Synergistic *in vitro* cytotoxicity of adociaquinone B and heptyl prodigiosin against MCF-7 breast cancer cell line

Zenaida P. Bojo[1*], Cyril D. Deaño[1], Sonia D. Jacinto[2], and Gisela P. Concepcion[1]

[1] The Marine Science Institute,
[2] Institute of Biology, University of the Philippines, Diliman, Quezon City 1101, Philippines

A major limitation of cancer chemotherapy is the inability to escalate doses of an anticancer drug due to intolerable cytotoxic side effects. This limitation can be overcome by using a combination of drugs administered at lower doses. In this study, we investigated the effects of combining two compounds isolated from marine organisms for *in vitro* cytotoxicity to the MCF-7 breast cancer cell line by the MTT assay. Adociaquinone B, from the marine sponge *Xestospongia* sp., and heptyl prodigiosin, from the marine bacterium *Pseudovibrio denitrificans* strain Z143-1, were combined in three molar proportions and the combinatorial effect was determined by isobologram analysis and the fractional inhibitory concentration (FIC) of each compound. To determine if these compounds individually caused apoptosis in MCF-7 cells, we performed brightfield microscopy to investigate cell morphology and Hoechst 33342 staining to check for chromatin condensation. Apoptosis is the preferred mode of cell death in cancer therapy because it causes minimal inflammation. Initial findings of this study suggest a synergistic cytotoxic effect of combinations of these two compounds against MCF-7 breast cancer cells, and their ability to induce apoptosis individually.

* Corresponding author
Email Address: zenybojo@yahoo.com
Submitted: June 22, 2010
Revised: October 19, 2010
Accepted: October 20, 2010
Published: November 12, 2010
Editor-in-charge: Eduardo A. Padlan
Reviewers:
Fancisco S. Chung, Jr.
Marina Gálvez-Peralta

KEY WORDS

adociaquinone B, heptyl prodigiosin, marine natural products, MCF-7 breast cancer cell line, MTT assay, combinatorial synergy, isobologram analysis, apoptosis

INTRODUCTION

The control of proliferation of cancer cells without harmful side effects to normal cells of the patient remains a major goal in the continuing search for improved methods of treating cancer. Toxicity leading to immunosuppression limits the dose of anticancer drugs that can be administered. Improved therapeutic efficacy and reduced toxicity can be achieved by a combination of drugs with different mechanisms of action, administered at lower doses, in synchrony or in sequence. Metronomic low dose (MLD) chemotherapy, the infusion of drugs at lower flow rates and lower doses but at faster cycles, is proving to be an effective regimen that could overcome the problem of toxicity (Chow et al. 2001, Kerbel et al. 2002, Allegrini et al. 2008, Zhang et al. 2009, Satti 2009)

In cancer chemotherapy, apoptosis is the preferred mode of effecting cell death. Unlike in necrosis, inflammatory response is not mounted, thus the neighboring cells are not injured (Soini et al. 1998). Apoptosis is a complex, tightly regulated cellular process of programmed cell death in which cells receive instructions to commit suicide for a variety of reasons (Nagata

1997, Soini et al. 1998,, Elmore 2007). It serves as a mechanism of cell clearance in processes such as embryogenesis and tumor regression (Peitsch et al. 1993). Morphological hallmarks of apoptosis include cell shrinkage, chromatin condensation, nuclear fragmentation, DNA fragmentation, formation of apoptotic bodies, and loss of membrane phospholipid asymmetry leading to the exposure of phosphatidylserine molecules, among others (Allen et al. 1997). Cell systems which follow these hallmarks are said to undergo "classical apoptosis"; however, an absence of one or more of these markers, as detected by a particular method, does not exclude the occurrence of apoptosis (Darzynkiewicz et al. 1997).

Apoptosis signaling occurs through three interconnected pathways, depending on where the damage or the need for cell death originates: the extrinsic receptor-mediated pathway, and the intrinsic mitochondrial and endoplasmic reticulum stress pathways. All pathways involve the action of a cascade of pro-apoptotic proteases known as caspases, counter-regulated by anti-apoptotic proteins of the Bcl2/Bclx family.

The death receptor (extrinsic) pathway involves binding of death receptors such as Fas or the tumor necrosis factor receptors to specific multimeric ligands. This binding results in the recruitment of adaptor molecules involved in the activation of caspase-8 which facilitates cleavage of caspase-3 (Rao et al. 2004). The extrinsic pathway also facilitates the involvement of the mitochondria via caspase-8 dependent cleavage of Bap31 and recruitment of Bcl-2 family members which target the mitochondria, resulting in the release of cytochrome c and subsequent formation of a caspase-9 complex, leading to the apoptotic cascade (Rao et al. 2004). On the other hand, the mitochondrial (intrinsic) pathway releases cytochrome c into the cytoplasm from the mitochondrial intermembranous space, activating caspase-9. The intrinsic pathway may also directly activate caspase-9 by mobilizing caspase-12 without the involvement of mitochondria. Similar to the mitochondria, the endoplasmic reticulum (ER) is a repository for both proapoptotic and antiapoptotic molecules. Endoplasmic reticulum stress pathway is generally mediated by ER stress receptors such as PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6). These in turn activate c-Jun N-terminal kinase (JNK) and induce C/EBP homologous proteins (CHOP) which inhibit the antiapoptotic proteins Bcl-2 and activate proapoptotic members of the BCL 2 family, Bax and Bad. As a result cytochrome c will be released from the mitochondria to initiate the caspase cascade (Szegezdi et al. 2006).

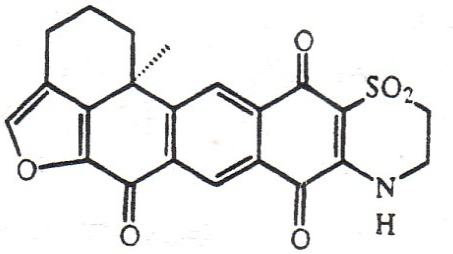
Although programmed cell death is generally brought about by caspase-3, the most important effector caspase, a number of cells have been demonstrated to go through caspase-3-independent pathways such as that observed with MCF-7 which has a functional deletion in its caspase-3 gene (Janicke et al. 1998, Kurokawa et al. 1999).

In our continuing efforts to discover potential cancer and other therapeutic agents from marine organisms, we have isolated a number of cytotoxic marine compounds from marine sponges, other marine invertebrate organisms and their associated microorganisms (Foster et al. 1992, Concepcion et al. 1995, de Guzman et al. 1999, Mangalindan et al. 2000, Lazaro et al. 2002, Tasdemir et al. 2002, Pimentel et al. 2003, Sandoval et al. 2004, Davis et al. 2004, Lazaro et al. 2006, Sertan-de Guzman et al. 2007). The oceans represent a largely untapped pharmacological resource, particularly for cancer (Simmons et al. 2005). Marine organisms are known to produce cytotoxic as well as growth inhibitory and regulatory secondary metabolites for their protection, defense and survival in the competitive marine environment. Our studies include the determination of molecular targets or mechanisms of action of marine compounds in order to identify more specific, effective and less toxic drug candidates, with focus on apoptotic compounds.

