RESEARCH ARTICLE

Inhibition of Topoisomerase IIα and Induction of Apoptosis in Gastric Cancer Cells by 19-Triisopropyl Andrographolide

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Abstract

Gastric cancer is the most common cancer in Eastern Asia. Increasing chemoresistance and general systemic toxicities have complicated the current chemotherapy leading to an urgent need of more effective agents. The present study reported a potent DNA topoisomerase IIα inhibitory activity of an andrographolide analogue (19-triisopropyl andrographolide, analogue-6) in gastric cancer cells; MKN-45, and AGS cells. The analogue was potently cytotoxic to both gastric cancer cell lines with the half maximal inhibitory concentration (IC_{50} values) of 6.3±0.7 µM, and 1.7±0.05 µM at 48 h for MKN-45, and AGS cells, respectively. It was more potent than the parent andrographolide and the clinically used, etoposide with the IC_{50} values of >50 µM in MKN-45 and 11.3±2.9 µM in AGS cells for andrographolide and 28.5±4.4 µM in MKN-45 and 4.08±0.5 µM in AGS cells for etoposide. Analogue-6 at 2 µM significantly inhibited DNA topoisomerase IIα enzyme in AGS cells, induced DNA damage, activated cleaved PARP-1, and Caspase3 leading to late cellular apoptosis. Interestingly, the expression of tumor suppressor p53 was not activated. These results show the importance of 19-triisopropyl-andrographolide in its emerging selectivity to primary target on topoisomerase IIα enzyme, inducing DNA damage and apoptosis by p53- independent mechanism. Thereby, the results provide insights of the potential of 19-triisopropyl andrographolide as an anticancer agent for gastric cancer. The chemical transformation of andrographolide is a promising strategy in drug discovery of a novel class of anticancer drugs from bioactive natural products.

Keywords: Gastric cancer- andrographolide analogue- topoisomerase IIα inhibitors- DNA damage- apoptosis

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Introduction

Gastric cancer (GC) is the fifth most common malignancy and the second leading cause of cancer-related deaths (Ferlay et al., 2013). Its incidence varies greatly across different geographic location amongst Eastern Asia, being the highest and substantially contributes to 6.8 % of the burden of cancer worldwide (Karimi et al., 2014). The mortality rate is also higher owing to the fact that early GC is asymptomatic, and diagnosed in advanced stage resulting to poor prognosis (Shi et al., 2014). In addition to surgery, the combination of radiotherapy and chemotherapy has been employed to suppress the recurrent cancer growth. However, the five-year survival rate for advanced stage GC is less than 20% due to the systemic toxicities and resistance to the current neo-plastic drugs (Yuan et al., 2016; Adham et al., 2017). Therefore, there is an urgent requirement of more effective agents to overcome these limitations in chemotherapy of GC.

DNA Topoisomerase has been established as an important molecular target of anticancer drugs. Topoisomerase II (Topo II) enzyme has critical functions to maintain the topology of the DNA during the vital cellular process such as DNA replication, transcription and recombination (Pommier et al., 2016). It exists in two homologous forms; alpha and beta forms. Topo IIα is abundantly expressed in proliferating cells more than in the normal quiescent cells (Hande, 2008). Moreover, GC cells highly express Topo IIα, which becomes an important drug target in gastric cancer (Miura et al., 2015). Inhibition of Topo IIα induces DNA damage response (DDR) characterized by recruitment of many repair proteins (Darzynkiewicz et al., 2009). Phosphorylation of histone (γ-H2A.X) at S139 is an early event and has been widely accepted as a surrogate marker (Darzynkiewicz et al., 2009; Podhorecka et al., 2010). The un-repairable DNA damage further induces poly (ADP-ribose) polymerase-1 (PARP-1) expression thereby directing the cells to induce

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apoptosis via activation of caspase 3 (Debatin, 2004). Apoptosis cell death is a crucial process, which has been implicated in cancer and the apoptotic-based therapy that causes minimal inflammation and damage to the tissues thereby becoming a powerful approach in cancer drug discovery (Baig et al., 2016; Koff et al., 2015). Apoptosis is also resulted either from tumor suppressor protein p53 dependent or independent mechanism (Khoo et al., 2014). DNA damage triggers the activation of p53 leading to p53 dependent apoptosis, and that without the p53 activation machinery (Fridman and Lowe, 2003).

