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Inhibition of colon tumor cell growth by 8-chloro-cAMP is dependent upon its conversion to 8-chloro-adenosine

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Recent interest in site-selective cAMP analogs has focused on the role of 8-chloro-adenosine (8-Cl-adenosine) in the inhibition of tumor cell growth by 8-chloro-cAMP (8-Cl-cAMP) (Van Lookeren Campagne, *et al. Cancer Res* 1991; 51: 1600–5). We have evaluated 8-Cl-cAMP and 8-Cl-adenosine for their growth inhibitory activity against two human colon adenocarcinoma cell lines, HCT116 and FET. Because these cell lines have been adapted to grow in chemically defined medium we were able to evaluate the effect of serum on 8-Cl-cAMP's growth inhibitory activity. In addition, cells grown in serum-free medium were tested for their sensitivity to 8-Cl-cAMP, serum-activated 8-Cl-cAMP and 8-Cl-adenosine. IC_{50} values, determined by measuring cell growth using a MTT colorimetric assay, showed that 'serum activation' of 8-Cl-cAMP was required to achieve inhibition of HCT116 ($IC_{50} = 1.3 \pm 0.1$ μM) and FET ($IC_{50} = 2.0 \pm 0.1$ μM) cell growth. IC_{50} values were not reached at the highest concentrations tested ($IC_{50} > 500$ μM) in the absence of serum, permitting us to conclude that 8-Cl-cAMP does not have growth inhibitory activity between 1.0 and 500 μM doses. HCT116 and FET cells grown in media containing serum and in the presence of 8-Cl-adenosine had IC_{50} values of 0.6 ± 0.1 and 0.9 ± 0.2 μM, respectively. HCT116 and FET cells grown in chemically defined medium containing 8-Cl-adenosine exhibited IC_{50} values of 1.0 ± 0.1 and 3.1 μM, respectively. Reversed-phase HPLC analysis showed an 11.4 ± 0.7% conversion of 8-Cl-cAMP to 8-Cl-adenosine in 1 h at 37°C in the presence of 10% fetal bovine serum (FBS). Analysis of the continued conversion of 8-Cl-cAMP after 72 h in media containing 10% FBS revealed that 69.5 ± 0.7% of the 8-Cl-cAMP was converted to 8-Cl-adenosine. These results strongly support the conclusion that enzymatic conversion of 8-Cl-cAMP to 8-Cl-adenosine occurs in the presence of serum and that 8-Cl-adenosine is the active inhibitory compound.

Key words: 8-Chloro-adenosine, 8-chloro-cAMP, colon tumor.

Introduction

Initial studies evaluating the growth inhibitory action of various site-selective cAMP analogs established that 8-chloro-cAMP (8-Cl-cAMP) was the most efficacious of the 24 cyclic nucleotide analogs studied.[1] The 8-Cl-cAMP analog had the ability to inhibit cell growth for a variety of cell types using a single 20 μM dose.[1] Further studies showed that site-selective cAMP analogs exhibit a major regulatory effect upon growth in a broad spectrum of human cancer cell lines and xenografts.[1–4] 8-Cl-cAMP is thought to function by binding to the regulatory subunit of type I or type II cAMP dependent protein kinases causing differential regulation of their activities.[1,4–5] Ally *et al.* have reported that 8-Cl-cAMP suppresses cell growth in breast cancer and binds, selectively, to the site 1 (Site B) receptor of the type II cAMP dependent protein kinase regulatory subunit ($RII\alpha/RII_{54}$ or $RII\beta/RII_{51}$).[6]

