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ORIGINAL ARTICLE

Cytogenetic effects of 8-Cl-cAMP on human and animal chromosomes

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Summary

Purpose: *To assess the cytogenetic effects in vitro and in vivo of a non-cytotoxic antitumor agent with biomodulator activity, 8-chloro-3',5'cyclic adenosine monophosphate (8-Cl-cAMP).*

Materials and methods: *Cytogenetic effects of 8-Cl-cAMP where evaluated using the in vitro chromosome cytogenetic assay (CA) on human peripheral blood lymphocytes of healthy individuals and by bone marrow micronucleus assay in adult BALB/c mice.*

Results: *In the in vitro chromosome CA, 8-Cl-cAMP (in all respective doses; 1.5 and 15 μm) induced mitotic inhibition and premature centromere separation (PCS) but no chromosomal damage in cultured human peripheral blood lymphocytes. In the in vivo test, single intraperitoneal (i.p.) injection of 8-Cl-cAMP in doses of 10, 80 and 150 mg/kg showed a dose-related effect on the frequency of micronuclei, detected in murine polychromatic erythrocytes (PCE).*

Conclusion: *The results of the present study show that genotoxicity of 8-Cl-cAMP has a different matrix of response when comparing results in vitro and in vivo, suggesting that high metabolic activity in vivo is responsible for the clastogenic potential of 8-Cl-cAMP. These comparative results indicate a need of having an available battery of genotoxic tests in order to evaluate possible cytogenetic effects of novel antitumor agents.*

Key words: 8-Cl-cAMP, antitumor agents, micronuclei, premature centromere separation

Introduction

Interest on antitumor agents' genetic toxicity has increased over the past years due to better patient treatments, combinational therapies and increased survival rate vs. higher incidence of secondary tumors after primary therapies and genetic defects due to mutagenic activity on gonadal cells [1-3].

8-Cl-cAMP is a antitumor agent synthesized more than 2 decades ago, based on a strategy to reduce toxicity to normal cells and increase specificity towards cancer cells by modulating the cells' signaling pathways [4,5]. cAMP is implicated in the regulation of a variety of cell functions. The actions of cAMP and cAMP analogues are supposed to be mediated by the cAMP-dependent protein kinase protein kinase A (PKA). The differential activity of 8-Cl-cAMP towards protein kinase isozymes expresses inhibition of cell growth, differentiation and neoplastic reversion of a wide variety of cancer cell lines [5,6].

PKA is an enzyme in mammalian cells, which is present in two isoforms: PKA I and PKA II [5-7]. Structurally, these isoforms are composed from catalytic and regulatory subunits. Every isoform in its composition has two types of regulatory units by which it can be differentially distinguished: regulatory subunit (R) I α and R I β, i.e. R I α and R I β go into the R I isoform and R II α and R II β go into the R II isoform [7,8]. The occurrence of R I and R II types of regulatory units differentiate by the phase of the cell cycle, and between cells of different tissues. Still, most abundant are the R I α and R II β subunits [7,8].

The isoforms of PKA can be also differentiated by their affinity towards cAMP, subcellular distribution and function [8]. On every regulatory subunit there are 2 places for cAMP attachment, the attachment place A

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and the attachment place B. 8-Cl-cAMP as a specific cAMP analogue is attached on both places (A and B), the same as cAMP, but with different affinity. With the strongest affinity 8-Cl-cAMP is attached to place B on the R II subunit and for place A with the weakest. On the other hand 8-Cl-cAMP has a high affinity towards place A on the R I subunit and moderate affinity for place B. In micromolar concentrations 8-Cl-cAMP is connected only on the B place of the R II subunit, so that PKA II stays in the form of a holoenzyme, which, by a negative biofeedback pool, brings a decrease in the synthesis of PKA I. The antiproliferative activity of 8-Cl-cAMP is based on the decrease or downstream regulation of PKA I in malignant transformed cells [8-10].

In vivo 8-Cl-cAMP as a sole therapy has shown a good antitumor effect on xenografts of colorectal carcinoma, breast, ovarian and pancreas carcinoma, and in combination therapy it has shown that it can increase the antiproliferative capacity and antitumor activity of taxanes and cisplatin analogues [11-15].

The rationale for combination therapy comes from the overall knowledge of the mechanisms from individual action of these substances, i.e. 8-Cl-cAMP and taxanes in which a crucial role in the synergistic action plays PKA. Taxanes are known for their genotoxic potential [16], but cAMP analogues have been relatively underrepresented for their clastogenic potential in human and animal cells.

