Cytotoxic, Antiproliferative and Pro-apoptotic Activities of Spirobisindole Alkaloids from the Philippine Medicinal Plant *Voacanga globosa*

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Abstract

Cancer remains a public health burden which is currently exacerbated by the occurrence of chemotherapeutic resistance and adverse effects associated with present drugs. Owing to their structural diversity and well-established pharmaceutical activities, natural products have been tapped in anticancer drug discovery. Herein, we investigated the cytotoxic and antiproliferative activities of spirobisindole alkaloids from the Philippine medicinal plant *Voacanga globosa*, including the pro-apoptotic activity of the most potent alkaloid. Previously isolated alkaloids globospiramine (**1**), deoxyvobtusine (**2**) and vobtusine lactone (**3**) were subjected to *in vitro* MTT and CellTiter Blue assays. Alkaloids **1-3** showed cytotoxicity and antiproliferative activities against tested cell lines (L929, KB3.1, A431, MCF-7, A549, PC-3, and SKOV-3) with **1** being the most biologically active. Western blot analysis revealed **1** promoted increased expression of cleaved caspase-3 and PARP, which are indicators that the treated cancer cells underwent the caspase-dependent apoptosis. Overall, our study demonstrated the anticancer potentials of spirobisindole alkaloids, especially globospiramine (**1**), from *Voacanga globosa*. The pro-apoptotic activity of globospiramine (**1**) is also reported.

Keywords: *Voacanga globosa*; globospiramine; spirobisindole alkaloid; cytotoxicity; apoptosis

Introduction

Cancer is still recognized as among the top global public health burdens. In 2020, almost 10 million cancer-related deaths have been recorded (Sung et al. 2021). Metastasis, which allows tumor cells to spread to other tissues and organs, as well as innate tumor heterogeneity, has been implicated in chemotherapeutic resistance (Rajesh et al. 2015; Siddiqui et al. 2022). Over the years, resistance to current treatment armamentarium, especially chemotherapy, has led to an increased number of treatment failures aside from the associated adverse effects. Therefore, continuous search for more anticancer agents is warranted.

Natural products (NPs) have been and are still tapped in drug discovery research due to their efficacious biological activities and low toxicity risks (Davison and Brimble 2019). NPs are well-established pharmacophore templates and drug prototypes given their remarkable chemical diversity and purported biological activities (Huang et al. 2020). Currently, more than 20% of recently approved anticancer chemotherapeutic agents are derived from or related to NPs (Huang et al. 2018; Newman and Cragg 2020). In addition, several studies have reported natural compounds with promising anticancer activities (Macabeo et al. 2021; Garcia et al. 2021; Garcia et al. 2022). In developing nations, traditional and folk medicinal practices, including use and consumption of ground medicinal plant parts, are still prevalent especially in far-flung areas (Cordell and Colvard 2012; Robinson and Zhang 2011; Fridlender et al. 2015). Investigations on NPs in the context of drug discovery therefore may provide ethnomedicinal validation on the use of such plants while contributing to the drug discovery pipeline.

Apoptosis or programmed cell death is a cellular process necessary to remove toxic and unwanted cells including tumor cells. In cancer pathophysiology, evasion to apoptosis is always implicated as an important event in disease progression and even in chemotherapeutic resistance. Thus, among

potential therapeutic mechanisms for anticancer drugs, including bioactive NPs, is either induction of pro-apoptotic or inhibition of anti-apoptotic proteins of cancer cells (Fesik et al. 2005; LaBarbera et al. 2009). Apoptotic pathways, especially those mediated by caspases, are also considered favorable therapeutic targets (Wen et al. 2012; Boice & Bouchier-Hayes 2020).

Relevant to this study, alkaloids are nitrogen-bearing heterocyclic compounds and are among the mostly studied compounds in anticancer drug discovery (Cordell et al. 2001; Daley and Cordell 2021). Generally, alkaloids can be further classified into indole, benzylisoquinoline, isoquinoline, purine, pyrrole, and quinolone alkaloids based on associated structural moieties (Lu et al. 2012; Rosales et al. 2020). Taxol and *Vinca* alkaloids, including their derivatives, are well-known examples of alkaloids that are now clinically utilized drugs in cancer therapy (Suffness & Wall 2021; Verma et al. 2022; Cech & Oberlies 2023). Recently, biologically active spirobisindole alkaloids globospiramine (**1**), deoxyvobtusine (**2**), and deoxyobtusine lactone (**3**) from the medicinal plant *Voacanga globosa* have been isolated. *V. globosa* compounds **1-3** were previously reported for their anti-HIV, anti-*Mycobacterium tuberculosis* and anticholinesterase properties (Macabeo et al. 2011; de Jesus et al. 2022). In this study, we report the *in vitro* cytotoxic and antiproliferative activities of compounds **1-3** (Figure 1) including the pro-apoptotic potentials of the most bioactive alkaloid **1**.

