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Evaluation of the Anti-Cancer Potential of Amphidinol 2, a Polyketide Metabolite from the Marine Dinoflagellate *Amphidinium klebsii*

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Abstract

The increasing incidence of new cancer cases and the appearance of cancer cells resistant towards standard chemotherapeutic drugs have prompted active research on finding novel compounds with promising anti-cancer properties. In this regard, marine organisms could provide interesting and unique compounds that may be of use in the treatment of this disease. Amphidinols (AMs) belong to a class of polyketide metabolites isolated from the marine dinoflagellate *Amphidinium klebsii*. These compounds are known to perforate the membrane via sterol interaction ultimately leading to pore formation and cell death. Herein, the activity of amphidinol 2 (AM2) against HCT-116, HT-29, and MCF-7 cancer cells was evaluated and compared with normal HDFn cells. Cell viability assays revealed that AM2 was cytotoxic to all cells tested, but it was significantly lower in normal cells; its IC_{50} against HDFn cells was 135.5 µg/mL compared with 1.2–8.5 µg/mL for the three cancer cell lines. Gene expression experiments showed that the presence of AM2 resulted in the upregulation of the pre-apoptosis markers *cfos* and *cjun* in all cancer cell lines tested, which may explain its observed cytotoxic action. These results demonstrate the potential of AM2, and possibly this class of compounds, as an effective anti-cancer therapeutic.

Key words: *Amphidinium klebsii*, Amphidinol 2, Apoptosis, Cytotoxicity.

1. Introduction

The incidence of cancer has increased significantly over the past decades transforming it into a major public health concern worldwide, both in terms of human and financial costs. In the United States, for example, it was projected that over 1.7 million new cases will be diagnosed in 2016 that will result in approximately 600,000 deaths (Siegel *et al*., 2017). This group of related diseases is characterized by 8 hallmarks including resisting apoptosis, sustained proliferative signaling, insensitivity to growth suppressors, replicative immortality, angiogenesis, invasion and metastasis, altered energy metabolism, and evasion of the body's immune responses (Hanahan and Weinberg, 2011). Understanding these traits will provide a better insight into this disease and consequently, the development of new ways to treat it, such as increasing the vulnerability of cancer cells to apoptosis. One of the promising research related to this is on the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is known to promote cancer cell death but not of normal cells. It was previously demonstrated that *cfos* and *cjun* protein products repress the transcription of the anti-apoptotic molecule c-FLIP(L), thus sensitizing prostate cancer cells to TRAIL-induced apoptosis (Li *et al.*, 2007). Furthermore, resistance to TRAIL-induced apoptosis was also observed upon binding to and repression of *cfos* by the anti-apoptotic molecule FBXL10 (Ge *et al.*, 2011). In addition to these, a number of studies also provided support for the notion that protein products of *cfos* and *cjun* are involved in inducing cancer cell death (Chan *et al.*, 2010; Shyu *et al.*, 2014).

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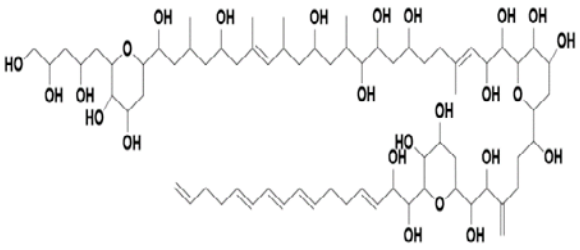