Andrographolide, a major bioactive diterpenoid lactone from *Andrographis paniculata* (Burm.f) Nees (Acanthaceae), has been reported to have potential anticancer activities (Kumar, et al., 2004; Singh et al., 2013; Hossain et al., 2014). Recently, a number of andrographolide analogues have been synthesized to improve potency and efficacy for anticancer activity (Sirion et al., 2012; Nateewattana et al., 2014). The analogues are mainly modified at the α, β-unsaturated-γ butyrolactone moiety, two double bonds and three hydroxyl groups, which result in compounds with emerging new activities (Kumar et al., 2004; Fridman and Lowe, 2003). In our earlier studies, a series of semi-synthetic andrographolide analogues were obtained by changing the functional groups at C-3, C-12, C-17, and C-19, by which the analogues exhibited varying degrees of cytotoxicity against a panel of cancer cell lines and inhibition of topoisomerase enzymes (Sirion et al., 2012; Nateewattana et al., 2013). Interestingly, 19-triisopropyl-andrographolide analogue (analogue-6) exhibited potent cytotoxic activities against a panel of six cancer cells (Sirion et al., 2012) with an emerging new activity by inhibiting the enzyme topoisomerase IIα activities in an in vitro cell-free system (Nateewattana et al., 2013). In the present study, we further examined the topoisomerase IIα inhibitory activities of the analogue-6 and its underlying anticancer mechanism in gastric cancer cells. The results provide insights of the potential of 19-triisopropyl-andrographolide as a chemotherapeutic agent for gastric cancer.

Materials and Methods

Chemicals and reagents

Rosewell Park Memorial Institute 1640 medium (RPMI), and antibiotic-antimycotic agents were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum, RIPA, proteinase inhibitor, and Super Signal West Pico chemi-luminescent substrate were purchased from Thermoscientific (Cramlington, UK). SDS, 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and etoposide were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo, USA). Annexin V FITC apoptosis kit was obtained from BD Biosciences,(San Jose, CA, USA). Anti-Topoisomerase II alpha and anti-PARP-1 antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-γ.H2A.X, anti-p53, anti-caspase 3 and anti-β-actin antibodies were from Cell signaling Technology (Beverly, MA, USA). HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG antibodies were from Cell signaling Technology (Beverly, MA, USA). All other chemicals unless otherwise stated were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Andrographolide analogue (Analogue-6)

Andrographolide was isolated from dried aerial part of *Andrographis paniculata* and 19-triisopropyl-andrographolide analogue (analogue-6) was prepared by changing the functional group at C-19 as previously described (Nateewattana et al., 2013). Figure 1 shows the chemical structure of analogue-6. The compound was identified by IR, NMR and HRMS (EST) and the purity was approximately 99%.

Cell culture

Human gastric cancer from Asian (MKN-45) and from Caucasian (AGS) cells were purchased from Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Japan), and American Type Culture Collection (ATCC, Manassas, VA, USA), respectively. They were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum and 1% antibiotic (100 U/ml of penicillin and 100 µg/ml of streptomycin) in a humidified 5% CO_2 incubator at 37 °C.