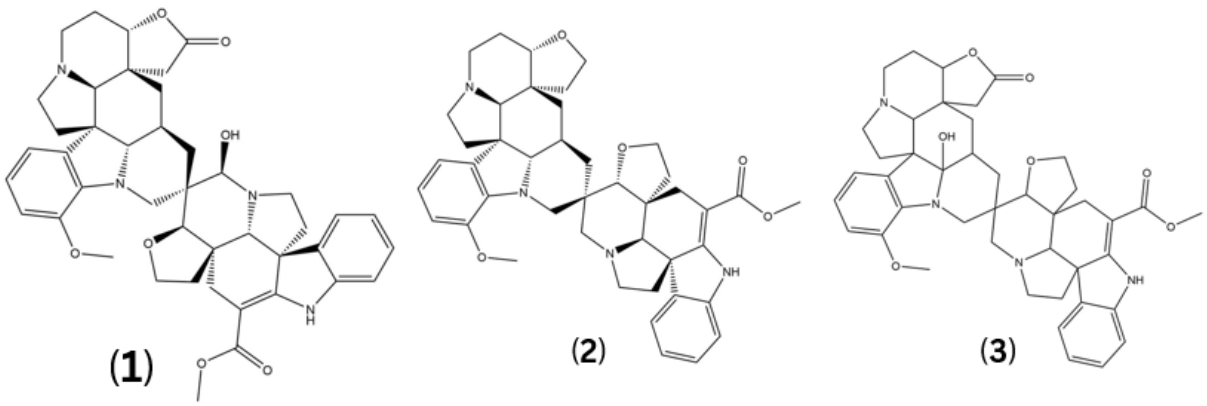

Figure 1. Structures of isolated *V. globosa* compounds globospiramine (**1**), deoxyvobtusine (**2**), and deoxyvobtusine lactone (**3**).

Materials and Methods

Test compounds. Test compounds globospiramine (**1**), deoxyvobtusine (**2**), and deoxyobtusine lactone (**3**) from the medicinal plant *Voacanga globosa* were isolated using previously reported methods (Macabeo et al. 2011). These alkaloids were dissolved in dimethyl sulfoxide (DMSO) to yield desired concentrations.

Biological assays.

MTT assay. The cytotoxicity of test alkaloids **1-3** were assessed using non-tumorigenic cell line L929, and cancer cell lines KB3.1 (or HeLa), A431m MCF-7, PC-3, SKOV-3, and A549 using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay (Becker et al. 2020). Briefly, cell lines were treated with test alkaloids. Incubation took place for 5 days. MTT was added and IC_{50} (half-maximal inhibitory concentration) was calculated based on the readings on fluorescence intensity of the purple color in microplate reader. For positive control, epothilone B was used (Garcia et al. 2022).

CellTiter Blue assay. Antiproliferative assessment of alkaloids **1**-**3** was carried out using CellTiter Blue assay (Promega, Mannheim, Germany) based protocol set by Krauth et al. (2010) with minor modifications. Around 10,000 cells per well were seeded in 96-well plates with 24 hr incubation time. Test alkaloids were then added after washing of medium, and cells were cultured at 37 ºC. CellTiter Blue reagent was added and allowed cell absorption for 2 hr. Readings obtained from microplate reader were analyzed and GI_{50} was calculated in GraphPad PRISM 5. Imatinib was utilized as positive control (Malaluan et al. 2022).

