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ORIGINAL ARTICLE

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8-Cl-adenosine is an active metabolite of 8-Cl-cAMP responsible for its in vitro antiproliferative effects on CHO mutants hypersensitive to cytostatic drugs

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Abstract 8-Cl-cAMP has been undergoing clinical trials as a potential chemotherapy agent, but there is much discussion in the literature as to whether the active agent is 8-Cl-cAMP itself, or its major metabolite, 8-Cl-adenosine. 8-Cl-cAMP is susceptible to the action of serum enzymes such as phosphodiesterases, and its metabolism when administered to cancer patients raises questions as to the mechanism of action of 8-Cl-cAMP. The stability of 8-Cl-cAMP when incubated with serum, and the effects of both 8-Cl-cAMP and 8-Cl-adenosine on the proliferation of variant lines of CHO cells hypersensitive to 8-Cl-cAMP were investigated. A solid-phase extraction (SPE) purification protocol and the HPLC method previously developed were used to determine 8-Cl-cAMP and 8-Cl-adenosine. Heat treatment of serum inactivated the enzymes in the culture medium responsible for activating 8-Cl-cAMP. Under these conditions 8-Cl-cAMP remained stable and there were no traces of its metabolite, 8-Cl-adenosine. Cell culture experiments showed that 8-Cl-cAMP only affected cell growth in medium that contained untreated serum. In contrast, 8-Cl-adenosine was shown to be growth inhibitory in medium containing either heat-treated or untreated serum. HPLC analysis of the culture medium from the cell culture experiments supported the hypothesis that 8-Cl-cAMP was only effective in inhibiting cell growth after metabolism to 8-Cl-adenosine. Thus further studies of this drug and its mechanism of action should focus on 8-Cl-adenosine.

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Introduction

The target of the second messenger cyclic 3′,5′-adenosine monophosphate (cAMP) is cAMP- dependent protein kinase (PKA) that is able to promote the phosphorylation of a wide range of substrates. Reversible protein phosphorylation plays a vital role in the control of cellular processes that are essential for normal cell growth and viability (Shenolikar 1988). The interplay between second messenger pathways illustrates the cooperation between the regulatory systems to coordinate and integrate the multiple cellular signals. Since PKA plays a pivotal role in cell growth regulation, it has been implicated in the proliferation of tumour cells (Cho-Chung 1990). Analogues of cAMP modify the activity of PKA and have shown antitumour effects in vitro (Ally et al. 1989). The analogue 8-Cl-cAMP has undergone phase I clinical trials as a chemotherapy agent (Propper et al. 1999; Tortora et al. 1995).

The structure of PKA has been described by Corbin et al. (1982) and at least two isozymes of PKA, called PKAI and PKAII, are known to exist (Ally et al. 1988; Foss et al. 1994). cAMP analogues modified at C-8 on the adenine ring are generally selective for site 1 of the two cAMP binding sites on the PKA regulatory (R) subunits (Cho-Chung 1988; Tortora et al. 1988) and 8-Cl-cAMP selectively binds to site 1 of RIIβ PKA (Cho-Chung 1988; Tortora et al. 1989).

A number of studies have shown that cancer cells exhibit elevated levels of PKAI (Bradbury et al. 1990; Nakajima et al. 1984; North et al. 1994; Rohlff et al. 1993), so investigations into ways of modifying the activity of the PKAs by using cAMP analogues have been undertaken (Ally et al. 1988, 1989; Cho-Chung et al. 1991). These have shown that 8-chloro substitution of the adenine ring of cAMP causes the greatest growth inhibition in a range of human cancer cell lines and that the growth inhibition is not due to increased cellular

cAMP levels (Cho-Chung 1989, 1991; Cho-Chung et al. 1989). The analogues elicit their inhibitory effect at low concentrations, for example 1–30 μM for 8-Cl-cAMP. Also, the effect of 8-Cl-cAMP on growth inhibition appears to be selective towards transformed cancer cells rather than non-transformed cells (Cho-Chung 1989; Cho-Chung et al. 1989; Tagliaferri et al. 1988).

