Preclinical Evaluation of Safety of Fucoidan Extracts From *Undaria pinnatifida* and *Fucus vesiculosus* for Use in Cancer Treatment

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Abstract

Objectives: To evaluate potential hepatic metabolism-mediated drug interactions with fucoidan from *Undaria pinnatifida* (UPF) or *Fucus vesiculosus* (FVF) and potential growth inhibition activity with either fucoidan alone or with chemotherapy. In vivo studies were done to confirm safety and investigate fucoidan-mediated immune modulation. **Methods:** Cytochrome P450 (CYP450) 3A4, 2C8, 2C9, and 2D6 inhibition experiments were conducted in vitro followed by an ex vivo human hepatocytes model to evaluate the CYP450 induction potential of each fucoidan at highest theoretical concentrations. Four hepatic metabolism phase II pathways—glutathione S transferase (GST), quinone oxidoreductase (QOR), catechol-O-methyltransferases (COMT), and uridine di-phosphate (UDP)-glucuronosyltransferase (UGT)—were evaluated with validated immunoassays. Growth inhibition assays were performed with each fucoidan alone and in combination with chemotherapy agents in a panel of human cancer cell lines. In vivo studies evaluated safety and immune moduation. **Results:** CYP450 inhibition was observed with FVF. The GST, QOR, and UGT pathways had no changes. UPF and FVF both interacted with COMT. No growth inhibitory activity in cancer cell lines was observed. UPF and FVF had synergistic activity with paclitaxel or tamoxifen and additive activity with topotecan. In vivo, FVF decreased HeLa human cervical tumor growth and both FVF and UPF decreased TOV-112D human ovarian tumor growth. Otherwise, no significant change in tumor growth was observed. FVF immune modulation of IgG and IL-6 was observed (p<0.03). **Conclusion:** At higher doses, UPF and FVF may have limited potential for drug-supplement interactions, with either CYP450 or COMT hepatic metabolism pathways. Additional studies are warranted to evaluate to confirm findings of fucoidans in combination with chemotherapy.

Keywords

fucoidan, *Fucus vesiculosus*, *Undaria pinnatifida*, chemotherapy, cancer, drug interactions

Introduction

Fucoidans are polysaccharides containing substantial contents of fucose and sulfate ester groups, which are constituents of brown seaweed and marine invertebrates, like sea urchins and sea cucumbers.[1] Fucoidans are composed of monosaccharides (mannose, galactose, glucose, xylose), uronic acids, acetyl groups, and proteins. They show a wide spectrum of biological effects, such as anticoagulant and antithrombotic properties, and antiviral, antitumor, antioxidant, and anticomplement functions.[2-4] Fucoidans also have a variety of immune-modulation effects, such as promoting activation of macrophages, induction of dendritic cells, natural killer cells, and T-cells, and enhancing antiviral and antitumor responses.[2-5]

Polysaccharides display the highest biological properties among macromolecules. Many polysaccharides obtained

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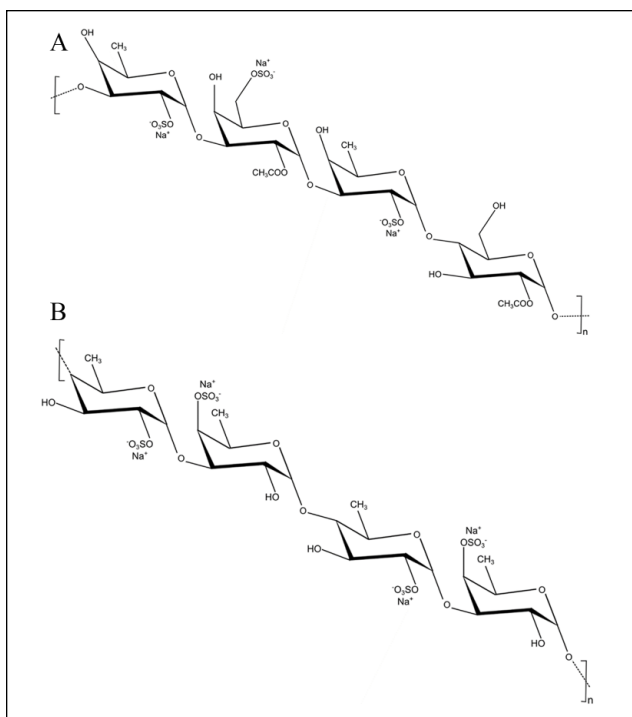
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Figure 1. (A) Chemical structure of FVF. (B) Chemical structure of UPF.

