Mutagenesis vol.11 no.6 pp.553–557, 1996

Cytotoxic and genotoxic effects of cleistanthin B in normal and tumour cells

Chhalliyil Prabhakaran Pradheep Kumar, Natarajan Panneerselvam, Sunderasan Rajesh and Govindaswamy Shanmugam[1]

Department of Biochemistry and Cancer Biology, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

[1]To whom correspondence should be addressed

Cleistanthin B, one of the toxic constituents of *Cleistanthus collinus*, was found to be cytotoxic to normal and tumour cells. In comparison with normal cells, tumour cells were sensitive to lower doses of toxin. The 50% growth inhibition (GI_{50}) values for normal cell lines were from 2×10^{-5} to 4.7×10^{-4} M and for tumour cells the values ranged from 1.6×10^{-6} to 4×10^{-5} M. Short exposure (30 min) of Chinese hamster ovary (CHO) cells to cleistanthin B at 1–6 μg/ml resulted in extensive chromatid and isochromatid breaks and gaps. However there was no significant increase in cell death and DNA strand breaks in cells treated under the above conditions. Cleistanthin B induced micronucleus formation in cultured lymphocytes in a dose-dependent manner. CHO cells treated with high doses of cleistanthin B showed a decrease in cell viability and a concomitant increase in DNA strand-breaks. The cell death appears to be due to apoptosis since nucleosome-like ladders were observed in the treated cells when the DNA was electrophorized in agarose gels.

Introduction

Cleistanthin B is a naturally occurring compound isolated from the plant *Cleistanthus collinus* (Roxb) Benth and Hook (family Euphorbiaceae). It is a small tree found in dry forests of central and southern India. The plant is extremely poisonous and astringent. Extracts of leaves, roots and fruits are used in acute gastro-intestinal disorders. The extracts are also used as suicidal and homicidal poisons. The active principles isolated from the leaves of *C.collinus* are cleistanthin B, cleistanthin A, collinusin, diphyllin and elagic acid (Chopra *et al.*, 1965).

Cleistanthins cause neutrophilic granulocytosis in rats, mice, cats and monkeys (Rao and Nair, 1970). Granulocytopenia induced by i.p injection of cyclophosphamide could be prevented by the glycoside cleistanthin (Rao and Nair, 1971). The alcoholic extract of the whole plant exhibited anti-proliferative activity against human epidermal carcinoma of the nasopharynx in tissue culture (Bhakuni *et al.*, 1969). All patients who succumbed to *C.collinus* poisoning had cardiac signs in the form of tachycardia followed by brachycardia, low pulse rate, hypotension and arrhythmias (Nagaraj, 1987). The LD_{50} value of cleistanthin B in mice is 1.2 mg/kg body weight. I.v. and i.p. administration of cleistanthin B to rats and rabbits resulted in decreased serum levels of alkaline phosphatase, lactate dehydrogenase, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase. The isoenzyme pattern of lactate dehydrogenase showed that the toxin affects heterologous isoenzymes while the homologous isoenzymes remain unaltered (Annapoorni *et al.*, 1986).

Since the crude extract of *C.collinus* was shown to possess anti-proliferative activity (Bhakuni *et al.*, 1969), we initiated this study to determine the cytotoxic and genotoxic effects of the chief toxic principle, cleistanthin B, in a panel of normal and tumour cell lines, with the objective of exploring this toxin as an anti-tumour agent. We investigated the genotoxic effects of cleistanthin B in Chinese hamster ovary (CHO) cells and cultured blood lymphocytes. Genotoxicity was evaluated using DNA strand breaks assay (Olive *et al.*, 1988), chromatid aberration (CA) analysis (Preston *et al.*, 1981; Ishidate *et al.*, 1988) and micronucleus (MN) assay using cytochalasin B (cyt-B) (Fenech and Morley, 1985). In addition, we observed that cleistanthin B induces apoptosis in cultured cells.