Reports from other laboratories have shown that 8-Cl-cAMP can decrease transforming growth factor α mRNA levels without changing epidermal growth factor (EGF) or c-Ha-*ras* mRNA populations.[7] These effects are thought to occur largely as a result of 8-Cl-cAMP's ability to increase levels of cyclic nucleotide responsive element binding protein in addition to its induction of c-*fos* mRNA.[7] The mechanisms by which 8-Cl-cAMP and N^6-benzyl-cAMP exert their growth inhibitory actions on sensitive cells were studied by Pepe *et al.*[8] and found to differ. 8-Cl-cAMP and N^6-benzyl-cAMP were both shown to produce a G_2/M cell cycle block by different mechanisms that were unrelated to 8-Cl-adenosine.[8]

In a report from Van Lookeren Campagne *et al.*[9] it was shown that 8-Cl-adenosine production occurred from 8-Cl-cAMP in the presence of fetal bovine serum (FBS) as a result of the actions of

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serum phosphodiesterase and 5′-nucleotidase activities. Various supplies of FBS, calf serum and equine serum were compared and found to convert 8-Cl-cAMP to 8-Cl-adenosine with efficiencies ranging from 28 to 72% when incubated for 3 days at 37°C. These authors hypothesized that 8-Cl-cAMP was, in fact, serving as a 'pro-drug' that underwent enzymatic conversion to the 'active' nucleoside form. They further demonstrated that cells could be protected against the inhibitory effects of 8-Cl-cAMP if adenosine deaminase was added to their culture medium. This result led them to the conclusion that the adenosine derivative was the active inhibitory compound. Although 8-Cl-cAMP was clearly shown to undergo enzymatic conversion, it was not clear whether the pro-drug, 8-Cl-cAMP, had inhibitory activity of its own.

In these studies we have tested the influence of serum upon the sensitivity and resistance of human colon tumor cell lines to growth inhibition by 8-Cl-cAMP. We have taken advantage of the adaptation of the HCT116 and FET cell lines to grow in chemically defined medium.[10,11] The following three issues have been addressed. (i) Examination of colon tumor cell sensitivity, or resistance, and/or its enzymatically derived adenosine derivative in a set of cell lines that differ widely in their differentiation class and autocrine regulatory phenotypes.[10,12,13] (ii) Comparison of 8-Cl-cAMP effects upon cell growth in the presence and absence of serum. It is important to determine whether 8-Cl-cAMP can inhibit growth under conditions where enzymatic drug conversion can be shown to be absent. (iii) Analysis of the degree of enzymatic conversion of 8-Cl-cAMP occurring in media derived from growth inhibited and non-growth inhibited cells.

We found no growth inhibitory activity for 8-Cl-cAMP when cells were cultured in the absence of serum. Furthermore, we found an absolute requirement for serum-activation of 8-Cl-cAMP, establishing that the predominant cAMP analog present after 3 days of incubation in culture medium containing 10% FBS is the 8-Cl-adenosine derivative.

Materials and methods

Culture of human colon tumor (HCT) cell lines

The HCT116 and FET HCT cell lines were cultured in supplemented McCoy's 5A medium containing 10% FBS,[10] or in chemically defined McCoy's 5A medium supplemented with 4 μg/ml transferrin, 20 μg/ml insulin, 10 ng/ml EGF and 48 μg/ml gentamicin.[11] Cells were grown at 37°C in a humidified 5% CO_2/95% air atmosphere. Cells were routinely screened for the presence of mycoplasma contamination using the Gen-Probe rapid detection system (Gen-Probe Inc., San Diego, CA).

