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# Growth Inhibition by 8-Chloro Cyclic AMP of Human HT29 Colorectal and ZR-75-1 Breast Carcinoma Xenografts is Associated with Selective Modulation of Protein Kinase A Isoenzymes

**A.D. Ramage, S.P. Langdon, A.A. Ritchie, D.J. Burns and W.R. Miller**

**Significant dose-related inhibition of growth of HT29 human colorectal cancer xenografts and ZR-75-1 breast cancer xenografts in immune-suppressed mice was induced by the cyclic AMP analogue, 8-chloroadenosine 3′,5′-cyclic monophosphate (8-Cl-cyclic AMP) when given by alzet mini-pumps over a 7-day period at doses of either 50 or 100 mg/kg/day. Levels and types of cyclic AMP binding proteins were measured by ligand binding and photoaffinity labelling, respectively, in tumours harvested at the end of the treatment period. Compared with levels in tumours from control animals, values of tumour cyclic AMP binding proteins from treated animals were significantly reduced. These effects were associated with an apparent modulation of the types of cyclic AMP binding proteins, 8-Cl-cyclic AMP-treated xenografts displaying a reduced ratio of RI/RII isoforms compared with untreated control tumours.**

**Key words: 8-Cl-cyclic AMP, growth inhibitors, breast/colorectal xenografts, protein kinase A**
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## INTRODUCTION

CYCLIC AMP is a ubiquitous regulatory molecule whose major actions appear to be modulated through cyclic AMP-dependent protein kinases protein kinase A [1]. Two major subtypes of the cyclic AMP-dependent protein kinases have been identified, differing in their regulatory subunits and referred to as type I (RI) and type II (RII) cyclic AMP-dependent regulatory subunits or cyclic AMP binding proteins [2, 3]. It has been suggested that the relative proportion of RI to RII expressed in cells may influence states of proliferation and differentiation [4]. Evidence has also been presented that malignant cells tend to overexpress the RI subunit at the expense of RII [5–8] and that strategies based upon redressing the balance of isoforms might reduce cellular proliferation and tumour progression [5, 9, 10]. Amongst such approaches has been the use of the cyclic AMP analogue 8-chloroadenosine 3′,5′ cyclic monophosphate (8-Cl-cyclic AMP) [11, 12]. This drug has been shown to cause growth inhibition in a range of cancer cell lines maintained in culture [8, 9, 11, 13].

The aim of the present study was to determine whether 8-Cl-cyclic AMP given *in vivo* to mice bearing xenografts of cancer cells was capable of exerting anti-tumour effects and whether these were associated with change in levels and types of cyclic AMP binding proteins.

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## MATERIALS AND METHODS

*Reagents*

8-Chloro cyclic adenosine monophosphate (sodium salt) was supplied by Dr Y.S. Cho-Chung (NIH, Bethesda, U.S.A.). 8-Chloro-adenosine was obtained from Biolog (Bremen, Germany), adenosine 3',5'-cyclic phosphate, sodium salt, from Sigma (Poole, U.K.), 5',8'-[$^3$H] adenosine 3',5'-cyclic phosphate, ammonium salt (45–56 Ci/mmol) from Radiochemical Centre (Amersham, U.K.) and 8-azidoadenosine 3',5'-cyclic [$^{32}$P] monophosphate (56–62 Ci/mmol) from ICN Radiochemicals (High Wycombe, U.K.).

*Xenografts*

Two different xenografts were studied: one derived from the HT29 colonic cancer cell line and the other derived from the ZR-75-1 breast cancer cell line. Xenografts were initiated by subcutaneous injection of $10^7$ cells into the flanks of nude mice (Harlan, U.K.). Once established, the xenografts were maintained by passage of fragments from the tumour-bearing animal to a recipient animal. The pathology of both these xenografts was consistent with that of poorly differentiated adenocarcinomas.

