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Andrographolide Induces G2/M Phase Cell Cycle Arrest in Human Head and Neck Cancer Cells

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Abstract—*Worldwide, head and neck squamous cell carcinoma (HNSCC) is one of the major cancer problems with limited treatment options. It can quickly progress to metastasis causing higher mortality among HNSCC patients. Hence, finding effective and alternative approaches to control the growth and progression of head and neck cancer are desirable for the management of HNSCC. Herein, we have studied the effect of andrographolide, the main constituent of a medicinal herb Andrographis paniculata, against growth and progression of head and neck cancer cells. Growth and viability of the HNSCC cancer cells were found to be significantly (P<0.001) decreased with AG treatment in dose-dependent manner at 48 h of treatment in Cal 33 and UM-SCC-22B HNSCC cells. AG treatment on HNSCC cells at 5 to 25 µM concentrations for 24 and 48 h caused (P<0.001) G2/M phase cell cycle arrest. G2/M phase cell cycle arrest sustained at 48 h in UM-SCC-22B cells while it was not observed for Cal 33 cells at this time point. This arrest was followed by an increase in sub-G1 phase cell population which is an indicator of apoptotic cell death. At molecular level, AG treatment downregulated (up to 80%) the protein levels of Cyclin B1 and CDK1 and enhanced (up to 3.7 fold) the expression of CDKI namely Cip1/p21 and Kip1/p27. It also increased apoptosis which was accompanied with PARP cleavage in cancer cells. These results provide new insights into the molecular mechanisms of anti-proliferative properties involving cell cycle arrest for AG against HNSCC cells.*

1. INTRODUCTION

Human squamous cell carcinoma (HNSCC) is one of the common cancer by incidence worldwide arising mostly in the epithelial lining of the oral cavity, larynx, pharynx and hypopharynx [1,2]. Unfortunately, there has been no improvement in the 5-year overall survival rate (40-50%) of HNSCC patients. The treatment modalities like surgery, chemotherapy and radiation are effective only in the early stages of head and neck cancer. The advanced HNSCC (stage III and stage IV) are more complicated and treatment options are very few and choice is dependent on a large number of factors like the site of the lesion and its accessibility for excision [3]. However, there are only marginal effects with long-term complications. These necessitates the look for an alternative approach which are affordable, quite safe, easily available that can control the growth and progression of HNSCC. Of all the cancers arising from different sites of head and neck, the cancer of hypopharynx has one of the worst prognoses. More than two-third incidences and deaths due to head and neck cancer are contributed by the cancers of oral cavity and pharynx [4]. They are often correlated with certain lifestyle risk factors like tobacco and alcohol consumption and there are evidences of dose relationship of their consumption and the occurrence of cancer. Thus, external factors are the predominant cause of this disease. Following this reasoning, it is plausible that chemoprevention with phytochemicals may also contribute to the inhibition of progression of HNSCC including oral and pharyngeal cancer. Several epidemological researches have reported that consumption of fruits, vegetables and grains as well as phytochemicals from non-dietry sources confers protection against wide variety of malignancies [5]. *Andrographis paniculata* Nees, a commonly used plant in Indian traditional system of medicines, has evoked interest in researchers in recent times. Studies have reported its preventive potential against chemotoxicity including carcinogenicity [6]. Andrographolide is derived from this plant and has shown a wide-range of anti-cancer activity or chemopreventive efficacy on several cancer cell lines including cervical HeLa, hepatoma HepG2, lymphocytic leukemia P388 cells, etc. It may offer a lead for novel anti-cancer agent against HNSCC. Whether AG can have any effect on cell cycle progression of HNSCC remains elusive, though. Hence, it was the main objective of this study.

Herein, we have used hypopharyngeal carcinoma UM-SCC-22B cells and tongue squamous cell carcinoma Cal 33 cells to evaluate the ability of AG to inhibit the growth of HNSCC cell lines. The main objective of the present study was to evaluate the potential of AG to suppress HNSCC growth and cell cycle progression as its underlying molecular mechanism.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Andrographolide (AG), sodium chloride, acetic acid, ethylenediamine tetra acetic acid (EDTA), hydrochloric acid, ,sodium hydroxide, methanol, crystal violet, bromophenol blue, MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide} , Trizma Base, calcium chloride, sodium dodecyl sulphate (SDS), ethidium bromide (EtBr) and β-mercaptoethanol (BME). Molecular biology grade DMSO was purchased from CalBiochem, USA. Protease and phosphatase inhibitors were procured from Roche Molecular Biochemicals (Indianapolis, IN, USA). Nitro-cellulose (NC) membrane/ PVDF and enhanced chemiluminescence (ECL) detection system was provided by Amersham Biosciences USA, Millipore USA and MDI India.