from natural sources such as plants and algae have anticancer properties. These include polysaccharides extracted from medicinal mushrooms and yeast glucans.[6] The structure of these natural polysaccharides allows them to be used in conjugation with anticancer agents; however, the activity may vary depending on the unique extraction and purification processes leading to variations in physiochemical and biopharmaceutical properties.[7]

The overall objective in this study was to determine the safety of *Undaria pinnatifida* and *Fucus vesiculosus* to support potential evaluation in prospective clinical trials in combination with chemotherapy in the oncology arena to ultimately improve patient outcomes. Specifically, studies were done to define the hepatic metabolism pathways for *fucoidans* and potential for drug-herbal interactions. Furthermore, the activity of the *fucoidan extracts* in a panel of human cancer cell lines was evaluated to define the growth inhibitory concentration (IC_{50}) of each fucoidan extract alone and/or in combination with commonly used cytotoxic chemotherapy agents. Finally, in vivo studies were completed to confirm safety of fucoidans in the presence of tumors and to investigate fucoidan-mediated immune modulation.

Materials and Methods

Fucoidans

In this study, the fucoidan extract from *Undaria pinnatifida* (UPF; Figure 1A) and the fucoidan extract from *Fucus*

vesiculosus (FVF; Figure 1B) were prepared by Marinova Pty Ltd (Cambridge TAS, Australia). The carbohydrate profiles were defined using a gas chromatography (GC)–based method for the accurate determination of individual monosaccharide ratios in a sample. This method relies on the preparation of acetylated alditol derivatives of the hydrolyzed samples established by Morvai-Vitányi and colleagues.[8] The uronic acid content was determined by spectrophotometric analysis of the hydrolyzed compound in the presence of 3-phenylphenol, based on a method described by Filisetti-Cozzi and Carpita.[9] Sulfate content was analyzed spectrophotometrically using a barium sulfate ($BaSO_4$) precipitation method (barium chloride [$BaCl_2$] in gelatin), based on methods developed by Dodgson and Price.[10] Finally, cations, including sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^+), were determined by flame atomic absorption spectroscopy. Table 1 is a summary of the composition of UPF and FVF that were used in the preclinical studies in this research.

Cell Culture

All human cancer cell lines including HeLa (HPV 16+) and SiHa (HPV 16/18+) cervical cancer cell lines, MCF-7 (ER+/PR+, COMT wild type) and ZR-75 (ER+/PR+, COMT wild-type) breast cancer cell lines, and TOV-112D (platinum-sensitive) ovarian cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). SKOV-3 GFP-Luc (platinum-resistant) ovarian cancer cell line was obtained from Cell Biolabs, Inc (San Diego, CA). The SiHa squamous cell cervical carcinoma, HeLa cervical adenocarcinoma, and the MCF-7 breast cancer cell lines were propagated in a media consisting of EMEM. The $SKOV_3$-GFP-Luc ovarian adenocarcinoma cell line was propagated with media consisting of McCoy's 5a medium. The ZR-75 breast cancer cell line was propagated in a media consisting of RPMI 1640, and TOV-112d was propagated in a mixture of 1:1 MCDB 105 and medium 199 with 2 mM L-glutamine and Earl's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% FBS. 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 in order to prevent major changes in cell line characteristics.