Figure 1. Chemical structure of AM2

Together with the increasing number of new cancer cases is the problem associated with resistance of cancer cells to chemotherapy and molecularly targeted therapies, prompting active research into finding new molecules with anti-cancer potential. Marine dinoflagellates are promising sources in the continuing search for new and unique bioactive secondary metabolites to combat cancer and other diseases. One of the very interesting bioactive natural products obtained from these organisms are the amphidinols (AMs), polyketide metabolites first reported from the dinoflagellate *Amphidinium klebsii* (Satake *et al*., 1991). This class of compounds is defined by unique structural features, namely a linear polyhydroxy moiety, two tetrahydropyran rings, and a polyene chain of varying length. In addition to *A. klebsii*, AMs have also been isolated from *A. carterae*, and currently 19 homologues are known (Satake *et al.*, 1991; Paul *et al.*, 1995; Paul *et al.*, 1997; Murata *et al.*, 1999; Echigoya *et al.*, 2005; Morsy *et al.*, 2005; Morsy *et al.*, 2006; Meng *et al.*, 2010; Nuzzo *et al.*, 2014), as well as a number of structurally-related compounds from other dinoflagellate species (Doi *et al.*, 1997; Huang *et al.*, 2004; Washida *et al.*, 2006; Suguhara *et al.*, 2011; Inuzuka *et al.*, 2014; Waters *et al.*, 2015). Amphidinols have been shown to exhibit antifungal and hemolytic activities which are believed to arise from its ability to permeabilize the membrane via preferential interaction with 3β-hydroxysterols, ultimately leading to cell death (Morsy *et al.*, 2008; Espiritu *et al.*, 2014). Previous investigations on membrane permeabilization by AMs suggest that the molecule could form both toroidal and barrel-stave pores (Houdai *et al.*, 2005; Espiritu *et al.*, 2014). Amphidinol 2 (AM2, Figure 1) is unique among the known AM homologs since this molecule has shown permeabilization of the cell membrane even in the absence of sterols (Morsy *et al.*, 2008). Furthermore, in addition to being hemolytic, AM2 was also previously reported to be cytotoxic against primary rat hepatocytes (Qi *et al.*, 2007), prompting us to investigate whether this molecule can be used as an effective anti-cancer agent.

Thus, the aim of this study is to explore on the chemotherapeutic potential of AM2 against HCT-116 human colorectal carcinoma, HT-29 human colorectal adenocarcinoma and MCF-7 human breast adenocarcinoma, and determine its effects on *cfos* and *cjun* gene expression, the protein products of which are critical in cancer progression. To the best of our knowledge, this is the first report on the anti-cancer activity of this class of molecules as well as on their effect on the aforementioned cancer-related genes.

2. Materials and Methods

2.1. Materials

Amphidinol 2 (AM2) was isolated as reported previously (Paul *et al.*, 1995). Human primary fibroblasts, neonatal HDFn (Invitrogen, USA) and cancer cell lines HT-29, HCT-116 and MCF-7 (American Type Culture Collection, USA) was provided by the Molecular Science Unit Laboratory of the Center for Natural Science and Ecological Research, De La Salle University. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) and 1x antibiotic-antimycotic (Invitrogen, USA) and incubated at 37C with 5% CO_2 and 98% humidity.

2.2. Cell Viability Assay against Amphidinol 2

HDFn, HT-29, HCT-116 and MCF-7cells, previously cultured to 90% confluence in a T-flask, were seeded into wells (2.4 x 10^5 cells/well) of a 96-well culture plate (Falcon, USA) and incubated for 24 hours to complete cell attachment. Afterwards, 100 µL of the compound, previously filter-sterilized, were subjected to two-fold serial dilution in the corresponding wells. Similar serial dilutions (two-fold) of colchicine (Sigma Aldrich, USA) were used as positive control. The plates were then incubated for 4 days, followed by addition of 10 µL of PrestoBlue® (Molecular Probes, Invitrogen) into each well, and an additional incubation of 30 minutes to 1 hour. Absorbance measurements were performed on a microplate reader (Biotek ELx800, BioTek Instruments, USA) at 570 nm and normalized to 600 nm values (reference wavelength). Background color was corrected by including wells containing only DMEM. Untreated wells with no added AM2 served as untreated controls.

Optical density readings obtained were used to calculate the cell viability index of the drugs using the equation, cell viability (%) = 100 – [100 – ($A_{treated}$ / $A_{untreated}$ x 100)], where $A_{treated}$ and $A_{untreated}$ is the absorbance of the treated and untreated cells, respectively. This was plotted against the corresponding treatment concentrations to derive IC_{50} (defined as the concentration of the drug necessary to inhibit cell growth by 50%) values whenever applicable.