2.2 Cell culture and treatments

Cal 33 and UM-SCC-22B were from American Type Culture Collection (Manassas, VA, USA). Cal 33 and UM-SCC-22B were maintained in DMEM media. The cell lines were grown supplemented with 10% FBS and 1% penicillin-streptomycin-amphotericin B cocktail in a humidified atmosphere under 5% CO2 and 37^0C temperature in CO_2 incubator. FBS was heat inactivated at 56^0C for 30 minutes before adding to media to be used for UM-SCC-22B. Cells were seeded and grown onto tissue culture plates till desired confluencies were achieved. For immunoblot assays cells were seeded and treated with agents at 70% confluency. Cells were treated with the specific doses of agent under specific growth conditions for specific time periods.

2.3 MTT assay

In 96-well culture plate, 10,000 cells were seeded and grown for 24 h in specific culture media and then subjected to different treatments for the specific time periods. Before the end of the treatments, 100 µl 0.5 mg/ml MTT was added to each well for 4 h. After incubation time was over, plates were removed from incubator and MTT solution was aspirated out. Cells were gently washed with 1x PBS once and incubated at R.T. in 100 µl DMSO for 5 minutes to dissolve the formazan crystals. Purple color was observed after dissolving formazan crystals in DMSO. Wells were read at 570 nm after brief pre-mixing using a Synergy H1 Hybrid Reader, BioTekmicroplate reader (USA) [7].

2.4 Flow cytometric analysis for cell cycle phase distribution

UM-SCC-22B and Cal-33 cells were similarly seeded and treated as for cell growth assay mentioned above. At the end of each treatment, total cells were collected by brief trypsinization and centrifugation followed by processing for cell cycle analysis using saponin-PI solution (0.3% saponin (w/v), 25 µg/ml PI (w/v), 0.1 mM EDTA and 10 mg/ml RNase (w/v) in PBS), as reported earlier [7]. Cell cycle distribution was then analyzed by flow cytometry using BD FACS Aria III flow cytometer from BD Biosciences (San Jose, CA) [7].

2.5 Apoptotic cell death assay by flow cytometry

Induction of apoptosis in UM-SCC-22B cells caused by andrographolide was quantitatively determined by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA) following the manufacturer's instructions as previously described [7]. Cells were treated with different doses of andrographolide (5, 10, 25 µM) for 48 h, and then harvested, washed with PBS and incubated with Alexa488 and propidium iodide and analyzed by flow cytometry (BD Biosciences, San Jose, CA) [7].

2.6 Cell lysate preparation and immunoblotting

For preparing lysate, cells were treated with andrographolide (5, 10 and 25 µM) for required time periods. Non-denaturing lysis buffer was used to prepare the lysate [13]. After the treatment was over the cells were washed and incubated in lysis buffer and then centrifuged at 4°C for 30 min at 14,000 rpm [7]. The concentration of protein in lysates were determined using Bradford reagent from Bio-Rad Laboratories (Hercules, CA). Anti- Cyclin B1 and CDK1 antibodies were from Cell Signaling Technology, USA. A primary antibody against cleaved PARP was purchased from BD Biosciences and Anti-CDC25C, p21 and p27 were purchased from Santa Cruz Biotech, CA, USA. Both anti-rabbit and anti-mouse secondary antibodies used were from Cell Signaling (Beverly, MA).

Whole cell lysates (60-80 µg) were mixed with 2x sample buffer by mixing equal volumes of lysate and 2x denaturing sample buffer and were loaded on 8, 10,12 or 15% SDS-PAGE gels, which were run at constant voltage. Proteins from the gel were transferred on to nitrocellulose membrane and blots were put in blocking buffer for 1 hr at R.T and incubated with specific primary antibody overnight and subsequently incubated with corresponding HRP-linked secondary antibody and the blots were visualized with ECL detection system [7].

2.7 Statistical analysis

Statistical analysis of the data were done using Graphpad Prism version 5 software. Student's test was done to evaluate the statistical significance of difference among control and treatment groups. A statistically significant difference was considered when P value was less than 0.05. All experiments were repeated at least three times with similar results.