Serum activation of 8-Cl-cAMP

8-Cl-cAMP was obtained from Dr George Johnson at the NCI; a reference kit containing 8-Cl-cAMP, 8-Cl-adenosine-5′-*O*-monophosphate, 8-Cl-inosine-5′-monophosphate, 8-Cl-adenosine, 8-Cl-inosine, 8-Cl-hypoxanthine, 8-Cl-adenine, 8-Cl-xanthine, 8-Cl - xanthosine, 8 - Cl - xanthosine - 5′ - *O* - monophosphate and 8-Cl-5′-adenosine diphosphate standards was obtained from Bio-Log Life Science Institute (La Jolla, CA). The purity of kit standards was confirmed by analytical HPLC as described under 'Extraction and reversed-phase HPLC of 8-Cl-cAMP and its enzymatically derived product(s)' below. 8-Cl-cAMP was dissolved to a final concentration of 1.0 mM in McCoy's 5A medium with or without 10% FBS. Stock 8-Cl-cAMP solutions containing 10% FBS were incubated at 37°C for 1 h, passed through a filter with a 0.22 μm pore size and stored at 0–4°C. cAMP, dibuteryl-cAMP and 8-Cl-adenosine were also dissolved to final concentrations of 1.0 mM in McCoy's 5A medium with or without 10% FBS.

Measurement of cell growth and growth inhibition

Cells were seeded at a density of 6.2×10^3 cells/cm^2 for HCT116 cells and 1.0×10^4 cells/cm^2 for FET cells in Costar 6-well Tissue Culture Clusters (Costar Corp., Cambridge, MA) and fresh medium, containing cAMP analog, was added 24 h later. Old media and drug were changed for fresh at day 4 and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay of Mosmann[14] performed on day 6 (cells were maintained in culture for a total of 5 days in the presence of drug). MTT assays were read at 570 nm in a Dynatech MR700 Microelisa reader (Dynatech Labs, Chantilly, VA).

Extraction and reversed-phase HPLC of 8-Cl-cAMP and its enzymatically derived product(s)

Media, with and without 8-Cl-cAMP, were removed from cells in culture after 0, 24, 48 and 72 h of incubation. Media controls, incubated without cells, were also obtained for the corresponding time points. Protein and other trichloroacetic acid (TCA) precipitable materials were removed following a 30 min incubation at 0–4°C after bringing the culture medium to a final concentration of 5% TCA. Centrifugation at 10 000 × *g* for 3 min, followed by three extractions with 5 volumes of water saturated diethyl ether, were performed as described by Van Lookeren Campagne *et al.*[9] Samples and nucleotide analog standards were dissolved in HPLC mobile phase buffer, i.e. 0.2 M NaH_2PO_4/0.025 M tributylamine/pH 6.0/18% methanol (v/v) to a final concentration of 10 mM, diluted 100-fold and 20 μl injected onto a 4.6 × 250 mm Beckman Ultrasphere ODS (5μ) column (Beckman Instruments, Palo Alto, CA). Tributylamine was selected over tributylammonium hydroxide[9] for its retention time reproducibility and its capacity to resolve 8-Cl-adenosine from 8-Cl-inosine. Columns were equilibrated and eluted at a flow rate of 0.75 ml/min and absorbance was monitored at 254 nm. HPLC was performed using dual Waters Model 510 pumps and a Model 441 absorbance detector (Waters Chromatography Division, Milford, MA). Retention times and areas under curves were determined using a Waters 740 Data Module.

Calculation of cell doubling time and IC_{50} values

Changes in cell doubling time were based upon the $\log_2$ of MTT absorbance at 570 nm as described by Patterson.[15] IC_{50} values were calculated from computer drawn lines based upon multiple experiments and triplicate data points. IC_{50} values are reported with error values that represent standard errors of the mean (SEM), i.e. $SD/\sqrt{n}$.[16] Reported IC_{50} values were compared using a two-tailed *t*-test. In all cases the calculated sample mean IC_{50} values differed significantly with $p \leq 0.05$.

Table 1. Inhibition of HCT cell growth by 8-chloro-cAMP in the presence and absence of FBS

	IC_{50} (μM)	
Cell line	(+) serum	(−) serum
HCT116	1.4 ± 0.1	>500
FET	2.0 ± 0.1	>500

The *p*-value calculated for these values using a two-tailed *t*-test was 0.0401.