2.3. cfos and cjun Transcript qRT-PCR Assay

Expression of the early apoptosis genes *cfos* and *cjun* was determined for HCT-116, HT-29, and MCF-7 cells, where AM2 exhibited significant cytotoxic activity, following the protocol reported previously (Shyu *et al.*, 2014). Briefly, 100 µL of the corresponding cells (2.4 x 10^5 cells/mL) were seeded separately into 96-well microplates and were incubated for 24 hours to attach the cells as monolayers. The cells were then exposed to AM2 for 30 minutes by adding the appropriate sample volume corresponding to the IC_{50} value for each cell. The positive control used was bleomycin, while the negative control included only the cancer cells. Afterwards, the total RNA was extracted from the cells with the TriZol Reagent (Invitrogen, USA) following the manufacturer's protocol.

All qRT-PCR reactions were performed using the Rotor-Gene 3000 thermocycler utilizing a final volume of 10 µL which contains the following: RNA template (1 µL),

2x KAPA FAST SYBR (5 µL; KAPA Biosystems, USA), 10 µM of the forward and reverse primers for *cfos* and *cjun* (0.3 µL each), and diethylpyrocarbonate-treated water (3.4 µL; Invitrogen, USA). The primer sequences used were: F: 5'-AAGGAGAATCCGAAGGGAAAGGAATAAGA-TGGCT-3',R: 5'-AGACGAAGGAAGACGTGTAAGCA GTGCAGCT-3' for *cfos*, and F: 5'-GCATGAGGAACC GCATTGCCGCCTCCAAGT-3',R: 5'-GCGACCAAGTCCTTCCCACT-CGTGCACACT-3' for *cjun.*

Synthesis of cDNA was carried out at 50 °C for 3 minutes, and subsequent amplification consisting of 40 cycles of the cDNA was performed for 20 seconds at 95 °C, for 30 seconds at 55 °C, and for 35 seconds at 72 °C, while melting analysis was carried out between 72 and 95 °C. The amplified transcript levels were quantified using an internal standard, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was also amplified at the same time, at different known magnitudes, specifically, 10^9, 10^8, 10^7, 10^6, and 10^5 copies. Quantification was done using the Rotor-Gene 3000 software ver. 6.1.93, where the critical threshold values were determined from the obtained standard curve.

2.4. Statistical Analysis

The average value of the treatment responses for the different assays were compared and analyzed with one-way analysis of variance ($p < 0.05$) and Tukey multiple comparisons test ($p < 0.05$). For nonlinear regression analysis (least squares method), the concentrations used were transformed to logarithmic scale to determine the IC_{50} values. GraphPad Prism ver. 7.02 was used for these analyses.

3. Results

In this study, the anti-cancer potential of AM2 was investigated, as well as on how it affects the gene expression of *cfos* and *cjun*, whose proteins products have been implicated as being crucial in cancer progression.

3.1. Cytotoxicity of AM2 against Various Cell Lines

The cytotoxicities of the aqueous solutions of AM2 on the four cell lines, based on cell viability assay, are shown in Figures 2 and 3. For the assays conducted, colchicine, an anti-cancer alkaloid that destabilizes microtubules (Lin *et al.*, 2013), served as the positive control. Results showed that addition of either AM2 or colchicine resulted in a concentration-dependent decrease in cell viability for all cell lines tested (Figure 2). However, analysis of the data revealed a notable difference between the two compounds' activities: significantly higher cell viability was recorded in normal cells compared with all three cancer cell lines upon treatment with AM2, starting at a concentration of 3.125 µg/mL (Figure 2A). In contrast, colchicine exhibited a comparable activity across all four-cell lines, except at a concentration of 100 µg/mL against HT-29 colon cancer cells (Figure 2B) where it was significantly higher.