Metabolism Studies

During the designing and planning of this preclinical research study, the achievable FVF or UPF human systemic plasma concentration data were unavailable to investigators. Hence, for all of the metabolism studies, the 0.2 mg/mL concentration was selected as an estimate of the clinically relevant concentration. This was based on the current estimated maximum recommended dosage of 3 g total daily

Table 1. Summary of the Composition of UPF and FVF.

	Total Carbohydrates (%)	Uronic Acid (%)	Polyphenol (%)	Sulfate (%)	Cations (%)	Peak MW (kDa)
UPF2012565	48.4	2.9	<2	21.5	5.6	51.7
FVF2012501	65.1	6	3.6	20.9	3.9	82.5

	Neutral Sugar Breakdown (w/w %)				
	Fucose	Xylose	Mannose	Galactose	Glucose
UPF2012565	24.0	0.0	1.5	18.6	0.8
FVF2012501	44.6	9.1	1.2	2.3	0.4

Abbreviations: UPF, *Undaria pinnatifida*;' FVF, *Fucus vesiculosus*; MW, molecular weight.

dose as instructed by the manufacturer, assuming 100% bio-availability and using an estimated total blood volume of an average adult of 7 L. However, a recent study by Irhimeh and colleagues reported less than 0.6% oral bioavailability in human fucoidan pharmacokinetic studies.[13] Therefore, concentrations used in these metabolism studies were above achievable plasma concentrations in humans so may not translate to the clinical setting. The concentrations of FVF or UPF used in the metabolism studies might be achievable in animal models with smaller total volume or in the human gut for short-term (2-4 hours) during transit through gastro-intestinal tract, but animal plasma concentration of FVF or UPF data were also not available.

Phase I Inhibition Assay. The highest analytical grade available chemicals and reagents were used. As mentioned earlier, the UPF and FVF were provided by Marinova Pty Ltd (Cambridge TAS 7170, Australia). All other chemicals and reagents were obtained from Sigma-Aldrich (St Louis, MO).

Cytochrome P450 enzyme microsomes. The group of isoenzyme microsomes, CYP450 3A4, 2C8, and 2C9, were acquired from BD Biosciences (Gentest) Discovery Labware (Woburn, MA). The stated total protein content for the isozymes packaged in 0.5 mL aliquots is 5.8 mg/mL in 100 mM potassium phosphate (pH 7.4) and corresponding CYP450 content is 1000 pmol/mL. These isozymes were utilized in accordance with provided Material Safety Data Sheets and technical bulletins.

High-throughput CYP450 inhibition assays. A validated high-throughput method for measuring CYP450 inhibition method that was provided by BD Gentest was used to develop the assay protocol. In brief, the test compounds (UPF and FVF), positive controls (ketoconazole, quercetin, quinidine, and sulfaphenazole), and substrates (dibenzylfluorescein and 3-(2-(N,N-diethyl-N-methyl-ammonium)ethyl)-7-methoxy-4-methylcoumarin iodide [AMMC]) were used to study this potential inhibition of