The aim of this study was to contribute to the characterization of the genetic toxicity of 8-Cl-cAMP by studying chromosomal damage in human lymphocytes and in the bone marrow of BALB/c mice. Chromosomal aberrations are direct indications of genetic damage by various xenobiotics including antitumor agents. Therefore, the two cytogenetic endpoints, chromosomal aberration test *in vitro* and micronuclei (MN) test in polychromatic erythrocytes (PCE) of mice *in vivo* serve as excellent markers to assess the genotoxic potential of 8-Cl-cAMP.

Materials and methods

Chemicals

For the cytogenetic analysis during the last 2 h of incubation colcemide was added to the medium in a concentration of 0.05 μg/ml. For the cytogenetic assay, the investigated substance 8-Cl-cAMP (Sigma Chemical Co., St. Louis, MO, USA; CAS No 41941-56-4) in doses of 1, 5 and 15 μM was added 40 h after the incubation and stayed in culture to the end of cultivation (72 h). A known clastogen, mitomycin C (Sigma Chemical Co., St. Louis, MO, USA; CAS No 50-07-7), was used as positive control at a dose of 0.15 μM using the same protocol as for 8-Cl-cAMP.

Chromosome aberration analysis in human lymphocytes in vitro

Metaphase chromosome analysis for the detection of chromosomal aberrations (CAs) was performed according to conventional techniques [17]. Human peripheral blood lymphocytes (PBLs 2×10^6 cells) obtained from 10 healthy donors were isolated by density gradient, cultured in 10 ml RPMI tissue culture medium (Gibco, Grand Island, NY, USA) containing 20% fetal bovine serum (inactivated), 1% phytohaemagglutinin, glutamine 1 mM and 500 IU of penicillin and streptomycin incubated at 37° C and 5% CO_2 for 72 h. After 48 h of incubation, the cultures were treated with 8-Cl-cAMP dissolved in PBS. The dose levels of 8-Cl-cAMP were 1, 5 and 15 μM. The dose levels were estimated by the highest tolerant dose (15 μM) in phase I clinical studies [18]. The positive control substance was mitomycin C at a dose of 0.15 μM. The lymphocyte cultures were treated with 8-Cl-cAMP for 24 h. Chromosomes were prepared for observation according to the conventional hypotonic –Giemsa schedule. Scoring was done by a single observer with randomized slides. CAs were scored in 200 metaphases per sample according to the classification criteria suggested by Savage (1976) [19]. All samples where pooled and the mean value was analyzed. The number of aberrant metaphases, referred to the text as damaged cells, and the number of CAs were counted.

Micronuclei analysis

The investigated substance was tested at 3 experimental concentrations. The lowest concentration should correspond to its level in the environment, but, as this is a synthetic substance, the level of 10 mg/kg of body weight (b.w.) was taken on the basis of postnatal growth toxicity induced by 8-Cl-cAMP [20]. The median concentration was established from the maximal tolerance dose, a dose which shows clinical manifestation of mild toxicity (loss of weight, diarrhea, ataxia, somnolence). The high concentration dose is a sublethal dose of 8-Cl-cAMP (150 mg/kg b.w.) based on preliminary toxicological investigation (acute toxicity) on BALB/c mice. Experimental design for both *in vivo* tests included 3 groups: positive control, negative control and experimental groups. The experimental groups were divided into 3 subgroups based on the chosen doses of 8-Cl-cAMP.

The negative control group was treated with normal saline solution. A known mutagen, cyclophosphamide,

at a dose of 60 mg/kg b.w. was used for the positive control group because of its known clastogenic and mutagenic properties [21]. Cyclophosphamide and 8-Cl-cAMP were dissolved in normal saline solution immediately prior to i.p. administration and the volume injected was 0.1 ml/kg/b.w. All groups had equivalent numbers of animals per test. For the MN test 8 animals were used per dose/group (both sexes). We used BALB/c mice of 6 weeks of age with an average weight of 19±2 g. The animals were kept under uniform conditions, housed under 12/12 h photoperiod at constant temperature (21° C) with free access to standard laboratory chow and water. Mice were sacrificed 24 h after single i.p. treatment, in accordance to the EEC convention guidelines. The frequency of MN in the bone marrow was detected according to the classical technique by Schmid and Tinwell [22,23]. Femoral cells were flushed out with fetal calf serum (Gibco, UK). The collected cells were sedimented by centrifugation (1800 rpm) and resuspended in fetal calf serum on microscopic slides. Before cells were fixed in methanol for 15 min they were air-dried and then dipped in phosphate buffer, pH 6.4. Slides were stained in a solution of acridic orange (12.5 mg/100 ml buffer) for 60 sec [23], were exposed to buffer for 10 min and then observed for the presence of MN in PCE. Data were summarized as the mean number of micronucleated PCE per 1000 PCE. In addition, the ratio between PCE and normochromatic erythrocytes (NCE) was also determined.