The PKAI (RIα) regulatory subunit has been implicated in cell proliferation and transformation, and the PKAII (RIIβ) subunit in inhibition of cell growth (Cho-Chung 1990; Cho-Chung and Clair 1993; Cho-Chung et al. 1991; Tortora et al. 1988). These authors proposed that the potency of 8-Cl-cAMP in the regulation of cell proliferation and growth depends on a perturbation of the functional balance between RIα and RIIβ protein subunits.

An alternative hypothesis put forward to explain the observed growth-inhibitory effect of 8-Cl-cAMP on cells in culture is that the active agent is not 8-Cl-cAMP, but its major metabolite, 8-Cl-adenosine, that results from the action of serum phosphodiesterases and 5′ nucleotidases (Kessin et al. 1991; Lange-Carter et al. 1993; Van Lookeren Campagne et al. 1991). 8-Cl-cAMP is metabolized extracellularly to 8-Cl-adenosine by phosphodiesterases present in the growth medium used to culture cells. Dipyridamole, which is an inhibitor of nucleoside uptake by cells, can protect cancer cells from growth inhibition by 8-Cl-cAMP. Therefore the site of 8-Cl-adenosine action is likely to be inside the cell. In this case, the mode of action of 8-Cl-cAMP on different cell lines will depend on (1) the quantity and nature of serum components of cell culture medium, (2) the nucleoside uptake system, (3) the permeability of the cells to 8-Cl-cAMP, and (4) the intracellular pathway of 8-Cl-adenosine and its toxicity to the cell. Therefore the mechanism of action by which 8-Cl-cAMP can bring about growth inhibition of cells in tissue culture may well vary widely (Van Lookeren Campagne et al. 1991). Since 8-Cl-cAMP is undergoing further clinical trials, it is necessary to understand its mechanism of action in more detail.

It is clear that there is a great deal of uncertainty over the mechanism by which 8-Cl-cAMP exerts its effect on the growth of tumour cells. To address this problem, we established the stability of 8-Cl-cAMP in cell growth medium under the conditions used in cell culture experiments. We found that enzymes present in the serum component of the culture medium metabolize 8-Cl-cAMP to 8-Cl-adenosine. We therefore designed a quantitative cell culture system for distinguishing between the activity of 8-Cl-cAMP and 8-Cl-adenosine on cells in culture by inactivating the enzymes that break down 8-Cl-cAMP. Then we analysed the role of 8-Cl-adenosine versus 8-Cl-cAMP using a panel of CHO cells that we had previously isolated as hypersensitive to cytostatic drugs (Davies et al. 1989), and that were found to be cross-hypersensitive to 8-Cl-cAMP. Our results showed that metabolism of 8-Cl-cAMP to 8-Cl-adenosine was needed for antiproliferative activity and that the CHO mutants were hypersensitive to 8-Cl-adenosine compared with the wild type. Since 8-Cl-cAMP is undergoing further clinical development, it may be more appropriate to use the active metabolite.

Materials and methods

Cell culture

Cell lines CHO K1, ADR4 and ADR5, and 8-Cl-cAMP were supplied by the Imperial Cancer Research Fund Clinical Oncology Laboratory, The Institute of Molecular Medicine, Oxford, UK. ADR4 and ADR5 are among five CHO mutants that have previously been derived from the parent K1 cell line by one-step mutagenesis (Davies 1992). The mutants were isolated on the basis of their hypersensitivity to Adriamycin and actinomycin D, both of which had been shown to act as topoisomerase II inhibitors. ADR4 and ADR5 have also been found to be sensitive to 8-Br-cAMP. For this reason they were selected for further study using the apparently more potent cAMP analogue, 8-Cl-cAMP. North et al. (1994) have shown that ADR5 cells overexpress RIα PKA protein, a feature exhibited by tumour cells (Cho-Chung 1990; Cho-Chung and Clair 1993; Cho-Chung et al. 1991; Tortora et al. 1988), making ADR5 a suitable model for comparing the susceptibility of normal and transformed cells to 8-Cl-cAMP and 8-Cl-adenosine.