Cell viability assay

Colorimetric MTT assay was used to determine the cell viability. MKN-45 at 2 x 10^4 cells/well, and AGS cells at 7 x 10^3 cells/well were seeded in 96-well plates with varying concentration (0.1-50 µM of compounds at 24, 48 and 72 h. Cells in control group were cultured in medium containing an equivalent amount of DMSO required to dissolve the compounds (less than 0.05%). Medium containing the compound and DMSO was removed at the end of every specific duration of treatment and incubated with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium dissolved with medium in a humidified 5% CO_2 incubator at 37 °C for 3 h. DMSO was used to dissolve the dark blue formazan and then measured at the wavelength of 540 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermoscientific, Cramlington, UK). The result was calculated as % of cell viability.

Analysis of apoptosis

Fluorescence activated cell sorting analysis was used to analyze an induction of cell apoptosis. For the detection of externalization of phosphatidylserine (PS), Annexin V-FITC Apoptosis Detection Kit was used. Briefly, AGS cells were seeded in 6-well plates for 24 h and treated with concentration of 1, 2 or 5 µM of analogue-6, 10 µM of andrographolide or etoposide (positive control) for 48 h. Cells were trypsinized with 0.05 % trypsin and then washed with ice-cold phosphate buffer saline (PBS). Then cells were co-stained with propidium iodide and Annexin V for 15 min at room temperature in the dark and analyzed by BD FACSCanto flow cytometry.

Western blotting analysis

MKN-45 and AGS cells were plated in a 6-well plate and treated with 0.5-10 µM of andrographolide,

analogue-6, and etoposide for 24 h. Cells were washed with cold 1X PBS and lysed with lysis buffer. Total proteins were extracted and measured using bicinchoninic acid biuret (BCA) assay. Proteins were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were washed, blocked with 5% non-fat dried milk, and incubated overnight with primary antibodies. The membranes were further washed and incubated with anti-horseradish peroxidase conjugated secondary antibodies. Chemi-luminescence substrate was used to visualize the immuno-reactive signals. Beta-actin was used as the loading control.

Statistical analysis

The data are all expressed as means ± the standard error of the mean (SEM). One-way analysis of variance (ANOVA) were used for analysis and the differences among treatment groups were compared by Tukey's method, using GraphPad Prism version 5.01 (GraphPad software Inc, CA). $P < 0.05$ was considered to be significant.

Results

Effects of andrographolide and analogue-6 on the growth inhibition in gastric cancer cells

The cytotoxic effects of andrographolide and analogue-6 in gastric cancer cells of both MKN-45 and AGS cells were examined at 24, 48 and 72 h using MTT assay. The half maximal inhibitory concentration (IC_{50} value) of the analogue at 48 h was 6.3±0.7 μM in MKN-45 cells and 1.7±0.05 μM in AGS cells (Table 1) whereas those of the parent andrographolide was >50 μM in MKN-45 and 11.3±2.9 μM in AGS cells, respectively. Of note, clinically used etoposide (positive control) was less cytotoxic than the analogue with IC_{50} values of 28.5±4.4 μM in MKN-45 and 4.08±0.5 μM in AGS cells, respectively.

Effects of andrographolide and analogue-6 on DNA damage and inhibition of topoisomerase IIα expression

To further investigate the molecular mechanism of cell death in gastric cells, the expression of γ-H2A.X, a DNA damage marker and Topo IIα were determined using Western blotting. As shown in Fig. 2a, 10 μM of the analogue significantly increased the expressions of γ-H2A.X in MKN-45 cells treated for 24 h ($P<0.01$).