Western blot of pro-apoptotic proteins. To determine the level of Bcl-2, full length PARP-1, cleaved PARP-1 cleaved caspase-3, and α-tubulin in A549 cells, proteins were extracted and analyzed as follows. Briefly, A549 cells ($2x10^5$ cells/well) were cultured in 6-well plates. After 20 h of incubation, the cells were treated with DMSO or various concentrations (0.2, and 0.1 µM of globospiramine (**1**). Treatment incubation lasted for 36 h, followed by washing the cells twice with ice-cold PBS and solubilized with radioimmuno-precipitation assay (RIPA) buffer containing cocktail protease inhibitors and phosphatase inhibitors (0.02% NaF, 0.5% $NaVO_4$, and 5% $Na_4P_2O_7$). Protein concentrations of the cell lysates were determined using Bradford protein assay (BioRad). Equal amounts of protein (40 µg) per sample were cooked and pipetted in the gel, then separated by gel electrophoresis, followed by electroblotting onto a polyvinylidene difluoride (PVDF) membrane. Blots were incubated in PBS with 0.1% Tween (PBST) (pH 7.6) containing 5% BSA. The cut-up membranes were then incubated with specific primary antibodies overnight, and then incubated with their specific secondary antibodies. After washing with PBST, the blots were developed using NBT/BCIP before exposure to photographic films. The following antibodies were used: Anti-Cleaved PARP1 antibody (ab32064), Anti-PARP1 antibody (ab191217), Cleaved-Caspase 3 (Asp175), Bcl-2 antibody (AF0769), and α-Tubulin antibody (1224-1AP).

Results and Discussion

Continuous discovery of anticancer agents is scientifically warranted especially with the occurrence of chemotherapeutic resistance and adverse effects associated with current drugs. Over the years, natural products (NPs) have played an essential role in the development of new chemotherapeutic agents, either as NP scaffolds and drug pharmacophores or as its structure itself (Kaur et al. 2015; Klijun et al. 2017; Davison & Brimble 2019). Among NP classes, alkaloids or nitrogen-bearing molecules especially from plants are well-reported for their biological activities (Magpantay et al. 2021; Malaluan et al. 2022; Manzano et al. 2023). Indole alkaloids are among alkaloidal classes that have gained recognition in the design of anticancer agents (Wan et al. 2019).

In our study, previously isolated spirobisindole alkaloids globospiramine (**1**), deoxyvobtusine (**2**), and deoxyobtusine lactone (**3**) from the Philippine medicinal plant *Voacanga globosa* were tested for their *in vitro* cytotoxicity and antiproliferative activities against tested mammalian tumorigenic and non-tumorigenic cell lines using MTT and CellTiter Blue assays. These alkaloids **1**-**3** have been previously reported to confer antimycobacterial, anticholinesterase, and antiviral properties (Macabeo et al. 2011; de Jesus et al. 2022; Africa et al. 2022). The attempt to investigate its potential anticancer potentials boils down to the use of *V. globosa* as an alternative and/or traditional medicine used by natives in the Philippines against several diseases including cancer (Canoy et al. 2011; Acebedo et al. 2014).

The results showed the alkaloid **1** demonstrated the most potent cytotoxicity (IC_{50} = 0.009 to 0.13 ug/mL) and antiproliferative activity (GI_{50} = 1.4 to 5.4 ug/mL) while alkaloid **3** showed moderate cytotoxicity (IC_{50} = 1.7 to 8.1 ug/mL) and antiproliferative activity (GI_{50} = 5.6 to 16.2 ug/mL). Alkaloid **2** conferred cytotoxicity to only two cell lines (non-tumorigenic L929 and HeLa KB3.1).

Compared to the positive control epothilone B, the cytotoxic and antiproliferative activities of the test compounds **1-3** were generally relatively lower (Table 1).

Table 1. Cytotoxicity and antiproliferative activities of alkaloids **1**-**3**.

Cell Lines	Test Compounds			Positive Controls	
	Globospiramine (**1**)	Deoxyvobtusine (**2**)	Vobtusine lactone (**3**)	Epothilone B	Imatinib
Cytotoxicity IC_{50} (ug/mL) | MTT Assay					
L929	0.04	30	3.4	0.00085	-
HeLa KB3.1	0.09	8.5	3.4	0.000024	-
A431	0.13	>50	7.5	0.0001	-
MCF-7	0.08	>50	4	0.00007	-
A549	0.13	>50	7.8	0.000031	-
PC-3	0.009	>50	1.7	0.000043	-
SKOV-3	0.06	>50	8.1	0.0001	-
Antiproliferative activity GI_{50} (ug/mL) | CellTiter Blue Assay					
HUVEC	5.4	>50	16.2	-	10.9
K-562	1.4	>50	5.6	-	0.1

(-) = not determined; MeOH served as negative control and showed no bioactivity.