Results

Sensitivity of HCT cells to 8-Cl-cAMP in the presence and absence of FBS

Initial efforts were focused upon the extension of those results reported by Katsaros *et al.*[1] to HCT cell lines. The HCT116 and FET cell lines were selected for these studies because they exhibit many of the collective biological properties of poor and well differentiated human adenocarcinoma cell lines, respectively.[12,13] Using doses of 8-Cl-cAMP that ranged from 0 to 500 μM, it was possible to show that HCT116 and FET cells grown in the presence of 10% FBS were growth inhibited by 8-Cl-cAMP with IC_{50} values of 1.4 ± 0.1 and 2.0 ± 0.1 μM, respectively (Table 1). Despite numerous attempts, IC_{50} values were not reached at the highest concentrations of 8-Cl-cAMP tested (IC_{50} > 500 μM) in the absence of serum (Table 1). These results, together with the report of Van Lookeren Campagne *et al.*,[9] prompted us to evaluate the role of serum in activating 8-Cl-cAMP on cells that were grown in the presence and absence of serum.

Cell sensitivity and reversed-phase HPLC analysis of serum-activated 8-Cl-cAMP

In order to establish whether 8-Cl-cAMP or metabolites of 8-Cl-cAMP were responsible for the inhibition of HCT cell line growth, two approaches were taken. The first approach involved the pre-activation of 8-Cl-cAMP in 10% FBS for 1 h prior to incubation with cells in culture. It was found that HCT116 and FET cells grown in chemically defined medium could now be growth inhibited (Figure 1) with IC_{50} values of 7.6 ± 0.5 and 17.0 ± 1.2 μM, respectively (Table 2). A comparison of the sensitivity of HCT116 and FET cells grown in the presence of 10% FBS to serum-activated 8-Cl-cAMP shows no significant difference.

The second approach involved the analysis of serum-activated 8-Cl-cAMP and its products after 0, 24, 48 and 72 h of incubation in the conditioned medium obtained from HCT116 cells cultured in

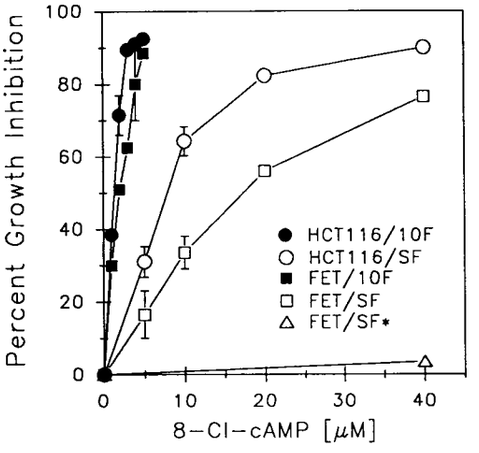

Figure 1. Inhibition of HCT116 and FET cell growth by serum-activated 8-Cl-cAMP. Stock solutions (1.0 mM) of 8-Cl-cAMP were made 10% in FBS and 'serum-activated' for 60 min at 37°C. Activated 8-Cl-cAMP was added to cells in culture wells in fresh medium 24 h after seeding. Wells were assayed with the MTT assay of Mosmann[14] after 5 days of treatment. All values are the average of four independent experiments; each experimental value is the average of three sample wells. Error bars represent the SEM calculated for each data point. The symbols are presented as an inset to the figure. FET/SF* denotes a control in which FET cells grown in serum-free medium were treated with non-activated 8-Cl-cAMP. Cell viability was determined using Trypan Blue dye exclusion for cells treated for 72 h with 10 μM serum-activated 8-Cl-cAMP and found to be 91.0 ± 1.5%.

the presence and absence of 10% FBS. After removal of cells by centrifugation and TCA precipitation (5% TCA), supernatants were analyzed relative to 8-Cl-cAMP and authentic metabolites of 8-Cl-cAMP obtained as a standard kit. The results of these studies are illustrated in Figure 2. They show that 69.5 ± 0.7% of the 8-Cl-cAMP was converted to 8-Cl-adenosine within 72 h of incubation in the presence of 10% FBS. Conversion was largely independent of the presence of cells, indicating that components present in serum, and not cell membrane or secreted cellular products, were responsible for the conversion of 8-Cl-cAMP to its adenosine metabolite. Studies designed to examine the amount of conversion of 8-Cl-cAMP occurring in McCoy's 5A medium lacking serum, with and without cells, showed essentially no conversion beyond the initial 11.4 ± 0.7% that occurred during serum-activation of the stock solution (Figure 2). Hence, the dominant cAMP analog present after 72 h of culture in 10% FBS is the adenosine metabolite.