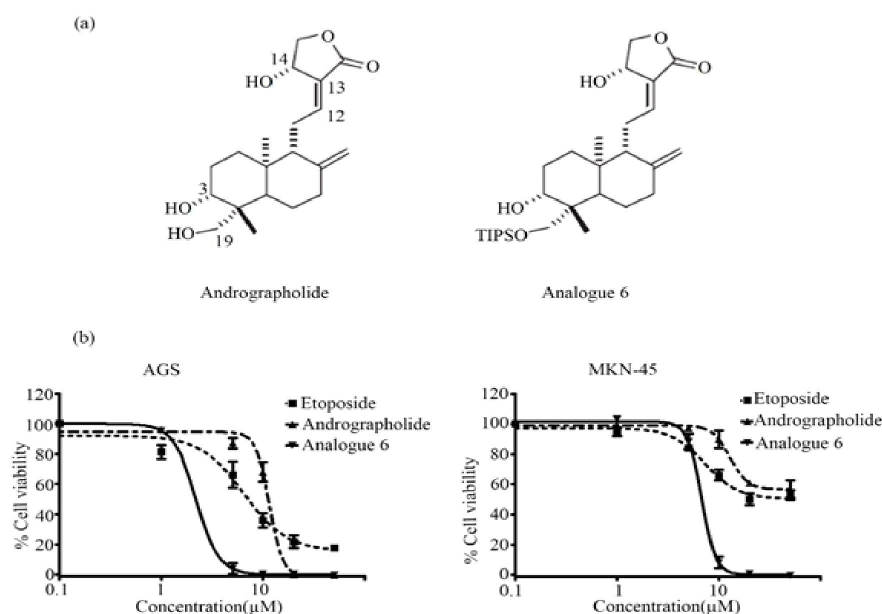

Figure 1. The Chemical Structure of Andrographolide and Analogue-6 (a). The cytotoxic effects of andrographolide, analogue-6, and etoposide on AGS and MKN-45 cells at 48 h after treatment, using MTT assay (b).

Table 1. IC_{50} Values of Andrographolide, Analogue 6 and Etoposide

Cell line	Incubation time (Hours)	IC_{50} (μM)		
		Etoposide	Andrographolide	Analogue 6
AGS	24	19.6 ± 0.9	21.5 ± 6.0	1.9 ± 0.09
	48	4.08 ± 0.5	11.3 ± 2.9	1.7 ± 0.05
	72	1.9 ± 0.3	13.3 ± 1.4	1.8 ± 0.1
MKN 45	24	>50	>50	8.8 ± 2.2
	48	28.5 ± 4.4	>50	6.3 ± 0.7
	72	12.6 ± 0.2	36.3 ± 5.9	5.6 ± 0.2

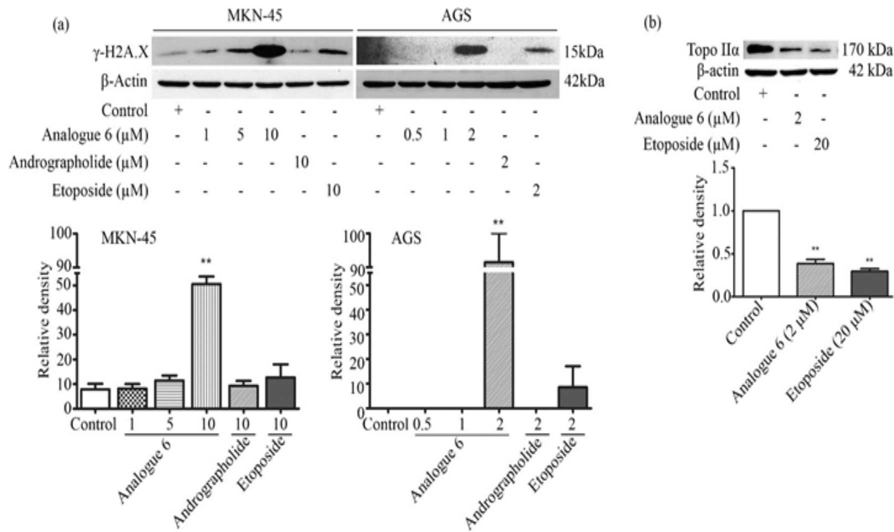

Figure 2. Effects of Andrographolide, Analogue-6, and Etoposide Treatments in Gastric Cancer Cells on the Induction of DNA Damage (a), and inhibition of Topoisomerase IIα (b). The DNA damage was represented by the expression of γ-H2A.X protein after treatment for 24 h whereas expressions of human topoisomerase IIα enzyme were detected at 4 h after treatment. The bar graph represents the mean normalized densitometry values of γ-H2A.X with β-actin. Data are means ± SEM of three independent experiments. ** P <0.01; significantly different from the vehicle control.