Statistical analysis

The clastogenic action of 8-Cl-cAMP was analyzed by using the analysis of variance (one way) and the x^2 test.

Results

Table 1 shows the distribution of total aberrations and related changes in the frequency of aberrant cells induced by doses of 1.5 and 15 μM of 8-Cl-cAMP. Results in Table 1 show that 8-Cl-cAMP didn't induce a statistically significant difference in total CAs and in different chromatid type aberrations in PBLs when compared with the negative control group.

Table 2 shows results concerning the occurrence of premature centromere separation (PCS) in PBLs induced by increasing doses of 8-Cl-cAMP. PCS is not a classical parameter of xenobiotic clastogenicity, but it can lead to chromosome instability and aneuploidy. To address these findings we estimated the frequency of PCS occurrence on one chromosome, more than one, and on all chromosomes (Figure 1).

In Table 2 PCS occurrence is more determined by its distribution pattern than by the percentage of total metaphases with PCS. The highest dose of 8-Cl-cAMP (15 μM) showed a shift from more than one chromosome to be affected by PCS to the group where all chromosomes are affected by PCS. Still, the overall presence of PCS in the 15 μM group is decreased compared with the 5 μM group, meaning that increasing doses of 8-Cl-cAMP and the occurrence of PCS is a specific phenomenon concentrated to the centromere region of the affected chromosomes and not to 8-Cl-cAMP cytotoxicity.

Table 1. Presentation of different chromatide type aberrations in peripheral blood lymphocytes of control groups and cells treated with increasing doses of 8-Cl-cAMP

Concentration	*Total no. of cells analyzed (pooled)*	*Chromatid breaks*		*Gaps*		*No. of iso-chromatid breaks*		*Total no. of aberrant cells*		*Mitotic index*	
		No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*	*No./SD**	*%*
Negative control (A)	2013	12	0.59	21	1	7	0.35	40	1.98	92±7	9.2
1 μM 8-Cl-cAMP (B)	2005	13	0.64	21	1	7	0.35	41	2	30±5.3	3*
5 μM 8-Cl-cAMP (C)	2012	14	0.7	23	1.14	6	0.30	43	2.1	17±3.1	1.6*
15 μM 8-Cl-cAMP (D)	2000	15	0.75	28	28	9	0.45	52	2.6	7,5±2.2	0.7*
Positive control (E) MMC 0.15 μM	2000	76*	3.8	152*	7.6*	86*	4.3	314*	15.7	2±1.7	0.1*

(A:B:C:D) without significant difference (NS)(A/B/C/D:E); *p <0.001, highly statistically significant difference of the negative control and experimental groups compared to positive control (x^2 test); The mitotic index represents the mean number of cells gone into mitosis based on 1000 cells scored per sample/subject; SD: standard deviation; MMC: mitomycin C

Table 2. Comparative estimation of premature centromere separation (PCS) induction in control and experimental groups treated with increasing doses of 8-Cl-cAMP

Concentration (μM)	*Average no. of analyzed mitoses*		*PCS induced on less than 2 chromosomes*		*PCS induced on more than 2 chromosomes*		*PCS induced on all chromosomes*		*Overall number of mitoses with PCS*	
	X±SD *(pooled)*	%	X±SD	%	X±SD	%	X±SD	%	X±SD	%
Negative control (K)	121±9.7	100	–	–	–	–	2±1.41	1.65	2±1.41	1.65
1 μM (A) 8-Cl-cAMP	110±10.2	100	23.7±2.8	21.5	10.1±1.2	9.2	0.2±0.4	0.18	33.8±3.3	31*
5 μM (B) 8-Cl-cAMP	125±10.5	100	41±3.2	32.8	19.4±2.2	15.5	/	/	60.5±4.6	48.4*
15 μM (C) 8-Cl-cAMP	115±9.5	100	23±2.6	20	10±1.91	8.7	0.5±0.5	0.4	33.5±4.2	29.1*

*indicates statistically significant difference, $p < 0.001$ (Student's t-test was used for statistical analysis of the overall number of mitoses with PCS)
K:A* K:B* K:C*
A:B=* B:C=* A:C = non significant
SD: standard deviation