Sensitivity of HCT cell lines to 8-Cl-adenosine

HCT cells grown in the presence of 8-Cl-adenosine exhibited IC_{50} values of 0.6 ± 0.1 and 0.9 ± 0.2 μM

Table 2. Inhibition of HCT116 and FET Cells with serum-activated 8-Cl-cAMP in the presence and absence of 10% FBS

		IC_{50} (μM)	
Cell line	Culture conditions	8-Cl-cAMP*[a]	8-Cl-adenosine
HCT116	10% FBS	1.3 ± 0.1[b]	0.6 ± 0.1[c]
HCT116	serum-free	7.6 ± 0.5	1.0 ± 0.1
FET	10% FBS	2.0 ± 0.1	0.9 ± 0.2
FET	serum free	17.0 ± 1.2	3.1[d]

[a] Individual values were obtained for drug doses that correspond to 5 times the IC_{50} reported in Table 2 for the respective cell line/culture condition combinations. For example, HCT116 cells treated with serum-activated 8-Cl-cAMP in the presence of 10% FBS were treated with a final drug concentration of 6.5 μM. The asterisk indicates 8-Cl-cAMP that has been activated by incubation with 10% FBS as described in Methods and materials.
[b] Means ± SEM.
[c] Incubation of stock 8-Cl-adenosine solutions in the presence and absence of 10% FBS at 37°C did not influence its activity.
[d] This value is from a single experiment with $n = 3$. Two-tailed p-values calculated for 8-Cl-cAMP* IC_{50} values for the comparison of 10% FBS with serum-free conditions were 0.001 (extremely significant) for HCT116 and 0.0064 (very significant) for FET. When a similar statistical analysis was performed for 8-Cl-adenosine IC_{50} values obtained in the presence and absence of serum, a p-value of 0.0086 (very significant) was obtained for HCT116 cells.

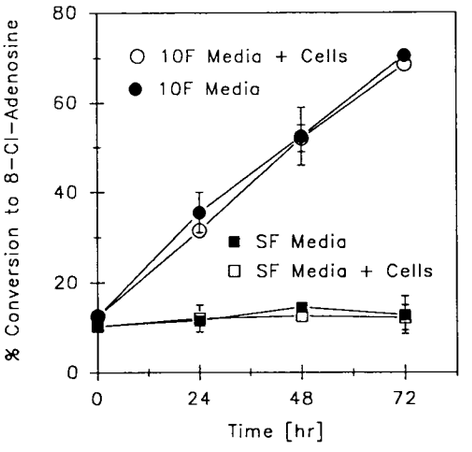

Figure 2. Dependence upon serum for the further conversion of serum-activated 8-Cl-cAMP to 8-Cl-adenosine. Serum-activated 8-Cl-cAMP was added to McCoy's 5A medium with and without 10% FBS and incubated at 37°C in a CO_2 incubator both in the presence and in the absence of HCT116 cells. $T = 0$, 24, 48 and 72 h aliquots were removed, cleared of cells, protein precipitated with a final concentration of 5% TCA and ether extracted. Samples were analyzed by HPLC relative to migration of nucleotide analog standards. Final identifications were made by co-chromatography. Areas under curves were integrated by a Waters 740 Data Module. Results are reported as the percent of total analog material converted to the 8-Cl-adenosine derivative.