Notably, the parent andrographolide and etoposide at the concentration of 10 µM were not potent to induce DNA damages as compared to the analogue. Similar results were obtained in AGS cells when treated with 2 µM of the analogue. As shown in Figure 2b, the expression of Topo IIα enzyme was significantly decreased in AGS cells at 4 h after treatment with 2 µM of the analogue (P<0.01). AGS is a rapid growing cell line and more sensitive to our tested compounds than MKN-45 cells. A well-known topoisomerase II poison etoposide at 20 µM, used as positive control, also inhibited the expression of Topo IIα enzyme.

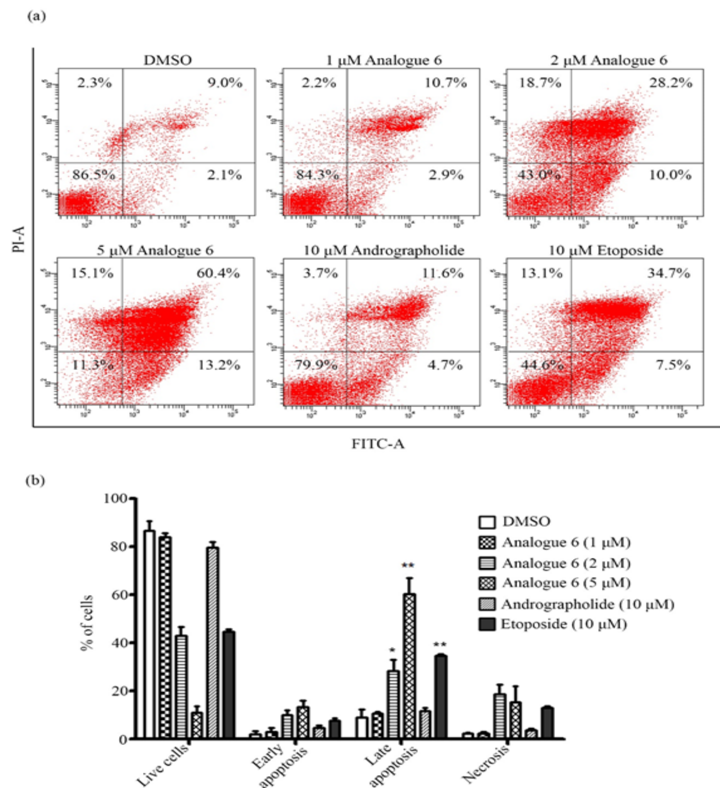

Figure 3. Andrographolide and analogue-6 Induce Apoptosis of AGS Cells. (a) AGS cells were treated with andrographolide, Analogue 6, or etoposide at the indicated concentrations for 48 h. The cells were then stained with Annexin V/PI and subjected to fluorescence activated cell sorting on BD FACS canto machine. Lower left and lower right panels represent live cells (An-/PI-) and early apoptotic cells (An+/PI-). Similarly, upper left and upper right panels represent necrotic cells (An-/PI+) and late apoptotic cells (An+/PI+). (b) Data are means ± SEM of three independent experiments. **P<0.01 significantly different from vehicle DMSO control.