In this study, we examined the combined cytotoxic effects of two marine compounds that had been isolated in our laboratory – adociaquinone B and heptyl prodiogiosin. The crude methanol extract of a yellow orange *Xestospongia* sp. sponge collected at Cape Bolinao in northern Luzon, Philippines, was purified and yielded yellow adociaquinone B (m/z 424) (1) as the major compound. Adociaquinone B is a thiazine ring derivative of a furano pentacyclic quinone belonging to the xestoquinone class of compounds isolated from *Xestospongia* and *Adocia* spp. sponges (Concepcion et al. 1995). Activities reported for the xestoquinones and the related halenaquinones and adociaquinones include cardiotonic activity, anticancer and inhibitory activity against enzymes such as protein tyrosine kinase, topoisomerase II, and myosin Ca^{2+} ATPase (Nakamura et al. 1985, Kobayashi et al. 1992, Bae et al. 1993, Alvi et al. 1993). Some simplified adociaquinone B analogs were found to exhibit significant inhibitory activity against Cdc25B. Expression of Cdc25B is uniquely increased after DNA-damage induced by carcinogens. Adociaquinone B was also shown to have antiproliferative activity against the A2780 human ovarian cancer cell line (Cao et al. 2009).

Adociaquinone B was shown to inhibit the enzyme DNA topoisomerase II in catalytic DNA unwinding and/or decatenation assays, and was active in a potassium-SDS (KSDS) assay, suggesting that it acts by freezing the enzyme-DNA cleavable complex (Concepcion et al. 1995). It did not displace ethidium bromide from DNA or unwind supercoiled DNA, implying that it does not intercalate DNA. Adociaquinone B showed significant cytotoxicity against the human colon tumor cell line HCT 116. Due to its more specific targeting, adociaquinone B is a potentially more effective and less toxic anticancer compound than DNA-intercalating topoisomerase II inhibitors such as doxorubicin.

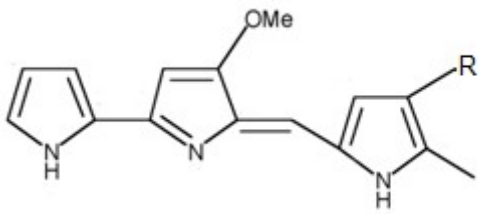
Heptyl prodigiosin (HPDG) (m/z 351) (16-methyl-15-heptylprodigionine or 2-methyl-3-heptyl-6-methoxyprodigiosene) (2) was purified from a *Pseudovibrio denitrificans* bacterium strain Z143-1 isolated from an unidentified tunicate



adociaquinone B (1)

collected in Zamboanga del Norte, Philippines. This new strain of *P. denitrificans* is similar to *P. ascidiaceicola* and *P. denitrificans* morphologically, physiologically and biochemically, except for its ability to ferment glucose and produce a characteristic red pigment. Fractionation of the hexane extract and further purification yielded the red pigment HPDG. HPDG showed anti-*Staphylococcus aureus* activity (Sertan-de Guzman et al. 2007) and *in vivo* antimalarial and *in vitro* nonmutagenic activity (Lazaro et al. 2002). In Jurkat cells, heptyl prodigiosin triggered apoptosis in a caspase 3-dependent and CD95 receptor-mediated manner, and inhibited the protection mechanism of the antiapoptotic proteins Bcl-2 and Bcl-x_L. HPDG also caused remarkable DNA fragmentation in SK-OV-3 ovarian cancer cells (Ranches et al. 2008, unpublished results).

HPDG belongs to the family of natural red pigments called prodigiosins, characterized by a common pyrrolylpyrromethene skeleton, and produced by various bacteria (Montaner and Perez-Tomas 2003). HPDG differs from the parent compound prodigiosin (3) at only one position. Some members have apoptotic effects, antimalarial and immunosuppressive properties *in vitro* and have also displayed antitumor activity *in vivo* (Tsuji et al. 1992, Yamamoto et al. 1999, Kim et al. 1999, Perez-Tomas 2003, Williamson et al. 2007). Prodigiosin was reported to induce apoptosis in human colon cancer cells (Montaner and Perez-Tomas 2001), in hematopoietic cancer cell lines (Montaner et al. 2000), and in a gastric cancer cell line HGT-1 (Diaz-Ruiz et al. 2001). It can also act as a dual DNA topoisomerase I and II inhibitor which may be used for the treatment of certain cancers (Montaner et al. 2005). Prodigiosin induces apoptosis of B and T cells from B-cell chronic lymphocytic leukemia (Campas et al. 2003) and caspase-9 and caspase-8 activation and release of cytochrome C in Jurkat cells (Montaner and Perez-Tomas 2002). Some naturally occurring members of the prodigiosin family, as well as synthetic analogs, possess potent and specific immunosuppressant properties acting through JAK3, a tyrosine kinase associated with the cytoplasmic tail of a cell surface receptor component called common gamma-chain, which is exclusive of all IL-2 cytokine family receptors (Boger and Patel 1988, Lee et al. 1995, Kawauchi et al. 1997, Mortellaro et al. 1999, D'Alessio et al. 2000).



R

heptyl prodigiosin (2): - $(CH_2)_6CH_3$

prodigiosin (3): - $(CH_2)_4CH_3$

In this study, we determined the cytotoxicity of the two marine compounds, adociaquinone B and heptyl prodigiosin, combined in different proportions, to the MCF-7 breast cancer cell line, to see if they act in synergy. We also investigated the ability of the two compounds to induce apoptosis in MCF-7 cells individually.

MATERIALS & METHODS

Cell culture

The human breast cancer cell line MCF-7 (ATCC catalog number HTB22) was obtained from the American Type Culture Collection (Manassas, VA) and was grown as a monolayer in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and 1% 1X of antibiotic-antimycotic penicillin-streptomycin and fungizone (PSF). Cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere (ATCC reference paper). MCF-7 cells over several passage numbers (10-15) were used for the different experiments. Morphology of the MCF-7 cells was checked regularly.

