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Evaluation Fucoidan Extracts From *Undaria pinnatifida* and *Fucus vesiculosus* in Combination With Anticancer Drugs in Human Cancer Orthotopic Mouse Models

Maryam Burney, BS[1], Lata Mathew, BS[1], Anjali Gaikwad, MS[1], Elizabeth K. Nugent, MD[1,2], Anneliese O. Gonzalez, MD[1,2], and Judith A. Smith, BS, PharmD[1,2,3]

Abstract

Objective: To determine the activity of fucoidan from *Undaria pinnatifida* (UPF) and *Fucus vesiculosus* (FVF) when given in combination of chemotherapy drugs using selected human breast or ovarian cancer orthotopic mouse models. **Methods:** Mice were inoculated with 1×10^6 cells of TOV-112d, MCF-7, or ZR-75 subcutaneously or $SKOV_3$-GFP-Luc intraperitoneally on day 0. MCF-7 and ZR-75 mice were administered with estradiol valerate 2 mg/kg in 0.2 mL castor oil subcutaneously two days prior to cell inoculation. Mice were randomized to one of six arms (N = 10/arm) paclitaxel, UPF/paclitaxel, FVF/paclitaxel, tamoxifen, UPF/tamoxifen, or FVF/tamoxifen. Tumors were measured three times per week for 28 days. **Results:** Improved activity was observed with UPF or FVF in combination with tamoxifen in both the MCF-7 and ZR-75D breast cancer mouse models. Decreased activity of paclitaxel was observed when given in combination with UPF or FVF in both breast cancer mouse models. The combination of FVF/tamoxifen in the TOV-112d ovarian cancer mouse model had improved activity but no there was difference observed with the UPF/tamoxifen in either ovarian cancer mouse model. No difference was observed with combination of UPF or FVF with paclitaxel in human ovarian cancer $SKOV_3$ or TOV-112d orthotopic mouse models. **Conclusion:** This study did confirm that UPF/FVF in combination with tamoxifen did not decrease tamoxifen activity in both breast and ovarian cancer, with some potential to improve activity compared to tamoxifen alone in breast cancers. Previous *in vitro* studies had suggested UPF and FVF had overall synergistic activity with paclitaxel; however, in the current *in vivo* human cancer mouse model studies there was no change in paclitaxel activity when given in combination with UPF or FVF in either of the two human ovarian cancer models. Furthermore, this study demonstrated that UPF or FVF given in combination with paclitaxel had a potential antagonistic effect in breast cancer models. Additional studies are warranted to delineate mechanisms contributing to variation in the *in vivo* activity when given in combination with paclitaxel. As a first step, a clinical pharmacokinetic study evaluating impact of FVF/UPF given in combination with chemotherapy in patients with solid tumors is underway.

Keywords
fucoidans, Fucus vesiculosus, Undaria pinnatifida, paclitaxel, tamoxifen

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Introduction

Fucoidans are fucose-containing sulfated polysaccharides derived from brown seaweeds and marine invertebrates like sea urchins and sea cucumbers.[1] Fucoidans consist of different combinations of monosaccharides such as galactose, glucose, and mannose, and other groups, including proteins, uronic acids, and acetyl groups. Natural polysaccharides such as fucoidans display the highest biological properties, notably anticancer properties, among macromolecules. Studies

[1]University of Texas Health Science Center McGovern Medical School at Houston, Houston, TX, USA
[2]UTHealth–Memorial Hermann Cancer Center-TMC, Houston, TX, USA
[3]Department of Pharmacy, Memorial Hermann Hospital-TMC, Houston, TX, USA

Corresponding Author:
Judith A. Smith, Department of Obstetrics, Gynecology & Reproductive Sciences, Division of Gynecologic Oncology, UTHealth McGovern Medical School, 6431 Fannin Street, Room 3.152, Houston, TX 77030, USA.
Email: judith.ann.smith@uth.tmc.edu

on fucoidans have enumerated numerous biological properties that include anticoagulation and antithrombotic properties, antiviral, antitumor, immunomodulatory, antioxidant, and anticomplement functions.[2-4]