CYP450 substrates.[11] The manufacturer's methods were used to prepare the common solutions, enzyme/substrate mixes, cofactors stocks, and positive control solutions. A final cofactor solution was prepared consisting of 1.3 mM of NADP+, 3.3 mM of glucose-6-phosphate, 0.4 Units/mL of glucose-6-phosphate dehydrogenase, and 3.3 mM of magnesium ion and then 200 μL of the cofactor solution was added in each reaction well of a 96-well titer plate. After the addition of CYP450 enzyme, the appropriate substrate (DBF at 100 μM or AMMC at 500 μM) and 58.3 μM of the inhibitor positive control were added to the reaction mixture and serially diluted 1: 3 for 8 wells. The experimental wells on each plate had either the low or high concentrations of FVF of either 285 μg/mL or 1.300 μg/mL or low or high concentrations of UPF of either 14 μg/mL or 285 μg/mL that also was then serially diluted 1:3 for 8 wells. The reactions were incubated for 30 to 60 minutes at 37°C and reactions were stopped with the addition of "stopping solutions" consisting of 80:20 ratio of acetonitrile–TRIS base solution (CYP450 2D6 only) or 2M NaOH solution for all of the other CYP450 isoenzymes. In each reaction well, IC_{50} values were used to quantify and report CYP450 inhibition. These data were produced by comparing the metabolism during assay reactions containing varied concentrations in the presence and absence of the known positive control inhibitor. The amount of substrate metabolized for the control comparison reactions was determined via fluorescence emission detection with FL600 Dual-Band plate reader from BioTek Instruments, Inc (Winooski, VT). The wavelength measured was at 528 nm (excitation 485 nm) of fluorescein (metabolite product of DBF metabolism by CYP450) or at 460 nm (excitation 360) of 3-(2-(N,N-diethyl-N-methyl ammonium)ethyl)-7-hydroxy-4-methylcoumarin (AMHC; metabolite product of AMMC).

Phase I enzyme induction assay. Cryopreserved human hepatocytes were obtained from BD Biosciences (Gentest) Discovery Labware. The cells were handled

according to the supplier's instructions and were thawed, isolated, and plated according to the supplier's protocol. The cells were incubated for at least 48 hours at 37°C (5% CO_2) to allow stabilization before use. Hepatocytes were replated using supplemented Hepatozyme SFM media (Gibco Invitrogen Corporation, Carlsbad, CA) containing 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) and 250 μM ascorbic acid (Sigma-Aldrich). Hepatocytes were then maintained in SFM media for the 7-day duration of the study. Primary hepatocyte cells were plated into separate 6-well Collagen I–coated plates for the CYP450 induction and substrate experiments, respectively. The cultures were prepared in triplicate for each experimental time point.

An ex vivo model of cryopreserved human hepatocytes was used to evaluate the ability of each fucoidan extract, UPF or FVF, to induce CYP450 metabolism for CYP450 3A4, 2C8/2C9, and 2D6 enzymes. A known substrate for each isoenzyme was selected, including diclofenac (CYP450 2D6), dextromethorphan (CYP450 2C8/2C9), or docetaxel (CYP450 3A4). The experiment was set up in triplicate using each known substrate alone, a control inducer of all 4 CYP450 isoenzymes, rifampicin 25 μM (Sigma-Aldrich) with the known substrate, then a low and high concentration of each of the fucoidan extracts UPF 14 μg or 285 μg/mL and FVF 285 μg or 1300 μg/mL and using known substrates diclofenac (2D6), dextromethorphan (2C8/2C9), and docetaxel (3A4). The hepatocytes were treated for a total of 72 hours with either the control inducer rifampicin or either FVF or UPF with media changes every 24 hours. After 72 hours, rifampicin or fucoidan products were removed and the appropriate substrate concentration specific to the CYP450 isoenzyme of interest or specific to the test substrate (fucoidan products) were added. Samples were taken at different time points, starting at time 0, 2.0 6.0, and 24 hours. Known inducer, substrate, and test sample were set on a 96-well plate and absorbance was read at the appropriate wavelength for the test substrate.

Phase II Enzyme Inhibition Studies

GST metabolism inhibition assay. GST activity was evaluated using a slightly modified method described by Mannervik and Guthenberg.[12] The assays were carried out in 200 μL total volume, 96-well ultraviolet-visible (UV-Vis) plates (Fisher Scientific, Waltham, MA). The reaction of 1 mM GSH with 1 mM 1-chloro-2,4-dinitrobenzene in the presence of 2 μL human liver S9 fraction (20 mg/mL), diluted in 1 M potassium phosphate buffer (pH 6.5), was measured by UV absorbance on Synergy HT multi-detection microplate reader (BioTek Instruments, Inc) at 340 nm. UPF 14 μg or 285 μg/mL and FVF 285 μg or 1300 μg/mL were added as the test/experimental agents and serially diluted 1:3 for 8 wells. Ethacrynic acid (6 μM) is a broad inhibitor of GST activity and was selected as a positive control inhibitor of GST activity and was added to wells serially diluted 1:3 for 8 wells as well.[14]

