Andrographolide Induced Apoptosis in NALM-6 Cells Mediated Through the Cell Cycle Arrest and Nuclear Fragmentation

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ABSTRACT

Introduction: Andrographis paniculata is an herb widely cultivated in South and Southeastern Asia. It has been traditionally used to treat infections and other physiological disorders for several hundreds. We investigated the anti-leukemic potential of Andrographolide (AGP) isolated from the leaves of this plant against an array of cancer cells to investigate its most efficacies in a particular cancer type. Methods: AGP was isolated from Andrographis paniculata leaves by using column chromatography. The structure was further determined by LC-MS, 1H NMR and 13C NMR. AGP was initially tested against four different cancer cell lines, namely NALM-6 (pre B-ALL), K562 (CML), A549 (lung carcinoma) and MCF-7 (breast carcinoma) using MTT assay at different time points and different concentrations. The effect of the isolated bio-molecule was also investigated in inducing apoptosis through the study of cell cycle progression using flow cytometry by PI staining and nuclear fragmentation pattern by DAPI staining and fluorescence microscopy. Results: the spectral analysis of the isolated bio-molecule assured that the compound was AGP. MTT assay data indicated that AGP was most potent to induce cytotoxicity in NALM-6 cells. Further investigation revealed that it effectively induced apoptosis by arresting cell cycle progression and increased the nuclear break down in NALM-6 leukemic cells. Conclusion: Our study efficiently demonstrated that the AGP isolated from Andrographis paniculata induced apoptosis in NALM-6 cells, which could be used in the therapeutic intervention of leukemia in the future.

Key words: Andrographis paniculata, Andrographolide, Apoptosis, Cell cycle, Cytotoxicity, Leukemia.

INTRODUCTION

Leukemia is the most common childhood cancer and associated with increased proliferation and decreased apoptosis in neoplastic blood cells. According to Cancer Research (UK), 9634 new cases and 4584 deaths occurred in 2014 from leukemia. Induction of apoptosis is the best strategy to destroy these malignant cells. These cells carry a number of mutations in their genome/proteome, that results in the up-regulation of anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL etc.) as well as down-regulation of different pro-apoptotic proteins (e.g. Bid). Therefore, utilization of multi-target drugs is most important for treating leukemia. Now-a-days a higher percentage of commercial drugs contain active principles from natural sources, particularly from plants for its multiple targets and least side-effects e.g. Paclitaxel, Podophyllotoxin etc. Andrographis paniculata Nees. (Acanthaceae), commonly known as ‘Kalmegh’, is a wonder drug and very popular in ‘Aurvedic’ and ‘Siddha’ systems of medicine in India for its wide spectrum of biological activities. Three major labdane type diterpenoids to which the main biological activities of the plant are attributed, are Andrographolide (AGP), 14-Deoxy-11,12-didehydroandrographolide and Neandrographolide (NAGP). AGP, the major diterpenoid of this plant, has hepatoprotective, hepatostimulant, anti-inflammatory, anti-viral and anti-leukemic activities. 14-deoxy-11,12-didehydroandrographolide, the second major diterpenoids of this plant, has cardiovascular and anti-inflammatory effects. Anti-leukemic activity of this compound has not yet been established, although its anti-cancer activity has been reported in the last few years. In the present paper, we have isolated AGP (diterpenoids) from the aerial parts of Andrographis paniculata plant by column chromatography and repeated crystallization. The structure of the isolated compound was established by spectral analysis and screened against different types of cancer cell lines, namely NALM-6 (pre B-ALL), K562 (CML), A549 (lung carcinoma) and MCF-7 (breast carcinoma). NALM-6 appeared to be the most sensitive in response to AGP treatment in comparison to the other types of cancer cells, as evidenced by MTT assay. In this article we first time investigated the anti-apoptotic activity of AGP against NALM-6 which was established by cell cycle analysis and nuclear break down assay.

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METHODS

Collection of plant materials
The leaves of the plants were collected from the Garmirjapur village of West Bengal during the September-October of 2014 and identified by Prof. N. D. Paria, Department of Botany, University of Calcutta. Plant materials were air dried in the shade at a 35°C-40°C temperature for several days, so that no residual water remains in the sample. Dried samples were grounded into fine powder (1kg) and stored in vacuum desiccator until use.

Chemicals
RPMI 1640 and fetal bovine serum (FBS) were purchased from Gibco/Invitrogen. Penicillin, streptomycin, DAPI, Propidium iodide and thiazoyl blue tetrazolium bromide (MTT) were purchased from Sigma. Silica gel (100-200) and all the solvents, e.g. methanol, chloroform, petroleum ether used for column chromatography were purchased from Merck, India.

Extraction and isolation of the compound
1Kg of powdered plant material of *Andrographis paniculata* was extracted with 95% methanol by a hot-extraction method using soxlet apparatus. The methanolic extract was evaporated using a rotary evaporation apparatus. The methanolic extract was evaporated using a rotary evaporation apparatus and treated with AGP at different concentrations for 24h and 48h. After treatment, cells were 17μM and 50μM respectively. We found that cell viability after AGP treatment was decreased in a concentration and time dependent manner at both time points. We also tested the cytotoxic action of AGP against normal PBMC and found that AGP did not show any significant cytotoxic action against PBMC.