The potential toxicity and acquired drug resistance of certain cancers to chemotherapy drugs have warranted other options to improve the effectiveness of these drugs. New strategies have been proposed that use the cancer-treating properties of natural polysaccharides in conjunction with anticancer agents that lack physiochemical and biopharmaceutical properties to increase the success of these drugs.[5-7] Current treatment for ovarian and breast cancers includes paclitaxel and tamoxifen. Paclitaxel is a taxane compound extracted from the bark of the Pacific yew tree with antimitotic activity that will induce cell cycle arrest and ultimately apoptosis in treated cells. Tamoxifen, a selective estrogen receptor modulator (SERM) agent, is used to treat ER-positive breast cancer in both early and late stages. Tamoxifen has shown limited success in patients with ovarian cancer as a primary treatment, however, has had some limited use in management of recurrent ovarian cancer.[8] Studies have demonstrated that fucoidan has improved treatment efficiency with taxane compounds through enhancing apoptosis and cell growth induction.[9,10]

Previously, an *in vitro* screening of the *Undaria pinnatifida* (UPF) and *Fucus vesiculosus* (FVF), demonstrated that both compounds appeared to have overall synergistic activity on inhibition of cancer cell growth given in combination with paclitaxel or tamoxifen.[11] Mathew and colleagues[11] conducted combined agent growth inhibition assays using IC_{50} concentration of chemotherapy agents, which included paclitaxel and tamoxifen, and 0.3 mg/mL fucoidan UPF and 1.3 mg/mL fucoidan FVF. The interaction indices of these drug combinations were used to confirm the synergistic activity of fucoidan with paclitaxel and tamoxifen *in vitro*. Hypothetically, *in vitro* the fucoidans' antiapoptotic activity contributes to the paclitaxel cytotoxicity and cytostatic activity of tamoxifen. The current study also proposed that *in vivo* the fucoidans' immune modulating activity may improve the overall antitumor benefits that were observed in the *in vitro* studies.[11] The overall objective of this study was to evaluate the potential for synergistic activity between fucoidan compounds and paclitaxel and tamoxifen for possible clinical use in cancer treatment regimens and for potential use in prevention of breast/ovarian cancer. This can be studied using *in vivo* orthotopic mouse models to evaluate and confirm synergistic activity in the presence of tumors.

Materials and Methods

Chemicals and Reagents

Fucoidans (UPF and FVF) were provided by Marinova Pty Ltd, 249 Kennedy Drive, Cambridge, TAS 7170 Australia. Fetal bovine serum (FBS), cell media, and trypsin-EDTA were purchased from GIBCO In*vitro*gen Co (Carlsbad, CA) were purchased from Sigma-Aldrich Co (St Louis, MO).

Cell Culture

All human cancer cell lines, comprising MCF-7 and ZR-75 (breast cancer cell lines), $SKOV_3$-GFP-Luc and TOV112d (ovarian cancer cell lines), were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The MCF-7 breast cancer cell line was propagated in a medium consisting of EMEM and the ZR-75 breast cancer cell line was propagated in a medium consisting of RPMI 1640. The $SKOV_3$-GFP-Luc adenocarcinoma cell line was propagated with media consisting of McCoy's 5a medium and TOV-112d was propagated in a mixture of 1:1 MCDB 105 and medium 199 with 2 mM L-glutamine and Earle's BSS (balances salt solution) adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. All cell lines were grown in 75-cm^2 culture flasks in 5% CO_2 in air at 37°C to 90% confluence. Cell lines used for this study were maintained for less than 15 passages to minimize the changes in cell line characteristics.

Orthotopic Mouse Models

The protocol was reviewed and approved by the institutional animal care and use committee (IACUC) prior to initiating any animal work. For this study, 40 female nude mice, 6 to 8 weeks old, were obtained from Charles River Laboratories (Wilmington, MA, USA). All the mice weighed from 22 to 26 g. They were maintained 5 per cage in specific pathogen free (SPF) barrier room, with a temperature of 22°C ± 3°C and 45°C ± 3°C relative humidity% and with free access to autoclaved food and reverse osmosis autoclaved water. The experiment procedures and the handling of the mice were in strict accordance with the guidance for the care and use of laboratory animals. MCF-7, ZR-75, TOV-112d cells (10×10^6) were dispersed in phosphate buffered saline with 20% matrigel and were injected subcutaneously whereas $SKOV_3$-GFP-LUC (10×10^6) cells were injected intraperitoneally in female nu/nu mice on day zero. Each mouse grew one tumor on the dorsal surface. Tumor measurements were obtained three times per week with electronic calipers (Mitutoyo, Utsunomiya, Japan), intraperitoneal tumors were imaged under Kodak Imaging Station (Kodak, Rochester, NY) once in a week and abdominal girth was measured by using measuring tape twice a week. Mice were monitored daily for signs/symptoms of morbidity, including but not limited to, lethargy, weight loss, anorexia, hunching, and so on. Mice were sacrificed via CO_2 inhalation followed by cervical dislocation, when tumor diameter was greater than 12 mm^2, or if a 10% or