UGT metabolism inhibition assay. UGT activity was evaluated following a method described by Liu and Franklin.[15] Inhibition assays were carried out in 200 μL total volume, 96-well UV-Vis plates. The 3 primary isoforms of UGT associated with drug metabolism were evaluated: UGT1A3, UGT1A6, and UGT2B17. Substrates for each isoform included estrone (UGT1A3), 1-napthol (UGT1A6), and testosterone (UGT2B17). In short, the reaction of 2 mM uridine 5′-diphosphoglucuronic acid (UDPGA) with each respective substrate in the presence of 2 μL human liver microsomes (20 mg/mL), diluted in 50 mM potassium phosphate buffer (pH 8.0), was measured by UV absorbance on a FL600 Dual-Band plate reader (BioTek Instruments, Inc) at either 220 nm (UGT1A6) or 230 nm (UGT1A3 and UGT2B17). UPF *or* FVF was added as the test/experimental agents using either UPF 14 μg or 285 μg/mL or FVF 285 μg or 1300 μg/mL and serially diluted 1:3 for 8 wells. Removal of UDPGA was used as a negative control.

QOR metabolism inhibition assay. QOR activity was evaluated using the method described by Benson and colleagues.[16] Assays were carried out in 96-well UV-Vis plates with total volume of 200 μL per well. The reaction mixtures contained 0.7 mg of bovine serum albumin, 25 mM Tris-HCl (pH 7.4), 0.2 mM NADPH, 40 μM 2,6-dichlorophenolindophenol (DCPIP), and control inhibitor, dicoumarol 10 mM. As with the UGT assay, UPF *or* FVF was added as the test/experimental agents using either UPF 14 μg or 285 μg/mL or FVF 285 μg or 1300 μg/mL, then serially diluted 1:3 for 8 wells. NADPH was measured by UV absorbance at 200 nm or DCPIP at 600 nm on a FL600 Dual-Band plate reader from BioTek Instruments, Inc.

COMT metabolism induction study. The cryopreserved human hepatocytes obtained from Corning (Gentest Discovery Labware) were replated using supplemented hepatozyme SFM media (Gibco Invitrogen Corporation) containing 10% fetal bovine serum (Gemini Bio-Products) and 250 μM ascorbic acid (Sigma-Aldrich) in 6-well collagen-coated culture plates. Cells were allowed to adhere for 8 hours prior to removal of seeding media. Hepatocytes were maintained in un-supplemented hepatozyme SFM media for at least 24 hours prior to the study being initiated. For the COMT method, primary hepatocyte cells were incubated with 500 ng/mL 17-β estradiol in the presence of UPF 14 μg or 285 μg/mL and FVF 285 μg or 1300 μg/mL. Fucoidans were compared to the control known inducer, folic acid 150 ng/mL.[17] Estradiol concentrations remaining at each time point were determined by employing a validated high-pressure liquid chromatography assay with ultraviolet absorbance detection according to parameters described in the CDER Guidance for Industry Bioanalytical Assay Method Validation detection.[18] Briefly, 17-β-estradiol was isolated from spiked hepatozyme media with liquid-liquid

extraction with *n*-hexane. Liquid chromatographic separation was achieved by isocratic elution on a Waters Bondpak C18, 4.6 × 250 mm, 10 μm particle size analytical column (Milford, MA). The mobile phase consisted of an isocratic flow of 50:50 deionized water-acetonitrile at a flow rate of 1.0 mL/minute and total run time of 15 minutes. The 17-β-estradiol peak was positively identified from other peaks using UV absorbance at a wavelength of 200 nm. Assay had sensitivity with lower limit of detection of 0.05 ng/mL and was linear from 0.25 μg/mL to 25 μg/mL.