Materials and methods

Chemicals

Cleistanthin B was purified from *C.collinus* leaves as previously described (Govindachari *et al.*, 1969). Cytochalasin B was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 2 mg/ml and stored at –70°C. At the time of experiment the stock solution was diluted with phosphate-buffered saline (PBS) and added to cultures at a final concentration of 6 μg/ml. Erythrosin B, cytochalasin B, phytohaemagglutinin (PHA) and 3-(4,5 dimethyl thiazol-2-yl)-2,5 tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

Primary cultures of human bone marrow fibroblasts (from healthy donors) were from the National Centre for Cell Science, Pune, India. They were maintained in 20% Iscove's modified Dulbecco's medium. For culturing human peripheral blood lymphocytes, venous blood from healthy donors aged 23–31 years were collected using heparinized vacutainer tubes. Lymphocytes were isolated using Ficoll-paque and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 2% PHA.

All cell lines, except the CHO were cultured in Dulbecco's modified medium plus 10% fetal calf serum and 2 mM L-glutamine and gentamycin sulphate (50 μg/ml) at 37°C in an incubator containing 5% CO_2. The CHO cells were maintained in Eagle's modified minimum essential medium (Flow Laboratories, Irvine, UK) supplemented with 10% adult bovine serum, 2 mM L-glutamine and gentamycin sulphate (50 μg/ml) (Sigma Chemical Co.).

Cytotoxicity assay

Cells were seeded on 96-well plates (Nunc, Kamstrup, Denmark), cultured for 1 day and then treated with different concentrations of cleistanthin B for 72 h at 37°C in 5% CO_2 in a humidified atmosphere. Control cells were grown under identical conditions. At the end of incubation, medium was removed and MTT (5 mg/ml) was added and the cells were further incubated for 4 h. After the incubation, the MTT solution covering the cells was removed, DMSO was added to the wells and cell viability was determined by measuring the absorbance in a MR 700 microplate reader with a test wavelengh of 570 nm and a reference wavelengh of 630 nm (Mosmann, 1983). The experiment was repeated three or four times and the variations were <7%.

Chromatid aberration analysis

Subconfluent cultures of CHO cells in 25 cm^2 flasks were incubated with cleistanthin B (1–6 μg/ml) at 37°C for 30 min. Solvent (ethanol 0.05%) and negative controls (without treatment) were maintained separately. After treatment the cells were washed twice with PBS and incubated with fresh medium for 3 h for recovery. Colcemid (Sigma Chemical Co.) at a final concentration of 0.2 μM was added and the cells were incubated further

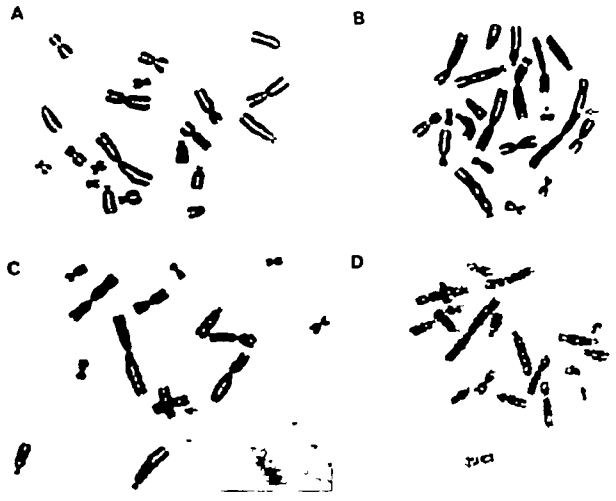

Fig. 1. Effects of cleistanthin B on chromatids. CHO cells were treated with cleistanthin and the chromatids were stained with Giemsa as described in Materials and Methods (see CA analysis) (**A**) control (untreated); (**B**) 6 μg/ml of toxin treatment showing a break; (**C**) 6 μg/ml of toxin treatment showing chromatid exchange; (**D**) 6 μg/ml of toxin treatment showing chromatids with multiple gaps.

Table I. Cytotoxicity of cleistanthin B in normal and tumour cell lines

Cell line	$GI_{50}(M)^a$
Normal cell lines	
Human peripheral blood lymphocytes (HPBL)	4.7×10^{-4}
Human bone marrow fibroblast (HBMF)	3.9×10^{-4}
Mouse embryo fibroblast (MEF)	3.6×10^{-4}
Human oral fibroblast (Tig-7)	2.3×10^{-4}
Chinese Hamster Ovary (CHO)	2.0×10^{-5}
Cancer cell lines	
T cell leukaemia (MOLT-4)	4.0×10^{-5}
Cervix carcinoma (HeLa)	2.5×10^{-5}
Breast carcinoma (MCF-7)	2.4×10^{-5}
Colon (Colo320)	1.6×10^{-5}
Larynx (HEp-2)	1.2×10^{-5}
Melanoma (SK-MEL-28)	1.0×10^{-5}
Oral (KB)	9.5×10^{-6}
Neuroblastoma (SK-N-MC)	5.5×10^{-6}
Fibrosarcoma (HT 1080)	4.0×10^{-6}
Cervix carcinoma (SiHa)	1.6×10^{-6}