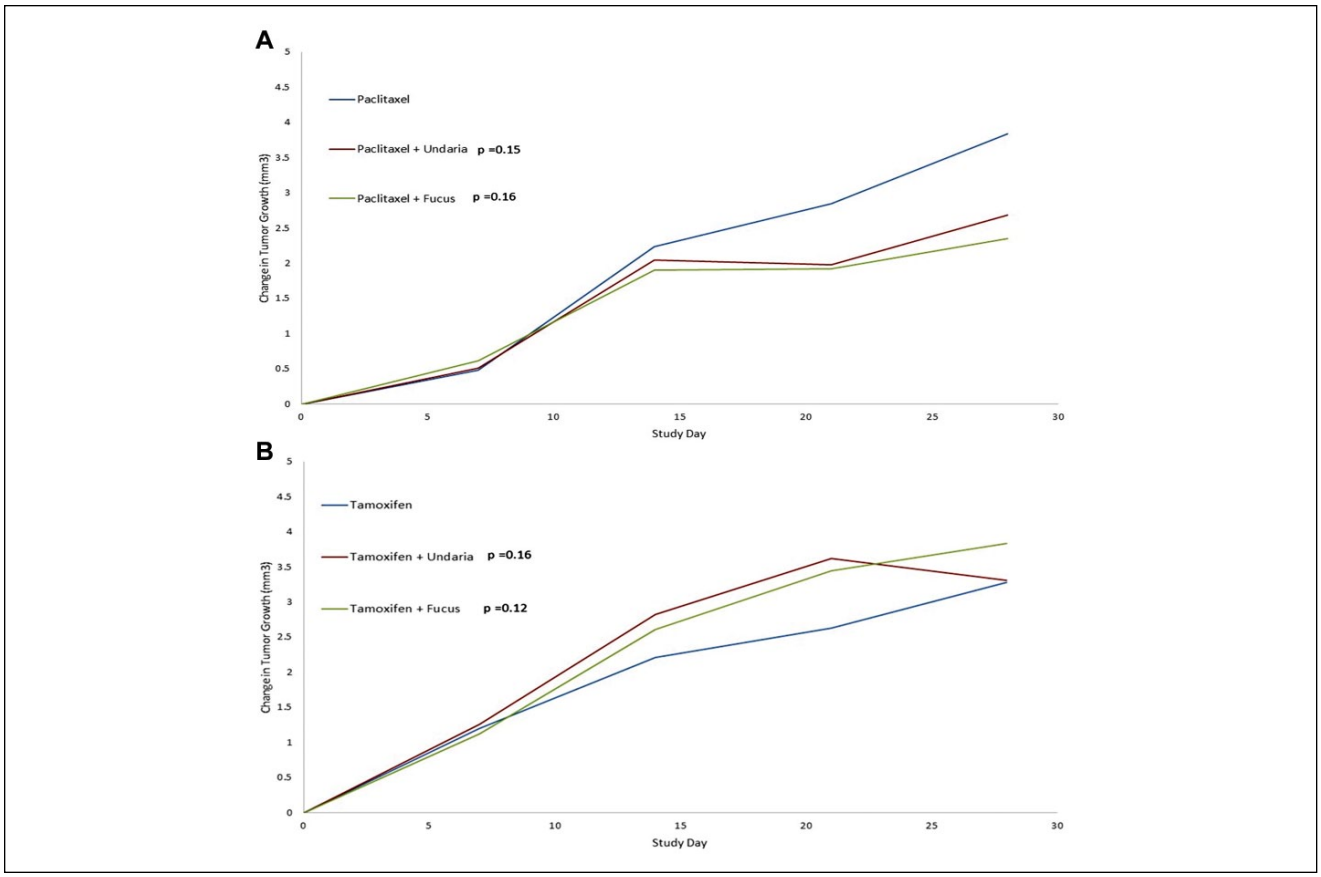

Figure 1. (a) Summary of the combination of fucoidans in combination with paclitaxel in $SKOV_3$-GPF-Luc human ovarian cancer orthotopic model. No significant change in tumor growth was seen. (b) Summary of the combination of fucoidans in combination with tamoxifen in $SKOV_3$-GPF-Luc human ovarian cancer orthotopic model. There was no significant change in tumor growth.