3. RESULTS

3.1 Andrographolide decreased proliferation and survival of HNSCC cells

The cytotoxic effect of AG treatment on Cal 33 and UM-SCC–22B cells were studied by MTT assay (Fig. 1A & B) for 24 and 48 h. MTT assay measures the formazan product at 570 nm. It was observed that AG significantly inhibited growth in all the three cell lines at 48 h in a concentration and time-dependent manner. The percentage inhibition with AG (5–25 µM) treatment for 48 h in Cal 33 cells were 9 - 58% ($P<0.01$-0.001) and in UM-SCC-22B cells were 10 - 37% ($P<0.01$-0.001). These results indicate that AG strongly inhibits the growth and survival of HNSCC cells.

3.2 Andrographolide induces G2/M phase arrest in HNSCC cells

To gain insight into the significant growth inhibitory effect of andrographolide in Cal 33 and UM SCC-22B cells and to explore the possible mechanism of its antiproliferative activity, we analyzed its effect on cell cycle progression. Graphical representation of the cell cycle distribution without or with treatment with 5-25 μM AG showed that it causes G2-M arrest at 24 h and 48 h in both UM-SCC-22B cells and Cal 33 cells, found to be more prominent in 24 h which was accompanied by a decrease in cell population in both G1 and S phases (Fig. 2B & C). Andrographolide treatment resulted in accumulation of cells in G2/M phase by 17.2% to 20.8% to 8.3% (P< 0.01-0.001) at 5 to 25 μM doses in UM-SCC-22 B cell line at 24 h and 1.3% to 12% to 27.34% at 5 to 25 μM doses in Cal 33 cell line.

3.3 Andrographolide modulate the levels of G2/M phase regulators in HNSCC cells

The G2-M is regulated by a complex between cyclin B1 and CDK1. AG treatment (5-25 μM for 24 h) caused a marked decrease in protein levels of cyclin B1 and CDK1 in both UM SCC-22B and Cal 33 cells (Fig. 3A & B). These results indicated that AG induced cell cycle arrest in G2-M phase in UM-SCC-22B and Cal 33 cells could be mediated *via* a decrease in protein levels of cyclin B1 and CDK1. The CDK1 is dephosphorylated by the phosphatase, CDC25C that results the entry of cells in M phase. It was observed that AG treatment decreased the level of CDC25C at 24 h in both the cell lines (Fig. 3A & B). We also analyzed the levels of Cip/Kip proteins. Our results revealed that treatment of UM-SCC-22B cells with varying concentrations of andrographolide (5-25 M) for 24 h resulted in an increase in expression of Cip1/p21 and Kip1/p27 in a dose-dependent manner (Fig. 3C).

3.4 Andrographolide induces cell death *via* apoptotic pathway in HNSCC cells

Further, we proceeded to characterize whether the decrease in viability observed in UM-SCC -22B cells upon treatment with andrographolide was due to the induction of apoptosis. Therefore, we treated the cells with andrographolide and apoptotic cells were analyzed using the Annexin V-conjugated FITC Apoptotic Detection Kit, as described earlier [7]. During apoptosis, the phospholipid present on the inner membrane of cells namely phosphatidylserine is translocated from inner to outer leaflet of membrane, which can bind with Annexin V–FITC and the conjugates can be examined with flow cytometry [23]. Consistent with the cell cycle data, 25 μM andrographolide treatment for 48 h showed increase in the apoptotic cells (Fig. 4A). Andrographolide (25 μM) after 48 h of treatment increased apoptotic cells to 16% (P<0.001) as compared to 4.5% in control. Activation of caspases can subsequently lead to apoptotic cell death through cleavage of a wide range of cellular proteins, including PARP. Next, we examined whether induction of apoptosis is associated with the activation of PARP proteins. Andrographolide (5-25 μM) treatment for 48 h showed a concentration-dependent increase in the cleaved levels of PARP (Fig. 6C).

4. DISCUSSION

The treatment modalities for HNSCC, like surgery, chemotherapy and radiation are found to be success only in the early stages of head and neck cancer. Locally advanced HNSCC (stage III and stage IV) are more complicated and treatment options are less. Majorly, HNSCC is homogenous disease in compared to other cancer types, as 95% of HNSCC is squamous cell carcinomas (SCC) [8]. Herein, we have selected two SCC model cell lines that are Cal-33 and UM SCC-22B. Both are representing two different site of origin and stages of cancer. Cal-33 is tongue SCC cells isolated from localised tumor. Whereas UM-SCC-22B is hypo-pharyngeal SCC cells derived from metastatic site at neck lymph node.

