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Induction of Apoptotic Cell Death in Human Jurkat T Cells by a Chlorophyll Derivative (Cp-D) Isolated from *Actinidia arguta* Planchon

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Abstract The chloroform and methanol (2:1, v/v) extract from an edible plant, *Actinidia arguta* Planchon, appeared to possess antitumor activity against human leukemias Jurkat T and U937 cells through inducing apoptosis. The substance in the solvent extract was purified by silica gel column chromatography, preparative TLC, and Sephadex LH-20 column chromatography. Characteristics of the substance analyzed by UV scanning analysis, and ^{1}H and ^{13}C NMR spectra suggested that the substance belongs to the chlorophyll derivatives-like group. The IC_{50} value of the chlorophyll derivative (Cp-D) determined by MTT assay was 15 μg/ml for Jurkat, 10 μg/ml for U937, and 11.4 μg/ml for HL-60, and was more toxic to these leukemias than to solid tumors or normal fibroblast. In order to elucidate cellular mechanisms underlying the cytotoxicity, the effect of the Cp-D on Jurkat T cells was investigated. When cells were treated with the Cp-D at a concentration of 15 μg/ml, [^{3}H]thymidine incorporation declined rapidly and was undetectable in 1 h. However, no significant changes were made in the cell cycle distribution of the cells by 24 h. The sub-G1 peak representing apoptotic cells began to be detectable in 36 h, at which time apoptotic DNA fragmentation was also detected on agarose gel electrophoresis, demonstrating that the cytotoxic effect of the Cp-D is attributable to the induced apoptosis. Under the same conditions, although the protein level of cyclin-dependent kinases such as cdc4, cdk6, cdk2, and cdc2 was not significantly changed until 24 h, the kinase activity of all cdks rapidly declined and reached a minimum level within 1–6 h and then recovered to the initial level by 12 h and sustained until 24 h. These results suggest that inactivation of cdks at an inappropriate time during the cell cycle progression in Jurkat T cells following a treatment with the Cp-D leads to induction of apoptotic cell death.

Key words: *Actinidia arguta* Planchon, a chlorophyll derivative (Cp-D), human T-cell leukemia, apoptosis, cdks

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Significant progress has been made in the field of chemotherapeutic treatment for cancer using anticancer drugs. However, as most anticancer drugs developed to date are also toxic to normal cells and tissues and thus have limited selectivity for cancer cells, practical use of these anticancer drugs has been restricted to only a few cases due to various side effects observed during clinical trials [14]. To overcome these limitations and to further improve the effectiveness of chemotherapy for cancer, it has been required not only to isolate a new anticancer drug that is specifically toxic to cancer cells but also to identify a new target in cancers, which induces destruction of cancers upon chemotherapy.

It has been suggested that the induction mechanism of apoptosis in tumors could be an efficient target for an anticancer drug, because malignant tumors can be removed by their own destruction upon the induced apoptosis [1, 2, 19, 25]. Apoptosis represents a form of cell death that is morphologically characterized by cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation with internucleosomal DNA digestion [26]. Since a considerable amount of attention has been focused on the role that apoptosis may play in mediating the cytotoxic effect of chemotherapeutic agents in tumor cells, it is known that several anticancer drugs such as etoposide, VM26, *m*-AMSA, dexamethasone, vincristine, *cis*-platium, cyclophosphamide, paclitaxel, 5-fluorodeoxyuridine, 5'-fluorouracil, and adriamycin induce extensive apoptosis rapidly in tumor cells [3, 5, 6, 9, 11, 21, 23]. However, the biochemical mechanisms involved in the drug-induced apoptotic process are still poorly understood.

Recently, we have initiated a project of isolating the antitumor agent that is specifically toxic to tumors through induction of apoptosis. In previous studies, we have shown that aburatubolactam C isolated from a marine *Streptomyces* sp. SCRC A-20 has a cytotoxicity against various malignant tumor cells of human murine origins, and rapidly induces apoptosis through upregulation of Fas ligand expression

and subsequent activation of Fas death signal [1, 2]. The cytotoxicity of aburatubolactam C appeared to be more effective on leukemias and lymphomas compared to solid tumors or normal fibroblast cells. More recently, we have also examined various edible plants to isolate a cytotoxic substance that is more specifically toxic to tumors, based on a simple concept that cytotoxic components to tumors from edible plants may not be severely toxic to normal cells or tissues.

In the present study, we describe that a cytotoxic substance isolated from an edible plant, *Actinidia arguta* Planchon, possesses an antitumor activity through inducing apoptosis. Purification and instrumental analysis have revealed that the substance is a chlorophyll derivative (Cp-D). Since the Cp-D has shown cytotoxicity against various tumor cells and can induce apoptosis in Jurkat T cells, the mechanism underlying induced apoptosis in Jurkat T cells were sequentially investigated. These results suggest that inappropriate regulation of the kinase activity for cyclin-dependent kinases (cdks) in the cell cycle of Jurkat T cells following the drug treatment results in triggering the apoptotic cell death.