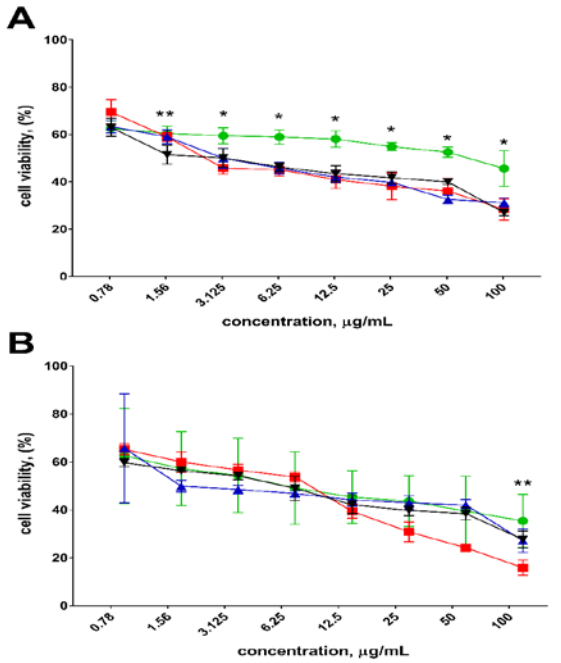

Figure 2. Average cytotoxicity values of AM2 (A) and colchicine (B) against normal (HDFn) and cancer (HCT-116, HT-29, MCF-7) cells. Green, black, red, and blue lines correspond to HDFn, HCT-116, HT-29, and MCF-7 cell viability, respectively. Error bars indicate the standard deviation of three independent trials. * indicates significant difference between normal and all cancer cell lines, while ** indicates significant difference between normal cell and only one cancer cell line (for A is HCT-116, and for B is HT-29).

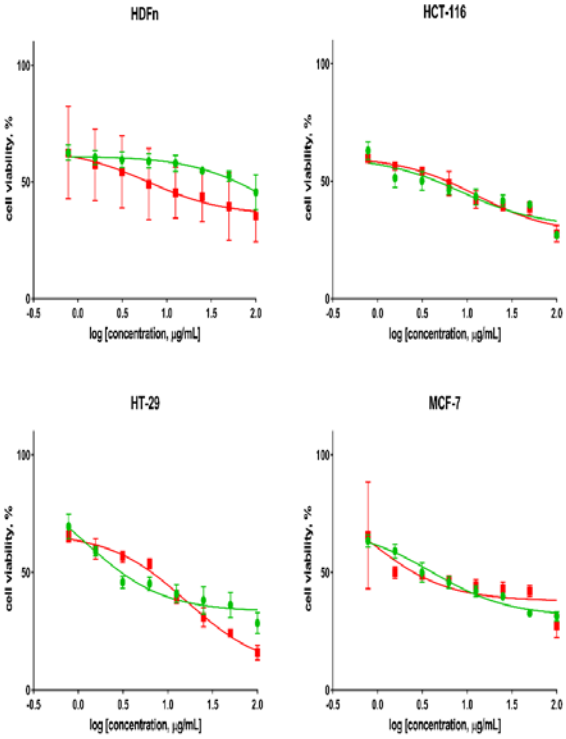

Figure 3. Nonlinear regression analysis of the average cytotoxicity values of AM2 and colchicine against the different cell lines used for IC_{50} determination. Green and red lines correspond to AM2 and colchicine curve-fitting, respectively. Error bars indicate standard deviation of three independent trials.

Nonlinear regression analysis of the dose-response curves (Figure 3) to determine IC_{50} values, or the half-maximal inhibitory concentration, provided more evidence of a better selectivity of AM2 towards cancer cells. The compound exhibited an IC_{50} value of 135.5 µg/mL against the normal HDFn cell, which was around 15- to 112-fold higher against the three cancer cells. In contrast, colchicine was roughly 22-fold more cytotoxic towards normal cells than AM2. Moreover, AM2 showed an approximately 13-fold and 1.5-fold greater activity against HT-29 and HCT-116 cancer cells, respectively, than colchicine, although it was less active against MCF-7 as shown in Table 1.

