributes to the growth-inhibitory effect of 8Cl-adenosine (e.g. by interference with protein synthesis or DNA replication) remains to be investigated.

In conclusion, it appears that the growth-inhibitory effect of 8ClcAMP on human glioma cells can be largely attributed to its metabolite 8Cl-adenosine. Therefore, we suggest that subsequent studies focus on 8Cl-adenosine rather than on 8ClcAMP, unless the use of 8ClcAMP *in vivo* (as a "caged compound") appears to have advantages over the use of 8Cl-adenosine. The growth-inhibitory effect of 8Cl-adenosine seems to be related to a block of the cell cycle progression in the G_2M phase. Apart from incorporation of 8Cl-adenosine into RNA or DNA, other mechanisms of action, such as inhibition of protein synthesis and distortion of purine nucleotide pools or ATP metabolism, may be involved in the growth-inhibitory effect of 8Cl-adenosine. Finally, the growth-inhibitory effect of 8Cl-adenosine on human glioma cells *in vitro* warrants *in vivo* studies using an animal model for human glioma to determine its potential as an antineoplastic drug.

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