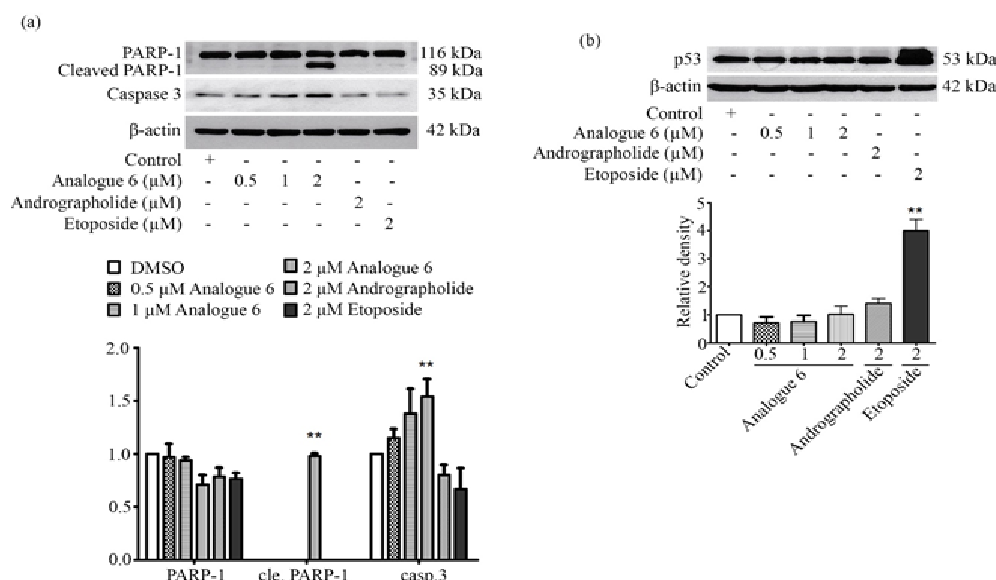

Figure 4. Effects of Andrographolide and Analogue-6 on the Proteins Involved in Apoptosis Pathway in AGS Cells. (a) Expressions of PARP-1, cleaved PARP-1, and Caspase 3; (b) expression of p53. Cells were treated with analogue-6, parent andrographolide and etoposide for 24 h. The bar graph represents the mean normalized densitometry values with β-actin. Data are means ± SEM of three independent experiments. ** P <0.01; significantly different from the vehicle control.

Induction of AGS cell apoptosis by andrographolide and analogue-6

The effects of andrographolide and analogue-6 on the induction of apoptotic cell death in gastric cancers was further investigated by detection of externalization of phosphatidylserine after staining with Annexin V and propidium iodide. In Figure 3, analogue-6 shows a concentration dependent increase in late apoptosis of AGS cells. At 48 h the analogue at concentration of 1, 2 and 5 μM induced late apoptosis of AGS cells up to 10.7±0.5, 28±4.6 and 60.4±6.5%, respectively, as compared to DMSO control (9.0±3.2%) (Figure 3a). The parent andrographolide, and etoposide (positive control) at 10 μM induced late apoptosis at approximately 11.6±1.4% and 34.77±0.5%, respectively. Analogue-6 was more effective in inducing apoptosis than the parent andrographolide, and the topoisomerase II poison; etoposide. These results suggest that analogue-6-mediated cell death was resulted from the induction of apoptosis mechanism.

Effects of andrographolide and analogue-6 on proteins involved in apoptosis

Involvement of proteins participating in apoptosis pathway, particularly those related to Topo IIα inhibition were determined. The expression of cleaved PARP-1 protein resulting from DNA damage was markedly increased at 24 h after treatment with 2 μM of analogue-6 (Figure 4a). Activation of Caspase 3, which is the hallmark of apoptosis, was increased (P<0.01) after treatment. On the other hand, the expression of p53 was not altered following the treatment by the analogue as compared with that after etoposide, which is a known inducer of p53 activation.