Andrographolide (AG), the main diterpenoid isolated from *A. paniculata*, was previously shown to have cytotoxic activity against human epidermoid carcinoma KB cells and lymphocytic leukemia P388 cells [9]. Even though, the previous studies have shed light on the anticancer potential of AG, there is no direct evidence showing its effect on HNSCC and the detailed molecular mechanisms are yet to be elucidated. Therefore, in the present study, we first analyzed the effect of AG on the viability on Cal 33 and UM-SCC-22B, HNSCC cells lines and the results revealed that AG significantly suppressed HNSCC cancer cell proliferation at higher doses at 24 h of treatment and in a dose-dependent manner at 48 h in these two HNSCC cell lines.

Cell cycle progression and it regulation is the key event to generate two daughter cells with ensure accuracy but in cancer misbalance in cell cycle regulation will lead uncontrolled cell proliferation. Duplication of chromosome and generation of two ideal daughter cells is ensured by cell cycle ‘checkpoints’. In cancer cells, many cell cycle regulators are been observed to be mutated or inactivated including p53. Due to this, regulation of uncontrolled cell proliferation via controlling cell cycle events using small molecules is become a key strategy for cancer therapeutics and prevention [10]. Here, AG treatment restrict the cells in G2/M phase which is associated with down regulation of Cyclin B1 and CDK1 expression. The formation of Cyclin-CDK complex is a key event for G2/M phase transition. This cycle B1 and CDK1 complex formation is activated by phosphatase CDC25C [11]. AG treatment also down regulates the expression of CDC25C. Around 67% of HNSCC shows p53 mutation [8] and cell lines used durian this study are having mutated p53 R273H and Y220C respectively in Cal-33 and UM-SCC-22B cell lines. P53 regulates CDKI-p21 & p27 which regulated kinase activity of Cyclin B1-CDK1 and regulates them. We evaluated whether AG treatment induces the expression of p21 and p27 in a p53-independent manner. We found that, AG treatment up-regulate

the expression of p21 and p27. Based on this data we could say that AG treatment causes G2/M phase arrest *via* p21 and p27, which is followed by CDC25C and Cyclin-CDK down-regulation.

During cell cycle arrest, cells try to rescue the damages and if not they activate the cell death mechanism to exclude those cells. Prolonged cell cycle arrest leads to the induction of programmed cell death or apoptosis. AG cased G2/M phase arrest, which is sustained till 48 h, and this was followed by apoptotic cell death. Cleavage of PARP at Asp214 disassembles cellular indicators of apoptosis. Activation of PARP depletes the cellular ATP level in response to DNA damage. This activates the mitochondrial death machinery for programmed cell death. AG treatment caused apoptotic cell death and activation of PARP.

In summary, present study suggests that AG-caused decrease in survival of HNSCC cells is associated with down-regulation Cyclin-CDK and CDC25C, and G2-M phase cell cycle arrest and apoptosis. Further, this arrest and apoptosis may be associated with up-regulation of p53-independent p21 and p27. Further, *in vitro* mechanistic studies and *in vivo* study may prove AG as a preclinical therapeutic candidate agent for HNSCC treatment.