greater decrease in body weight was found during the study period. At the end of the study, all the remaining mice were sacrificed. After sacrifice, the total tumor burden was determined by macroscopic dissection. Immediately after sacrifice, tumors were surgically removed from all mice and stored at −80°C.

Study Treatment

The mice were divided into six groups of ten for each cell line (N = 60 for four cell lines). There were six arms in this study: paclitaxel 10 mg/kg intravenous (IV) given once (n = 10); paclitaxel 10 mg/kg IV with UPF 30.4 mg/kg in 0.2 mL per os (PO) once daily via gastric gavage (n = 10); paclitaxel 10 mg/kg IV with FVF 30.4 mg/kg in 0.2 mL PO once daily via gastric gavage (n = 10); tamoxifen 10 mg/kg in 0.2 mL PO once daily via gastric gavage (n = 10); tamoxifen 10 mg/kg PO in 0.2 mL PO once daily via gastric gavage with UPF 30.4 mg/kg in 0.2 mL PO once daily via gastric gavage (n = 10); or tamoxifen 10 mg/kg in 0.2 mL PO once daily via gastric gavage with FVF 30.4 mg/kg in 0.2 mL PO once daily via gastric gavage (n = 10). Treatment was continued for total of 28 days starting from day one.

Results

The UPF/paclitaxel combination did not alter tumor growth in human ovarian cancer orthotopic models with $SKOV_3$-GFP-Luc or TOV-112d cell lines (Figure 1a). Similarly, there was no significant change or reduction of tumor growth with FVF/paclitaxel combination in human ovarian cancer orthotopic models with $SKOV_3$-GFP-Luc or TOV-112d cell lines (Figure 2a). The $SKOV_3$-GFP-Luc human ovarian cancer mouse model demonstrated no significant change when treated with either UPF or FVF in combination with tamoxifen compared with tamoxifen alone (Figure 1b). In the TOV-112d human ovarian cancer models, FVF/tamoxifen showed a statistically significant ($P < 0.05$) increase in tumor growth. However, UPF/tamoxifen had no significant effect on tumor growth rate in the TOV-112d group (Figure 2b).

In the MCF-7 breast cancer mouse model, the combination of UPF/paclitaxel and FVF/paclitaxel resulted in a

