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Molecular Mechanisms Underlying the Inhibition of Cell Proliferation by Cleistanthins

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Cleistanthus collinus, a toxic plant, contains three glycosidic compounds, Cleistanthin A, B and C. Cultured K562 cells were treated with 1μg/ml and 10μg/ml of each Cleistanthins (A and B). At higher concentration a significant dose dependent decrease has been noted in the incorporation of thymidine and uridine into DNA and RNA, respectively. However, no significant difference in leucine incorporation into proteins was discerned. Hence present study shows Cleistanthin A and B inhibition nucleotides (DNA, RNA) with no difference in aminoacid transportation into macromolecules (Protein) into cell cytoplasm of K562 cells. However it dos not affect transport of nucleotides and aminoacids into the cytoplasm. Thus, these results showed that the cell growth/ proliferation is checked by these compounds at the replication and transcriptional level, but not at the translational level.

Introduction :

Cleistanthus collinus (Family: Euphorbiaceae) is an extremely poisonous and astringent plant frequently employed in suicidal purposes (homicidal poison), which contains three identical compounds, namely *Cleistanthin A* (Govindachari *et al*., 1967), *Cleistanthin B* (Lakshmi *et al*., 1970) and *Cleistanthin C* (Anjaneyulu *et al*., 1975) in addition to other secondary metabolites. The alcoholic extracts of leaves, roots and fruits of this plant, are used to treat gastro intestinal disorders (Chopra *et al*., 1965). The extracts also show significant anticancer activity against human epidermaoid nasopharynx (Bhakuni *et al*., 1969) and cause tumor regression in mice by inducing apoptosis (Pradheepkumar *et al*., 1996, 2000). However it has alsobeen reported to induce significant neutrophilic granulocytosis (Rao and Nair, 1970, 1971; Annapoorani *et al*., 1984).

Since most of the anti cancer drugs (both natural and synthetic) execute their action by targeting nucleic acids, microtubular spindle, hormone receptors and enzymes, the present study was designed to evaluate the molecular mechanism of Cleistanthin A and B on cell proliferation in tumor cell lines keeping in view its anticancer potential .

Material and Methods

Extraction of Cleistanthin A and B :

Cleistanthin A and B were extracted from *C. collinus* leaves (Govindachari *et al*., 1967; Annapoorani *et al*., 1984) by alcoholic soxhalation. The alcoholic extract was processed and purified by thin layer chromatography by different solvent system. The purified crystals were dissolved in absolute ethanol and suitable aliquots were prepared in 0.5% dimethyl sulphoxide (DMSO) for further experiment. The compounds were confirmed by TLC analysis against authentic samples.

Cultivation of tumour cell line (K562 - Chronic myelogenous leukemic cells) :

The cells from the primary culture were washed thrice with phosphate buffer saline (PBS). Few drops of trypsin (0.25% in PBS) were added to cultured cells and the monolayer was disturbed by gentle flushing with Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal calf serum (FCS). The cells were then resuspended in 10% adult bovine serum (ABS) containing medium and transferred to sterile culture bottles and incubated at 37°C.

Radioisotope incorporation assay :

The K562 cells in the sub-confluent stages were chosen, transferred to 75cm^2 Falcon petridishes and incubated under 5% CO_2 atmosphere with a relative humidity of 98%. Cells were synchronized to quiescent stage by maintaining them in 0.5% FCS for 24hrs and stimulated again by treating with 10 % FCS containing RPMI 1640 medium before drug treatment.

Radioisotope incorporation assay was performed as described by Russel *et al*. (1984) with slight modifications. Equal numbers of exponentially growing K562 cells were seeded onto 75cm$_2$ petridishes and cell monolayers were maintained in 10% FCS containing RPMI 1640 medium. The sub-cultured K562 cells were treated individually with Cleistanthin A and B at the concentration of 1μg/ml and 10μg/ml and incubated at 37°C. At the end of 2, 4, 6 h treatment, an abrupt of cells were pulse labeled with 0.1 μCi [^{3}H] - thymidine, [^{3}H] - uridine and [^{3}H] - leucine for 30 min. At the end of labeling the cells were washed with cold PBS thrice and fixed with 5% TCA (twice

for 5min each on ice). The TCA insoluble fraction were collected and pooled. The test tubes were air dried and then lysed with 0.1 N NaOH - 1% SDS mixture and the lysate was removed and spotted onto whatman filter paper strips. The filter paper strips were air dried, placed in a scintillation vial and 2 ml of scintillation fluid (omny fluor) was added and radioactivity was determined. An aliquot from the TCA soluble fraction was added to scintillation vial and mixed with 2 ml of scintillation fluid and the amount of radioactivity was determined.