MATERIALS AND METHODS

Reagents, Antibodies, and Cells

Protein G-agarose and E64 were purchased from Calbiochem (San Diego, U.S.A.), and α-casein, tween-20, β-glycerophosphate, p-nitrophenyl phosphate, sodium orthovanadate, RNase, proteinase K, and propidium iodide were from Sigma (St. Louis, U.S.A.). Radioactive material of [^{3}H]thymidine (2 Ci/mM) was obtained from NEN Biotechnolgy System (Boston, MA, U.S.A.), and [γ-^{32}P]ATP (≈3,000 Ci/mM) was obtained from Amersham (Arlington Heights, U.S.A.). Silica gel (Merck Kieselgel 60, 70–230 mesh) and silica TLC plates (Silica gel 60 F_{254}) were purchased from Merck Company (Darmstadt, Germany). Rabbit polyclonal anti-cdk4, anti-cdk6, anti-cdk2, and anti-cdc2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, U.S.A.), and monoclonal anti-FasL and anti-Fas antibodies were from Transduction Laboratories (Lexington, U.S.A.). Human leukemia Jurkat T cells, human myeloid leukemia U937 cells were kindly supplied by Dr. Albert A. Nordin (Gerontology Research Center, NIA/NIH, Baltimore, U.S.A.). Human promyelocytic leukemia HL-60 cells, human colon adenocarcinoma COLO 320DM cells, and murine fibroblast NIH 3T3 cells were from KRIBB culture collection (KRIBB, Taejon, Korea). Jurkat, U937, HL-60, and COLO 320DM cells were maintained in RPMI 1640 (Life Technologies, Gaithersburg, U.S.A.) containing 10% FBS (UBI, Lake Placid, U.S.A.), 20 mM HEPES (pH 7.0), 5×10^5 M β-MeOH, and 100 μg/ml gentamycin. The culture medium used for NIH 3T3 cells and Sarcoma 180 were Dulbecco's Modified Eagle's Medium (Life Technologies, Gaithersburg, U.S.A.) supplemented with 10% FBS, 20 mM HEPES (pH 7.0), 1 mM sodium pyruvate, 5×10^5 M β-MeOH, and 100 μg/ml gentamycin. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Screening of Edible Plants for Isolation of Antitumor Substance

Approximately 50 edible plants in Korea were collected and air-dried at room temperature. To examine the presence of an effective constituent possessing antitumor activity in these edible plants, 10 g of individual plants were extracted with 10 ml of a solution composed of $CHCl_3$ and MeOH (2:1, v/v) and evaporated under reduced pressure. The residue was dissolved in DMSO, and assayed for the cytotoxicity against Jurkat T cells and U937 cells using the MTT assay as described previously [1].

Instrumental Analysis

Ultraviolet (UV) spectrometric analysis was completed by using a UV spectrophotometer (Shimadzu, Japan). Briefly, 1 μg of the purified cytotoxic substance was dissolved in 1 ml of methanol, and then the UV spectrum from 200 to 800 nm was taken by scanning. ^{1}H-NMR and ^{13}C-NMR spectra were measured with a GSX-400 spectrometer (JEOL, Japan) using 10 mg of the sample dissolved in chloroform-*d* as a solvent.

Cytotoxicity Assay and DNA Fragmentation Analysis

The cytotoxic effect of the Cp-D on several tumor cells was analyzed by either the MTT assay or [^{3}H]thymidine-incorporation as described previously [1]. For MTT assay, tumor cells (5×10^4) were grown for 2 days with serial dilutions of the Cp-D in a 96-well plate. After incubation, 50 μl of MTT solution (1.1 mg/ml) was added to each well and then incubated for an additional 4 h. The colored formazan crystal produced from MTT was dissolved in 150 μl of DMSO and then the OD value was measured at 540 nm by a plate reader. The incorporation of [^{3}H]thymidine into DNA by Jurkat T cells treated with the Cp-D was determined by adding 5×10^4 cells to 15 μg/ml of the Cp-D in a 96-well plate. At each time point after incubation, 1 μCi/well of [^{3}H]thymidine was added for 4 h and assayed for the incorporation of [^{3}H]thymidine by liquid scintillation.

In order to determine apoptotic DNA fragmentation induced in Jurkat T cells following the treatment by the Cp-D, the isolation of apoptotic DNA fragments was performed as previously described [1, 2, 7]. Briefly, the cells were harvested by centrifugation and then treated with a lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). After centrifugation for 5 min at 1,600 ×g, the supernatant was collected and brought to 1% SDS, and

treated for 2 h at 50°C with RNase A and subsequently with proteinase K for 2 h at 37°C. The DNA fragments were precipitated with 2.5 volume of ethanol in the presence of 5 M ammonium acetate. The DNA fragmentation was visualized by electrophoresis on a 2% agarose gel.

Flow Cytometry Analysis

Any changes of the cell cycle progression of Jurkat T cells following the treatment by Cp-D was analyzed by flow cytometry as described elsewhere [12]. Approximately 1×10^6 cells were suspended in 100 μl of PBS, and then 200 μl of 95% cold ethanol was added while vortexing. The cells were incubated at 4°C for 1 h, washed twice with PBS containing 2% FCS, and resuspended with 12.5 μg of RNase in 250 μl of 1.12% sodium citrate buffer (pH 8.45). Incubation was continued at 37°C for 30 min before staining of the cellular DNA with 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed on a FACScan flow cytometer for the relative DNA content, based on an increased red fluorescence.

Western Blot Analysis

Cellular lysates were prepared by suspending 4×10^6 Jurkat T cells in 100 μl of HB lysis buffer (137 mM NaCl, 15 mM $MgCl_2$, 60 mM β-glycerophosphate, 15 mM p-nitrophenyl phosphate, 0.1 mM sodium orthovanadate, 1 mM DTT, 15 mM EGTA, 1 mM PMSF, 4 μg/ml E64, 0.1% Triton X-100, 25 mM MOPS, pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. Equivalent amounts of cell lysates were subjected to electrophoresis on an 11% SDS-polyacrylamide gel and electrotransferred to Immobilon-P membrane (Millipore Co., Bedford, MA, U.S.A.). The membrane was treated with a blocking buffer (3% nonfat skim milk, 0.1% tween-20, 100 μg/ml gentamycin) at room temperature for 1–2 h. The membrane was probed with rabbit polyclonal anti-cdk4, anti-cdk6, anti-cdk2, or anti-cdc2 antibody at 4°C overnight, and then with a horse-radish peroxidase conjugated goat anti-rabbit IgG at 4° for 1 h. Detection of each protein was visualized on the X-ray film using an ECL Western blotting detection system according to the manufacturer's instructions.