**RESULTS**

Spectroscopic data of the isolated compound

*Andrographolide* (C_{30}H_{46}O_{4})

\[ ^1H \text{NMR (C}_{\text{D},N}, 300MHz): \delta 7.18 (1H, t, J = 1.5, 7.0 Hz), 5.37 (1H, m), 4.88 (1H, d, J = 1.0 Hz), 4.85 (1H, d, J = 1.0 Hz), 4.60 (1H, dd, J = 6.0, 10.0 Hz), 4.50 (1H, dd, J = 2.5, 10.5 Hz), 4.43 (1H, d, J = 10.5 Hz), 3.64-3.60 (2H, m), 2.73 (t, J = 7.0 Hz), 1.90 (1H, m), 1.51 (3H, s), 0.70 (3H, s); \]

\[ ^{13}C \text{NMR (C}_{\text{D},N}, 75 MHz): \delta 170.7, 147.9, 147.0, 130.2, 108.8, 79.8, 75.4, 66.0, 64.1, 56.3, 55.3, 43.2, 39.1, 38.1, 37.3, 29.0, 25.0, 24.3, 23.7, 15.2. \]

MS (ESI) 373.23 [M+Na]·

**Differential cytotoxic activity of Andrographolide**

We investigated the anti-proliferative effects of AGP against different cancer cell lines. Each cell line was treated with and without AGP for 24h and 48h, followed by MTT assay. AGP exhibited cytotoxic activity against all the cell lines tested, but NALM-6 was most sensitive. The IC_{50} values of AGP at 48h of incubation to NALM-6, K562, A549 and MCF-7 cells were 17μM, 25μM, 22μM and 21μM respectively. We found that cell viability after AGP treatment was decreased in a concentration and time dependent manner at both time points. We also tested the cytotoxic action of AGP against normal PBMC and found that AGP did not show any significant cytotoxic action against PBMC.

**Cell cycle analysis after AGP treatment**

To investigate the underlying mechanism of anti-leukemic activity of AGP, we performed cell cycle analysis after AGP treatment (control and IC_{50} value) by using flow cytometry after staining with Propidium Iodide. AGP mediated apoptosis was evidenced by the elevated number of sub G0/G1 cell population in comparison to the control set.

**Nuclear fragmentation analysis**

Occurrence of nuclear fragmentation, is one of the hallmark evidence of apoptosis. We performed DAPI staining after AGP treatment and cell cycle analysis with the most sensitive cell line NALM-6.

**DISCUSSION**

In the present study, we isolated AGP from Indian medicinal plant *Andrographis paniculata* in a new protocol. At first we prepared the methanolic extract of the leaves, as it is reported to possess the most promising anti-cancer properties. This methanolic extract was run through column chromatography using a combination of solvent system and isolated pure compound by repeated crystallization. The structure of the isolated compound was determined by spectral analysis. Although extensive works have been performed to establish anti-cancer activity of AGP and its mechanism of action in several cancer types, anti-leukemic action of this diterpenoid has not yet been explored thoroughly. Therefore, we investigated the anti-leukemic activity of the isolated compound by studying its cytotoxic efficacy towards two leukemic cell lines viz. NALM-6, K562 along with A549 (lung carcinoma) and MCF-7 (breast carcinoma) as a positive control as it is sensitive towards the AGP. The result of the MTT assay showed a differential cytotoxic effect of AGP.
Figure 1: Schematic representation of isolation of AGP from the methanolic extract of *Andrographis paniculata* leaves through column chromatography.

Figure 2: Chemical Structures of the isolated diterpenoid Andrographolide (AGP).
activity of AGP towards four cancer cell lines and NALM-6 was found most sensitive. Therefore, we further explored the mechanistic detail of anti-cancer activity of AGP against most sensitive NALM-6 cells. Apart from the anti-cancer activity, AGP interestingly exhibited nominal cytotoxicity towards the normal PBMC, which helped us to conclude that AGP has a potential to target the cancer cells leaving the normal healthy cells apart. Cell cycle arrest is one of the most promising evidence of apoptosis. Previous study entails that AGP effectively arrest cell cycle progression at G0/G1\textsuperscript{16} as well as G2-M\textsuperscript{17} phase of the cell cycle. We performed cell cycle analysis of the AGP treated and untreated NALM-6 cells. Our result correlates with the Banerjee and her co-workers data and any G2-M arrest was not found. We further studied the nuclear fragmentation analysis because it is another hallmark event in apoptosis. We treated NALM-6 cells with AGP and after 48h of incubation period, we visualized nuclei under a fluorescence microscope after staining with DAPI stain. We found an appreciable amount of cells with fragmented nuclei in comparison to the control untreated cells.

**CONCLUSION**

In conclusion, we can say that AGP, a diterpenoid isolated from *Andrographis paniculata*, effectively inhibited NALM-6 pre-B-ALL leukemic cells along with other cancer cells and thereby holds a great promise in
its anti-leukemic potentiality and can be included in therapeutic interventions of leukemia treatment in the days to come.

CONFLICT OF INTEREST
The Authors have no conflict of interest

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ABBREVIATIONS USED
LC-MS: Liquid chromatography-Mass spectrometry; NMR: Nuclear magnetic resonance; B-ALL: B-cell acute lymphoblastic leukemia; CML: Chronic myeloid leukemia; DAPI: 4,6-diamidino-2-phenylindole; Bel-2: B-cell lymphoma 2; Bel-XL: B-cell lymphoma-extra large; FITC: Fluorescein isothiocyanate; PI: Propidium iodide; RPMI: Roswell Park Memorial Institute; PBS: Phosphate buffered saline; AGP: Andrographolide.

REFERENCES
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