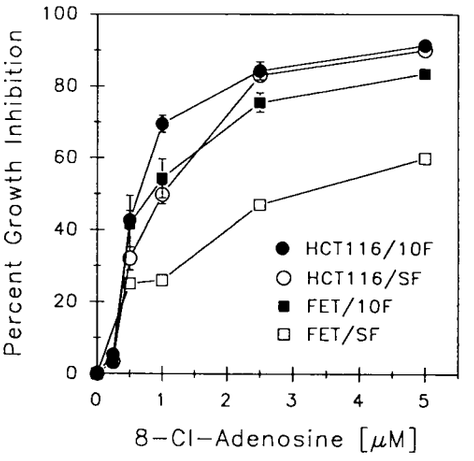

Figure 3. Inhibition of HCT116 and FET cell growth by 8-Cl-adenosine. See the legend to Figure 1 for conditions and procedures. Cell viability was analyzed as described in the legend to Figure 1. A value of 91.3 ± % was determined for cells treated for 72 h with 10 μM 8-Cl-adenosine. Comparison of the percent viability values determined for the data presented in Figure 1 and in this figure revealed that there was not a significant difference ($p = 0.8764$).

for HCT116 and FET cells, respectively, grown in the presence of 10% FBS (Figure 3 and Table 2). These values are slightly lower than the previous data obtained for cells inhibited with serum-activated 8-Cl-cAMP (Table 1) and merely reflect the incomplete conversion that occurred under our conditions for serum activation. HCT116 and FET cells grown in the presence of 8-Cl-adenosine exhibited IC_{50} values of 1.0 ± 0.1 and 3.1 μM, respectively, when grown in chemically defined medium. These data suggest that the HCT116 and FET lines adapted to grow in chemically defined medium are approximately 2–3 times less sensitive to the nucleoside derivative than their counterparts grown in serum containing media.

Characteristics of the growth inhibition for cells treated with serum-activated 8-Cl-cAMP and 8-Cl-adenosine

To further characterize the effects of serum-activated 8-Cl-cAMP and 8-Cl-adenosine upon the inhibition of HCT cells, the reversibility of the growth inhibition was examined. As shown in Table 3, when the growth of HCT116 and FET cells was examined immediately after removal of serum-activated 8-Cl-cAMP or 8-Cl-adenosine, at doses 5-fold higher than their IC_{50} concentration, several types of growth responses were observed. None of the HCT116 cells exhibited a full reversal of inhibition, i.e. none of the treated HCT116 cells exhibited doubling times of 20 or 31 h, characteristic of untreated controls.[17] 8-Cl-adenosine treated HCT116 cells grown in the absence of serum were irreversibly inhibited by 8-Cl-adenosine. FET cells, however, grown in media containing 10% FBS were reversibly inhibited by serum activated 8-Cl-cAMP and by 8-Cl-adenosine. It is apparent from these results that cellular responses following the removal of 8-Cl-cAMP or 8-Cl-adenosine will be highly dependent upon the individual target cell line and the conditions of cell culture.

Discussion

These studies definitively establish the inability of 8-Cl-cAMP to undergo conversion to nucleoside metabolites after it is added to cell culture supernatants lacking serum. More importantly, the

Table 3. Comparison of cell doubling times for cells inhibited with 8-Cl-cAMP* or 8-Cl-adenosine following removal of drug

Cell line	Culture conditions	Cell doubling time[a] (h)		
		control[b]	8-Cl-cAMP*	8-Cl-adenosine
HCT116	10% FBS	20	34–68	34–51
HCT116	serum-free	31	42–57	279–733
FET	10% FBS	42	36–58	37–44

[a] Cell doubling times were calculated from $\log_2$ values for MTT absorbance changes at 570 nm.[14,15]
[b] Cell doubling rates for HCT116 cells grown in the presence and absence of 10% FBS were determined by Boyd *et al.*[17]

approach we have taken, i.e. the use of cells cultured in chemically defined medium, shows that 8-Cl-cAMP is incapable of inhibiting tumor cell growth in the absence of serum over a very wide range of concentrations (Table 1). A closer examination of the metabolites present at early and late times during incubation in serum-containing culture medium shows a continual and progressive increase in the generation of the adenosine metabolite. Interestingly, HPLC patterns obtained for drug that was present in culture supernatants for various periods of time did not reveal significant amounts of additional UV absorbing metabolites. This occurred in a chromatography system with the capacity to resolve most 8-Cl-cAMP metabolites.