*Tumour growth*

Tumour fragments were implanted subcutaneously into the flank of nude mice (Harlan, U.K.) and allowed to grow to about 4–6 mm in diameter (over a period of about 1 month). Animals were then allocated to treatment or control groups, with a minimum of six animals per group. Treatment consisted of implanting alzet mini-pumps delivering for 1 week either 8-Cl-cyclic AMP (50 or 100 mg/kg/day) or vehicle (water). Tumour size was measured twice a week using calipers and the volume calculated. After 7 days of treatment tumours were harvested and stored in liquid nitrogen until the assay for level and type of cyclic AMP binding proteins.

*Cytosol preparation*

Tumours were removed from liquid nitrogen and processed on ice. Aliquots of tumour (100–200 mg) were minced with scissors and homogenised (Silverson, Chesham, U.K.) in 1:10 w/v of buffer A (20 mM Tris, 0.25 M sucrose, 2 mM magnesium chloride, 1 mM calcium chloride, 10 mM potassium chloride, 16.26 mM HCl, pH 7.5). The resulting homogenate was centrifuged for 1 h at 105 000*g* in a Beckman L7-65 Ultracentrifuge at 4°C. The supernatant was retained and used as the cytosol in cyclic AMP binding protein assays and for measurement of cyclic AMP-like material.

*Cyclic AMP binding assay*

Unless stated otherwise, cytosol (50 μl) was incubated overnight at 4°C with 5',8'-[$^3$H] cyclic AMP (100 μl, 25 nM to give a final concentration in the incubation of 10 nM) in buffer A (55 mM potassium phosphate to which 11 mM theophylline was freshly added) and 100 μl buffer B containing radioinert cyclic AMP (Sigma) at increasing final concentrations of 0, 10, 20, 40, 80, 10 000 nM. Bound and free cyclic AMP were then separated by filtration through Millipore HAWP 0.45 μm filters. Filters were washed in assay buffer C (buffer B with addition of 10 nM magnesium chloride) and transferred to counting vials containing Micellar fluor NE260 liquid scintillant (NE Technology Ltd, Edinburgh, U.K.). The vials were incubated at 37°C for 2 h and radioactivity was measured using a Tricarb liquid scintillation counter (Canberra Packard, Pangbourne, U.K.). Results were analysed according to Scatchard [14] and binding expressed as fmol cyclic AMP bound per mg cytosol protein.

*Measurement of cytosol protein*

Cytosol protein content was measured by the method of Bradford [15] using bovine serum albumin as a standard.

*Typing of cAMP binding proteins using photoaffinity labelling*

Different types of binding proteins were determined by photoaffinity labelling with 8-azidoadenosine 3',5'-cyclic [$^{32}$P] monophosphate, as adapted from Pomerantz and associates. [16]. Cytosol samples (50 μl) were prepared as described above and incubated with 8-$N_3$ [$^{32}$P] cyclic AMP (15 μl, 400 nM), 15 μl of 0.27 M morpholino-ethane sulphonic acid (Sigma) and 53 mM magnesium chloride in a 0.4-cm well microtitre plate at room temperature for 1 h in the dark. Contents of the wells were then UV irradiated for 30 s at 254 nM by placing a Mineralight UVS-11 hand lamp directly over the plate.

The reactions were stopped by the addition of sodium dodecyl sulphate (SDS) buffer (3% SDS, 15% 2-mercaptoethanol, 30 mM Tris, 30% glycerol, 1% bromophenol blue). The samples were heated to 90°C for 3 min and the proteins resolved electrophoretically on a 12% SDS–PAGE gel for 3–4 h at 35 mA, according to the method of Laemmli [17]. Radioactively labelled ($^{14}$C) molecular weight markers were run with each gel. After electrophoresis, the gels were fixed overnight in 40% methanol, 10% acetic acid, 10% glycerol and dried under vacuum in a gel drier (Model 583-Biorad, Hemel Hempstead, U.K.). The dried gels were then exposed to preflashed X-ray film (Kodak X-omat AR or Fuji) for 5–15 h at −80°C in autoradiography cassettes fitted with intensifier screens (Hi-speed X-Genetic Research International). Autoradiograms were processed in a Kodak X-ray developer and fixer.