Immunoprecipitation and Kinase Assay

For immunoprecipitation of individual cdks, 60 μg of the cellular lysate at 1 μg/l was allowed to react with 2 μg of each cdk antibody at 4°C for 2 h. The immune complexes were recovered by adding 30 μl of protein G-agarose, and incubation was continued, with rotation, at 4°C for 1 h. The protein G-agarose was collected by centrifugation (2000 ×g for 3 min) and washed three times with the lysis buffer and then once with kinase assay buffer (137 mM NaCl, 5 mM EGTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 1 mM PMSF, 4 μg/ml E64, 25 mM MOPS, pH 7.2). The immunoprecipitates were asssayed for the α-casein kinase activity. Phosphorylation of α-casein was measured by incubating the protein G-agarose beads with 30 μl of hot kinase assay cocktail (1 mg/ml of α-casein, 10 μCi [γ-^{32}P]ATP, 100 μM ATP, 1× kinase assay buffer) for 30 min at 30°C. The reaction was stopped by boiling the mixture in 1× SDS sample buffer for 5 min, and the reaction mixture was resolved on an 11% SDS-polyacrylamide gel electrophoresis. The gel was dried and the presence of phosphorylated α-casein was detected by autoradiography after exposure at −70°C.

RESULTS AND DISCUSSION

Isolation, Purification, and Characterization

Among the fifty edible plants tested, the organic solvent (chloroform:methanol = 2:1, v/v) extract of *A. arguta* Planchon appeared to be the most cytotoxic to human leukemias Jurkat T and U937 cells. In order to purify the active principle, approximately 1 kg of air-dried *A. arguta* Planchon was extracted with 10 l of chloroform and

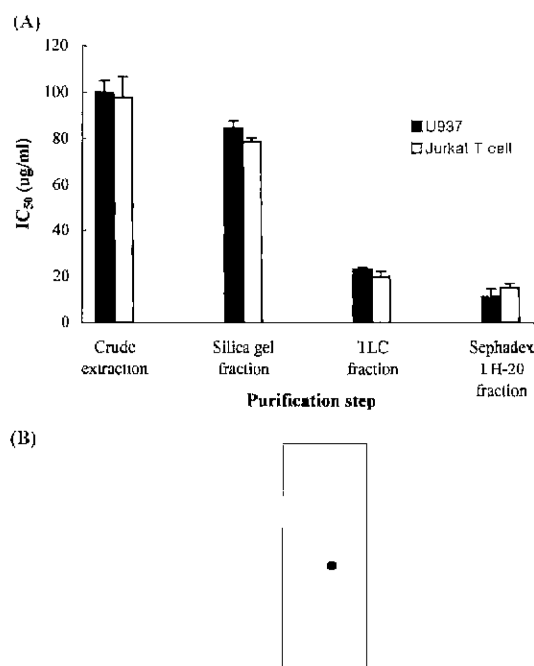


Fig. 1. Increase in the inhibitory activity of the cytotoxic substance on human leukemias Jurkat T and U937 cells during purification from *Actinidia arguta* Planchon (A), and a thin layer chromatogram of the purified cytotoxic substance (B).

For the inhibitory activity assay, the sample from each purification step was serially diluted and added into a 96-well plate containing 5×10^3 cells of Jurkat or U937. After 2 days, the cell proliferation was measured by MTT assay. The cytotoxic substance obtained from the final purification step was developed on Silica gel F_{254} TLC plates with 9:1 mixture of chloroform and methanol.

methanol mixture (2:1, v/v) for 6 h at room temperature with a continuous agitation. The extract was concentrated under reduced pressure to give 20 g of powder. The IC_{50} value of the crude extract against both Jurkat T cells and U937 cells was 100 μg/ml (Fig. 1A). The extract was applied on a silica gel column chromatography, which was eluted stepwise with chloroform and methanol. The active fraction eluted with chloroform and methanol (8:2, v/v) was evaporated to achieve dryness. The IC_{50} value of the resulted powder (4 g) against Jurkat and U937 was 80-85 μg/ml (Fig. 1A). After development on a TLC plate (chloroform:methanol = 9:1, v/v), the band corresponding to Rf 0.45 was scraped off to be extracted with a mixture of chloroform and methanol (2:1, v/v). Concentration of the extract recovered 400 mg of powder exhibiting 20-22 μg/ml of IC_{50} value. Further purification of this active fraction by Sephadex LH-20 column chromatography (eluted with chloroform:methanol = 1:2, v/v) afforded 50 mg of cytotoxic compound, of which the IC_{50} value was 10-15 μg/ml against Jurkat and U937. These results indicate that a specific activity of the cytotoxic compound increased by 7-10 folds during the purification process. To confirm the purity of the cytotoxic compound that was obtained from Sephadex LH-20 column chromatography, TLC analysis (Silica gel 60 F_{254}, chlorform:methanol = 9:1, v/v) was carried out. The purified substance appeared as a single spot with the R_f value of 0.45, suggesting that it is pure enough for a further instrumental analysis (Fig. 1B).