MTT assay

Cytotoxicity towards MCF-7 cells was assessed in a MTT-microtiter plate cytotoxicity assay, modified from the method of Mosmann (1983). Media was initially removed from culture flasks. The cells adhering to the culture flasks were washed twice by phosphate buffered saline (PBS), pH 7.4. Trypsin-EDTA (0.05%) was added to the culture flask to facilitate the harvesting of the adherent cells. The flask was incubated at 37°C in a humidified 5% CO_2 atmosphere. After 10 minutes of incubation with trypsin, the cells were centrifuged at 3000 rpm for 3 minutes. The number of cells was determined using a hemocytometer. Cells were seeded (20,000 cells/well) in 200 µL of growth medium in 96-well microtiter plates and allowed to adhere for 24 hours. Cells were treated with working solutions of the compounds of different concentrations. The compounds were tested in quadruplicates with a final DMSO concentration of 1.0 % or less in each well. The treated cells were incubated for 72 hours. The media was removed after 72 hours and 3-(4,5-

Table 1. Adociaquinone B (ado B) and heptyl prodigiosin (HPDG) concentrations (in μM) used for the synergy evaluation

		IC_{50}	~ 3 x IC_{50}		
	ado B	0.668	2		
	HPDG	0.677	2		
1:1		3:1		1:3	
ado B	HPDG	ado B	HPDG	ado B	HPDG
2.000	2.000	6.000	2.000	2.000	6.000
0.400	0.400	1.200	0.400	0.400	1.200
0.080	0.080	0.240	0.080	0.080	0.240
0.016	0.016	0.048	0.016	0.016	0.048
0.003	0.003	0.010	0.003	0.003	0.010
0.001	0.001	0.002	0.001	0.001	0.002

dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (15 μL, 5 mg/mL in PBS, pH 7.4) was added followed by 3 hours incubation. MTT is reduced by viable cells to a purple formazan product. Following removal of media, the formazan was solubilized by addition of DMSO (100 μL) to each well. Absorbance for each well at 570 nm was measured using an SLT Model Tecan Spectra III ELISA plate reader. The cell growth in the DMSO negative control wells was used to determine the zero inhibition growth level for each experiment. Doxorubicin, an established chemotherapeutic drug, was used as positive control. Average absorbance of the test sample wells was compared to the average absorbance of the control wells to determine the fraction of growth inhibition or fractional survival (fs) at a particular dose. Three independent experiments, using quadruplicate wells per trial, were performed to obtain reliable fractional survival values. Fractional survival was determined using the following formula: fractional survival = $(sample_{ave} - blank_{ave}) / (control_{ave} - blank_{ave})$

Synergy evaluation

Evaluation of synergy was patterned after the method of Roks et al. (1999). Initially, the individual IC_{50} values of the test compounds were determined using the MTT assay. A stock solution of 6 μM was prepared to provide a wide range of dose values. IC_{50} values were computed from the linear regression curve of fractional survival against concentration.

Different combinations of the compounds were prepared based on their individual IC_{50} values (Table 1); 1:1, 3:1 and 1:3 ratios of their IC_{50}s (in μM) were used. Each solution was serially diluted in a microtiter plate for IC_{50} determination. For the 1:1 combination, the stock solution was 2 μM of compound A and 2 μM of compound B, approximately three times the IC_{50} of each compound. Six doses of 5-fold dilutions were made from the 2μM stock solution. For the 3:1 combination, the stock solution was 6 μM of compound A and 2 μM of compound B. Six doses of 5-fold dilutions were made from the 6μM(A)/2μM(B) stock solution. For the 1:3 combination, the stock solution was 2 μM of compound A and 6 μM of compound B. Six doses of 5-fold dilutions were made from the 2μM(A)/6μM(B) stock solution.

To determine the cytotoxic effect of the different combinations of adociaquinone B and HPDG, the fractional inhibitory concentration (FIC) of each compound was determined. The FIC is the ratio between the IC_{50} of a compound when it is used in combination with another compound and the IC_{50} of the compound when used alone. FIC values indicate the nature of the interaction between the two compounds. The sum of the FICs for one particular combination is: (a) equal to 1 if the effects of the compounds are additive; (b) greater than 1 if the effects of the compounds are antagonistic; and (c) less than 1 if the effects of the compounds are synergistic (Hall et al. 1983, Roks et al. 1999, Tallarida et al. 2006).

The IC_{50} values were plotted on an isobologram of compound A versus compound B to graphically visualize any interactions between the two compounds. In an isobologram, the dose of one compound is represented on the abscissa; the dose of the other is represented on the ordinate. The line that connects the two plotted points of the pure single compounds is the isobolographic line. If experimentally determined data points lie on this line, the effects of the compounds are additive (no interaction). If the points lie below the line, there is supra-additivity or synergy. If they lie above the line, there is infra-additivity or antagonism (Roks et al. 1999, Tallarida 2006). Each experimentally determined data point represents the IC_{50} of a specific combination of compound A and compound B.

Data analysis by nonparametric ANOVA, followed by the Kruskal-Wallis Test, was used to assess the statistical differences of fractional survival and IC values generated by the MTT experiments. Tukey-Kramer Multiple Comparisons Test was also used to evaluate the differences in the IC_{50} values generated by each treatment combination. A p-value < 0.05 is considered significant, and $p<0.0001$ is extremely significant. Statistical analysis was performed using the GraphPad InStat software v3.05.

Morphological observations by bright field microscopy and Hoechst staining

The morphological markers of apoptosis can be observed in bright field microscopy and in fluorescence microscopy using the Hoechst 33342 stain. This dye is taken up by both live and apoptotic cells, but shows a more intense blue fluorescence in the latter, as DNA in condensed chromatin exhibits hyperchromasia (Crissmann et al. 1987, Darzynkiewicz et al. 1997). Briefly, MCF-7 cells were harvested by trypsinization

(see *MTT assay* section of Methodology) and seeded at 20,000 cells/well in 200 µL of growth medium in 96-well plates. The cells were incubated overnight and allowed to adhere. Cells were treated with working solutions of adocioaquinone B or HPDG at 1X IC_{50} concentration for 6, 12, 24 and 36 hours. Treatment was terminated at the prescribed time by aspiration of growth medium. Hoechst 33342 staining solution, with a final concentration of 10 µg/mL, was added, and cells were incubated for 90 minutes. Cells were observed using bright field and fluorescence microscopy and photographed.