[a]GI_{50} was determined using MTT as described in Materials and Methods.

for 3 h. Mitotic cells were collected in a centrifuge tube by gentle shake off. The cells were treated with hypotonic solution of 0.075 M KCl at 37°C for 2–3 min. The cells were then fixed with methanol : acetic acid (3:1). Air-dried chromosome preparations were stained with 3% Giemsa solution in Sorenson phosphate buffer (pH 6.8) for 5–7 min. The slides were coded and 200 well-spread metaphases were screened for chromatid aberrations (Evans *et al.*, 1984).

Micronucleus assay

Human peripheral blood lymphocytes were exposed to different concentrations of cleistanthin B for 24 and 48 h. After 18 h of culture in the presence of cyt-B (5 μg/ml), cells were fixed as described above. Two to three drops of fixed cell suspension were dispensed onto the surface of cold microslides. For each donor, 1500 binucleate cells per concentration were scored blind for MN frequency in each treatment (Preston *et al.*, 1981; Ishidate *et al.*, 1988).

Viability test

About 5×10^5 CHO cells were seeded in 6-well tissue culture plates and treated with desired amounts of the toxin and incubated at 37°C for 24 h in a humidified atmosphere containing 5% CO_2. After incubation, the cells were trypsinized and resuspended in 0.4% erythrosin B in PBS. The percentage of viable cells was determined by counting stained (dead) and unstained (live) cells. The experiment was repeated three times and the experimental variation was 2–5%.

DNA precipitation assay

Exponentially growing CHO cells were radiolabelled for 20 h with 0.1 μCi/ml [^{3}H]-thymidine, (17 Ci/mmole; Bhabha Atomic Research Centre, Bombay, India). DNA precipitation assay (Olive *et al.*, 1988) was carried out using labelled cells that were treated with different doses of cleistanthin B for 24 h. Briefly, cells were harvested with trypsin and about 3.5×10^5 cells were lysed for 1 min in 0.5 ml solution containing 2% sodium dodecyl sulphate (SDS), 10 mM EDTA,10 mM Tris and 0.05 M NaOH. Then 0.5 ml of 0.12 M KCl was added gently and the tubes were kept in a 65°C water bath for 10 min. The tubes were subsequently cooled in ice for 20 min and centrifuged for 10 min at 1000 *g*. The supernatant was decanted into a scintillation vial and 1 ml of 0.05 M HCl was added. The pellet was dissolved in 2×1 ml distilled water at 65°C, vortexed and poured into a scintillation vial. Radioactivities in the supernatant and the pellet fractions were measured in a liquid scintillation counter.

Assessment of apoptosis

CHO cells (2×10^6) were treated with indicated amounts of cleistanthin B for 24 h. The cells were washed twice in PBS and centrifuged at 700 *g*. The cell pellet was then treated with 100 μl of lysis buffer (1% NP-40, 50mM Tris, 20mM EDTA). After centrifugation for 5 min at 1600 *g*, the supernatant was collected and the pellet was treated again with the same amount of lysis buffer. The supernatants were brought to 1% SDS and incubated for 2 h with 5 μg/ml of RNase A at 56°C followed by digestion with proteinase K (2.5 μg/ml) for at least 2h at 37°C. After addition of 1/2 vol 10 M ammonium acetate the DNA was precipitated with 2.5 vol ethanol, dissolved in gel loading buffer and separated by electrophoresis in 1.5% agarose gels. (Herrmann *et al.*, 1994)

Statistical analysis

Student's *t*-test (Student–Newman Keul's test) was used to compare the aberrations between the control and treated cells.