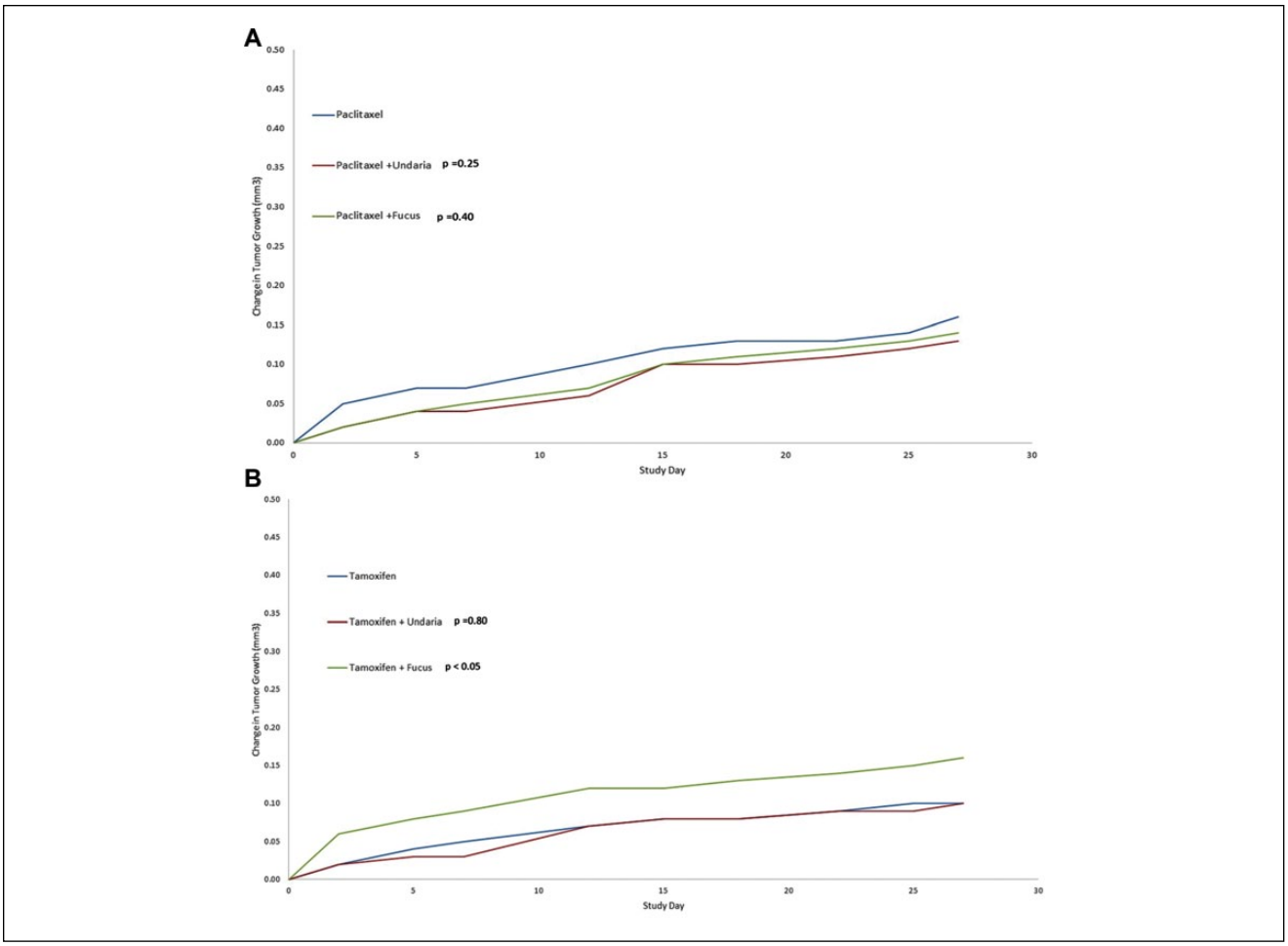

Figure 2. (a) Summary of the combination of the fucoidans in combination with paclitaxel in TOV-112d human ovarian cancer orthotopic model. No significant change in tumor growth was seen. (b) Summary of the combination of the fucoidans in combination with tamoxifen in TOV-112d human breast cancer orthotopic model. There was statistically significant change in tumor growth with FVF/tamoxifen.

statistically significant increase of tumor growth ($P < 0.05$) when compared with paclitaxel alone (Figure 3a). In the ZR-75 human breast cancer mouse model, UPF/paclitaxel and FVF/paclitaxel also showed a statistically significant increase in tumor growth ($P < 0.001$) compared with paclitaxel alone (Figure 4a). In the MCF-7 human breast cancer mouse model, the UPF/tamoxifen ($P < 0.001$) and FVF/tamoxifen ($P < 0.001$) combinations resulted in significant reduction of tumor growth compared with tamoxifen alone after 28 days supplementation (Figure 3b). Also, ZR-75 mouse models showed similar reduction of tumor growth after treatment with UPF/tamoxifen ($P < 0.001$) and FVF/tamoxifen ($P <0 .001$) in combination (Figure 4b).

Discussion

Evaluation of nutritional supplements with current anticancer agents is of considerable interest. Fucoidan, a brown seaweed extract, has gained a lot of attention for its antitumor properties, which include the apoptotic destruction of cells. Current literature has suggested additive or synergistic activity of fucoidan in combination with chemotherapy agents to improve clinical outcomes. Zhang and colleagues[9] studied the therapeutic efficiency of fucoidan in combination with cisplatin, tamoxifen, and paclitaxel and their findings showed an improvement in cell proliferation reduction in MCF-7 cell line. Another study by Atashrazm et al[12] determined the synergistic potential of fucoidan with lapatinib and results showed a reduction in cell growth of a breast cancer cell line, OE33, through activity comparable to tyrosine kinase inhibitors. Treatment with fucoidan alone in orthotopic mouse models resulted in downregulation of vascular endothelial growth factor expression in 4T1 breast cancer cell line and reduction in tumor weight.[13] These results are in accordance with this study which demonstrated the reduction of