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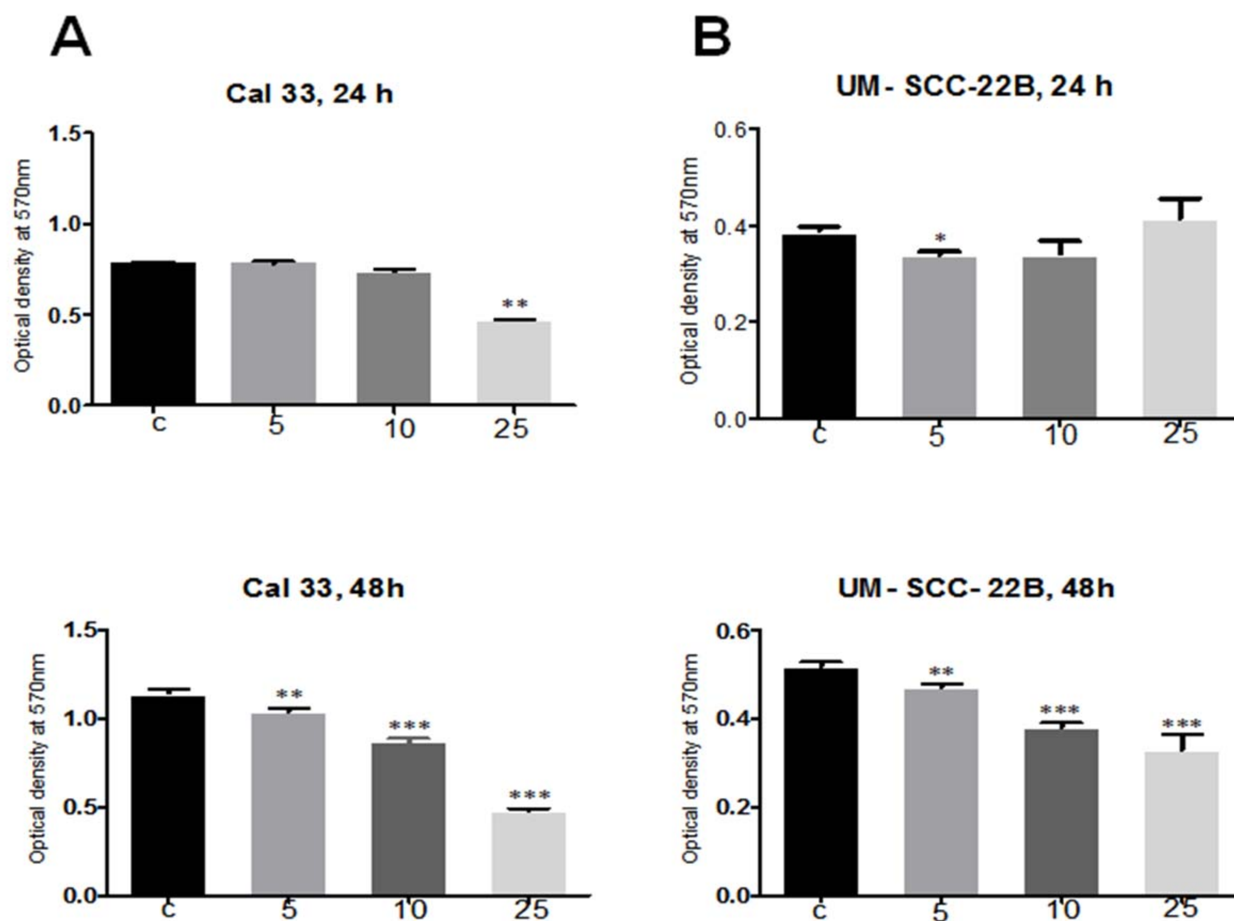
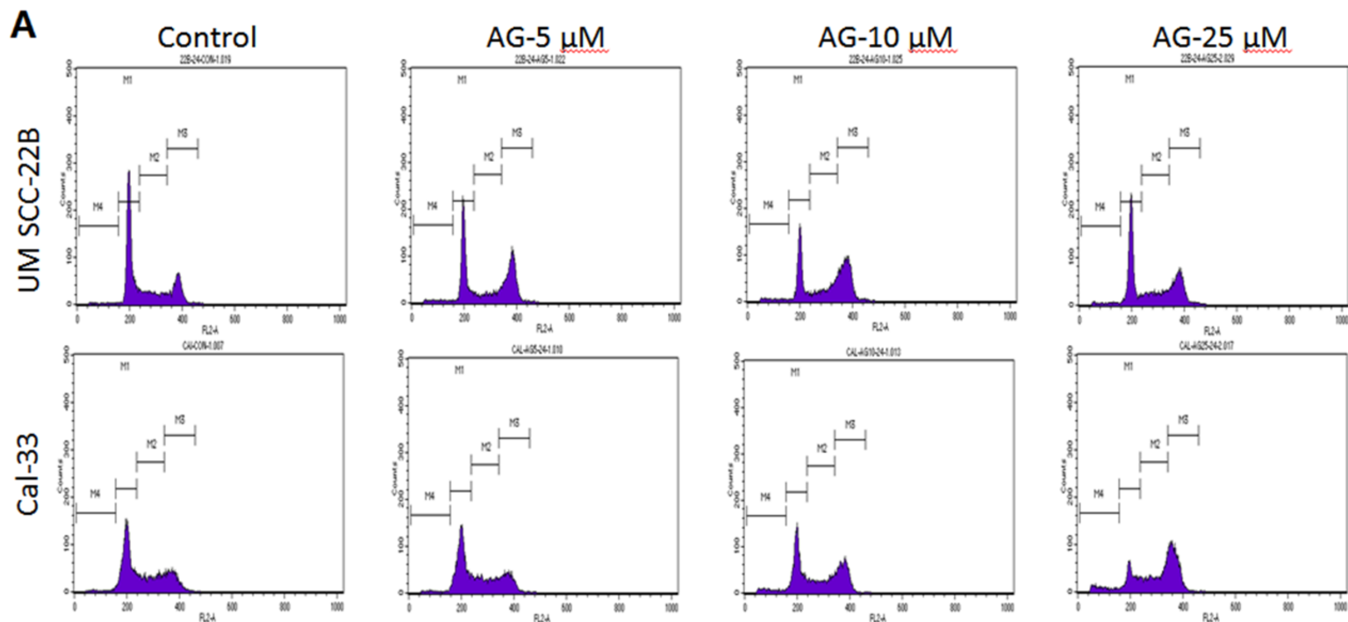
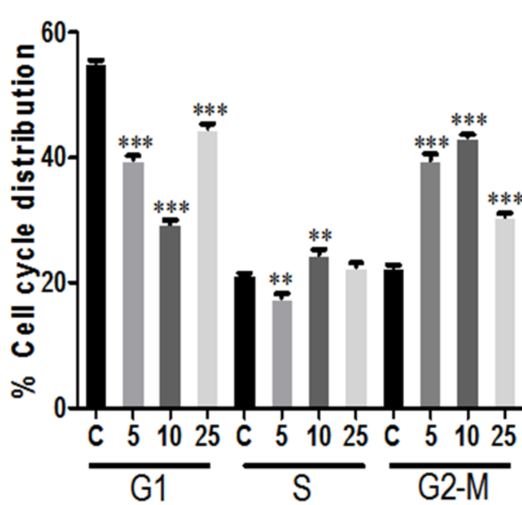