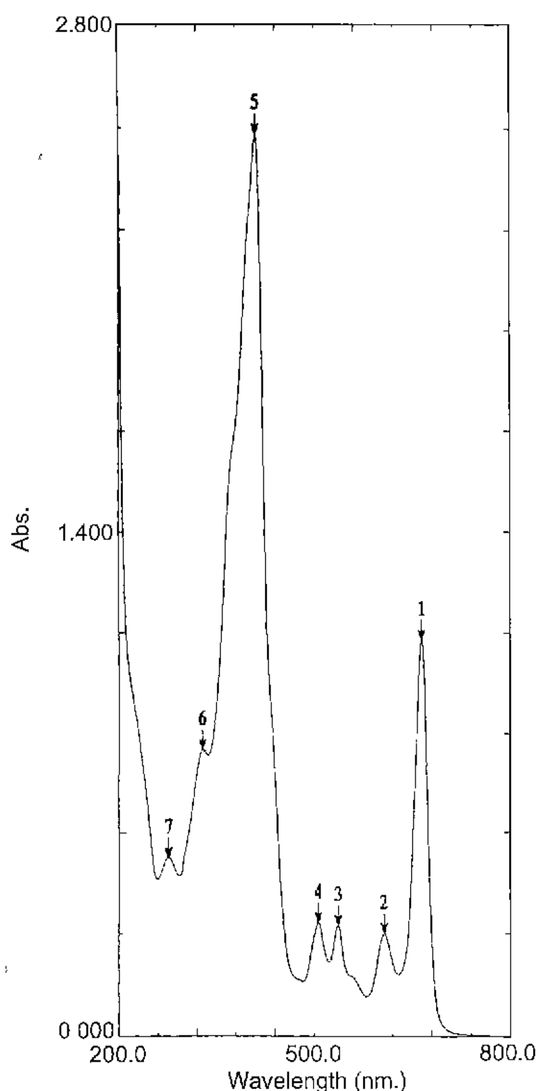


Fig. 2. UV spectrum of the purified cytotoxic substance.
One microgram of the substance was dissolved in 1 ml of methanol and analyzed by scanning from 200 to 800 nm

To elucidate the chemical structure of the purified substance, instrumental analyses including UV, ^{1}H-, ^{13}C-NMR were carried out. The substance showed a typical UV absorption of chlorophyll at 410 and 665 nm [27]. In the ^{1}H-NMR spectrum, aromatic singlet resonances at δ 7-10 ppm indicated the existence of a porphyrin skeleton, and overlapped methylene signals near δ 1.25 ppm showed signs of a phytol side chain being present [15]. In the ^{13}C-NMR spectrum, generally the middle portion of the aromatic-olefinic region, containing the signals of the quaternary α- and β-pyrrole carbons, is worth special attention because the chemical shifts of these carbons appear to show the highest sensitivity to structural alterations in the macrocyle. As a result of careful investigation into these signals of the cytotoxic substance, it corresponded well to the reported data of the chlorophyll derivative, pheophytin *a* [22]. However, signals near δ 177 ppm and the sp3 hybridized or aliphatic region with values in a range of 10-75 ppm seemed to be quite inconsistent with pheophytin *a*, suggesting that the cytotoxic compound had a different side chain to phytol. From these observations, the cytotoxic compound is presumed to be a derivative of chlorophyll (Cp-D), more likely pheophytin *a* with a different aliphatic alcohol side chain.

Cytotoxic Activity of the Cp-D and Apoptosis Induction

The cytotoxic effect of the Cp-D on various malignant tumor cells of human and murine origins was determined by MTT assay. The Cp-D appeared to have cytotoxicity for most tumor cells tested and the IC_{50} values determined by MTT assay were in the range from 10 μg/ml to ≥100 μg/ml (Table 1). The IC_{50} value appeared to be 15 μg/ml for Jurkat, 10 μg/ml for U937, 11.4 μg/ml for HL-60, 70 μg/ml for COLO 320DM, ≥100 μg/ml for sarcoma 180, and 25 μg/ml for murine normal fibroblast NIH 3T3. These results indicate that the cytotoxic substance Cp-D is more toxic to leukemia cells than to solid tumors or normal fibroblast cells.

To determine the cellular mechanisms underlying the cytotoxicity, the antiproliferating as well as apoptotic effects of the Cp-D on Jurkat T cells were investigated. When cells were treated with the Cp-D at a concentration of 15 μg/ml,