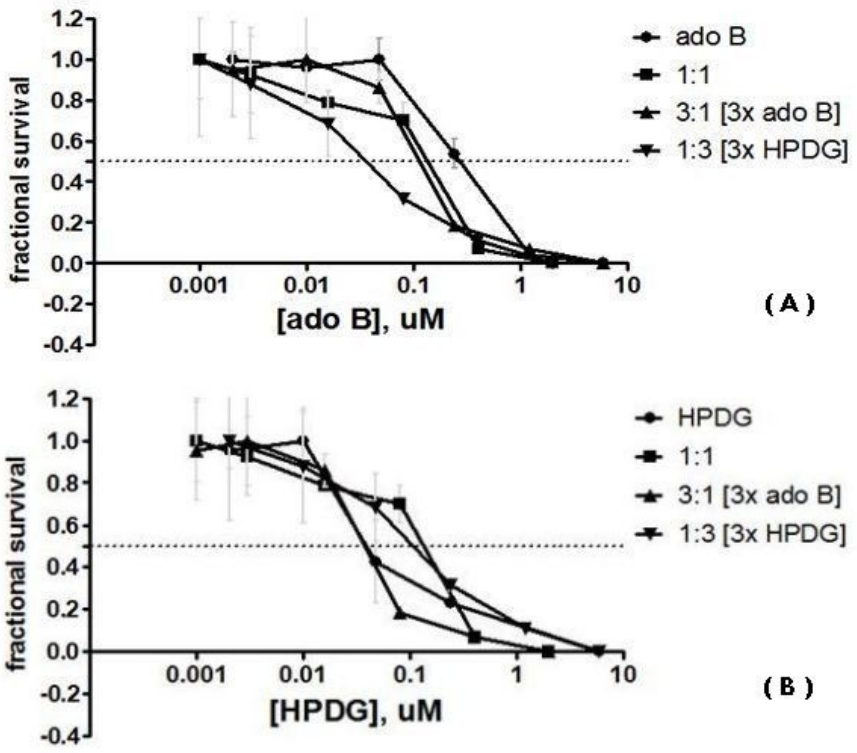


Figure 1. Cytotoxicity curves of adociaquinone B (ado B), heptyl prodigiosin (HPDG) and combinations of both in MCF-7 cells. Dotted lines indicate a fractional survival of 0.500. **(A)** Effect of different combinations compared to adociaquinone B alone. **(B)** Effect of different combinations compared to heptyl prodigiosin alone. All combinations (1:1, 3:1 and 1:3) shifted the cytotoxicity curve of adociaquinone B and heptyl prodigiosin to the left indicating greater cytotoxicity at lower combined doses.

RESULTS AND DISCUSSION

Synergy evaluation

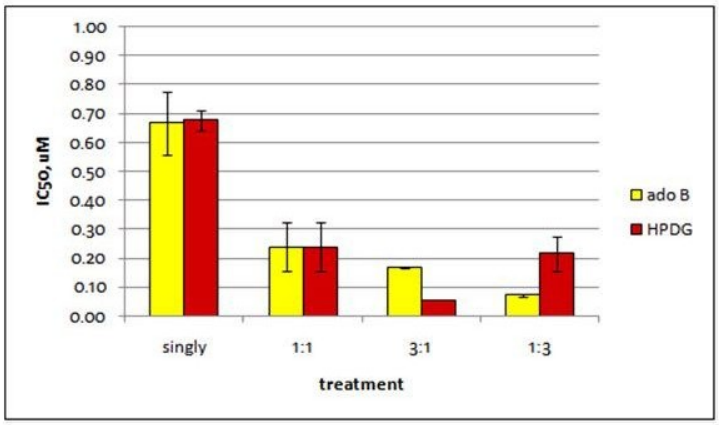
MCF-7 cells were treated with adociaquinone B alone, HPDG alone, and a 5-fold dilution series of 1:1, 3:1 and 1:3 ratios of the IC_{50}s (in µM) of adociaquinone B and HPDG. The final working concentrations ranged from 6 uM to 0.001 µM of each compound. Cell survival was determined using the MTT assay. Cytotoxicity curves are shown in Figure 1. IC_{50}s of 0.668 µM for adociaquinone B and 0.677 µM for HPDG were determined from the dose-response curves. The 1:1 IC_{50} ratio series, with a starting stock concentration of 2 uM for both adociaquinone B and HPDG, yielded an IC_{50} of 0.239 µM for both compounds, a ~3-fold decrease from their initial individual IC_{50} values. The 3:1 IC_{50} ratio series, with a starting stock concentration of 6 µM of adociaquinone B and 2 µM of HPDG, yielded an IC_{50} of 0.168 µM for adociaquinone B, a ~4-fold decrease, and an IC_{50} of 0.056 µM for HPDG, a ~12-fold decrease. On the other hand, the 1:3 IC_{50} ratio series, with a starting stock concentration of 2 µM of adociaquinone B and 6 uM of HPDG, yielded an IC_{50} of 0.072 µM for adociaquinone B, a ~9-fold decrease, and an IC_{50} of 0.217 µM for HPDG, a ~3-fold decrease. The effect of the different combinations is presented as bar graphs in Figure 2A. The individual IC_{50}s of the two compounds were significantly reduced for all the different combinations used (1:1, 3:1, and 1:3).

The changes in the IC_{50} values of the different combinations were further analyzed by determination of their fractional inhibitory concentrations (FIC). The sum of the FICs for all combinations of adociaquinone B and HPDG (1:1, 3:1 and 1:3) is less than 1; thus, the compounds when combined have a synergistic effect. The data shows that an increase in concentration of adociaquinone B (3:1 ratio combination) significantly reduced the IC_{50} of HPDG, and vice-versa (1:3 combination). Figure 2B shows the fractional inhibitory concentration (FIC) calculation of the interaction between adociaquinone B and HPDG on MCF-7 cells. For all three combinations, FIC was less than 1 which indicates synergy. Mean values of IC_{50} were determined by performing three trials using quadruplicate wells for each dose per trial. Statistical significance was analyzed using ANOVA and Tukey-Kramer Multiple Comparisons Test ($p < 0.05$). Doxorubicin was used as a positive control. To further identify the nature of this drug interaction, an isobologram, based on the IC_{50} of each compound, was generated (Figure 2C). All data points, corresponding to the IC_{50}s of the different ratios of adociaquinone B and HPDG, lie below the isobolographic line indicating synergy at all dose combinations. Based on the results, combinations of adociaquinone B and HPDG acted synergistically, and can be considered potentially useful for anticancer combinatorial chemotherapy.

Morphological observations

MCF-7 cells, derived from a human epithelial breast adenocarcinoma, express the estrogen receptor on the cell surface and are caspase-3-deficient. The MCF-7 cell line is one

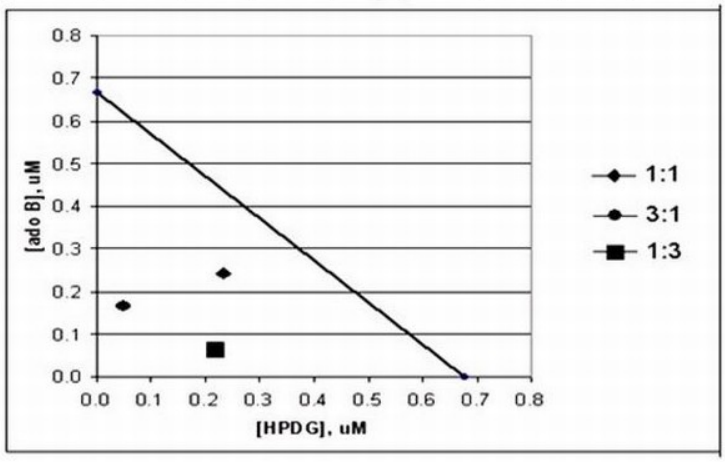
of the most frequently used breast cancer model systems especially for hormone-dependent breast cancer (Hamelers et al. 2003). It also resembles the most widely found phenotypes in the clinics since approximately 50% to 70% of breast cancers are estrogen receptor-positive without amplification of the *ERBB2*/*HER2* gene (Jordan and Brodie 2007). To further explore the potential use of the two marine compounds in chemotherapy, we examined their ability to induce apoptosis in these cells. For both compounds, a decrease in cell attachment as the treatment time was increased was observed from the bright field images (Figure 3). Whereas the cells in the negative control were intimately attached to each other and to the culture vessel, i.e., cells looked flattened, cells in the treated setups assumed a smaller, more rounded shape, presumably because cell dehydration, an early event in apoptosis, had been triggered (Darzynkiewicz et al. 1997). Using Hoechst staining, other hallmarks of apoptosis were observed. Condensed nuclei were seen as early as 6 hours after HPDG treatment of MCF-7 cells. Both adociaquinone B- and HPDG–treated cells exhibited sickle-shaped nuclei as early as 6 hours after treatment, while fragmented nuclei were observed 24 hours after treatment.