Figure 1: Effect of andrographolide (AG) on cell growth and viability of HNSCC, Cal 33 and UM-SCC-22B cells. (A) & (B) cell viability in Cal 33 and UM-SCC-22B, respectively, examined at 24 h and 48 h by MTT assay as described in Materials and Methods. Results are representative of three independent experiments. The data are presented as mean of triplicate samples for each treatment and data are shown as mean ± SEM of triplicate samples. *P<0.05, ** P<0.01 ***P< 0.001 versus control analysed using Student's t-test.

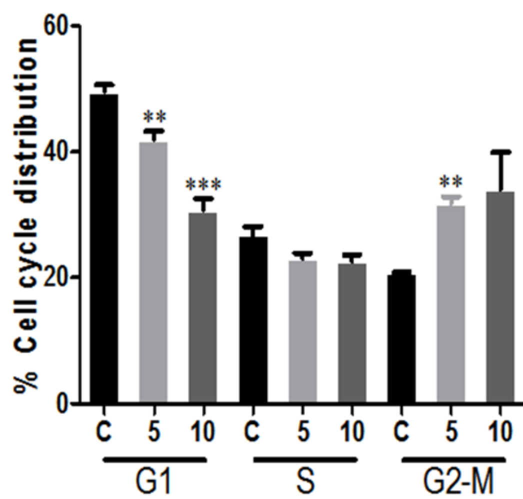


B

22B (24 h)



22B (48h)



C

Cal 33 (24 h)

Cal 33 (48 h)