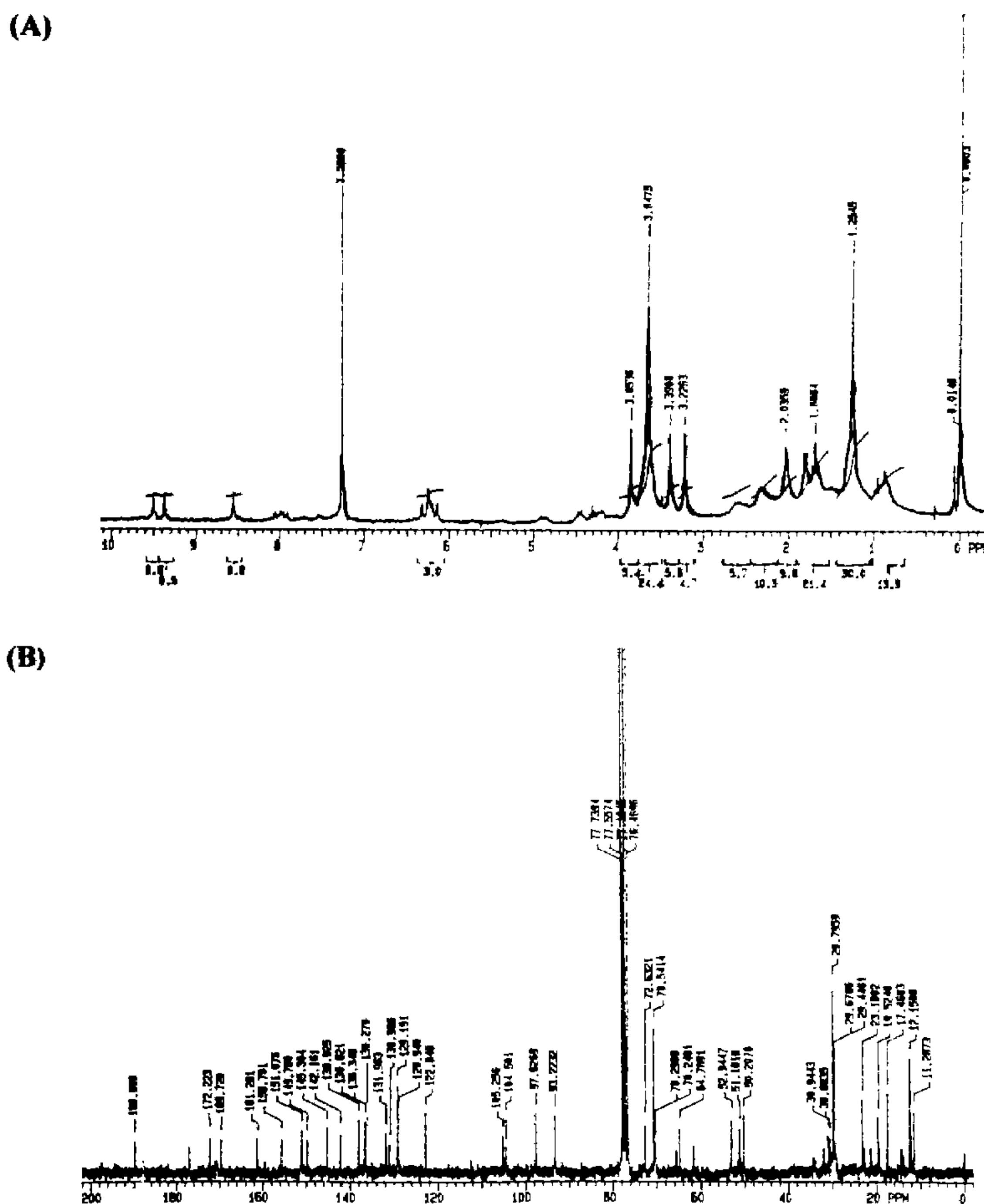


Fig. 3. ^{1}H-NMR spectrum (A) and ^{13}C-NMR spectrum (B) of the purified cytotoxic substance.
After 10 mg of the substance was dissolved in chloroform-*d*, the NMR spectrum was measured by 400 mHz NMR spectroscopy.

[^{3}H]thymidine incorporation declined very rapidly and was undetectable in 1 h and this undetectable level was sustained until 48 h after the Cp-D treatment. This demonstrates that the treatment of Cp-D results in a rapid blockage of cellular DNA synthesis (Fig. 4). However, under the same conditions, cell cycle distribution of the cells was not significantly changed until 36 h, at which time not only was the sub-G1 peak representing apoptotic cells detectable but apoptotic DNA fragmentation was also detected on an agarose gel electrophoresis (Figs. 5 and 6). Additionally, when Jurkat T cells were treated with the Cp-D at various concentrations ranging from 2.5 to 15 μg/ml for 48 h, apoptotic DNA fragmentation on an agarose gel electrophoresis was detectable even in the presence of 2.5

Table 1. Inhibitory concentration of the Cp-D against malignantly proliferationg tumor cells.

Cell line	IC_{50} (g/ml)
Jurkat	15.0
U937	10.0
HL-60	11.4
COLO320DM	70.0
Sarcoma 180	≥100
NIH3T3	25.0

The IC_{50} indicates a concentration of active fraction of *Actinidia arguta* Planchon which caused 50% decrease in the number of viable cells based on MTT assay.

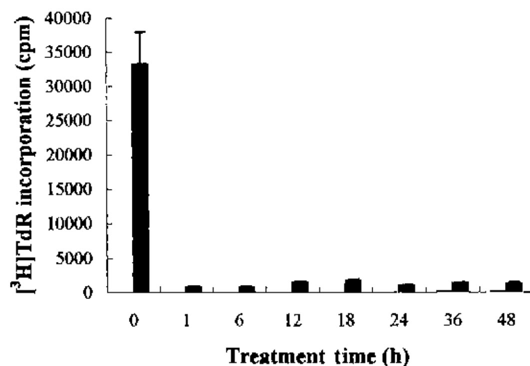
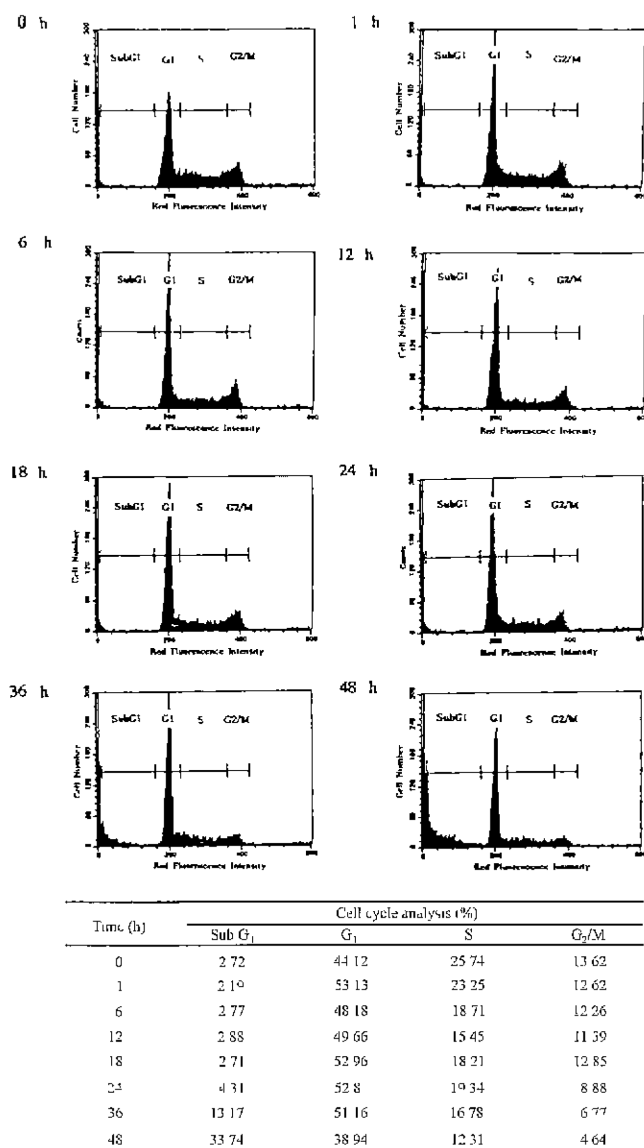