*Quantitation of cyclic AMP binding protein types*

The photoaffinity autoradiograms were scanned by computer-assisted densitometry. All bands present were assessed and the 48 and 52 kDa binding proteins were expressed as a ratio (48:52).

*Assay for cyclic AMP-like material*

Cytosols were heated to 90°C for 3 min to denature proteins and assayed using a commercial kit for cyclic AMP (Amersham). In brief, boiled cytosol (50 μl in duplicate) was incubated for 2 h at 4°C with 8'-[$^3$H] cyclic AMP (50 μl, 18 pM) in 150 μl of Tris/EDTA buffer. Bound and free cyclic AMP were separated by adding a charcoal supernatant (100 μl) and tubes centrifuged for about 5 min. Supernatant (200 μl) was then removed for scintillation counting. The concentration of cyclic AMP-like material in the sample was then determined from a standard curve and results expressed as nmol/l. (Whilst the assay formally measures cyclic AMP, a preliminary study indicated that 8-Cl-cyclic AMP had 100% cross-reaction and the result should be regarded as measuring cyclic AMP-like material.)

## RESULTS

*8-Cl-cyclic AMP effects on tumour growth*

The effects of treatment with 8-Cl-cyclic AMP on the growth of HT29 and ZR-75-1 xenografts are shown in Figure 1. In both tumour types, 8-Cl-cyclic AMP produced a dose-related inhibition of growth over the 7-day treatment period. The reduction in tumour growth produced by 8-Cl-cyclic AMP was

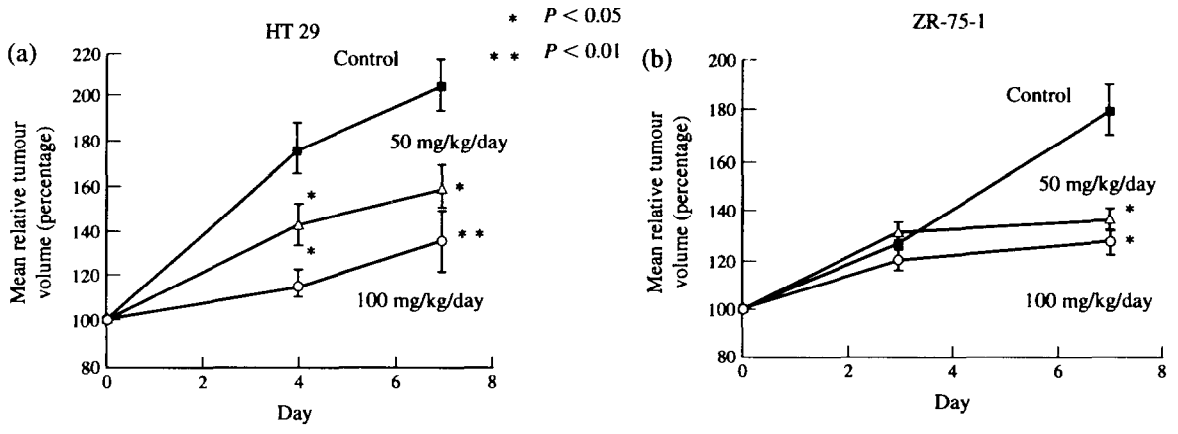


**Figure 1. The effects of treating immunocompromised mice bearing either (a) HT29 or (b) ZR-75-1 xenografts with 8-Cl-cyclic AMP or vehicle (control) on tumour size. Tumour size is expressed as a percentage of the starting volume and each point represents the mean of six xenografts. The bars are the standard errors of the means. Statistical comparisons are made with control tumours at the same time point.**

significant by the Wilcoxon rank test at the 7-day time point for both the 50 and 100 mg/kg/day doses. It should be noted, however, that even at the higher dose, both HT29 and ZR-75-1 continue to increase in size throughout treatment. The results in Figure 1 illustrate a representative experiment; similar results were obtained on at least six occasions for HT29 xenografts and four occasions for ZR-75-1 xenografts.