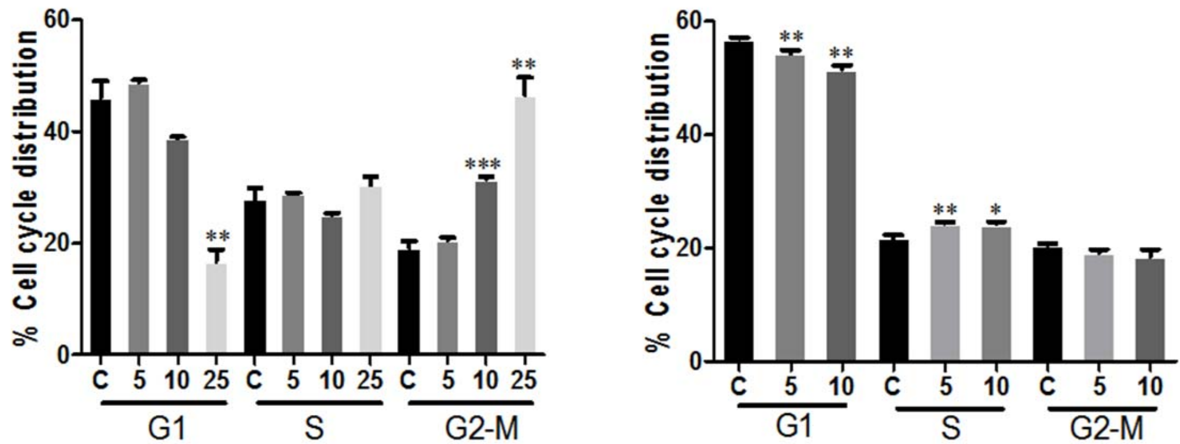
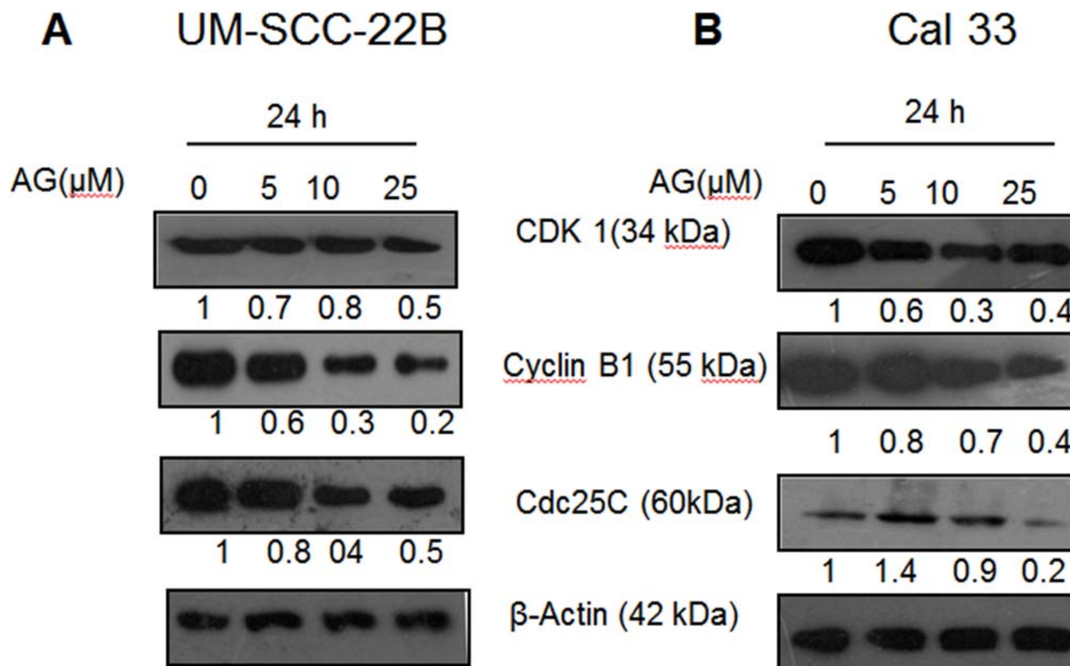


Figure 2: Effect of AG on cell cycle progression of UM-SCC-22B cells and Cal 33 cells. The cells were treated with DMSO (control), 5, 10 or 25 µM AG for 24 and 48 h. After indicated treatment times,cells were collected, washed with PBS, digested with RNase A, and then cellular DNA stained with propidium iodide. PI-stained cells were analyzed for cell cycle phase distribution as detailed in materials and methods. (A) Representative histogram of cell cycle distribution in UM-SCC-22B and Cal 33 cell lines in presence of different treatments. (B) The percentage distribution of cells in different phases analyzed by flow cytometry for 22B cells and (C) Cal 33 shown for 24 and 48 h. The percentage distribution of cells shown is the mean of triplicate samples for each treatment. The data are representative of three independent experiments. Bars, SEM; *P<0.05, **P<0.01, ***P<0.001 versus control.