Results

Cytotoxic effects of cleistanthin B

Cleistanthin B was tested for cytotoxicity against a broad spectrum of normal and tumour cell lines. Cytotoxic effect was monitored by determining the viability of cells based on their ability to reduce MTT. The GI_{50} values for five normal and 10 cancer cell lines were determined and the results are shown in Table I. The GI_{50} values of cleistanthin B range from 2×10^{-5}M to 4.7×10^{-4} M for the normal cells and 1.6×10^{-6} to 4×10^{-5}M for the tumour cells.

Effect of cleistanthin B on the induction of chromatid aberrations

Exposure of CHO cells for 30 min to cleistanthin B resulted in characteristic induction of chromatid-type aberrations (Table II and Figure 1). Statistical analysis showed that there is a significant difference in the extent of aberrations between the control and the treated cells, at all the concentrations ($P < 0.001$). In the toxin treated cells a dose-dependent increase in the yield of chromatid aberrations and isochromatid breaks was observed. The exchange aberrations were not dose-dependent. At 6 μg/ml, and above the toxin induced extensive gaps in chromatids (Figure 1). The chromatid aberrations occur at doses that are well below those showing significant loss of viability or double-strand breaks in DNA (Table II).

Effects of cleistanthin B on DNA strand breakage and cell survival

CHO cells when treated with low doses of cleistanthin B for a short time (30 min) caused CA, but neither significantly affected the cell viability nor DNA strand breaks (Table II). Therefore high doses of the toxin were used for viability and strand-break assays. The percent survival values for CHO cells treated with 20, 40, 60 and 80 μg/ml of the toxin for 24 h were 72, 61, 53 and 43% respectively (Figure 2). The results indicate a progressive decrease in the survival of cells in response to increasing doses of cleistanthin B. DNA precipita-

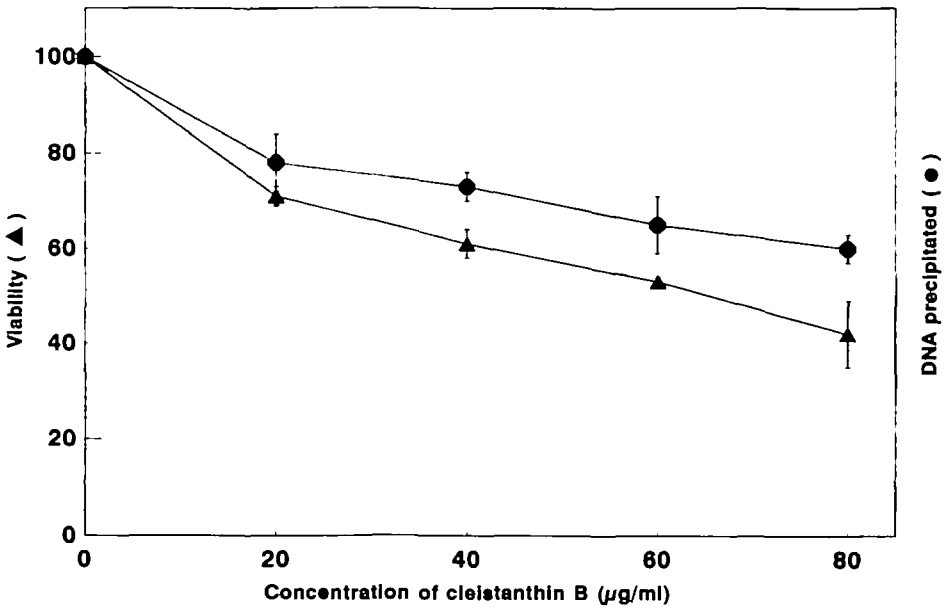

Fig. 2. DNA precipitation and viability assays. Cells were treated with 5–80 μg/ml of cleistanthin and percentage of DNA precipitated and percentage survival after 24 h were determined. The percentages were normalized to that of the control (untreated) cells. Results given are the means of triplicate measurements. Error bars represent SEM.