The finding that the more differentiated FET cells were slightly less sensitive to the growth inhibitory action of serum-activated 8-Cl-cAMP, as well as 8-Cl-adenosine, was not unexpected. The reduced sensitivity of FET cells to growth inhibition by serum-activated 8-Cl-cAMP and 8-Cl-adenosine probably contributes to the full reversibility of their growth inhibition following drug removal. Previous studies have shown that HCT116 cells are more sensitive to growth inhibition by sodium butyrate than FET cells.[18] It is thought that this may result from losses in one or more drug detoxification mechanisms from the less differentiated HCT116 cell line.

Our results extend the data reported by Van Lookeren Campagne *et al.*[9] indicating that the adenosine derivative was the growth inhibitory nucleoside in their system. It is of particular interest that the level of conversion of 8-Cl-cAMP to 8-Cl-adenosine reported by Van Lookeren Campagne *et al.* coincided closely with those results reported in this study, i.e. approximately 70% conversion to 8-Cl-adenosine.[9]

We only observed a complete reversal of growth inhibition for FET cells following treatment with serum-activated 8-Cl-cAMP and 8-Cl-adenosine, similar to results reported by Han *et al.*[19] These findings suggest that the inhibitory effects achieved on cancer cell growth in potential clinical applications of this compound, or its metabolites, might be of long duration. We cannot, however, ignore pharmacokinetic, systemic distribution and clearance differences between these two analogs. There may, in fact, be pharmacokinetic and/or pharmacodynamic properties that favor the administration of 8-Cl-cAMP. For example, 8-Cl-cAMP administration may blunt toxicities that limit the therapeutic use of the nucleoside metabolite. The incomplete reversibility of the 8-Cl-adenosine inhibition observed for HCT116 cells might be an indication of sustained duration for toxic effects upon non-tumor cells in hosts that undergo treatment with this agent.

Conclusion

The impact of these studies on the mechanism of action of 8-Cl-cAMP clearly focuses upon the effects of 8-Cl-adenosine. Since 8-Cl-adenosine is not phosphorylated, this provides some advantages in so far as drug uptake is concerned.[9] These results would also suggest that mechanisms involving cyclic nucleotide monophosphate analogs would probably occur soon after the administration of 8-Cl-cAMP and would probably not lead to growth inhibition. We cannot rule out the possibility that 8-Cl-cAMP or its analogs may have other reversible and/or non-inhibitory effects upon cells. However, in order to identify and characterize these drug effects, it will be necessary to utilize serum-free or stripped serum systems. Studies are in progress to determine those effects upon tumor cells reported for 8-Cl-cAMP[1,4–6] that accrue from the cyclic nucleotide analog as opposed to those that are either shared or unique

to 8-Cl-adenosine. Of particular interest will be the influence of the 8-Cl-adenosine derivative upon cyclic nucleotide kinase function and subunit translocation.