(A)

	single drug		1:1		3:1		1:3	
	ado B	HPDG	ado B	HPDG	ado B	HPDG	ado B	HPDG
IC_{50}, uM	0.668	0.677	0.239	0.239	0.168	0.056	0.072	0.217
FIC			0.358	0.353	0.251	0.083	0.108	0.321
Total FIC				0.711		0.334		0.428

(B)



(C)

Figure 2. The effect of combined treatment of MCF-7 cells with adociaquinone B (ado B) and heptyl prodigiosin (HPDG) with respect to their IC_{50} values. The combinations of 1:1, 3:1 and 1:3 ratios of their IC_{50}s were used. **(A)** Bar graphs showed individual IC_{50}s of the two compounds were significantly reduced ($p<0.05$) for all different combinations. **(B)** Fractional Inhibitory Concentrations (FICs) <1 for all three combinations indicate synergy. Mean values of IC_{50} were determined by performing three trials using quadruplicate wells for each dose per trial. Statistical significance was analyzed using ANOVA and Tukey-Kramer Multiple Comparisons Test. Doxorubicin was used as a positive control. **(C)** Isobologram analysis indicates synergy for all combinations. All data points are below the isobolographic line.

Previous studies showed that both compounds are cytotoxic to different cell lines and act on different targets. This study supports the finding that the primary mode of cell death induced by HPDG is apoptosis. Even if MCF-7 cells are known to be deficient in caspase-3, a critical effector protein in the apoptotic process, they still undergo apoptosis utilizing non-caspase proteins to degrade substrates otherwise degraded by caspase-3. Semenov et al. (2004) suggested that microsome-associated caspase-7 induces oligonucleosomal DNA fragmentation. Upon incubation with human placental chromatin, microsomes of apoptotic MCF-7 activate 40-50 kDa nucleases and induce oligonucleosomal fragmentation of chromatin in a cell-free system. Caspase-3/7 inhibitor Ac-DEVD-CHO is shown to suppress DNase activation and chromatin fragmentation.

Other features typical of apoptosis can be effected by other related molecules in caspase-3-deficient MCF-7 cells. Janicke et al. (1998) showed that one or more proteases or caspases other than caspases-2, 3, and 7 is activated in MCF-7 and plays a crucial role in the cleavage of substrates such as Rb, PAK2, DNA-PK_{cs}, gelsolin, and DFF-45. Leist and Jäätella (2001) state that a number of proteases such as cathepsins, calpains, serine proteases and the proteasome complex can bring about features typical of apoptosis in caspase-independent programmed cell death. Experimental data provide evidence of their roles as essential cofactors upstream or downstream of caspases in several cell death models. Moreover, many non-caspase proteases can cleave some of the classic caspase substrates, thus indicating that they might demonstrate the same effects on the cells as caspases. Examples include cathepsins D

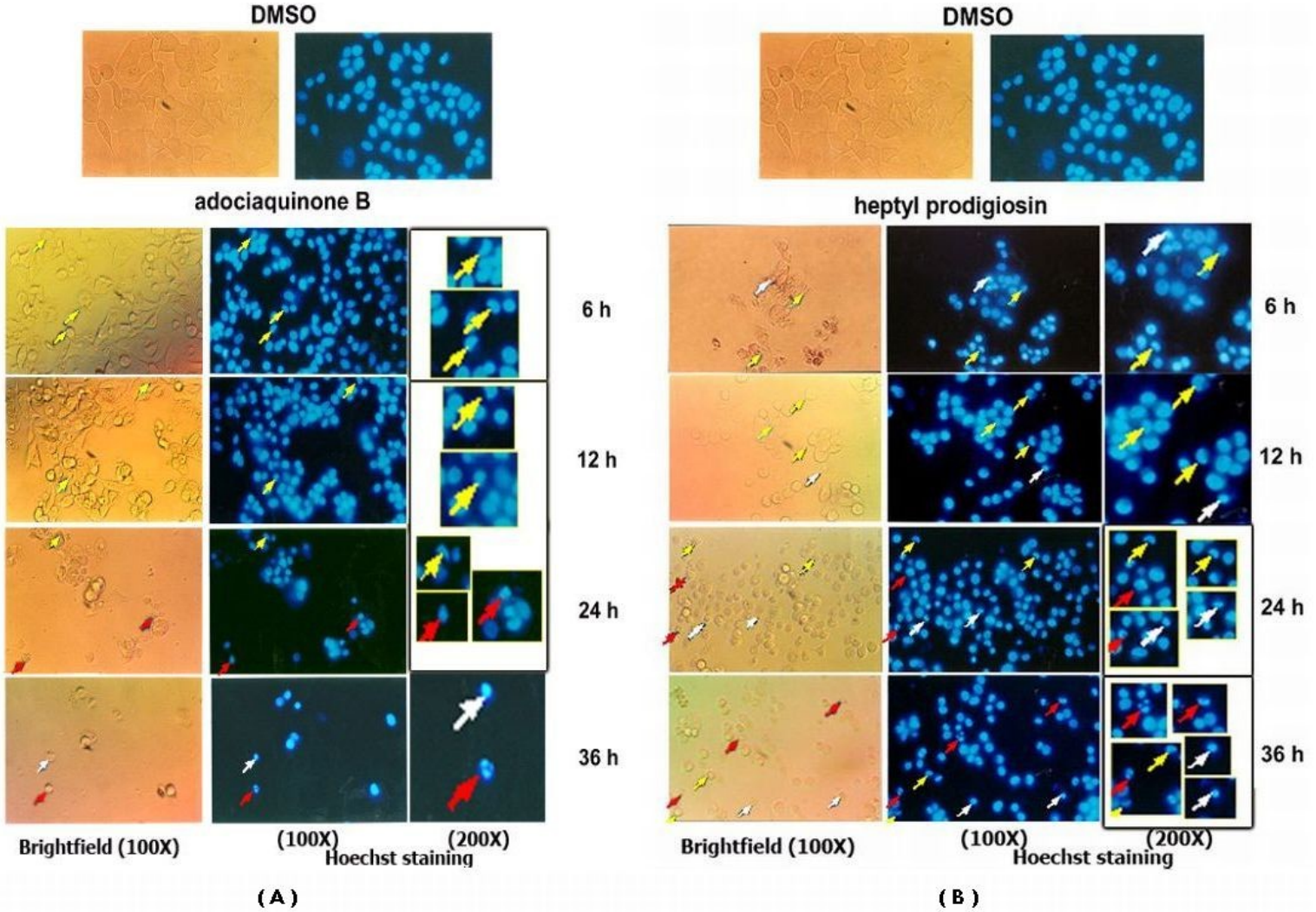


Figure 3. Bright field and fluorescence microscopy of Hoechst 33342-stained MCF-7 cells. In the treated setups, morphological features of apoptosis can be observed in condensed (white arrow), sickle-shaped (yellow arrow) and fragmented (red arrow) nuclei. DMSO was used as negative control. Concentrations of adociaquinone B (ado B) (1X IC_{50}) **(A)** and heptyl prodigiosin (HPDG) (1X IC_{50}) **(B)** were used for the treated set-ups.