Table II. Effects of cleistanthin B on the frequencies of chromatid-type aberrations, viability and DNA strand breaks in CHO cells

Cleistanthin B (μg/ml)	Normal Cells	Abnormal Cells	Aberrations per 100 cells[a]						% Viability[e]	% DNA strand breaks[f]
			Chromatid Aberrations	Isochromatid breaks	Chromatid exchange	Gaps	Total aberrations (-Gap)	MG		
Control-I[b]	199	1	0 5	0	0	0	0 5	0	-	0
Control-II[b]	196	44	2	0	0	0	2	0	100	0
1	168	32	14	2	4	2	20[c]	0	-	-
2	168	32	10	6	0	4	16[c]	0	98	3
3	160	40	14	4	4	2	22[c]	0	-	
4	148	52	18	6	2	10	26[c]	0	99	3
5	138	62	27	7	3	4	37[c]	0	-	
6	125	75	32	14	0	14	46[c]	10	99	4
8	90	110	NS[d]	-	-	-	-	-	97	7
10	47	153	NS	-	-	-	-	-	98	6

[a]200 cells were scored in each case after 30 min exposure to the toxin followed by 3 h incubation without toxin and a further 3 h with colcemid.
[b]Control 1 = negative control; control 2 = solvent control.
[c]$P < 0.001\%$ level (Student's t-test).
[d]N.S. = not scorable since the gaps were numerous.
[e]Viability of cells after 30 min exposure to the toxin and 6 h recovery in the absence of toxin was determined by the MTT assay.
[f]DNA strand breaks were monitored under the same conditions as for viability.

tion assay was done under identical conditions as that used for the cell viability assay. Treatment of CHO cells with cleistanthin B at concentrations of 20, 40, 60 and 80 μg/ml for 24 h showed 78%, 70, 65 and 62% precipitation of DNA respectively in comparison with the control (Figure 2). The dose-dependent decrease in the percentage of precipitated DNA indicate DNA strand breaks.

Induction of apoptosis by cleistanthin B

The induction of strand breaks in DNA and loss of cell viability caused by cleistanthin B may be explained by its ability to induce apoptosis. Cleistanthin B indeed induced programmed cell death in cells that were treated with 10–20 μg/ml of toxin for 24 h. Agarose gel electrophoresis of DNA from the treated cells showed nucleosome-like ladder pattern (Figure 3). The DNA fragments in the ladder ranged from 185 bp and its multiples up to 2500 bp.

Induction of micronuclei formation by cleistanthin

The frequency of occurrence of MN in cultured human blood lymphocytes following cleistanthin B treatment is shown in Table III and Figure 4. Human blood lymphocytes treated with 2–10 μg/ml of cleistanthin B for 24 and 48 h showed formation of micronuclei. At 24 h of treatment there was a definite increase in MN formation when the cells were exposed to 2–10 μg/ml of cleistanthin B (Table III). The dose-dependant

Table III. Effects of cleistanthin B on micronucleus formation in lymphocytes

S.No	Treatment μg/ml	Total No. of binuclei (BN) [a]	Total No. of micronuclei (MN)	No. of MN/BN 1 2 3 4	Average MN/1000 BN ± SD
	24 h				
1	Control I [c]	3000	12	12 0 0 0	4 ± 0.82
2	Control II [c]	3000	15	15 0 0 0	5 ± 0.82
3	2	3000	50	39 4 1 0	16.7 ± 2.30 [d]
4	4	3000	43	37 3 0 0	14.3 ± 2.49 [d]
5	6	3000	60	44 2 4 0	20.0 ± 2.60 [d]
6	8	3000	70	54 5 2 0	23.3 ± 4.1 [d]
7	10	3000	74	49 8 3 0	24.6 ± 3.8 [d]
	48 h				
8	Control I [c]	3000	14	14 0 0 0	4.7 ± 0.47
9	Control II [c]	3000	16	16 0 0 0	5.3 ± 0.94
10	2	3000	51	41 5 0 0	17.0 ± 2.16 [d]
11	4	3000	84	55 8 3 1	28.0 ± 0.82 [d]
12	6	3000	73	53 7 2 0	24.3 ± 2.49 [d]
13	8	3000	81	67 4 2 0	27.0 ± 1.4 [d]
14	10	2000 [b]	65	40 6 3 1	32.5 ± 1.2 [d]

[a]1500 BN were scored from each donor
[b]Cleistanthin B at 10 μg/ml caused appreciable loss of cells due to high toxicity, therefore, 1000 BM were used.
[c]Control 1 = negative control; control 2 = solvent control
[d]$P < 0.01$ (Student's *t*-test)