*Cyclic AMP binding levels in xenograft tumours*

Total cyclic AMP binding was measured in xenografts harvested from animals after 7 days of treatment with a 8-Cl-cyclic AMP. Results for HT29 are shown in Figure 2a. The mean level of binding was progressively reduced by increasing doses of the drug. Despite the overlap in values between individual tumours derived from control and treated animals, the reduction in binding was significant for both the 50 mg/kg/day ($P < 0.05$) and 100 mg/kg/day groups ($P < 0.01$). Similar determinations were performed in ZR-75-1 xenografts although only four tumours from the control and 100 mg/kg/day groups were compared. Nevertheless, as is shown in Figure 2b, all the tumours from the treated group displayed lower values than control xenografts. This difference in binding is again significant by the Wilcoxon rank test ($P < 0.05$).

*Photoaffinity labelling of cyclic AMP binding proteins*

The types of binding proteins present in tumour cytosols were determined by photoaffinity labelling with 8-azido-cyclic AMP.

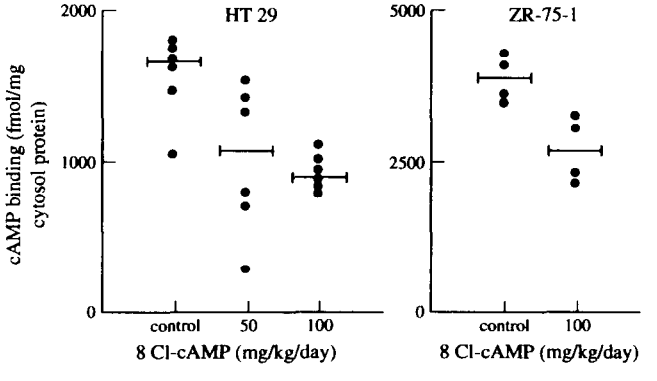


**Figure 2. Tumour cyclic AMP binding levels in xenografts treated with either vehicle or 8-Cl-cyclic AMP for 7 days. Bars represent median values.**

Results for the HT29 xenografts are shown in Figure 3. Four major proteins were identified with molecular weights of 52, 48, 43 and 37 kDa.

Treatment with 8-Cl-cyclic AMP produced a dose-related reduction in intensity of binding of all proteins. However, there was evidence of a differential effect in that the 48 kDa band appeared to be reduced more by treatment than, for example, the 52 kDa band. Scanning of the autoradiograms showed that the ratio of 48:52 kDa bands was reduced in a dose-related manner such that the 48 kDa band was not detected in tumours from the 100 mg/kg/day treated group (Table 1). Corresponding results for photoaffinity labelling of ZR-75-1 xenografts are shown in Figure 4. In contrast to HT29, five proteins were identified with molecular weights of 52, 48, 43, 39 and 37 kDa. Treatment with 8-Cl-cyclic AMP (100 mg/kg/day) produced apparent reductions in binding of each protein in three out of four xenografts. Scanning of the autoradiographs indicated that the ratio of 48:52 kDa was decreased with treatment (Table 1). The identity of the individual bands was not formally determined. However, others have suggested that the 48 and 52 kDa bands correspond, respectively, to the RI and RII regulatory subunits of protein kinase A and the 37 kDa band is a product of proteolytic cleavage.