Ham's F10 medium, fetal calf serum (FCS), newborn calf serum (NCS), phosphate-buffered saline (PBSA) were from Life Technologies, Paisley, UK; 8-Cl-adenosine was from Biolog, Bremen, Germany; 8-Cl-cAMP was from Dr. K. Miki, Tonen Corp, Kanagawa, Japan; and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was from Sigma, Poole, UK.

Cells were grown in Ham's F10 medium containing 5% FCS and 5% NCS in 15-cm culture dishes. The plates were incubated at 37°C in an atmosphere containing 5% carbon dioxide, and the cells were divided three times a week. Experiments were conducted using cells grown in 96-well plates at a seeding density of 500 cells/well for CHOK1 and 750 cells/well for ADR4 and ADR5. Cell survival was determined using the MTT assay for viable cells (Carmichael et al. 1987; Mosmann 1983; Plumb et al. 1989). The intensity of colour developed from MTT was determined using an automatic plate reader that reported means and standard deviations (Titertek Multiscan ELISA MK II, with Deltasoft Software; Biometallics, Princeton, N.J.).

Two drug treatments were used in the cell culture experiments: a 24-h exposure and a 4-day exposure. In both cases the drug was administered to the cells 3 days after setting up the 96-well plates. After 24 h the drug and medium were removed from wells of half of the plates, and the well contents for replicate treatments were pooled and saved for chemical analysis. The cells for the 24-h treatment were replenished with fresh growth medium and allowed to grow on for the remaining 3 days of the test. Meanwhile the cells in the other plates were left to grow for 4 days in wells containing the drug. The medium from these wells was harvested and stored for HPLC analysis immediately prior to applying the MTT assay to this treatment.

HPLC

Samples for HPLC analysis were first purified by solid-phase extraction (SPE) minicolumns using Bond-Elut SAX for 8-Cl-cAMP and Bond-Elut C-8 for 8-Cl-adenosine after the method by Cummings (1994) as modified by Robbins (1995) (Jones Chromatography, Pontypridd, UK). The previously developed HPLC system (Robbins 1995) was used to separate and quantify 8-Cl-cAMP and 8-Cl-adenosine using a reversed phase C-18 column (Lichrospher 100 RP18, Merck, Lutterworth, UK) and isocratic elution with mobile phase (pH 3.5) comprising 0.1 M KH_2PO_4, 0.52 mM tetrabutyl ammoniumhydrogensulphate and 12% methanol.

Results

8-Cl-cAMP stability in medium with or without serum

Initial investigations were carried out to establish the stability of 8-Cl-cAMP in the normal growth medium under the conditions used to culture the CHO cells. SPE and HPLC were used to quantify the 8-Cl-cAMP and 8-Cl-adenosine. Controls using 10 μ*M* 8-Cl-cAMP incubated in (a) PBSA or (b) Ham's F10 medium without serum showed it to be completely stable for 24 h. The treatment with 10% serum in Ham's F10 medium showed a 25% loss of 8-Cl-cAMP and concurrent appearance of 8-Cl-adenosine over the same time scale (data not shown). Other stability tests on 8-Cl-cAMP showed that it remained stable in PBSA at 37°C for at least 1 month (data not shown).

Heat treatment of the serum at 58°C for 1 h before addition to the growth medium showed little breakdown of 8-Cl-cAMP after 5 days incubation in medium containing heat-treated serum (Fig. 1).

Growth of cells in heat-inactivated serum and effects of 8-Cl-cAMP

Growth curves for the three cell lines cultured in 96-well plates showed that cells from all cell lines grew as well in medium containing heat-treated serum as in untreated serum, and the wild-type cells grew better than the two mutants.

Dose response curves to 8-Cl-cAMP were determined for both the 24-h and 4-day exposures for the three cell lines, CHO K1, ADR4 and ADR5 (Figs. 2 and 3). There were greater differences between the cell line sensitivities with the shorter exposure of 24 h and ADR5 was the more sensitive of the two derived cell lines.