Discussion

Andrographolide has a unique bioactive core structure that has been used as a lead compound for semi-synthesis of analogues with variety of superior therapeutic activities in drug discovery research, particularly for the anticancer activity (Hossain et al., 2014). By addition of triisopropyl at C-19, the compound inhibited DNA topoisomerase IIα enzyme (Topo IIα) using in vitro cell-free system (Nateewattana et al., 2013). The inhibition of DNA Topo IIα enzyme has been suggested as one of the most favorable properties required for anticancer agents. In the present study, we demonstrated the potent anticancer activity and the underlying mechanism of 19-triisopropyl-andrographolide (analogue-6) in two human gastric cancer cells. Analogue-6 potently inhibited Topo IIα in human gastric cancer cells which was accompanied by increasing DNA damage, PARP-1 cleavage, caspase 3 activation, and apoptosis without activation of p53. To our knowledge, this is the first report on the anticancer property of analogue-6 via the inhibition of Topo IIα expression in gastric cancer cells and the induction of p53-independent apoptosis.

Topoisomerase IIα enzyme plays a vital role in several cellular processes such as DNA transcription, replication, chromatin segregation and condensation, thereby renders itself as an important target for anticancer agents (Walker and Nitiss, 2002). It is also essential for survival of proliferating cells due to its ability to decatenate and disentangles DNA by passing an intact helix through a transient double stranded break in DNA (Byl et al., 2001). Re-ligation of DNA backbone is also carried out by Topo IIα after the strand passage. Topo IIα inhibitors/poison act on this process to induce breaking of DNA. In addition, inhibition of Topo IIα enzyme induces DNA damages which, in turn, recruits DNA repairing protein; γ-H2A.X (Andoh, 2000; Fragkos et al., 2009; Podhorecka et al.,

2010). In the present study, analogue-6 markedly increased the phosphorylation of histone H2A.X on S139 in gastric cancer cells which were greater than those by the parent andrographolide and etoposide, suggesting the induction of DNA damage as the result of Topo IIα inhibition.

After an un-repairable DNA damage, cells undergo apoptosis via several possible mechanisms (Baig et al., 2016). Activation of the cleavage of PARP-1 and Caspase 3 are the hallmarks of apoptosis (Wong, 2011). PARP-1 is activated in response to DNA damage and plays a role in DNA replication, transcription, and repair. In this study, analogue- 6 increased the expressions of γ-H2A.X, cleaved PARP-1, and activated Caspase 3. Since PARP-1 protein is a substrate of Caspase 3 (Chaitanya et al., 2010), it is likely that apoptotic cell death in gastric cancer AGS cells induced by the analogue was resulted from the downstream activation of Caspase 3 by γ-H2A.X, and the cleavage of PARP-1 occurred via the Caspase 3-dependent pathway.

The protein p53 is one of the most potent cancer suppressor proteins that provide its anticancer activity by governing transcription of many downstream target genes related in cell cycle arrest, DNA repair, apoptosis, and preventing cancer-causing mutations (Khoo et al., 2014). This protein can be activated by DNA damages and oxidative stress by which its activation caused an increase in the half-life of p53 and modification of its conformation (Williams and Schumacher, 2016). However, our analogue did not activate the p53 expression whereas the etoposide markedly increased the expression of p53 (Figure 4). Despite the useful function of p53, however, acute activation of p53 may lead to severe negative consequences such as systemic toxicities causing cell death in sensitive tissues such as hematopoietic system, and gastric epithelium (Komarova, 2001). Earlier studies showed that p53 inhibitors could overcome this issue resulting to protection from secondary toxicities (Komarova, 2001; McNamee and Brodsky, 2009; Strom et al., 2006). In addition, it has been reported that oncogenic potential of many cancer cells is related to the accumulation of oncogenic mutant p53. Any compound that has biological effect to restore p53 activity also has potential as anticancer agents. Notably, etoposide, which activates p53, has been reported to cause severe cardiotoxicity and secondary leukemia (Kobayashi and Ratain, 1994; Pai and Nahata, 2000). In our study, exposure to etoposide also markedly activated p53. However, it is not clear whether it is related to the secondary systemic toxicities as previously reported (Kobayashi and Ratain, 1994; Pai and Nahata, 2000). Analogue-6, which did not activate p53, may be able to over-come these adverse effects.