*Cytosol levels of cyclic AMP-like material*

Cytosol from HT29 xenografts analysed for cyclic AMP binding was assayed using a radioligand binding kit for cyclic AMP. Cross-reacting material was detected in all cytosols but levels progressively increased from a mean ± S.D. value of 4.32 ± 0.87 nmol/l in control xenografts to 12.36 ± 1.20 nmol/l in xenografts from animals treated with 50 mg 8-Cl-cyclic AMP/kg/day to 19.88 ± 6.33 nmol/l in xenografts from animals treated with 100 mg 8-Cl-cyclic AMP/kg/day. Although the nature of the crossreacting material was not definitively identified, it seems likely that 8-Cl-cyclic AMP may have contributed to the values in the xenografts from drug-treated animals. To determine the effects of endogenous levels of 8-Cl-cyclic AMP on the cyclic AMP binding, cytosols from control xenografts were diluted in buffer containing 8-chloro-cyclic AMP (10 and 20 nmol/l) before assay, to cover the concentration of cross-reacting material found in xenografts from treated animals. Whilst cyclic AMP binding was decreased, the degree of effect was minimal (< 10% for 10 nmol/l and 10–15% for 20 nmol/l—data not shown).

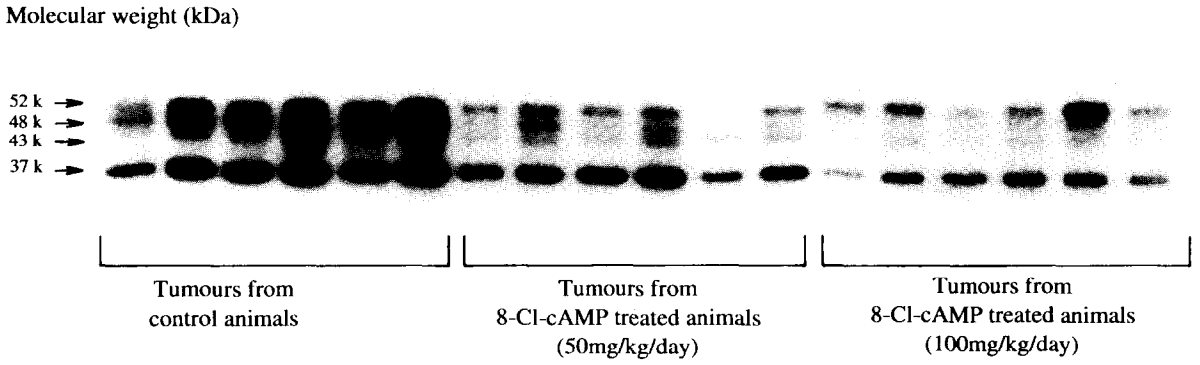


**Figure 3. Photoaffinity labelling of cyclic AMP binding proteins of tumour cytosols from HT29 xenografts treated with either vehicle or 8-Cl-cyclic AMP for 7 days. Proteins with molecular weights of 52, 48, 43 and 37 kDa were detected.**

*Table* 1. *Ratios of the* 48 *to* 52 *kDa bands measured from photoaffinity labelling (mean ± S.E.)*

| | Control | 8-Cl-cyclic AMP treatment 50 mg/kg/day | 100 mg/kg/day |
|---|---|---|---|
| HT29 | 1.6 ± 0.87 | 0.60 ± 0.20 | 0.0 ± 0.0 |
| ZR-75-1 | 9.0 ± 6.40 | 6.30 ± 5.00 | Not done |

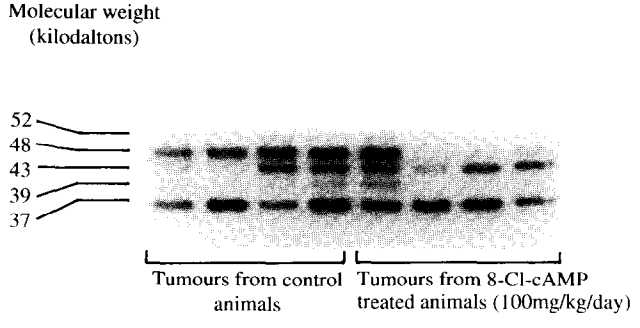


**Figure 4. Photoaffinity labelling of cyclic AMP binding proteins of tumour cytosols from ZR-75-1 xenografts treated with either vehicle or 8-Cl-cyclic AMP for 7 days. Proteins with molecular weights of 52, 48, 43, 39 and 37 kDa were detected.**

## DISCUSSION

The present investigations have shown that the cyclic AMP analogue, 8-Cl-cyclic AMP, is capable of exerting anti-tumour effects in two different types of human cancer xenografts and that in comparison with controls, treated xenografts have decreased levels and changed proportions of cyclic AMP binding proteins.