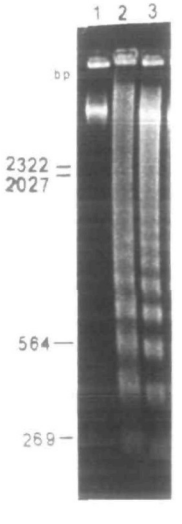

Fig. 3. Agarose gel electrophoresis of DNA from CHO cells treated with cleistanthin B. Cells were exposed to various amounts of toxin. DNA was isolated and analysed as described in Materials and Methods. Lane 1 DNA from untreated cells; lanes 2–4 respectively contained DNA from cells treated with 10 and 20 μg/ml of toxin.

increase was more pronounced at 48 h of treatment in which 4–6 μg/ml of toxin led to an increase of 54% in MN formation over that observed with 2 μg/ml.

Discussion

Many anti-tumour compounds are derived from plants. Crude extracts of *C.collinus* plant were reported to possess anti-tumour effects in epidermal carcinoma cells in culture (Bhakuni *et al.*, 1969). In this study, we have purified one of the toxic principles namely cleistanthin B from *C.collinus* and assessed its anti-proliferative activity using cultured cell lines. Cleistanthin B was found to be toxic to normal and tumour cell lines. Even in the tumour cell lines, some were less sensitive to cleistanthin B than others. The tumour cell line that showed the least sensitivity is the T cell leukaemia (Molt-4) while the cervical carcinoma (SiHa) cells are highly sensitive to this compound. Other tumour cells (Table I) show sensitivity in between the above. Cleistanthin B appears to have low cytotoxicity against normal human cells like bone marrow fibroblasts, peripheral blood lymphocytes and oral fibroblasts (Table I). CHO cells were as sensitive as human T-cell leukaemia, cervical carcinoma and breast cancer cells. However, it may not be appropriate to compare a hamster cell line with human cell lines. Although human cancer cell lines showed higher sensitivity to this toxin than the three human

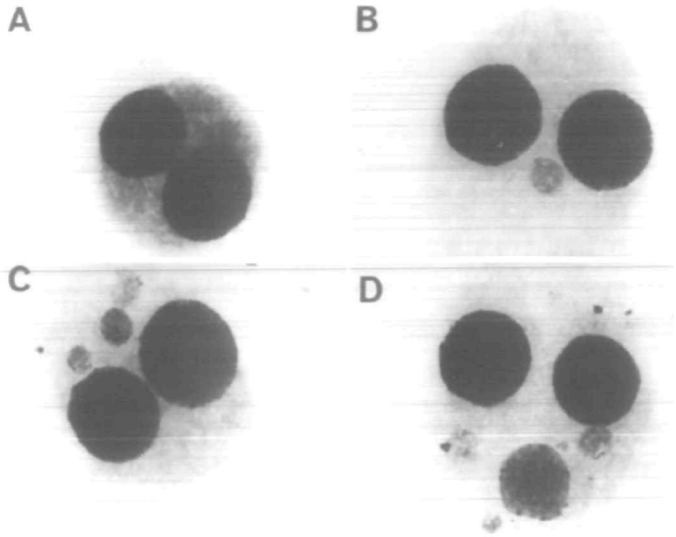

Fig. 4. Induction of micronucleus formation by cleistanthin B. Cultured lymphocytes were treated with 2–10 μg/ml of toxin and the micronuclei were monitored as described in Materials and Methods. (**A**) untreated control; (**B–D**) 4 μg/ml of toxin treatment for 48 h.

normal cell types examined (Table I), the best way of comparison would be with the corresponding normal cells such as comparison of carcinomas with normal epithelial cells.

Treatment of cultured cells with cleistanthin B resulted in the production of many chromatid aberrations. However, there was a significant difference in the survival doses and the dose which induced aberrations. The aberrations were induced at low concentrations of the chemical, well below that which induced significant cell death. At low doses the damage induced by this compound may be repairable and therefore the survival of the cells were not affected. This is also supported by the low induction of MN in cells treated with low concentrations of the toxin for longer time.