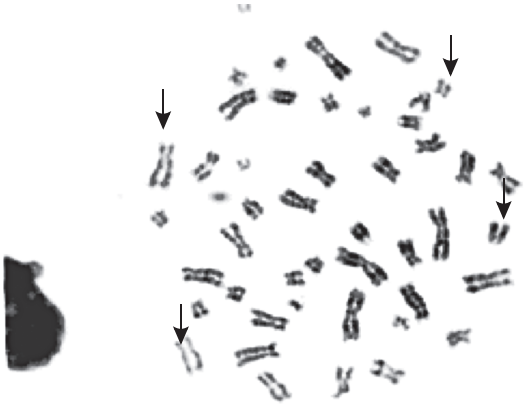

Figure 1. Metaphase nuclei showing premature separation in more than 2 chromosomes (arrows) induced by 8-Cl-cAMP at a dose of 5 μM.

In contrast to partially negative results in the CA test *in vitro*, the administration of 8-Cl-cAMP *in vivo* showed an ability to induce MN in PCE of bone marrow of BALB/c mice (Table 3). Statistical analysis demonstrated a statistically highly significant difference ($p < 0.001$) in MN induction (4.88±0.35; 8.32±0.57; 11.74±0.37) at all 3 concentrations of 8-Cl-cAMP (10, 80 and 150 mg/kg b.w., respectively) over the negative control (2.04±0.28) (Table 3). Also, these results demonstrated a dose-response relationship between exposure to 8-Cl-cAMP and the frequency of MN in PCE. The ratio between PCE and NCE did not reveal any toxic effect of 8-Cl-cAMP on erythropoiesis.

Discussion

The results obtained in the *in vitro* study demonstrated that 8-Cl-cAMP did not induce structural chromosomal damage in cultured lymphocytes, but induced a decrease in the mitotic index and induction of premature centromere separation. These results show a non clastogenic action of 8-Cl-cAMP with a note of cytotoxicity seen as an alteration of centromere dynamics or PCS. PCS was found in all doses. Also, the findings of chromosomal instability were accompanied by a decrease in the mitotic index. The significance of dose-response relationship for the mitotic index indicates that PCS may result from a comprised mitotic cell cycle control machinery.

The results of the mouse study with acute *in vivo* exposure was positive. MN induction due to 8-Cl-cAMP reached statistical significance at all doses tested when compared to the negative control group (Table 3).

The *in vivo* experimental results demonstrate a correlation between 8-Cl-cAMP cytotoxicity and genotoxicity. One of the possible reasons is that the plateau of overall cytogenetic changes already exists at the maximal tolerated dose (MTD) dose level (80 mg/kg b.w.) where animals clinically express signs of mild toxicity. Our results demonstrate genotoxic potential

Table 3. Frequency of polychromatic erythrocytes (PCE) with micronuclei (MN) induced by increasing doses of 8-Cl-cAMP

Treatment (mg/kg)	*PCE with MN/1000 cells*[a]	*PCE/NCE*[b]
Negative control/PBS	2.04±0.28	2.03±0.21
10 8-Cl	4.88±0.35[c]	2.10±0.27
80 8-Cl	8.32±0.57[c]	2.18±0.41
150 8-Cl	11.74±0.37[c]	1.87±0.35
Cyclophosphamide 60	19.4±3.75[c]	1.75±0.14

[a]each treatment group contained 8 mice and 1000 PCE were examined per animal. Data indicate mean ± SD; [b]data indicate percentage mean ± SD valued on 1000 erythrocytes; [c]$p < 0.01$, significantly different from the negative control results using x^2 test; NCE: normochromatic erythrocytes

of 8-Cl-cAMP on bone marrow cells of BALB/c mice strain considering the correlation between dose and effect in the MN test.