and B in camptothecin-induced death of liver cancer cells; of cathepsin B in fibrosarcoma cells treated with TNF; of the proteasome in colchicine-treated neurons and of calpains in vitamin D-treated breast cancer cells. However, more work is needed to define the role of the individual proteases in the complex process of programmed cell death.

Ranches et al. (2008, unpublished results) showed that HPDG triggered caspase-3 dependent apoptosis in CD95 (Fas/Apo-1)-sensitive S-Jurkat T cells and is a possible chemosensitizer to tumor cells with an overexpression of the death receptor CD95, a dysfunctional apoptosome and overexpressed Bcl-2 and Bcl-x_L proteins. Using flow cytometry, the study also showed that HPDG induces DNA fragmentation dose- and time-dependently in MCF-7 human breast and SK-OV-3 ovarian carcinoma cell lines, indicating that HPDG-induced apoptosis is not restricted to Jurkat T cells.

No studies have been done previously on the activity of adociaquinone B in MCF-7 breast cancer cells. In this study, adociaquinone B was shown to induce apoptosis in MCF-7 cells to a lesser extent and at a later time than HPDG. This supports the previous finding that adociaquinone B acts directly on the DNA-topoisomerase II complex (as a TOPO II poison) and induces TOPO II-mediated DNA cleavage (Concepcion et al. 1995), which could then trigger apoptosis. Several studies have suggested that topoisomerase II-targeted drugs act to elevate p53 protein levels in the nucleus (Binaschi et al. 1995, Cui et al. 2002). Activated p53, in turn, induces the expression of many proteins including p21, which is a universal inhibitor of the cyclin-dependent kinases (Cdks), and is required to arrest cells at the G_1 and G_2 checkpoints of the cell cycle after DNA damage. The cellular response to "p53 → p21" induction can either be cell cycle arrest or apoptotic cell death (Gartel et al. 2002).

CONCLUSIONS

Based on the results of this study, the different combinations of adociaquinone B and HPDG provide a synergistic cytotoxic effect against the MCF-7 breast cancer cell line. The 3:1 adociaquinone B: HPDG combination yielded the highest synergistic effect, i.e., the lowest total FIC value indicating the greatest reduction in their individual IC_{50} values. These synergisms can reduce dose requirements (with an increased effective therapeutic index), thus reducing toxicity when projected to possible clinical applications.

By observing characteristic morphological changes, the two compounds were shown to cause apoptosis in MCF-7 cells individually, with effects clearly observed after 24 hours of treatment. One of the early events of apoptosis is the loss of intracellular water which leads to a change of cell shape and size. This event is followed by nuclear condensation and fragmentation and the formation of apoptotic bodies. Chromatin condensation is seen as early as 6 hours for HPDG-treated cells, while it took 36 hours for adociaquinone B–treated cells to exhibit chromatin condensation.

The greatest synergy observed in the 3:1 adociaquinone B: HPDG combination suggests that administering a TOPO II poison like adociaquinone B may sensitize the breast cancer cells further to pro-apoptotic agents such as HPDG. Using a combination of drugs with different molecular targets provides improved therapeutic efficacy and thus therapeutic advantages over using a single anticancer agent. Two independently active compounds, at the optimal combinations, yield increased efficacy at significantly reduced doses, thus reducing toxic side effects which are generally brought about by the effects of high doses of chemotherapeutic agents on normal cells, and by nephrotoxicity and hepatotoxicity. Moreover, the use of effective synergistic combinations does not call for further modifications of the chemical structure of the compounds to improve efficacy. It may also reduce the overall cost of therapy with the reduced total amount or weight of drugs required.

Studies on the combinations of adociaquinone B and HPDG with known anticancer drugs, such as doxorubicin and paclitaxel, are currently being pursued. In *vitro* evaluation of schedule-dependent interaction of different combinations of HPDG with paclitaxel or doxorubicin is also being performed.

Isobologram analysis could be expanded to the complete dose-response matrix study of varying adociaquinone B concentrations for different fixed concentrations of HPDG, and vice versa. Furthermore, other apoptotic studies such as flow cytometry, DNA fragmentation and molecular immunoblot could be performed for the two compounds. These studies can be performed but require more compound whose available quantity is really limited at this point.

ACKNOWLEDGEMENT

The authors would like to acknowledge the Department of Science and Technology - Philippine Council for Health Research and Development (DOST-PCHRD) for the funding of this project. We thank John Phillip L. Fadul for editing the manuscript. This study is related to Philippine Patent No. 1-2002-000516: Microorganism and the Use Thereof for Producing Heptylprodigiosin and the Use of Heptylprodigiosin, Date Granted: February 24, 2009; and Philippine Patent No.1 -2004-000379: Synergistic Combination of Marine Compounds & Use Thereof in the Treatment of Cancer, Date Granted: June 23, 2009.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

ZPB performed the cell culture, MTT assay and synergy evaluation experiments, CDD performed the microscopy and Hoechst staining experiments, ZPB, SDJ, GPC analyzed the data and wrote the paper, SDJ and GPC provided the research design and directed the study, GPC provided the marine compounds.

REFERENCES

Allegrini G, Falcone A, Fioravanti A, Barletta MT, Orlandi P, Loupakis F, Cerri E, Masi G, Di Paolo A, Kerbel RS, Danesi R, Del Tacca M, Bocci G. A pharmacokinetic and pharmacodynamic study on metronomic irinotecan in metastatic colorectal cancer patients. Br J Cancer 2008; 98(8):1312–1319.

Allen R, Hunter W, Agrawal D. Morphological and biochemical characterization and analysis of apoptosis. J Pharmacol Toxicol Meth. 1997; 37(4):215-228.

Alvi KA, Rodriguez J, Diaz MC, Moretti R, Wilhelm RS, Lee RH, Slate DL, Crews P. Protein tyrosine kinase inhibitory properties of planar polycyclics obtained from the marine sponge *Xestospongia cf. carbonaria* and from total synthesis. J Org Chem 1993; 58:4871-4880.

Bae MA, Tsuji T, Kondo K, Hirase T, Ishibashi M, Shigemori H, Kobayashi J. Inhibition of mammalian topoisomerase I by xestoquinone and halenaquinone. Biosci Biotechnol Biochem 1993; 57:3301-331.