The interaction and correlation between PARP1 and p53 have been documented. Wieler et al., (2003) reported that PARP1 is a key regulator of the p53 response to DNA damage as the inhibition of endogenous PARP1 functions was found to suppress the transactivation function of p53 after treatment with ionizing radiation. However, the correlation of the overexpression of PARP1 and p53 was found only in the advance stage of tumor in meningioma (Csonka et al., 2014) and epithelial ovarian cancer (Godoy et al., 2011). It seems that the correlation of PARP1 with p53 might serve as a marker for aggressive behavior of disease (Godoy et al., 2011). In the present study, we observed the activation of PARP-1 cleavage without the activation of p53. It is possible that the activation of p53 is a later event in tumor progression than PARP-1 protein in consistent with the previous report (Csonka et al., 2014).

In considering the cytotoxicity of the analogue, andrographolide is a diterpenoid lactone with α, β-unsaturated lactone group, which is electrophilic in nature and belongs to Michael acceptor system category (Nguyen et al., 2015). Various analogues have been synthesized to improve the property of being Michael acceptor of the α, β-unsaturated γ-butyrolactone moiety. In the previous studies, our andrographolide analogues 3A, 1B and 2C showed potent anticancer activities with inhibitory activity on hTopo II α enzyme in cell-free system. These analogues are different in their core structures and alkyl-substituted groups on silicon atom at C-19 position (Nateewattana et al., 2013; Sirion et al., 2012). Analogue 3A contains epoxy ring at C-8 position and has tert-butyldiphenyl silyl ether group at C-19 position whereas analogue 2C is 14-deoxy-12-hydroxyandrographolide analog with tert-butyldimethyl silyl ether group at C-19 and two acetyl groups at C-3 and C-14. Analogue-6 (1B in our previous study) is the modified hydroxyl group at the C-19 of andrographolide structure by addition of silicon based molecule triisopropyl (TIPS). These analogues inhibited the activity of hTopo II α enzyme in cell-free system. Therefore, more investigations on the molecular mechanism in mammalian cancer cells are required. It is not clear what properties of the compound provide this important activity. Analogue 3A was reported to induce apoptosis in cholangiocarcinoma through topoisomerase II alpha inhibition and both epoxide at the C-17 position and TBDPS at the C-19 position of the andrographolide molecule are required for the activities (Nateewattana et al., 2014). However, the mechanism of analogue-6 or 1B in mammalian cancer cells has not been investigated. Interestingly, analogue-6 markedly increases the cytotoxicity with an emerging selective target on topoisomerase IIα enzyme, causing DNA damages. From the determination of lipophilicity (logP) value of analogue-6, it was increased approximately 3.8 folds compared to the parent andrographolide compound (data not shown). The increased lipophilicity might enhance penetration of the compound into the cells exerting the potential cytotoxic activities. Importantly, our analogue-6 inhibited the DNA Topo IIα activity in both cell-free system (Nateewattana et al., 2013) and in the AGS cells (Figure 2b). These results provide the scientific information to develop this compound for treatment gastric of cancer.

Taken together, we show that 19-triisopropyl andrographolide (analogue-6) exhibited potent anticancer activities against human gastric cancer cells by primarily targeting DNA Topo IIα enzyme and inducing cellular apoptosis. The anticancer activity of analogue-6 was more potent than the parent andrographolide and etoposide. The mechanism of the analogue was through the inhibition of Topo IIα enzyme causing DNA damages, PARP-1 cleavage and enhanced caspase 3 activity thereby

leading to apoptosis via a p53-independent mechanism. Analogue-6 may represent a novel class of Topo II inhibitor that has an immense potential for further development as new anticancer drug particularly for gastric cancer. More importantly, andrographolide is a naturally occurring compound and is abundant in our locality.

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