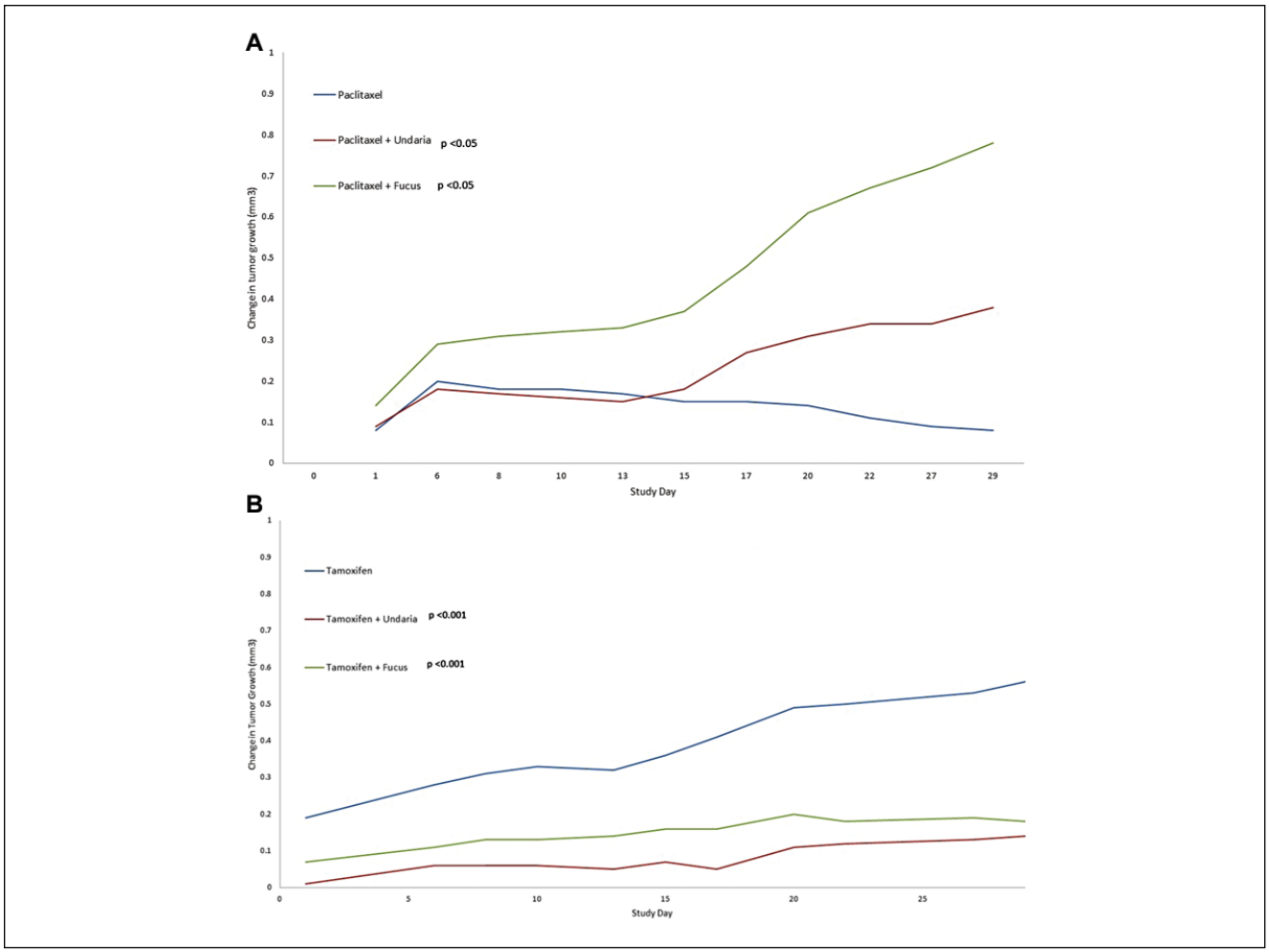

Figure 3. (a) Summary of the combination of the fucoidans in combination with paclitaxel in MCF-7 human breast cancer orthotopic model. There was statistically significant change in tumor growth with FVF/paclitaxel and UPF/paclitaxel. (b). Summary of the combination of the fucoidans in combination with tamoxifen in MCF-7 human breast cancer orthotopic model. There was statistically significant change in tumor growth with UPF/tamoxifen and FVF/tamoxifen. UPF, *Undaria pinnatifida*; FVF, *Fucus vesiculosus*.

tumor cell growth in MCF-7 and ZR-75 human breast cancer cell lines with tamoxifen in combination with both fucoidans, UPF and FVF.