Binaschi M, Zunino F, Capranico G. Mechanism of action of DNA topoisomerase inhibitors. Stem Cells 1995; 13:369-379.

Boger DL, Patel M. Total synthesis of prodigiosin, prodigiosene, and desmethoxyprodigiosin: Diels-Alder reactions of heterocyclic azadienes and development of an effective palladium (II)-promoted 2,2'-bipyrrole coupling procedure. J Org Chem 1988; 53:1405-1415.

Cámpas C, Dalmau M, Montaner B, Barragan M, Bellosillo B, Colomer D, Pons G, Perez-Tomas R, Gil J. Prodigiosin induces apoptosis of B and T cells from B-cell chronic lymphocytic leukemia. Leukemia 2003; 17:746-750.

Cao S, Murphy B, Foster C, Lazo J, Kingston D. Bioactivities of simplified adociaquinone B and naphthoquinone derivatives against Cdc25B, MKP-1, and MKP-3 phosphatases. Bioorg Med Chem 2009; 17(6):2276 – 2281.

Chow K, Boehrer S, Geduldig K, Krapohl A, Hoelzer D, Mitrou PS, Weidmann E. In vitro induction of apoptosis of neoplastic cells in low-grade non-Hodgkin's lymphomas using combinations of established cytotoxic drugs with bendamustine. Haematologica 2001; 86:485-493.

Concepcion GP, Foderaro TA, Eldredge GS, Lobkovsky E, Clardy J, Barrows LR, Ireland CM. Topoisomerase II-mediated DNA cleavage by adocia- and xestoquinones from the Philippine sponge Xestospongia sp. J Med Chem 1995; 38(22):4503-4507.

Crissmann HA, Steinkamp JA. A new method for rapid and sensitive detection of bromodeoxyuridine in DNA replicating cells. Exp Cell Res 1987; 173 (1):256–261.

Cui H, Schroering A, Ding HF. p53 mediates DNA damaging drug-induced apoptosis through a caspase-9-dependent pathway in SH-SY5Y neuroblastoma cells. Molec Cancer Ther 2002; 1:679-686.

D'Alessio R, Bargiotti A, Carlini O, Colotta F, Ferrari M, Gnocchi P, Isetta A, Mongelli N, Motta P, Rossi A, Rossi M, Tibolla M, Vanotti E. Synthesis and immunosuppressive activity of novel prodigiosin derivatives. J Med Chem 2000; 43(13):2557-65.

Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry 1997; 27:1-20.

Davis RA, Mangalindan GC, Bojo ZP, Antemano RR, Rodriguez NO, Concepcion GP, Samson SC, de Guzman DD, Cruz LJ, Tasdemir D, Harper MK, Feng X, Carter GT, Ireland CM. Microcionamides A and B, bioactive peptides from the Philippine sponge *Clathria (Thalysias) abietina*. J Org Chem 2004; 69 (12):4170-6.

de Guzman FS, Carte B, Troupe N, Faulkner DJ, Harper MK, Concepcion GP, Mangalindan GC, Matsumoto SS, Barrows LR, Ireland CM. Neoamphimedine: A New Pyridoacridine Topoisomerase II Inhibitor Which Catenates DNA. J Org Chem 1999; 64(4):1400-1402.

Diaz-Ruiz C, Montaner B, Perez-Tomas R. Prodigiosin induces cell death and morphological changes indicative of apoptosis in gastric cancer cell line HGT-1. Histol Histopathol 2001; 16(2): 415-421.

Elmore, S. Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol 2007, 35:495-516,

Foster MP, Concepcion GP, Caraan GB, Ireland CM. Bistratamides C and D: Two New Oxazole-Containing Cyclic Hexapeptides isolated from a Philippine *Lissoclinum bistratuma* Ascidian. J Org Chem 1992; 57:6671-6675.

Gartel, AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Molec Cancer Ther 2002; 1:639-649.

Hall MJ, Middleton RF, Westmacott D. The fractional inhibitory concentration (FIC) index as a measure of synergy. J Antimicrob Chemother 1983; 11:427–433.

Hamelers IH, van Schaik RF, Sussenbach JS, Steenberg PH. Estradiol responsiveness of MCF-7 laboratory strains is dependent on autocrine signal activating the IGF type I receptor. Cancer Cell International 2003; 3:1-10.

Janicke RU, Ng P, Spregart ML, Porter AG. Caspase-3 Is Required for α-Fodrin Cleavage but Dispensable for Cleavage of Other Death Substrates in Apoptosis, J Biol Chem 1998; 273(25):15540–15545,

Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 2007; 72:7-25.

Kawauchi K, Shibutani K, Yagisawa H, Kamata H, Nakatsuji S, Anzai H, Yokoyama Y, Ikegami Y, Moriyama Y, Hirata H. A possible immunosuppressant, cycloprodigiosin hydrochloride, obtained from *Pseudoalteromonas denitrificans*. Biochem Biophys Res Commun 1997; 237(3):543-547.

Kerbel RS, Klement G, Pritchard KI, Kamen B. Continuous *low-dose* anti-angiogenic/*metronomic chemotherapy*: from the research

laboratory into the oncology clinic. Annals Onco 2002; 13:12-15.

Kim H-S, Hayashi M, Shibata Y, Wataya Y, Mitamura T, Horii T, Kawauchi K, Hirata H, Tsuboi S, Moriyama Y. Cycloprodigiosin hydrochloride obtained from *Pseudoalteromonas denitrificans* is a potent antimalarial agent. Biol Pharm Bull 1999; 22:532-534.

Kobayashi J, Hirase T, Shigemori H, Ishibashi M, Bae MA, Tsuji T, Sasaki T. New pentacyclic compounds from the Okinawan marine sponge *Xestospongia sapra*. J Nat Prod 1992; 55:994-998.

Kurokawa H, Nishio K, Fukumoto H et al. Alteration of caspase-3 (CPP32/Yama/apopain) in wild-type MCF-7 breast cancer cells. Oncol Rep 1999; 6:33-37.

Lazaro JEH, Nitcheu J, Predicala RZ, Mangalindan GC, Nesslany F, Marzin D, Concepcion GP, Diquet B. Heptyl prodigiosin, a bacterial metabolite, is antimalarial *in vivo* and non-mutagenic *in vitro*. J Nat Toxins 2002; 11(4):367-377.

Lazaro JEH, Nitcheu J, Mahmoudi N, Ibana JA, Mangalindan GC, Black GP, Howard-Jones AG, Moore CG, Thomas DA, Mazier D, Ireland CM, Concepcion GP, Murphy PJ, Diquet B. Antimalarial activity of crambescidin 800 and synthetic analogues against liver and blood stage of *Plasmodium* sp. J Antibiot 2006; 59(9): 583-590.