Whilst antiproliferative effects of 8-Cl-cyclic AMP have been previously reported [10, 18, 19], most studies have been performed using *in vitro* cultures and there is controversy as to whether under these conditions the action of 8-Cl-cyclic AMP is mediated by its metabolite 8-chloroadenosine produced by exposure to serum-supplemented culture media [20–23]. To our knowledge, only two previous investigations have employed *in vivo* (and arguably more physiologically relevant) systems of human tumour xenografts growing in immunocompromised mice—the studies of Ally and colleagues [8] using the lung cancer cell line LX-1 and that of Parandoosh and colleagues [24] utilising the mammary cell line MX1. Both investigations, like the present observations, demonstrate that short-term administration of 8-Cl-cyclic AMP was able to inhibit the growth of tumour xenografts, although only Ally and colleagues [8] reported tumour regression. These results, therefore, suggest that 8-Cl-cyclic AMP is capable of producing anti-tumour effects in a spectrum of solid tumours, although there may be differences in degrees of responsiveness between them.

There are two major explanations which might account for the decrease in tumour values for cyclic AMP binding observed in 8-Cl-cyclic AMP-treated xenografts—either that there is a reduction in the levels of binding proteins or that treatment results in increased levels of endogenous cyclic AMP-like material which could compete within the assay to decrease apparent binding. To explore the latter possibility, we analysed heat-denatured cytosols from control and drug-treated xenografts for endogenous competitors using a commercial assay for cyclic AMP. These results indicated that xenografts from 8-Cl-cyclic AMP-treated animals do indeed have raised levels of cyclic AMP-like material and these were related to the dose of drug administered (as the assay was unable to distinguish between cyclic AMP and 8-Cl-cyclic-AMP, analysis of unfractionated cytosols was unable to define the nature of cross-reacting material). However, the addition of these levels of 8-Cl-cyclic AMP to control cytosols, whilst marginally reducing cyclic AMP binding (5–15%) did not elicit the magnitude of effect as was observed by *in vivo* treatment (40–50%). It seems more likely that administration of 8-Cl-cyclic AMP reduces the levels of tumour cyclic-AMP binding proteins and this would be consistent with the report that treatment causes a decrease in the mRNA for certain cyclic AMP binding proteins. Interestingly, these studies suggest that 8-Cl-cyclic AMP treatment appears to reduce the expression for RI while inducing the expression of RII [25]. If these effects were translated into protein, it would account, at least in part, for the decreased ratio of 48:52 kDa proteins as detected by photoaffinity labelling in the present study. Again, we cannot totally exclude the possibility that the changes in pattern between treated and control xenografts result from different levels of competing cyclic AMP-like material in the tumour cytosols.

It is not possible from the present study to determine whether the effects of 8-Cl-cyclic AMP on xenograft growth and cyclic AMP binding are associated coincidentally or causally (or indeed, if causally related, whether the effects on binding programme for cellular proliferation or result from change in growth pattern). However, molecular manipulations in which

cells are either transfected with cDNA or treated with antisense deoxyoligonucleotides suggest that a phenotype in which type I regulatory subunit expression is reduced relative to type II (such as was seen in 8-Cl-cyclic AMP-treated xenografts) results in reduced growth rates [26–27].

Irrespective of the mechanism of action of 8-Cl-cyclic-AMP, its anti-tumour effects as evidenced in the present study are both interesting and encouraging. As a result, the drug is now being used in phase I studies as a potential therapeutic agent in patients with cancer. Results from these studies may not only lead to novel treatment regimens but also provide further insights towards controlling malignant cell growth.


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