Results :

The radioisotope-incorporation assay revealed that there was 20 - 40% reduction in [^{3}H]-thymidine incorporation into DNA in the K562 cells treated with 1 μg/ml Cleistanthin A whereas, time dependant drastic reduction in thymidine incorporation (up to 70%) was noted in 10 mg/ml Cleistanthin A treated cells. However, the cells treated with Cleistanthin B at the concentration of 1 μg/ml showed no significant difference in reduction in all the time periods, but 65% reduction was observed in cells treated at the concentration of 10 μg/ml (Fig 1).

K562 cells treated at 1 μg/ml concentration of Cleistanthin A and B showed about 20% reduction in [^{3}H]-uridine incorporation in RNA, whereas cells treated at 10 μg/ml concentration of Cleistanthin A and B showed 50 % and 20% reduction in [^{3}H]-uridine incorporation respectively (Fig 3). The [^{3}H] leucine assay revealed no significant difference in reduction in [^{3}H]-leucine incorporation in the cells treated with Cleistanthin A and B (Fig 5).

The effect of Cleistanthin A and B on [^{3}H]-thymidine, [^{3}H]-uridine and [^{3}H]-leucine transport was measured by the amount of free nucleotides and aminoacid pool in the cytoplasm. There were no significant reduction in transportation of thymidine, uridine and leucine into the K562 cells treated with Cleistanthin A and B (Fig 2, 4 and 6).

Discussion :

It is evident from the results that there was a significant time and dose dependent decrease in the incorporation of thymidine and uridine into DNA and RNA, respectively in Cleistanthin A and B treated cells. It is understood that the decreased incorporation of thymidine and uridine is not

due to the blockage in transportation but due to the inhibition of incorporation by Cleistanthin A and B at molecular level. The exact mechanism of inhibition of incorporation is not yet known and it is difficult to pinpoint the mechanism behind this inhibition without any further experiments. Nevertheless, the protein synthesis or incorporation of amino acids was not affected in the cells that were treated with Cleistanthin A and B. The reason could be that the RNA molecules that were synthesized prior to the treatment (or at the G_1 phase of cell cycle) might carry out the translational process regardless of replication and transcription processes, which were affected after the treatment.

Failure of incorporation of thymidine and uridine after stimulation in the treated cells indicates that a significant population of cells was denied progression from G_1 phase to S phase of the cell cycle and still remains in G_0 phase. Based on this mechanism, most of the anticancer drugs have been designed in such a manner that they could target only the actively dividing cells. However, these drugs can also be applied to quiescent cells, but the cells die only after they enter the dividing phase.

Several attempts have been made to identify novel anticancer agents in the previous studies. But none of the drugs have come up with satisfactory potential and harmless effect in curing cancer. Bhakuni *et al.* (1969) for the first time showed the anticancer activity of alcoholic extract of *Cleistanthus collinus* plant against human epidermal carcinoma of nasopharynx. Similarly, Pradheepkumar *et al*., (1996; 1999; 2000) reported that the Cleistanthin A and B are effective on cancer cell lines by the variety of modes such as, inducing apoptosis, inhibiting DNA synthesis, causing DNA damage, chromosomal aberration and chromatid exchange in tumor cell lines. When comparing anti cancer potential and toxicity of Cleistanthin A and B with other known anticancer drugs, Cleistanthin A and B showed high potential in tumor regression, induction of low level of lipid peroxidation and generation of less free radical in tumor bearing mice (Pradheepkumar *et al*., 1999) than the other known anticancer agents.

From the available literature and the results from the current study, it can be concluded that Cleistanthin A and B have potential to hamper the cell growth and proliferation by interfering the replication and transcriptional processes and in turn can act as candidate for the regression of cancer cell proliferation and suppression of cell growth.

Fig. 1 : Effect of Cleistanthin A and B on [3H] thymidine incorporation into DNA in k562 cells