Table 1. Summary of IC_{50} values for AM2 and colchicine against normal and cancer cell lines obtained from curve-fitting

	AM2	Colchicine
HDFn	135.5 µg/mL	6.1 µg/mL
HCT-116	8.5 µg/mL	12.8 µg/mL
HT-29	1.2 µg/mL	16.3 µg/mL
MCF-7	4.1 µg/mL	0.7 µg/mL

3.2. Effect of AM2 on the Gene Expression of cfos and cjun

Gene regulation of the early apoptotic markers *cfos*/*cjun* significantly increased in all carcinoma cells incubated with AM2 (Figure 4). HCT-116 treated with colchicine and AM2 were statistically similar for both *cfos* and *cjun* whereas untreated HCT-116 cells gave significantly lower values. The *cfos*/*cjun* expression levels in both HT-29 and MCF-7 trials also followed a similar trend. These data are consistent with the comparable cytotoxicity of AM2 against these cancer cell lines. The expression of *cfos*/*cjun* in the aberrant cells incubated with AM2 and colchicine were approximately 100,000-fold higher than in untreated ones.

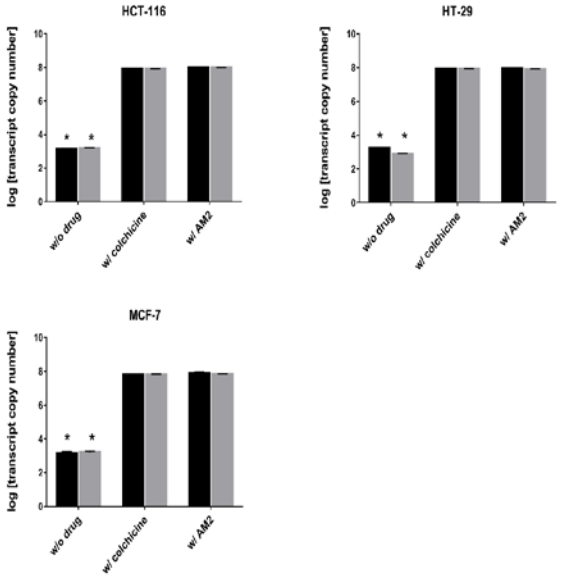

Figure 4. Average transcript copy numbers of the early apoptosis markers cfos (black) and cjun (gray) in untreated and treated HCT-116, HT-29, and MCF-7 cancer cell lines obtained using qRT-PCR. Error bars indicate standard deviation of three independent trials. * indicate significant difference ($p < 0.05$) between the untreated and treated cells.

4. Discussion

The results of the cytotoxicity assays revealed that cancer cells are more sensitive to AM2 than normal ones, pointing to a better selectivity of the compound towards aberrant cells. Furthermore, the results also suggest that normal cells are able to tolerate, and thrive at, AM2 concentrations that would otherwise kill malignant cells, as evidenced by the significantly higher IC_{50} value for normal, HDFn cells. Solid tumors and many malignancies are known to have elevated levels of cholesterol compared with normal cells, primarily brought about by an increased uptake of low-density lipoproteins and the enhancement of cholesterol biosynthesis (Cruz *et al.*, 2013; Silvente-Poirot and Poirot, 2014; Li *et al.*, 2016). This may account for the higher sensitivity of the cancer cells tested towards AM2 since amphidinols have been shown to preferentially interact with membrane sterols leading to more extensive membrane binding, leading to membrane disruption and ultimately, cell death (Morsy *et al.*, 2008; Espiritu *et al.*, 2014). Higher cholesterol content in the membrane will result in a greater accumulation of AM2 on the membrane surface resulting in critical biological effects, such as possibly pore formation. Although it is not possible with these data to ascertain pore formation as the mode of cytotoxic action of AM2, involvement of this mechanism in killing cancer cells have been reported earlier (Lopez *et al.*, 2013). Another distinct possibility to account for the observed selectivity of AM2 towards cancer cells is that its interaction with membrane cholesterol could prevent the sterol from exerting its proper physiological function, such as its role in lipid rafts. Lipid rafts are membrane microdomains rich in cholesterol and sphingolipids that are known to be platforms for various signalling processes, including cell survival, and have been reported to have a higher occurrence in cancer cells than in normal ones (Zhuang *et al.*, 2005; Li *et al.*, 2006; Mollinedo and Gajate, 2015). AM3, a homologue of AM2, has been previously demonstrated to interact with raft-forming liposomes suggesting that it also recognizes cholesterol in this liquid-ordered domain (Espiritu, 2017). Therefore, it is reasonable to suggest that AM2 might behave similarly, given their similarities in structure and bioactivity, resulting in impaired sterol function in lipid rafts that eventually results to cell death. In fact, lipid rafts have been proposed earlier to be viable targets for cancer management (Zhuang *et al.*, 2005; Li *et al.*, 2006). Moreover, the cytotoxicity of AM2 against the three cancer cell lines tested was comparable ($p < 0.05$), indicating that the observed effects of the compound does not depend on the cell type and most probably a general mechanism of cell killing may be involved.