Comparison of 8-Cl-adenosine and 8-Cl-cAMP

When cells were grown in untreated serum there was no difference in sensitivity between 8-Cl-cAMP and 8-Cl-adenosine. However, when the serum was heat treated, no growth inhibition by 8-Cl-cAMP was evident. Heat treatment of serum had no effect on growth inhibition by 8-Cl-adenosine. This effect was seen for all three cell lines with both the 24-h exposure (Fig. 2) and the 4-day exposure (Fig. 3).

HPLC analysis of the spent growth medium from the cell experiments at the end of the 24-h drug exposure or after the 4-day exposure showed that after 24 h 8-Cl-cAMP had begun to breakdown to 8-Cl-adenosine, and this was more pronounced after 4 days (Fig. 4). The HPLC data demonstrated that heat treatment of serum prior to adding it to growth medium prevented the breakdown of 8-Cl-cAMP during the cell culture experiments.

IC_{50} values for 8-Cl-adenosine were determined for the 24-h and 4-day drug exposures. For the 24-h treatment the IC_{50} of 8-Cl-adenosine was 17 ± 2.91 μ*M* for ADR5, 40 ± 3.91 μ*M* for ADR4 and 360 ± 2.5 μ*M* for CHO K1. For the 4-day treatment the IC_{50} values were 8 ± 1.25 μ*M* for ADR5, 14 ± 1.27 μ*M* for ADR4 and 15 ± 2.14 μ*M* for CHO K1. Therefore only a small breakdown in 8-Cl-cAMP, for example 5%, would have a significant effect on the cells in culture.

Discussion

8-Cl-cAMP has aroused a considerable amount of interest as a prospective anticancer agent, and investigations into its mode of action have led to the proposal of a variety of theories to explain the observed effects (Cho-Chung 1990; Cho-Chung and Clair 1993; Cho-Chung et al. 1991; Kessin et al. 1991; Lange-Carter et al. 1993; Langeveld et al. 1992; Tortora et al. 1988; Van Lookeren Campagne et al. 1991). Our studies showed that over the

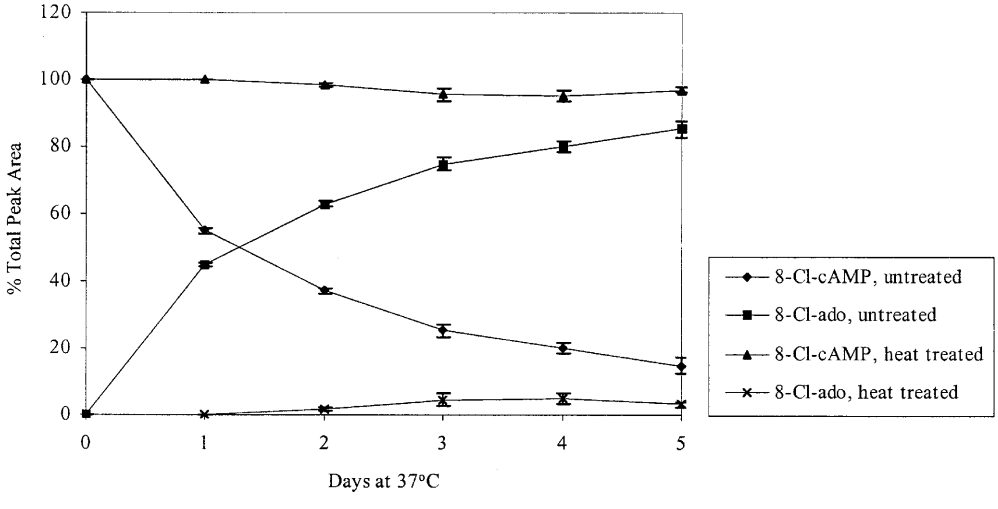

Fig. 1 Stability of 10 μ*M* 8-Cl-cAMP in cell growth medium at 37°C showing breakdown in the medium containing untreated serum, and the concurrent appearance of 8-Cl-adenosine. 8-Cl-cAMP remained stable in the medium when the serum had been heat treated at 58°C for 1 h. The mean values and standard errors from three independent experiments each with three replicates are shown