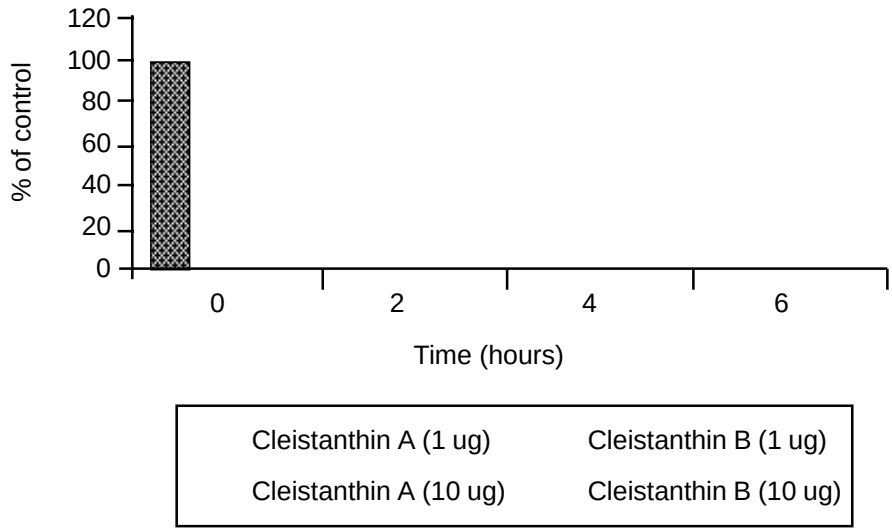


Fig. 2 : Effect of Cleistanthin A and B on [3H] thymidine transport in k562 cells

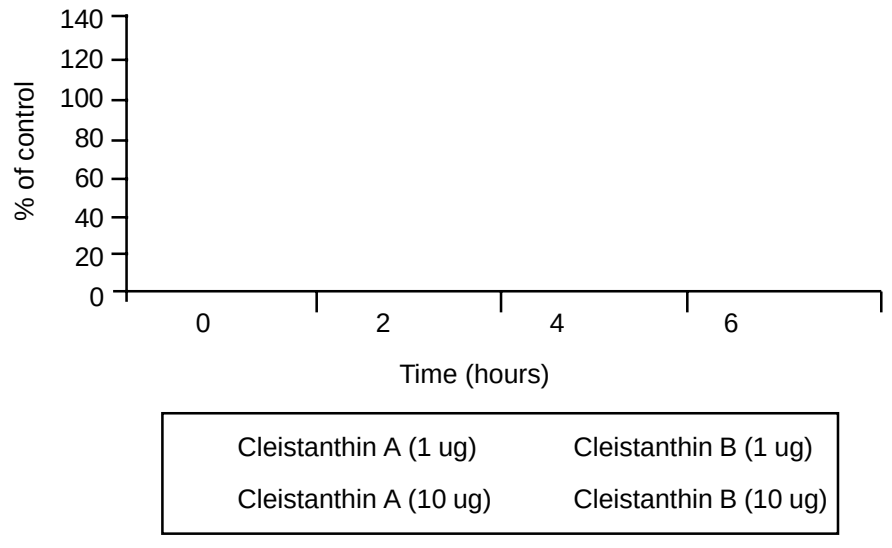


Fig. 3 : Effect of Cleistanthin A and B on [3H] uridine incorporation into RNA in k562 cells