Breast and colon adenocarcinomas are refractory and resistant to a number of broadly used anticancer agents which renders them ineffective. Deregulation of cell death pathways have been linked to the multifactorial mechanisms which have been associated to this inherently resistant phenotype (Holohan *et al.*, 2013). In HT-29, MCF-7, HCT-116 cell lines, it has been established that the integrity of the p53/p21 regulatory system or function thereof has been damaged causing a failure in the body's natural ability to rid itself of irreversibly damaged cells

(Mitkin *et al.*, 2015; Wang *et al.*, 2015). The stalemate between p53 and p21-driven genes and drug sensitivity remains controversial since cytotoxicity of these medical agents can injure both the targeted carcinoma cells and the normal ones. For example, upon interaction with DNA damaging agents, normal cells with intact p53/p21 function suggest the existence of a checkpoint that delays replication, and that may extend the time available for DNA repair. This lack of repair mechanism could suggest that the chemotherapeutic activity of AM2 could follow this process since a highly elevated concentration is needed to reach the IC_{50} for HDFn as compared to the p53-defective aberrant cells. Furthermore, previous research have demonstrated that impairment of the apoptotic pathway, for instance by activation and upregulation of the Akt pathway involved in cellular repair mechanisms, leads to increased survival of cancer cells (Mundi *et al.*, 2016).

The results of this experiment strongly suggest that the increased cytotoxicity for HCT-116, HT-29, and MCF-7 cells incubated with AM2 may be associated with a molecular pathway involving an upregulation of the early apoptotic gene markers *cfos* and *cjun*. Cellular survival pathways in the mutant cell lines seem to have been circumvented since the presence of elevated markers *cfos*/*cjun* have indicated that programmed cell death has ensued. This apoptosis-related cell death may also be caused by impairment of the cell's natural repair mechanisms, although further research needs to be done to confirm this. Finally, given the potent hemolytic activity of AM2 that limits its therapeutic potential (Paul *et al.*, 1995), among others, structure-activity relationship studies must also be conducted to obtain the most effective structure for anti-cancer use, while at the same time minimizing its unwanted side effects.

5. Conclusions

The results of the study showed that AM2 was cytotoxic against the mutant cell lines HCT-116, HT-29, MCF-7, as evidenced by their respective low IC_{50} values, but it was significantly less active against normal HDFn cells. The cytotoxic activity recorded here may be due to the observed upregulation of the early apoptotic gene markers *cfos* and *cjun*, which was significantly higher in the treated cells than in untreated ones, and similar to the positive control colchicine. These suggest that AM2 could result in the eventual activation of the apoptotic pathway as a means to kill cancer cells. These results provide support for the role of AM2 as a potential chemotherapeutic agent, especially for colorectal and breast adenocarcinoma.

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