Lee MH, Kataoka T, Magae J, Nagai K. Prodigiosin 25-C suppression of cytotoxic T cells in vitro and in vivo similar to that of concanamycin B, a specific inhibitor of vacuolar type H(+)-ATPase. Biosci Biotechnol Biochem 1995; 59(8):1417-1421.

Leist M, Jäättelä M. Four Deaths and A Funeral: From Caspases to Alternative Mechanisms. Nature Reviews, Molecular Cell Biology 2001; 2:1-10

Mangalindan GC, Talaue M, Cruz LJ, Franzblau S, Richardson A, Ireland CM, Concepcion GP. Agelasine F from a Philippine *Agelas* sp. Sponge Exhibits *In vitro* Antituberculosis Activity. Planta Medica 2000; 66:364-365.

Montaner B, Castillo-Avila W, Martinell M, Ollinger R, Aymami J, Girlt E, Perez-Tomas R. DNA interaction and dual topoisomerase I and II inhibition properties of the anti-tumor drug prodigiosin. Toxicol Sci 2005; 85:870-879.

Montaner B, Navarro S, Piqué M, Vilaseca M, Martinell M, Giralt E, Gil J, Pérez-Tomás R. Prodigiosin from the supernatant of *Serratia marcescens* induces apoptosis in haematopoietic cancer cell lines. Brit J Pharmacol 2000; 131:585-593.

Montaner B, Pérez-Tomás R. Prodigiosin-induced apoptosis in human colon cancer cells. Life Sci 2001; 68(17):2025-2036.

Montaner B, Pérez-Tomás R. Prodigiosin induces caspase-9 and caspase-8 activation and cytochrome C release in Jurkat T cells. Ann NY Acad Sci 2002; 973:246-249.

Montaner B, Perez-Tomas R. The prodigiosins: a new family of anticancer drugs. Curr Cancer Drug Targets 2003; 3(1):57-65.

Mortellaro A, Songia S, Gnocchi P, Ferrari M, Fornasiero C, D'Alessio R, Isetta A, Colotta F, Golay J. New immunosuppressive drug PNU156804 blocks IL-2-dependent proliferation and NF-kappa B and AP-1 activation. J Immunol 1999; 162(12):7102-7109.

Mosmann T. Rapid colorimetric assay for cellular growth and survival, application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65:55-63.

Nagata S. Apoptosis by death factor. Cell 1997; 88:355-365.

Nakamura H, Kobayashi J, Kobayashi M, Ohizumi Y, Hirata Y. Physiologically active marine natural products from Porifera. VII. Xestoquinone. A novel cardiotonic marine natural product isolated from the Okinawan sea sponge Xestospongia sapra. Chem Lett 1985; 6:713-716

Peitsch MC, Müller M, Tschopp J. DNA fragmentation during apoptosis is caused by frequent single-strand cuts. Nucleic Acids Research 1993; 21(18):4206-4209.

Pérez-Tomás R, Montaner B, Llagostera E, Soto-Cerrato V. The prodigiosins, proapoptotic drugs with anticancer properties. Biochem Pharmacol 2003; 66(8):1447-1452.

Pimentel SMV, Bojo ZP, Roberto AVD, Lazaro JEH, Mangalindan GC, Florentino LM, Lim-Navarro P, Tasdemir D, Ireland CM, Concepcion GP. Platelet aggregation Inhibitors from Philippine marine invertebrate samples screened in a new microplate assay. Marine Biotech 2003; 5(4):395-400.

Rao RV, Ellerby HM, Bredesen DE. Coupling endoplasmic reticulum stress to the cell death program. 2004. Nature 11;370 -380.

Roks G, Deckers CL, Meinardi H, Dirksen R, van Egmond J, van Rijn CM. Effects of polytherapy compared with monotherapy in antiepileptic drugs: an animal study. *J* Pharmacol Exp Ther 1999*;* 288(2):472-477.

Sandoval IT, Davis RA, Bugni TS, Concepcion GP, Harper MK, Ireland CM. Cytotoxic isoquinoline quinines from sponges of the genus Petrosia. Nat Prod Res 2004; 18(1):89–93.

Satti J. The Emerging Low-Dose Therapy for Advanced Cancers. Dose Response 2009; 7(3):208-220.

Semenov DV, Aronov PA, Kuligina EV, Potapenko MO, Richter VA. Nucleosides, Nucleotides and Nucleic Acids 2004; 23(6 & 7):831-836.

Sertan-de Guzman AA, Predicala RZ, Bernardo EB, Neilan BA, Elardo SP, Mangalindan GC, Tasdemir D, Ireland CM, Barraquio WL, Concepcion GP. *Pseudovibrio denitrificans* strain Z143-1, a heptyl prodigiosin-producing bacterium isolated from a Philippine tunicate. FEMS Microbial Lett 2007; 277(2):188-96.

Simmons TL, Andrianasolo E, McPhail K, Flatt P, Gerwick WH. Marine natural products as anticancer drugs. Mol Cancer Ther 2005; 4(2):333-342.

Soini Y, Paakko P, Lehto V-P. Histopathological evaluation of apoptosis in cancer. Am J Pathol 1998; 153(4):1041-1053.

Szegezdi E, Lougue SE, Gorman AM, Samali A. Mediators of Endoplasmic Reticulum Stress-induced Apoptosis. EMBO reports 2006; 9:880-885.

Tallarida RJ. An Overview of Drug Combination Analysis with Isobolograms. J Pharmacol Exp Therap 2006; 319:1-7

Tasdemir D, Mallon R, Greenstein M, Feldberg LR, Kim SC, Collins K, Wojciechowicz D, Mangalindan GC, Concepcion GP, Harper MK, Ireland CM. Aldisine alkaloids from the Philippine Sponge *Stylissa massa* are potent inhibitors of mitogen-activated protein kinase kinase-1 (MEK-1). J Med Chem 2002; 45:529-532.

Tsuji R, Magae J, Yamashita M, Nagai K, Yamasaki M. Immunomodulating properties of prodigiosin 25-C, an antibiotic which preferentially suppresses induction of cytotoxic T cells. J Antibiot (Tokyo) 1992; 45(8):1295-1302.

Williamson NR, Fineran PC, Gristwood T, Chawral SR, Leeper FJ, Salmond GP. Anticancer and immunosuppressive properties of bacterial prodiginines. Future Microbiol 2007; 2:605-618.

Yamamoto C, Takemoto H, Kuno K, Yamamoto D, Tsubura A, Kamata K, Hirata H, Yamamoto A, Kano H, Seki T, Inoue K. Cycloprodigiosin hydrochloride, a new H^+/Cl^- symporter, induces apoptosis in human and rat hepatocellular cancer cell lines *in vitro* and inhibits the growth of hepatocellular carcinoma xenografts in nude mice. Hepatology 1999; 30:894-902.

Zhang M, Tao W, Pan S, Sun X, Jiang H. Low-dose metronomic chemotherapy of paclitaxel synergizes with cetuximab to suppress human colon cancer xenografts. Anti-Cancer Drugs 2009; 20(5): 355-363.