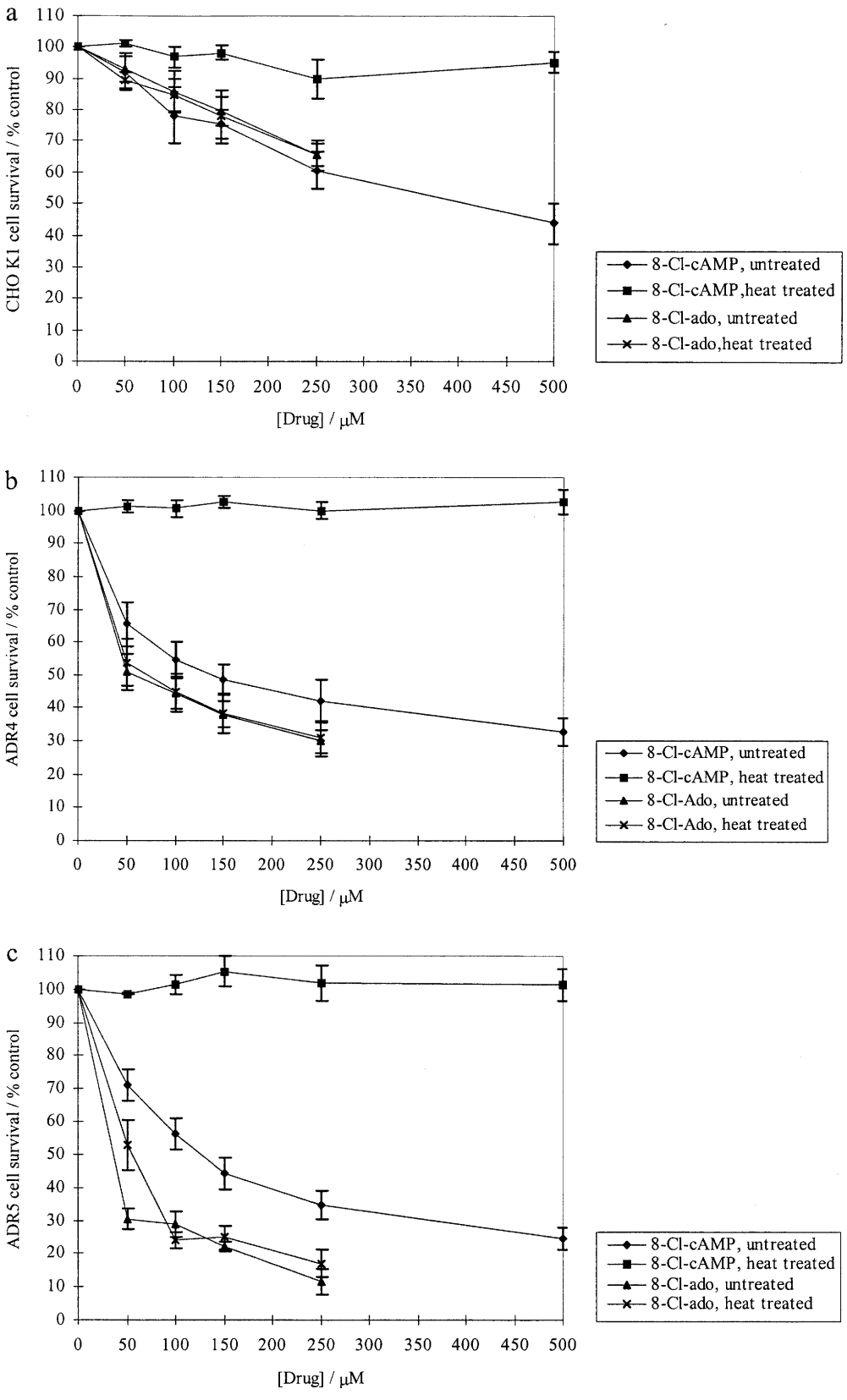

Fig. 2a–c Effects of a 24-h exposure of (**a**) CHO K1, (**b**) ADR4 and (**c**) ADR5 cells to 8-Cl-cAMP and 8-Cl-adenosine in medium containing either untreated or heat-treated serum. The mean values and standard errors from three independent experiments each with eight replicates are shown

course of 5 days, 80% of the 8-Cl-cAMP was broken down to 8-Cl-adenosine. HPLC analysis provided a sensitive quantitative method of analysing the growth medium for both 8-Cl-cAMP and 8-Cl-adenosine. The observed metabolic breakdown of 8-Cl-cAMP was attributed to phosphodiesterases and 5′-nucleotidases