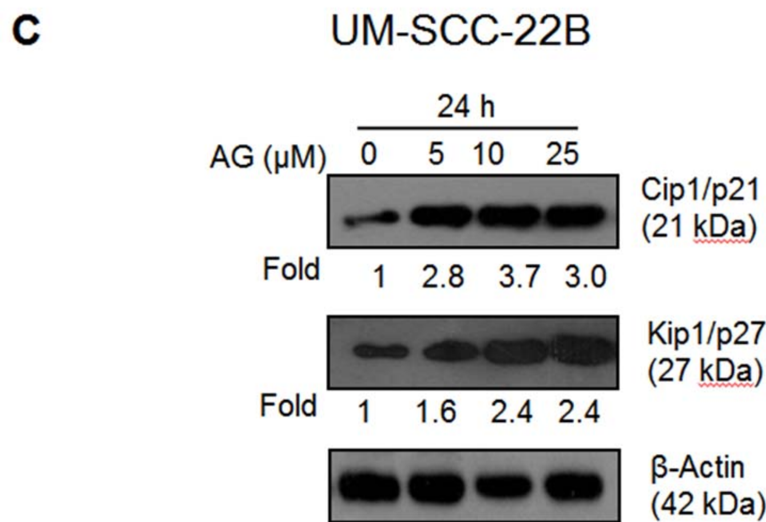


Figure 3: Effect of andrographolide on cell cycle regulators in UM-SCC-22B and Cal 33. Cells were treated with DMSO or 5 to 25 µM concentrations of AG for 24 h. Immunoblotting was done for cyclin B1, CDK1 and Cdc25C using lysates from both cells lines (A) UM-SCC-22B and (B) Cal 33 (C) expression of Cip1/p21 and Kip1/p27 in UM-SCC-22B cells treated with AG as detailed in Materials and Methods. Membranes were stripped and re-probed with anti-beta actin antibody to ensure equal protein loading. Numbers on top of the bands represent fold changes in protein levels as determined by densitometric analysis of the immunoreactive bands and corrected for beta-actin loading control.

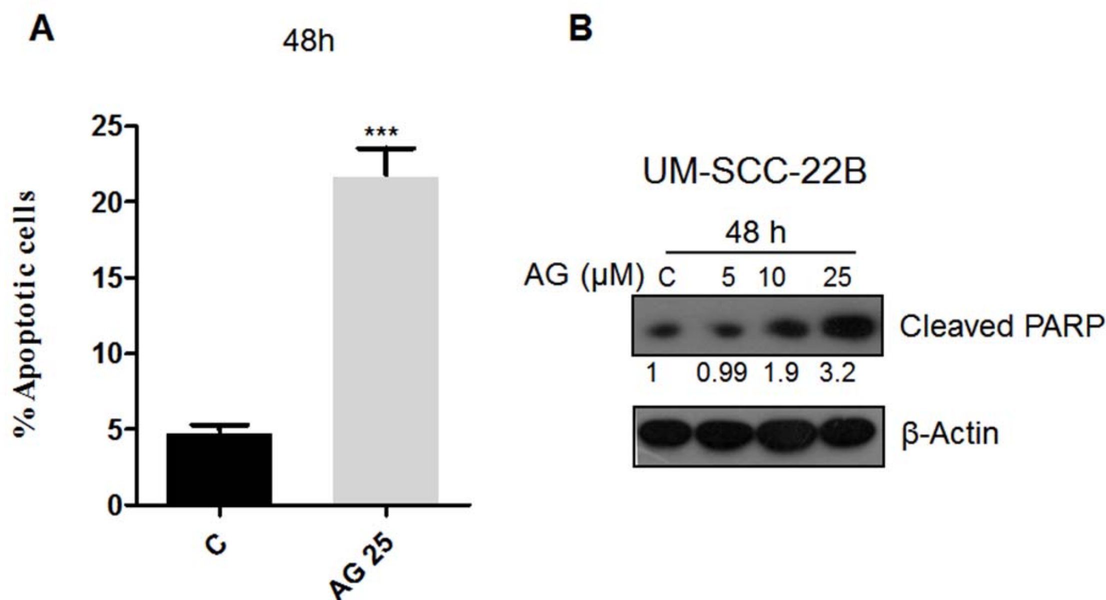


Figure 4: Effect of andrographolide on apoptotic death of HNSCC cells. Cells were treated with either DMSO (control) or 25 µM AG for 48 h. At the end of treatments, cells were harvested and stained with annexin V and PI. Apoptotic cells were analyzed by flow cytometry. (A) The percentage of apoptotic cells (annexin V-positive) as means of triplicate samples. (B) Western immunoblot analysis was performed for the expression level of cleaved poly (ADP-ribose) polymerase (PARP). Membrane was striped and re-probed with anti-β actin for loading control. Densitometric data shown above each band are fold-change of the protein levels from control and normalized to β-actin. The data are presented as mean of triplicate samples for each treatment and shown as mean ± SEM of triplicate samples. *P<0.05, ** P<0.01 ***P<0.001 versus control, analysed using Student's t-test.