UGT metabolism induction study. For the UGT method, primary hepatocyte cells were incubated with 150 μM estrone (for UGT1A3) and 600 μM 1-naphthol (for UGT1A6) in presence of UPF 14 μg or 285 μg/mL and FVF 285 μg or 1300 μg/mL. Fucoidan was compared to control β-naptholflavone 80 μM. The cultures were maintained in duplicate for each experimental time point, including 2 hours, 6 hours, and 24 hours. To determine UGT induction, remaining substrate concentrations in samples were measured with FL600 Dual-Band plate reader from BioTek Instruments, Inc using UV detection at 230 nm for estrone (UGT1A3) and at 280 nm for 1-naphthol (UGT1A6).

In Vitro Growth Inhibition Studies

Fucoidan Alone Study. Growth inhibition assays were conducted as previously described.[19] Briefly, cells were plated at 2500 to 5000 cells per well in 96-well plate and incubated at 37°C for 24 hours. Each of the cancer cell lines were treated with 50 μL of various concentrations of fucoidan extracts, UPF or FVF, starting at 300 μg/mL for low or 1300 μg/mL for high followed by serial dilutions for the remaining 9 columns of wells. For the cell growth inhibition studies fresh stock solutions were prepared in media of each respective cell line for each experiment. Control wells had media alone and blank wells had no cells, drug, or media. After a 72-hour incubation period, 25 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to obtain a final concentration of 300 μg/mL, and cells were incubated for 2 hours. Plates were then centrifuged and the supernatant was removed. Afterwards, 50 μL of dimethyl sulfoxide was added to each well and absorbance at 563 nm was measured. The inhibitory concentration to achieve 50% cell death (IC_{50}) for each fucoidan extract and each cell line were calculated.

Fucoidan Extracts in Combination With Selected Chemotherapy Agents. Combination agent growth inhibition assays were done using IC_{50} concentration of one of the selected chemotherapy agents including topotecan, gemcitabine, paclitaxel, carboplatin, 5-fluorouracil, tamoxifen, and letrozole with UPF 300 μg/mL or FVF 1300 μg/mL for total of 72 hours incubation and then growth inhibitory activity was

evaluated. The IC_{50} was selected to ensure that enough viable tissue was available for immunoblotting and flow cytometry experiments. After 72 hours growth inhibitory activity was evaluated as described above for the single agent studies. All experiments were done in quadruplicate.

To define synergy/additive activity for the in vitro growth inhibition assays the interaction index was used to determine antagonism, additive, or synergistic activity as described by Tallarida[20] was determined with the following equation: $\gamma = a/A + b/B$, where A = dose of drug A alone that gives the specified effect; B = dose of drug B alone that gives specified effect; a = dose of drug A used in combination to achieve specified effect; and b = dose of drug B used in combination to achieve specified effect. The interaction index measures drug combination as follows: γ value equal to one it is additive; γ value less than one is synergistic and γ value greater than one is antagonistic.

In Vivo Study With Orthotopic Human Cancer Mouse Models

The protocol was reviewed and approved by the Institutional Animal Care and Use Committee prior to initiating any animal work. For this study, 240 female nude mice, 6 to 8 weeks old, were obtained from Charles River Laboratories Wilmington, MA. All the mice weighed about 22 to 26 g and were maintained 5 per cage in specific pathogen free (SPF) barrier room, with a temperature of 22 ± 3°C and 45 ± 3°C RH% (relative humidity). They had 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 guide for the care and use of laboratory animals. The mice were divided into 4 groups of 10 for each cell line (N = 240 for 6 cell lines). There were 4 arms of this study: 2 treatment arm (N = 10 each), no treatment control arm (N = 10), and a vehicle control arm (N = 10). The treatment arm received an oral dose (30.4 mg/kg, in 0.2 mL, gastric gavage) of UPF or FVF, which was selected based on estimated equivalent total daily dose of fucoidan of 2125 mg/daily in a 70-kg adult, the control arm received an oral dose of autoclaved water (0.2 mL, gastric gavage) per day starting on day 1 and continued until completion of the study (day 28). The UPF and FVF were provided by Marinova Pty Ltd and were prepared to a final concentration of 152 mg/mL in deionized purified water once a week and stored in 2°C to 8°C for animal studies.