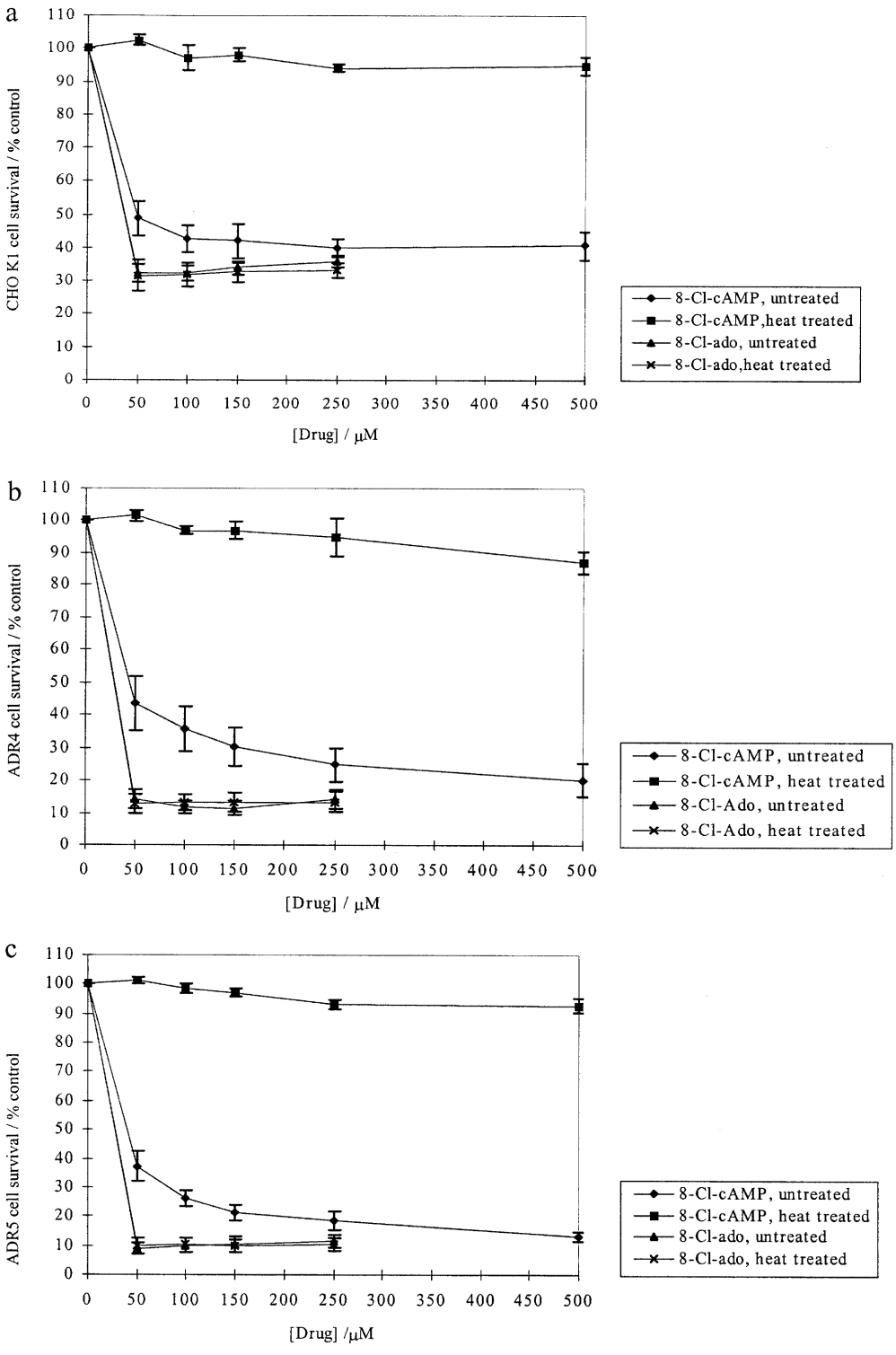

Fig. 3a–c Effects of a 4-day exposure of (**a**) CHO K1, (**b**) ADR4 and (**c**) ADR5 cells to 8-Cl-cAMP and 8-Cl-adenosine in medium containing either untreated or heat-treated serum. The mean values and standard errors from three independent experiments each with eight replicates are shown

present in the serum contained in the growth medium and heat treating the serum before addition to the medium was an effective way of denaturing these enzymes.

The ADR4 and ADR5 cell lines used in this study were mutants derived from the CHO K1 parent cell line and had previously been selected for their hypersensitivity to topoisomerase II inhibitors (North et al. 1994). They were also hypersensitive to 8-Cl-cAMP, and ADR5 exhibited elevated PKA RIα protein levels compared with ADR4 and the parent cell line. This made ADR5 