However, the results in this study also showed an increase in cell growth with fucoidans in combination with paclitaxel compared with paclitaxel alone in MCF-7 and ZR-75 cell lines. However, both fucoidans with paclitaxel or tamoxifen showed no significant reduction or inhibition of tumor cell growth in ovarian cancer cell lines, $SKOV_3$-GFP-Luc and TOV112d. It can be noted that in this study, treatment concentration of UPF and FVF in orthotopic mice models was 30.4 mg/kg, which translates to an estimated 0.76 mg/mL dose concentration considering average weight of mouse to be 25 g and assuming 100% oral absorption, which initial pharmacokinetic studies estimate to be less than 3%.[14] This is slightly above the concentration of UPF (0.3 mg/mL) and below the concentration of FVF (1.3 mg/mL) used to determine IC_{50} concentrations in the previous study.[11] Likely the *in vitro* application of concentrations above achievable concentrations *in vivo* contributed to the inconsistencies in the translation of the data from the *in vitro* studies to the *in vivo* human cancer mouse models.

Based on the findings of this study, the next step is to evaluate the benefits of adding supplementation with fucoidans with tamoxifen in clinical setting for patients undergoing treatment for breast cancer. Because of tamoxifen's unique metabolism, pharmacokinetic/safety studies should be completed to define efficacy and evaluate drug/supplement interactions before proceeding to phase II studies. Toxicology studies with UPF using dose of 1350 mg/kg/d for four weeks in rats showed it to be safe with regard to oral safety and with no abnormal clinical manifestations or alterations to body biochemistry.[15] Li et al[16] have shown no significant harmful changes when 300 mg/kg body weight