SiHa cells (3.0×10^6), HeLa, MCF-7, ZR-75, TOV-112D cells (5.0×10^6) were dispersed in PBS with 20% matrigel and injected subcutaneously (subQ), and each mouse grew one tumor on the dorsal surface. The SKOV$_3$-GFP-Luc (5.0×10^6) was injected intraperitoneal (IP) in female nu/nu mice on day zero. Tumor measurements were obtained 3 times per week. The subQ tumors were measured with electronic

Table 2. Phase I: Cytochrome P450 Metabolism of Fucoidans at High and Low Concentrations.

Table 2A. CYP450 Inhibition Summary[a].

	UPF Low (14 µg/mL)	UPF High (285 µg/mL)	FVF Low (285 µg/mL)	FVF High (1300 µg/mL)
2C8	−	+	+	+
2C9	−	+	+	+
3A4	−	−	+	+
2D6	−	−	+	+

Abbreviations: UPF, *Undaria pinnatifida* fucoidan; FVF, *Fucus vesiculosus* fucoidan.
[a]UPF at low concentration had no effect on all of the CYP450 isoenzyme activities. The UPF high concentration showed inhibition of the 2C8 and 2C9 pathway. FVF showed inhibition of all 4 enzyme pathways, 2C8, 2C9, 3A4, and 2D6, at both the high and low concentrations.

Table 2B. CYP450 Induction Summary[a].

	UPF Low (14 µg/mL)	UPF High (285 µg/mL)	FVF Low (285 µg/mL)	FVF High (1300 µg/mL)
2C8/2C9	−	−	−	−
3A4	−	−	−	−
2D6	+	+	+	+

Abbreviations: UPF, *Undaria pinnatifida* fucoidan; FVF, *Fucus vesiculosus* fucoidan.
[a]UPF and FVF at both low and high concentrations had no effect on CYP450 2C8, 2C9, or 3A4 isoenzyme activity. UPF and FVF at both low and high concentrations showed induction of the CYP450 2D6 pathway.

calipers (Mitutoyo, Utsunomiya, Japan) and the IP tumors were imaged under Kodak Imaging Station (Kodak, Rochester, NY) once in a week, and abdominal girth was measured by using a measuring tape twice in a week. Blood was collected from facial vein from each group every week during the treatment period, plasma were separated and kept in −80°C freezer to evaluate selected panel of immune markers including interleukin-6 (IL-6), immunoglobulin G (IgG), interferon-α/β (IFN-α/β), and interferon-γ (IFN-γ). Mice were monitored daily for signs/symptoms of morbidity including but not limited to lethargy, weight loss, anorexia, hunching, or any signs of distress. Mice were sacrificed via CO_2 inhalation followed by cervical dislocation, when tumor diameter was greater than 12 mm² or if there was greater than a 10% decrease in body weight found during the study period. At the end of the study all the remaining mice were sacrificed. When sacrificed, total tumor burden was determined by macroscopic dissection. Immediately after sacrifice, tumors were surgically removed from all mice and stored at −80°C.

Quantification of Selected Immune Markers

A selected panel of immune markers including IL-6, IgG, IFN-α/β), IFN-γ were evaluated from mouse blood samples collected weekly throughout the treatment. Standard validated ELISA assays for the detection of the selected immune marker including IL-6, IgG1, IFN-α, IFN-β, and IFN-γ were employed according to respective protocol from the manufacturer (eBioscience, San Diego, CA). For each series of immunomarker determinations, a standard curve was constructed with known concentrations of these markers. Sandwich ELISAs for the detection of total IL-6, IgG1, IFN-α, IFN-β, and IFN-γ were performed and plasma concentrations of these 4 immune markers were calculated from respective standard graphs.