Fig. 4. Effect of the Cp-D on [^{3}H]thymidine incorporation in Jurkat T cells.

Continuously growing Jurkat T cells (4×10^5) were incubated with 15 μg/ml of the Cp-D in a 96-well plate. At the indicated time points, cells were processed to assess the incorporation of [^{3}H]thymidine as an index of cell proliferation.



Time (h)	Cell cycle analysis (%)			
	Sub G_1	G_1	S	G_2/M
0	2.72	44.12	25.74	13.62
1	2.19	53.13	23.25	12.62
6	2.77	48.18	18.71	12.26
12	2.88	49.66	15.45	11.39
18	2.71	52.96	18.21	12.85
24	4.31	52.8	19.34	8.88
36	13.17	51.16	16.78	6.77
48	33.74	38.94	12.31	4.64

Fig. 5. Flow cytometric analysis of the cell cycle distribution in Jurkat T cells after treatment by the Cp-D.

After Jukat cells were incubated in the presence of 15 μg/ml of the Cp-D, and at each time point cells were harvested. Cell cycle analysis was performed on an equal number of cells (10^4) by flow cytometry after staining of DNA by propidium iodide.

μg/ml of Cp-D (data not shown). These results demonstrate that antitumor effect of the Cp-D purified from *A. arguta* Planchon is caused by the induced apoptotic cell death, and suggest that the rapid and sustained blocking of cellular DNA synthesis of continuously proliferating tumor cells may lead to induction of apoptotic cell death.

Changes of Cdks in Jurkat T Cells Following Treatment by the Cp-D

The eukaryotic cell cycle is typically divided into four phases. These are the periods of DNA synthesis (S phase) and mitosis (M phase) separated by gaps called G_1 and G_2. Progression through each of these phases in mammalian cells is governed to a large extent by the sequential activation and inactivation of a series of cdks. While the G_2/M transition is regulated by catalytically active cdc2 (cdk1)/cyclin B complexes, the G_1/S transition requires cdk2/cyclin E and possibly cdk2/cyclin A complexes [4, 10, 12]. The completion of progression through the G_1 phase also involves cdk4 and cdk6 complexed mainly with D type cyclins [18]. Involvement of dysregulation of cdks in the apoptotic cell death has been initially supported by the evidence that premature activation of $p34^{cdc2}$ kinase is associated with apoptosis [20]. Sequentially, as a potential mechanism in the antineoplastic drug-induced apoptosis of tumor cells, the perturbation and subsequent inappropriate regulation of the catalytic activity of cdks have been implicated [16, 24, 28, 29].

Since treatment of the Cp-D in Jurkat T cells rapidly blocks the cellular DNA synthesis that is the central event for the S phase and since there is no significant accumulation of the cells in a specific phase of the cell cycle before undergoing apoptosis under the same conditions, it is highly likely that perturbation of the catalytic activity of

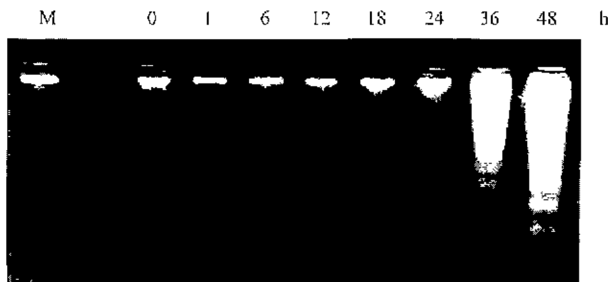


Fig. 6. Apoptotic DNA fragmentation of Jurkat T cells treated by the Cp-D.
Continuously proliferating Jurkat T cells (4×10^6 cells) were incubated with the Cp-D at a concentration of 15 μg/ml for the times indicated and then the cells were harvested to analyze apoptotic DNA fragmentation by the NP-40 lysis method as described in Materials and Methods.

cdks necessary for the G_1/S as well as G_2/M transitions may have occurred by the Cp-D. To confirm this prediction, the change in the protein levels of cdks as well as in their kinase activities was investigated in Jurkat T cells after treatment by 15 μg/ml of the Cp-D. As shown in Fig. 7, the proteins specific for cdk4, cdk6, cdk2, and cdc2 were easily detectable in continuously proliferating Jurkat T cells by Western blot analysis and the protein level was not changed significantly until 24 h after the drug treatment. Since the kinase activity of cdks is downregulated by the specific protein inhibitors (cdk inhibitors) and $p27^{Kip1}$ is believed to be a main cdk inhibitor in T cells [8, 13, 17],