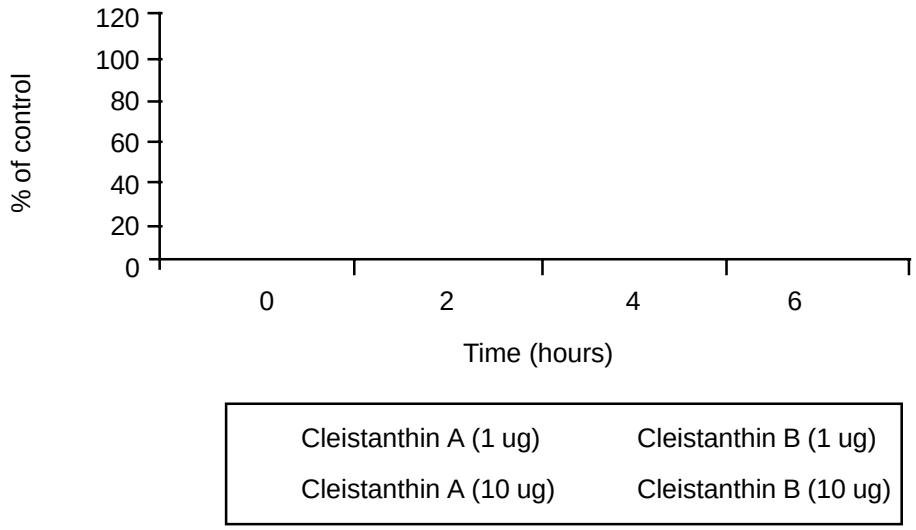


Fig. 4 : Effect of Cleistanthin A and B on [3H] uridine transport in k562 cells

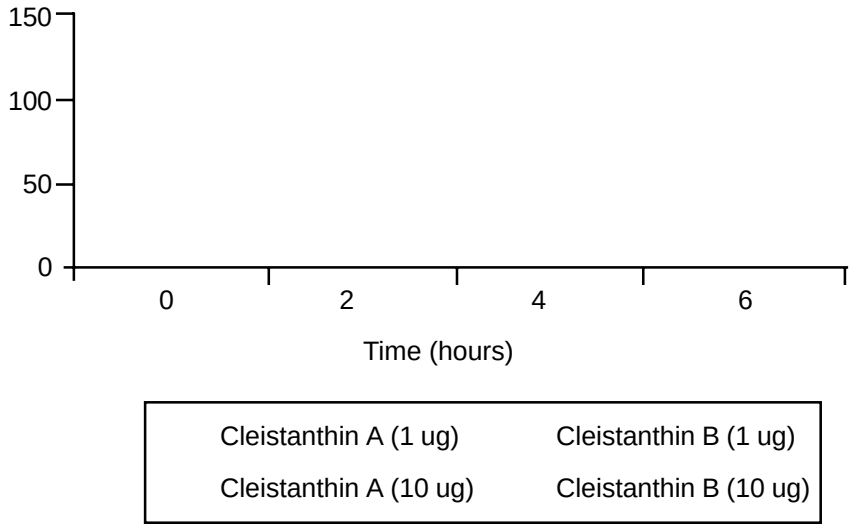


Fig. 5 : Effect of Cleistanthin A and B on [3H] leucine incorporation into protein in k562 cells

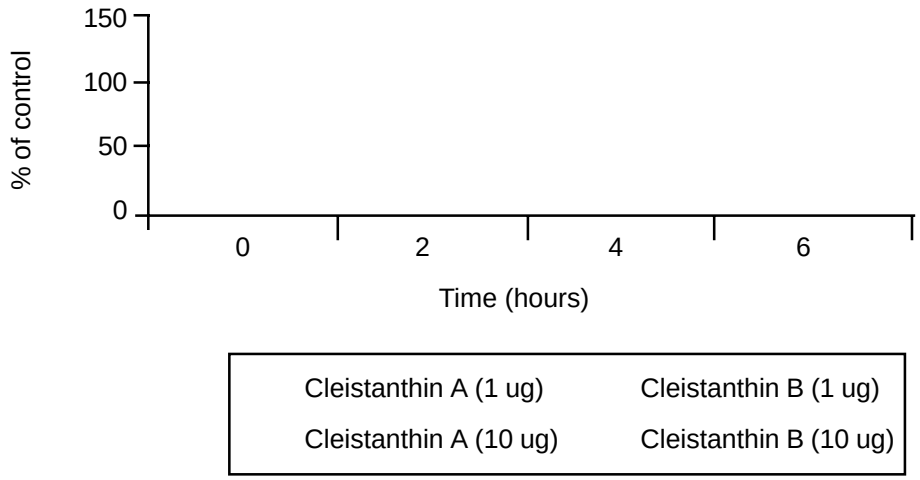
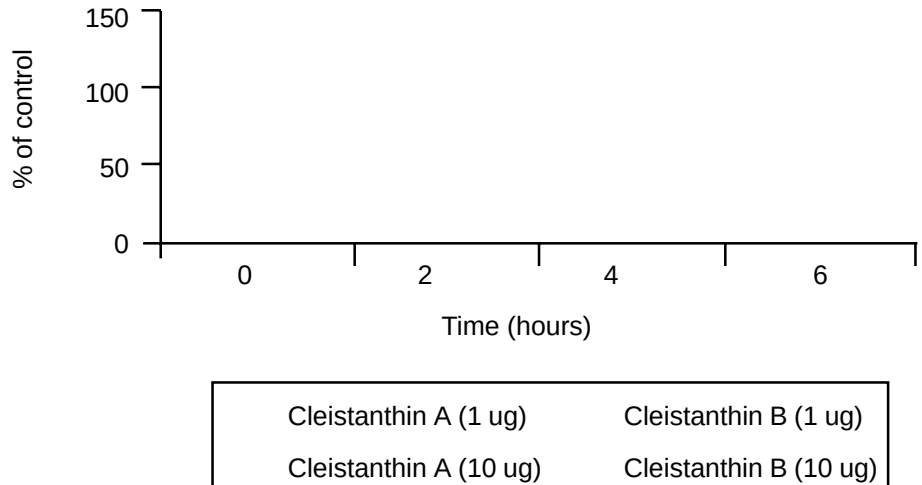


Fig. 6 : Effect of Cleistanthin A and B on [3H] leucine transport in k562 cells



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