suitable as a model for tumour cells in comparison with ADR4 and CHO K1 in investigations using 8-Cl-cAMP. Tests using 8-Cl-cAMP or 8-Cl-adenosine showed ADR5 to be more sensitive than CHO K1 as expected, and that growth inhibition by 8-Cl-cAMP for all three cell lines could be completely abolished by heat

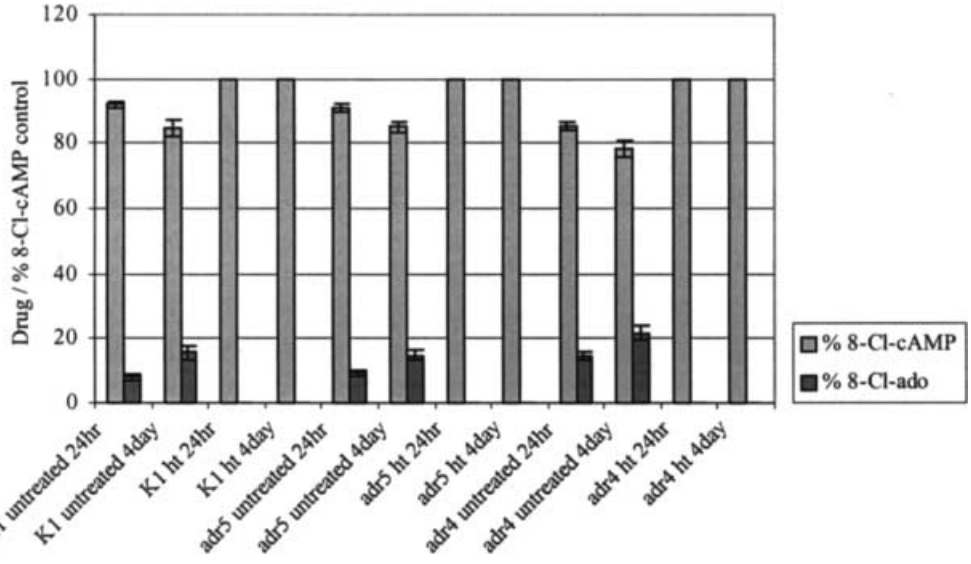

Fig. 4 HPLC analysis of growth medium from CHO K1, ADR4 and ADR5 cells treated with 8-Cl-cAMP, showing the percentage breakdown of the drug to 8-Cl-adenosine (*8-Cl-ado*) (*ht* heat-treated serum in the medium). The mean values and standard errors from three independent experiments per cell line, each with nine replicates are shown

treating the serum in the growth medium. Thus the observed growth inhibition by 8-Cl-cAMP in medium containing untreated serum could be attributed to the presence of 8-Cl-adenosine.