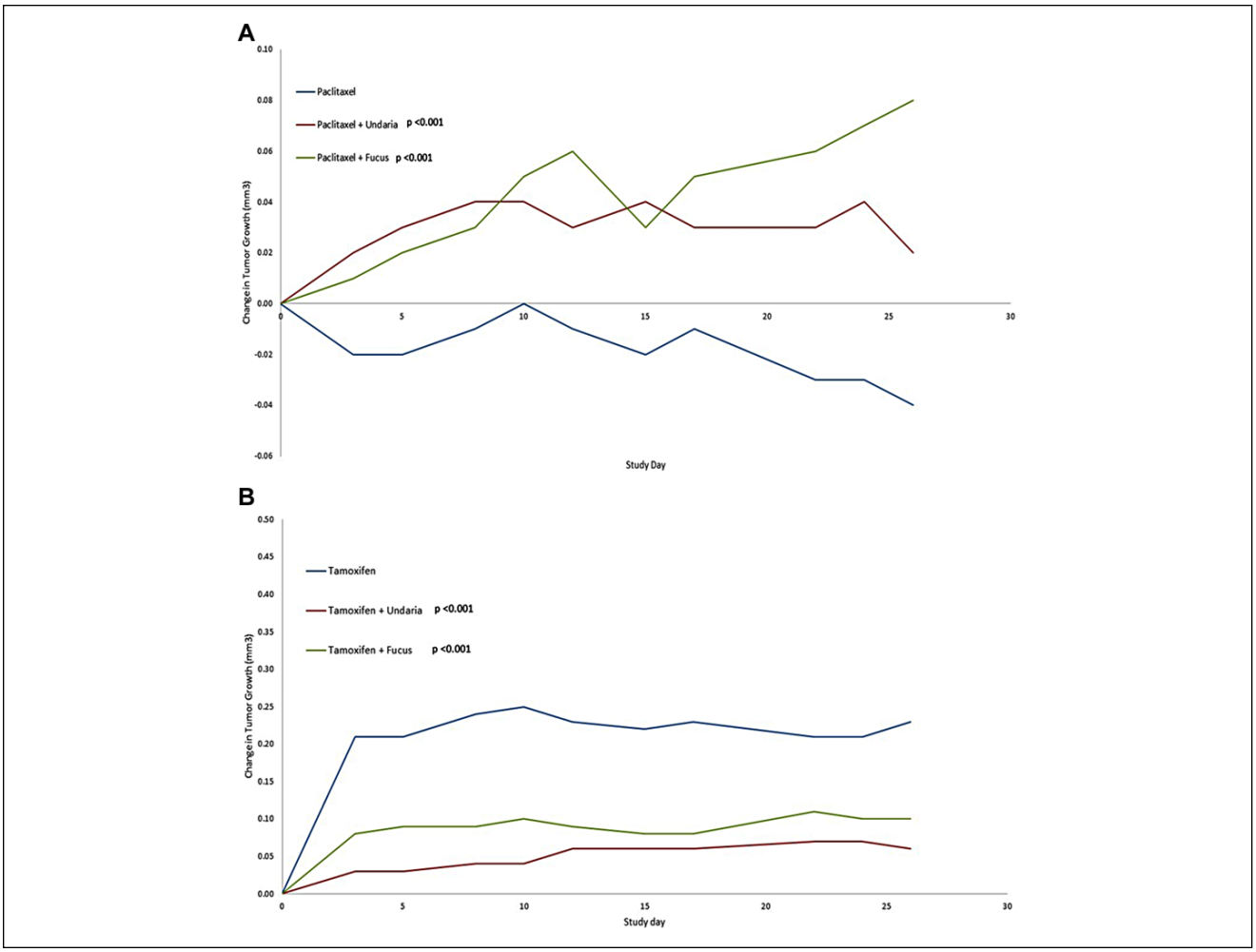

Figure 4. (a) Summary of the combination of the fucoidans in combination with paclitaxel in ZR-75 human breast cancer orthotopic model. The figure represents the tumor growth rate day 0 to 28 days. There was statistically significant change in tumor growth with FVF/paclitaxel but decrease in tumor growth with UPF/paclitaxel. (b) Summary of the combination of the fucoidans in combination with tamoxifen in ZR-75 human breast cancer orthotopic model. There was statistically significant change in tumor growth with UPF/tamoxifen and FVF/tamoxifen. UPF, *Undaria pinnatifida*; FVF, *Fucus vesiculosus*.

per day of fucoidans were administered in rat models. The results of the fucoidans in breast cancer models were statistically significant, supporting an avenue to continue to explore and define the role of fucoidans in optimizing cancer therapy. Furthermore, additional preclinical studies are necessary to provide statistical significance to support our finding that fucoidan in combination with paclitaxel and tamoxifen in ovarian cancer cell models does not have any therapeutic effect on reducing tumor growth.

The strengths of this study are that it was the first study to look at the tumor inhibitory effect of fucoidan in combination with chemotherapy agents as well as tamoxifen in both *in vivo* human ovarian cancer and breast cancer orthotopic mouse models. There are currently no data available to determine concentrations achieved in the gastrointestinal tract where most of the fucoidans' proposed immune modulation would occur. A limitation of this study was that tumor growth activity did not reach statistical significance in TOV-112d and $SKOV_3$-GPF-Luc human ovarian cancer cell models. Therefore, data to support the hypothesis that fucoidan in combination with paclitaxel and tamoxifen results in synergistic/additive activity was not achieved.

This study did confirm that UPF/FVF in combination with tamoxifen did not decrease tamoxifen activity in both breast and ovarian cancer with some potential to improve activity compared to tamoxifen alone in breast cancers. Previous *in vitro* studies had suggested UPF and FVF had overall synergistic activity with paclitaxel; however, in the current *in vivo* human cancer mouse model studies there was no change in paclitaxel activity when given in combination with UPF or FVF in either of the two human ovarian cancer models. Furthermore, this study demonstrated that UPF or FVF given

in combination with paclitaxel had a potential antagonistic effect in breast cancer models. Additional studies are warranted to delineate mechanisms contributing to variation in the *in vivo* activity when given in combination with paclitaxel. As a first step, a clinical pharmacokinetic study evaluating impact of FVF/UPF given in combination with chemotherapy in patients with solid tumors is currently underway.

Declaration of Conflicting Interests

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