The adverse effects of cleistanthin B on DNA and chromosomes and subsequent loss of viability suggest that the cell death caused by the toxin is probably by apoptosis. A number of anti-tumour agents including enediyene, cisplatin, bleomycin, camptothecin doxorubicin and etoposide have been shown to induce cell death by apoptosis (Wrasidlo *et al.*, 1994; Waldman *et al.*, 1996). The nucleosome-like DNA ladders observed upon electrophoresis of DNA from the toxin-treated cells suggest that cleistanthin also induces cell death by apoptosis. The higher sensitivity of tumour cells to cleistanthin and the apoptotic death caused by this toxin indicate that cleistanthin is a potential candidate for cancer chemotherapy, provided its selectivity against tumour cells versus normal cells can be maintained and enhanced in animal models and humans.

Acknowledgements

We thank Ayyanan and Mani for informing us of the use of *C.collinus* in suicides and for providing the plant material for the early phases of this study. We are grateful to Dr C.Damodaran, Forensic Sciences Department, Madras, India, for providing us the authentic samples of cleistanthin B and the National Centre for Cell Science, Pune, India, for providing the cell lines as well as for providing facilities for cytotoxic studies.

References

Annapoorni,K.S. and Damodaran,C. (1986) Novel inhibition of LDH isoenzymes by *Cleistanthus collinus* toxins. *Current Sci.*, **55**, 854–856.

Bhakuni,D.S., Dhar,M.L., Dhar,M.M., Dhawan,B.N. and Mehrota,B.N. (1969) Screening of Indian plants for biological activity part II. *Indian J. Exp. Biol.*, **7**, 250.

Chopra,R.N., Badhwar,R.C. and Gosh,S. (1965) *Poisonous Plants of India.* Vol. 2. Indian Council of Agricultural Research, New Delhi.

Evans,H J., Kilbey,B.J., Legator,M., Nichols,W. and Ramel,C., (1984) *Handbook of Mutagenicity Tests Procedures.* Elsevier Science Publishers BV, Amsterdam, The Netherlands, pp. 406–428.

Fenech,M. and Morley,A. (1985) Measurement of micronuclei in lymphocytes. *Mutat. Res.*, **147**, 29–36.

Govindachari,T.R , Sathe,S.S., Viswanathan,N., Pai,B.R. and Srinivasan,M. (1969) Chemical constituents of *Cleistanthus collinus. Tetrahedron*, **25**, 2815–2821.

Herrmann,M., Lorenz,H.M., Voll,R., Grunke,M., Woith,W. and Kalden,J.R. (1994) A rapid and simple method for the isolation of apoptotic DNA fragments. *Nucleic Acids Res.*, **22**, 5506–5507.

Ishidate,M.Jr, Harnois,M.C and Sofuni,T. (1988) A comparative analysis of data on the clastogenicity of 951 chemical substances tested in mammalian cell cultures. *Mutat. Res.*, **195**, 151–213.

Mosmann,T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *J. Immunol. Meth.*, **65**, 55–63.

Nagaraj,S. (1987) Cardiac toxicity of Oduvan thalai (*Cleistanthus collinus*) common leaves poisoning in Tamil Nadu (Report of 25 cases). *Antiseptic*, January, pp. 34–35.

Olive,P.L. (1988) The DNA precipitation assay: a rapid and simple method for detecting DNA damage in mammalian cells. *Environ. Mol. Mutagen.*, **11**, 487–495.

Preston,R.J., Au,W., Bender,M., Brewen,J., Carrano,A., Heddle,J., McFee,A., Wolff,S. and Wasson,J. (1981) Mammalian *in vivo* and *in vitro* cytogenetic assays: a report of the US EPA's Gene-Tox Program. *Mutat. Res.*, **87**, 143–188.

Rao,R.A. and Nair,T B.(1970) Investigations on induction of neutrophilic granulocytosis and toxicity of cleistanthin – CIBA GO.4350 – A new glycoside from *Cleistanthus collinus* (Roxb). *Pharmacology*, **4**, 347–358.

Rao,R.A. and Nair,T.B. (1971) Effect of cleistanthin, a new glycoside, on experimentally induced Leukopenia in rats and mice. *Drug Res.*, **21**, 828.

Waldman,T., Lengauer,C , Kinzler.K.W and Vogelstein.B.(1996) Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking P21. *Nature*, **381**, 713–716.

Wrasidlo,W., D.S.Johnson and D.L.Boger (1994) Induction of endonucleolytic DNA fragmentation and apoptosis by the Duocarmycins. *Bioorg. Med. Chem. Letts.*, **4**, 631–636.

Received on October 9, 1995; accepted on August 23, 1996