The results of previous preclinical investigations of 8-Cl-cAMP demonstrated a complicated action of 8-Cl-cAMP in various tumor cell lines *in vitro* and *in vivo* [24-27]. Based on the microarray expression profiles of 8-Cl-cAMP and 8-Cl-adenosine [28], and the differential expression of genotoxicity *in vivo* and *in vitro* we can conclude that 8-Cl-cAMP exerts a very complex cytotoxic, genotoxic and antiproliferative action in cancer and normal cells, i.e. 8-Cl-cAMP is active *per se* and in part through its metabolite, 8-Cl-adenosine. In our *in vitro* experiment we used heat-inactivated serum in order to exclude serum metabolism of 8-Cl-cAMP. We found no clastogenic action in the cytogenetic test in the proposed dose regime. The doses were estimated by evaluating the neutral red uptake (data not shown) and literature data, where IC50 (50% growth inhibition) showed a 50% growth inhibition in doses of 1-25 μM for 19 cancer cell lines with no signs of toxicity [24]. These doses appear to be concentrations below those at which degradation by phosphodiesterase can take place [27, 29- 31] and that the growth–inhibitory effect is not due to cell killing. Still, the findings of an increase in the frequency of MN in the highest dose and PCS in all doses confirm that 8-Cl-cAMP at these doses can induce chromosomal instability.

8-Cl-cAMP can induce a specific chromosomal alteration, i.e. PCS. PCS is known as a parameter of chromosome instability leading to aneuploidy [32-35]. The induction of PCS (31, 47 and 29%, respectively) by all doses of 8-Cl-cAMP (1, 5 and 15 μM) was statistically highly significant ($p < 0.001$) when compared to the control group (1.65%). Further analysis showed a distribution pattern of PCS induced by our investigated agent. This pattern is dependent on the dose. Still, there is no linear dose-effect correlation, i.e. at the highest dose (15 μM) we saw a decrease in the overall number of mitoses with PCS (Table 2). This suggests that 8-Cl-cAMP induction of PCS is probably due to its action through a mechanism involving PKA-II proteins which are present at the centromere region [36].

Today's clinical experience of 8-Cl-cAMP antitumor activity comes from 3 phase I clinical trials. Investigators have established that plasma concentration in the range of 1-5 μm of 8-Cl-cAMP can significantly inhibit tumor proliferation, and that this effect is attained at concentrations below the MTD, which gives a possibility to reduce toxicity and genotoxicity [37,38]. 8-Cl-cAMP is especially potent in combination therapies with other cytotoxic agents [39], as it does not increase their myelotoxic effect which is one of the main problems of combination therapies in oncology. Similar findings have also been recorded for the erythrocyte line toxicity in preclinical toxicological studies, i.e. 8-Cl-cAMP doesn't suppress erythropoiesis, as our results show in the mouse model.

Prediction of potential carcinogens and risk assessment are mainly toxicological problems, and human carcinogens - especially antitumor agents - are likely to operate through multiple mechanisms [39-41]. Mutational mechanisms can be proposed for most human carcinogens, but clear evidence also exists that epigenetic mechanisms may be involved. PCS can be classified as an epigenetic alteration expressing chromosomal instability leading to aneuploidy.

In studying a chemical, it is not uncommon to find a positive chromosomal aberration test *in vitro* and a negative test *in vivo* or *vice versa*, or even no evidence for mutagenicity in a battery of genotoxicity tests [42]. Chromosome aberrations may indicate alterations in cell homeostasis that are important in genome instability i.e., interference with DNA replication or condensation [20,42]. So, chromosomal instability by 8-Cl-cAMP as detected in PCS in human lymphocytes may not be the result of electrophilic interaction with DNA, but through microtubular dynamics by utilizing PKA II [36]. Results given by both tests may postulate that 8-Cl-cAMP has promoter activity exerted on the basis of its metabolic level. Numerous biochemical and molecular analyses have shown that an increase in the expression of 3', 5' phosphodiesterases by 8-Cl-cAMP *in vivo* indicate a potential for co-toxic activity of 8-Cl-cAMP metabolites. Still, these metabolites, especially 8-Cl-adenosine, and new data for 8-ATP show anticancer activity [25,29,37-41]. So, how do our tests help evaluate the risk-benefit ratio?

Biological effects of chemicals are dependent on the dose and the dose rate at which chemicals reach the target tissue. Even if 8-Cl-cAMP is shown to be a non clastogen in human lymphocytes, the antitumor agent might enhance instability or PCS initiated by other chemicals. The results of the complex cytogenetic activity of 8-Cl-cAMP show that *in vitro/ in vivo* correlations are most important in preclinical and clinical toxicological risk evaluations.

Our results of *in vitro* 8-Cl-cAMP genotoxicity demonstrates that PCS is a parameter of chromosome instability and should be evaluated solely as a parameter of genotoxicity in future risk-benefit assessments.

The results of 8-Cl-cAMP genotoxicity and the anti-proliferative property of 8-Cl-cAMP to act through various mechanisms *per se* and/or through its metabolites in low but effective doses promise new pathways for combination therapies that may be used in future treatments of cancer patients.

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