For the 24-h drug treatment, although 50–500 μ*M* 8-Cl-cAMP and no 8-Cl-adenosine was initially in the medium, the HPLC data showed that over the time course of the study at least 5% of the 8-Cl-cAMP had been converted to 8-Cl-adenosine, giving a concentration range of 2.5–25 μ*M* of 8-Cl-adenosine. This was comparable with the observed IC_{50} of 17 μ*M* for the 24-h exposure of ADR5 cells and would account for the observed inhibition of cell growth. Similarly, HPLC data for the 4-day treatment showed a depletion in 8-Cl-cAMP by 15%, with a concurrent appearance of 7.5–75 μ*M* 8-Cl-adenosine, comparable with the observed IC_{50} of 8-Cl-adenosine of 8 μ*M* for ADR5, 14 μ*M* for ADR4 and 15 μ*M* for CHO K1 cells.

It is therefore evident that 8-Cl-adenosine is the active agent in inhibiting cell growth in untreated growth medium when cells have been treated with 8-Cl-cAMP. Recently, other workers have also provided evidence to support this hypothesis. Juranic et al. (1998) have shown that heat treating various sera used in growth media depresses the action of 8-Cl-cAMP on three mammalian cell lines. Halgren et al. (1998), using multiple myeloma cell lines, have found that 8-Cl-cAMP is metabolized extracellularly to 8-Cl-adenosine by phosphodiesterases and 5′-nucleotidases, and that the 8-Cl-adenosine has to enter the cells in order to exert cytotoxicity. They found that the presence of a nucleoside uptake inhibitor reduces the cytotoxicity of both 8-Cl-cAMP and 8-Cl-adenosine, and their investigations indicate that 8-Cl-adenosine is likely to be causing apoptotic cell death. The same group has recently reported (Gandhi et al. 2001) that treating multiple myeloma cells with 8-Cl-cAMP leads to its extracellular metabolism to 8-Cl-adenosine that then enters the cells, is phosphorylated by adenylate kinase, and accumulates in the cells as 8-Cl-ATP. They also found (Gandhi et al. 2001) that the concentration of intracellular ATP falls as the level of 8-Cl-ATP rises, and that RNA synthesis is inhibited, though DNA synthesis is largely unaffected. They suggested that the inhibition of RNA synthesis may trigger apoptosis in multiple myeloma cells, either by inhibiting RNA polymerases or by incorporation into RNA as 8-Cl-ATP. The inhibition of RNA, rather than DNA, synthesis may be explained by the relatively long doubling time (72 h) for multiple myeloma cells, with 72% of the cells in G_1 phase of the cell cycle at any one time. This would imply that the cells are involved in protein synthesis rather than DNA replication, hence the pronounced effect on RNA production. Gandhi et al. (2001) concluded that for multiple myeloma cells, 8-Cl-ATP is the intracellular cytotoxic metabolite of 8-Cl-cAMP.

Investigations into the stability of 10 μ*M* 8-Cl-cAMP incubated in 10% human plasma has shown phosphodiesterase and 5′-nucleotidase activity, with 5% breakdown of the drug over a 5-day incubation (data not shown). Although this metabolism of 8-Cl-cAMP is considerably less than in bovine serum, stability studies using 100 μ*M* 8-Cl-cAMP incubated in undiluted human plasma showed conversion of 40% of the drug to 8-Cl-adenosine over 5 days at 37°C (data not shown). This activity should be taken into consideration when conducting drug trials using 8-Cl-cAMP with patients. However, the steady-state concentrations of 8-Cl-cAMP found in phase I trials (Cummings et al. 1996) have been found to be very low, and turnover time too fast to allow extensive metabolism to 8-Cl-adenosine. The demonstration of 20-fold hypersensitivity of the CHO cell lines ADR5 and ADR4 to 8-Cl-adenosine suggests that they will be useful in elucidating the mechanism of action of the drug. The ADR5 cell line differs from the ADR4 cell line because it has elevated RIα expression. That there is no differential effect of 8-Cl-adenosine on ADR4/ADR5 makes it unlikely that this compound has effects through relative RII/RI levels. Since ADR4 and ADR5 fall into

two different complementation groups there are clearly multiple mechanisms of 8-Cl-adenosine action.

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