Inducing apoptosis is among the initial mechanisms of anticancer agents, especially that most tumor cells are known to evade the process of programmed cell death (Fulda 2010; Hanahan and Weinberg 2011; Koff et al. 2015). In our study, we investigated the pro-apoptotic activities of globospiramine (**1**) through Western blot analysis on cleaved caspase 3 and PARP-1. Caspase-3 is considered a specific effector caspase. Cleavage and activation of caspase-3 yields apoptotic signal propagation through cleavage of downstream substrates like poly ADP ribose polymerase (PARP) and other target proteins (Soldani and Scovassi 2002; Liu et al. 2017; Eskandari and Eaves 2022). Therefore, the activation and cleavage of caspase 3 and PARP-1, which are well-established

indicators of apoptotic pathway, is a common therapeutic mechanism investigated in the context of drug discovery (Soldani et al. 2002; Mantena et al. 2006; Qiu et al. 2019; Yadav et al. 2021).

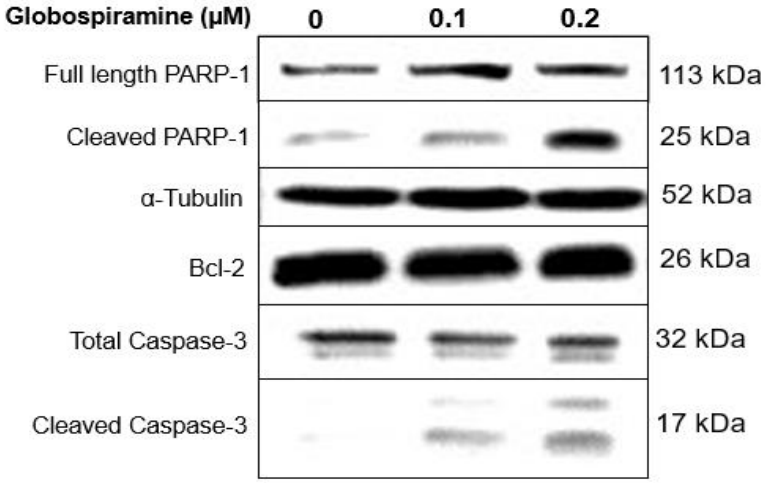

Figure 2. Western blot analysis showing increased expression of cleaved caspase-3 and PARP-1 which are indicators of apoptosis upon 36-hr exposure of A549 cells to globospiramine (**1**) in a concentration-dependent manner.

Western blot results showed that globospiramine (**1**) induced the cleavage of effector caspase-3 and increased presence of cleaved product of PARP-1, thereby indicating that A549 cells underwent cell death through the caspase-dependent apoptotic pathway (Figure 2). Induction of caspase-dependent apoptosis has been also reported in other natural products (Battle et al. 2005; Wang et al. 2016; Dai et al. 2017). In our study, the pro-apoptotic activity of **1** provided additional *in vitro* support to the use of *V. globosa* as a traditional anticancer regimen among natives, with putative mechanism of action of **1**. Furthermore, compounds that induce apoptosis have been reported to target specific molecular pathways and proteins upstream the caspase-dependent pathway (Liu et al. 2013; Zhou et al. 2018; Won and Seo 2020). The results therefore warrant a

more in-depth understanding on the therapeutic mechanisms of globospiramine (**1**), which may potentially target pathways and/or proteins leading to apoptosis.

Conclusion

The study reports the cytotoxic and antiproliferative spirobisindole alkaloids globospiramine (**1**), deoxyvobtusine (**2**), and deoxyobtusine lactone (**3**) from the Philippine medicinal plant *Voacanga globosa*. We also elucidated the pro-apoptotic activity of the most potent globospiramine (**3**) and provided baseline *in vitro* evidence to validate ethnomedicinal use of *V. globosa* against cancer. Overall, further investigations on globospiramine (**3**) are warranted to maximize its potential as a drug pharmacophore or natural product scaffold for the discovery of new generation anticancer agents.

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Conflict of Interest: The authors declare no conflict of interest.

Author Contributions

JAHM: Conceptualization, methodology, data collection analysis and interpretation of data, original draft preparation, review and editing of the draft. EAMA: Methodology, data collection analysis and interpretation of data, original draft preparation, review and editing of the draft. APGM: Conceptualization, supervision of methodology and experimentation, interpretation of data, review and editing of the draft. C-HY, NPGA: Conceptualization, supervision of

methodology and experimentation, review and editing of the draft. All authors have read and agreed to the final version of the manuscript.

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