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References

1. Katsaros D, Tortora G, Tagliaferri P, *et al.* Site-selective cyclic AMP analogs provide a new approach in the control of cancer cell growth. *FEBS Lett* 1987; **223**: 97–103.
2. Cho-Chung YS, Clair T, Tagliaferri P, *et al.* Basic science review: site-selective cyclic AMP analogs as new biological tools in growth control, differentiation and proto-oncogene regulation. *Cancer Invest* 1989; **6**: 161–77.
3. Cho-Chung YS. Commentary: site-selective 8-chloro-cyclic adenosine 3′,5′-monophosphate as a biologic modulator of cancer: restoration of normal control mechanisms. *J Natl Cancer Inst* 1989; **81**: 982–7.
4. Ally S, Clair T, Katsaros D, *et al.* Inhibition of growth and modulation of gene expression in human lung carcinoma in athymic mice by site selective 8-Cl-cyclic adenosine monophosphate. *Cancer Res* 1989; **49**: 5650–5.
5. Tortora G, Tagliaferri P, Clair T, *et al.* Site-selective cAMP analogs at micromolar concentrations induce growth arrest and differentiation of acute promyelocytic, chronic myelocytic, and acute lymphocytic human leukemia cell lines. *Blood* 1988; **71**: 230–3.
6. Ally S, Tortora G, Clair T, *et al.* Selective modulation of protein kinase isozymes by the site-selective analog 8-chloroadenosine 3′,5′-cyclic monophosphate provides a biological means for control of human colon cancer cell growth. *Proc Natl Acad Sci USA* 1988; **85**: 6319–22.
7. Takanashi A, Yasui W, Yoshida K, *et al.* Inhibitory effects of 8-chloro-cyclic adenosine 3′,5′-monophosphate on cell growth of gastric carcinoma cell lines. *Jpn J Cancer Res* 1991; **82**: 325–31.
8. Pepe S, Tortora G, Noguchi PD, *et al.* Effects of 8-chloroadenosine 3′,5′-monophosphate and N^6-benzyl-cyclic adenosine 5′-monophosphate on cell cycle kinetics of HL-60 leukemia cells. *Cancer Res* 1991; **51**: 6263–7.
9. Van Lookeren Campagne MM, Villalba Diaz F, Jastorff B, *et al.* 8-Chloroadenosine 3′,5′-monophosphate inhibits the growth of Chinese Hamster Ovary and Molt-4 cells through its adenosine metabolite. *Cancer Res* 1991; **51**: 1600–5.
10. Brattain MG, Brattain DE, Fine WD, *et al.* Initiation of cultures of human colonic carcinoma on confluent fibroblasts. *Oncodev Biol Med* 1981; **2**: 355–67.
11. Hoosein NM, Brattain DE, McKnight MK, *et al.* Characterization of the inhibitory effects of transforming growth factor-β on a human colon carcinoma cell line. *Cancer Res* 1987; **47**: 2950–4.
12. Taylor CW, Brattain MG, Yeoman LC. Rate of growth and extent of differentiation reflected by cytoplasmic proteins and antigens of human colon tumor cell lines. *Cancer Res* 1984; **44**: 1200–5.
13. Wan CW, McKnight MK, Brattain DE, *et al.* Different epidermal growth factor growth responses and receptor levels in human colon carcinoma cell lines. *Cancer Lett.* 1988; **43**: 139–43.
14. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55–63.
15. Patterson Jr MK. Measurement of growth and viability of cells in culture. *Methods Enzymol* 1979; **58**: 141–52.
16. Norman GR, Streiner DL. Parametric statistics: statistical inference. In: *PDQ statistics.* Philadelphia: BC Decker 1986: 27–40.
17. Boyd DD, Levine AE, Brattain DE, *et al.* Comparison of growth requirements of two human intratumoral colon carcinoma cell lines in monolayer and soft agarose. *Cancer Res* 1988; **48**: 2469–74.
18. Taylor CW, Kim YS, Childress-Fields KE, *et al.* Sensitivity of nuclear c-*myc* levels and induction to differentiation inducing agents in human colon tumor cell lines. *Cancer Lett* 1992; **62**: 95–105.
19. Han Z, Chatterjee D, Wyche JH. Characterization of an 8-Cl-cAMP resistant variant of HL-60 cells. *J Cell Biol* 1991; **115**: 435a.

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