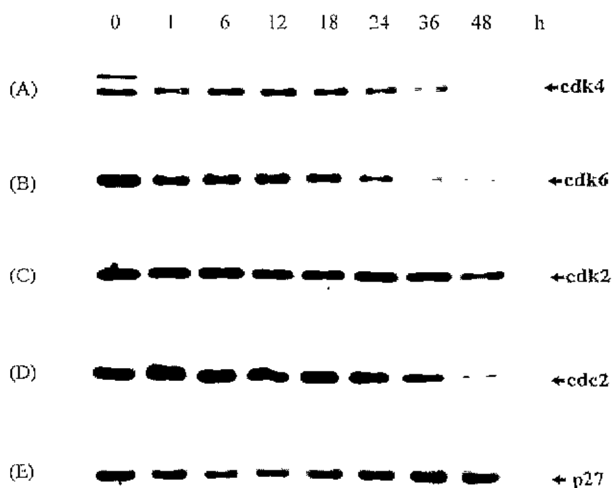


Fig. 7. Kinetic analysis of the protein levels of cdk4 (A), cdk6 (B), cdk2 (C), cdc2 (D), and $p27^{Kip1}$ (E) in Jurkat T cells following addition of the Cp-D.
The cells ($6-8\times10^6$) were incubated at a concentration of 2.5×10^5/ml with 15 μg/ml of the Cp-D for the indicated times and prepared for the cell lysates. Equivalent amounts of the cell lysate (20 μg) were subjected to electrophoresis on a 11% SDS-PAGE and electrotransferred to Immobilon-P. The membranes were probed with anti-cdk4, anti-cdk6, anti-cdk2, anti-cdc2, and anti-$p27^{Kip1}$, and then with a horse-radish peroxidase conjugated goat anti-rabbit IgG. Detection of each protein was performed using the ECL western blotting detection system

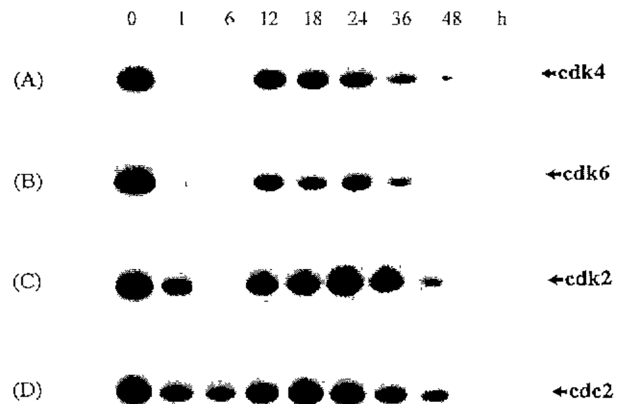


Fig. 8. Effect of the Cp-D on the catalytic activity of cdk4 (A), cdk6 (B), cdk2 (C), and cdc2 (D) in Jurkat T cells.
The cell lysate (60 μg), prepared as described in Fig. 7, was immunoprecipitated with each cdk antibody and then was subjected to α-casein kinase assay. The kinase reaction mixture was electrophoresed on an 11% SDS-polyacrylamide gel electrophoresis. The gel was dried and the phosphorylated α-casein was visualized by autoradiography after exposure at - 70°C.

the change in the protein level of $p27^{Kip1}$ was analyzed as well. There was a slight decrease in the protein level of $p27^{Kip1}$ within 6-18 h rather than upregulation of $p27^{Kip1}$ protein (Fig. 7). Under the same conditions, the catalytic activity of all cdks such as cdk4, cdk6, cdk2, and cdc2 rapidly and concomitantly declined and reached an undetectable or a minimum level in 1-6 h following the treatment by Cp-D (Fig. 8). Subsequently, the kinase activity of all cdks was simultaneously recovered to the initial level by 12 h and sustained until 24 h. However, since there was no significant change in the cell cycle distribution until 24 h, these results indicate that the Cp-D rapidly inhibits the catalytic activity of cellular cdks possibly through the post-translational regulation irrespective of cell cycle progression. It is noteworthy that changes in the protein levels of Fas and Fas ligand were not detected under these conditions (data not shown).

Taken together, these results suggest that an inappropriate regulation of the kinase activity of cyclin-dependent kinases (cdks) during the cell cycle progression in Jurkat T cells following the treatment by the Cp-D triggers the apoptotic cell death. The mechanism responsible for the rapid and concomitant inactivation of cdks including cdk4, cdk6, cdk2, and cdc2 in Jurkat T cells treated by the Cp-D remains to be elucidated.

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REFERENCES

1. Bae, M. A., H. S. Kang, S. W. Rue, J. H. Seu. and Y. H. Kim. 1998a. Enhanced expression of Fas ligand is associated with aburatubolactam C-induced apoptosis in human Jurkat T cells. *Biochem. Biophys. Res. Commun.* **246:** 276–281.
2. Bae, M. A., K. Yamada. D. Uemura, J. H. Seu, and Y. H. Kim. 1998b. Aburatubolactam C, a novel apoptosis-inducing substance produced by marine *Streptomyces* sp. SCRC-20. *J. Microbiol. Biotechnol.* **8:** 455–460.
3. Fisher, D. E. 1994. Apoptosis in cancer therapy: Crossing the threshold. *Cell* **78:** 539–542.
4. Grana, X. and E. P. Reddy. 1995. Cell cycle control in mammalian cells. Role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene* **11:** 211–219.
5. Hannun, Y. A. 1997. Apoptosis and dilemma of cancer chemotherapy. *Blood* **89:** 1845–1853.
6. Havrilesky, L. J., A. Elbendary. J. A. Hurteau, R. S. Whitaker. G. C. Rodriguez, and A. Berchuck. 1995. Chemotherapy-induced apoptosis in epithelial ovarian cancers. *Obstet. Gynecol.* **85:** 1007–1010.
7. Herrmann. M.. H.-M. Lorenz, R. Voll. M. Granke, W. Woith, and J. R. Kalden. 1994. A rapid and simple method for the isolation of apoptotic DNA fragments. *Nucleic Acids Res.* **22:** 5506–5507.
8. Hunter. T. and J. Pines. 1994. Cyclins and cancer: cyclin D and cdk inhibitors come of age. *Cell* **79:** 578–582.
9. Huschtscha. L. I., W. A. Bartier, C. E. Ross, and M. H. Tattersall. 1996 Characteristics of cancer cell death after exposure to cytotoxic drugs *in vitro*. *Br. J. Cancer* **73:** 54–60.
10. Jun, D. Y., H. K. Park, A. A. Nordin, J. E. Nagel, and Y. H. Kim. 1998. Characterization of the murine *cdc2* gene. *Mol. Cells* **8:** 731–740.
11. Kaufman, S. H. 1989. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: A cautionary note. *Cancer Res.* **49:** 5850–5878.
12. Kim. Y. H., J. J Proust, M. J. Buchholz, F. J. Chrest, and A. A. Nordin. 1992. Expression of the murine homologue of the cell cycle control protein $p34^{cdc2}$ in T lymphocytes. *J. Immunol.* **149:** 17–23.
13. Kwon, T. K., M. A. Buchholz, P. Ponsalle, F. J. Chrest, and A. A. Nordin. 1997. The regulation of $p27^{Kip1}$ expression following the polyclonal activation of murine G_0T cells. *J. Immunol* **158:** 5642–5648.
14. Lokich, J. J. 1980. *Clinical Cancer Medicine: Treatment Tactics*. pp. 1–15, G. J. Hall Press, Boston, U.S.A.
15. Lotjonen, S., T. J. Michalski. J. R. Norris, and P. H. Hynninen. 1987. High-field proton NMR study of the liquid solution structure of monomeric bacteriochlorophyll *a* and chlorophyll *a*. *Magn. Reson. Chem.* **25:** 670–674.
16. Meikrants, W., S. Gisselbrecht, S. W. Tam, and R. Schiegel. 1994 Activation of cyclin A-dependent protein kinases during apoptosis. *Proc. Natl. Acad. Sci. USA* **91:** 3754–3758.
17. Peter, M. and I. Herskowltz. 1994. Joining the complex; cyclin dependent kinase inhibitory proteins and the cell cycle. *Cell* **79:** 181–184.
18. Shi, L.. G. Chen. D. He, D. G. Bosc, D. W. Litchfield, and A. H. Greenberg. 1996. Granzyme B induces apoptosis and cyclin A-associated cyclin-dependent kinase activity in all stages of the cell cycle. *J. Immunol.* **157:** 2381–2385.
19. Shi, L., W. K. Nishioka, J. M. Th'ng, E. M. Bradbary. D W. Litchfield. and A. H. Greenberg. 1994. Premature $p34^{cdc2}$ activation required for apoptosis. *Science* **263:** 1143–1145.
20. Sherr, C. J. 1994. G1 phase progression: Cycling on cue. *Cell* **79:** 551–555.
21. Shinomiya, N , M. Shinomiya, H. Wakiyama, Y. Katsura, and M. Rokutanda. 1994. Enhancement of CDDP cytotoxicity by caffeine is characterized by apoptotic cell death. *Exp. Cell. Res.* **210:** 236–242.
22. Simo, L. and P. H. Hynninen 1989. Carbon-^{13}NMR spectra of chlorophyll *a* chlorophyll *a*', phrochlorophyll *a* and the corresponding pheophytins. *Org. Mag. Reson.* **21:** 757–765.
23. Walker, P. R , C. Smith, T. Youdale. J. Lebnaris. J. F. Whitfield, and M. Sikorska. 1991. Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res.* **51:** 1078–1085.
24. Wang, Q.. P. J. Worland. J. L. Clark, B. A. Carlson, and E. A. Sausbille. 1996. Apoptosis in 7-hydroxystaurospurine-treated T lymphoblasts correlates with activation of cyclin dependent kinases 1 and 2. *Cell Growth Differ.* **6:** 927–936.
25. Watabe. M.. Y. Mansuda. S. Nakajo, T. Yoshida, T. Kuroiwa. and K. Nakaya. 1996. The cooperative interaction of two different signalling pathways in response to bufalin induces apoptosis in human leukemia U937 cells *J. Biol. Chem.* **271:** 14067–14080.
26. Williams, G. T. 1991. Programmed cell death: Apoptosis and Oncogenesis. *Cell* **65:** 1097–1098.
27. Woo. W.-S. 1996. *Methods in Natural Products Chemistry*, pp. 377–378, 1st ed., SNU Publishing, Seoul. Korea.
28. Yao. S. L., K. A. Mekenna, S. J. Sharkis, and A. Bedi. 1996. Requirement of $p34^{cdc2}$ kinase for apoptosis mediated by the Fas/Apo-1 receptor and interleukin 1β-converting enzyme-related proteases. *Cancer Res.* **56:** 4551–4555.
29. Zhang, Q., H. S. Ahuka. and Z. F. Zakeri. 1997. Cyclin dependent kinase 5 is associated with apoptotic cell death during development and tissue remodelling. *Dev. Biol.* **183:** 222–233.