Statistical Analysis

All experiments were carried out at least in triplicate and repeated if the coefficient of variance was greater than 20%. Final results are described through appropriate summary statistics (eg, means, standard deviations, and correlation coefficients). ANOVA was employed to determine differences in metabolism activity for each respective metabolic pathway, and Pearson's correlation test was used to evaluate all correlations. A paired *t* test was used to evaluate the viability in continuous data as appropriate. Results were considered to be significant when $P > .05$. The program GraphPad Prism 5.02 (GraphPad Software Inc, San Diego, CA) was used to perform the analysis.

Results

Metabolism Studies

High-Throughput CYP450 Assays. At the 2 selected concentrations of UPF of 14 µµg/mL and 285 µg/mL was not an inhibitor or inducer of CYP450 2C8, 2C9, or 3A4 pathways (Table 2A and 2B) At the 2 selected concentrations of FVF (low [285 µg/mL] and high [1300 µg/mL]) inhibition of the CYP450 2C8, 2C9, 3A4, or 2D6 pathways was observed. Induction of

Table 3. Phase II: Evaluation of Fucoidan Effect on CYP450 Metabolism Isoenzymes[a].

	UPF Low (14 µg/mL)	UPF High (285 µg/mL)	FVF Low (285 µg/mL)	FVF High (1300 µg/mL)
QOR	NC	NC	NC	NC
GST	NC	NC	NC	INH
UGT	NC	NC	NC	NC
COMT	IND	IND	INH	INH

Abbreviations: INH, inhibition; IND, induction; NC, no change in metabolism.

[a]UPF high and low both caused induction in COMT pathway. FVF low is an inhibitor of the COMT pathway. FVF high resulted in inhibition of GST pathway and COMT pathway.

CYP450 2D6 pathway was observed when exposed to either UPF or FVF at the low and high concentrations.

Phase II Metabolism Pathways. UPF (low [14 µg/mL] and high [285 µg/mL] concentrations) and FVF (low [285 µg/mL] and high [1300 µg/mL] concentrations) were observed to inhibit or induce the GST, QOR, and UGT pathways. In the COMT pathway, the UPF extract exhibited induction of COMT, which was less than the positive control inducer (folic acid). However, the FVF extract exhibited inhibition of COMT pathway (see Table 3).

In Vitro Growth Inhibition Activity

As anticipated, none of the fucoidan compounds consistently achieved a concentration with 50% inhibition (IC_{50}) that was reproducible across all cell lines. We did observe slightly more activity with the FVF in 87% of cell lines compared to UPF in 53% of the cell lines across the concentration ranges. In the screening of UPF and FVF, both fucoidan extracts appeared to have overall synergistic activity given in combination with paclitaxel or tamoxifen and additive activity given in combination with topotecan (Table 4). There were some cell lines that despite overall antagonistic activity observed still had slight (10% to 15%) increase in growth inhibitory activity with the combination of fucoidan/chemotherapy compared to the chemotherapy agent alone.

Single Agent Animal Studies

The animal studies definitely confirmed the safety of both UPF and FVF extracts in the presence of cancer. As single agent given by mouth once daily, neither fucoidan extract affected the growth or growth rate of human cancer orthotopic mouse models including ovarian cancer cell line $SKOV_3$-GFP-Luc (platinum- resistant; Figure 2A) or TOV-112d (platinum sensitive; Figure 2B); or breast cancer cell lines MCF-7 (COMT variant; Figure 3A), ZR-75 (COMT wild type; Figure 3B); or cervical cancer cell lines HeLa (HPV 16+; Figure 4A) or SiHa (HPV 16/18+; Figure 4B). In fact, FVF statistically decreased tumor growth in the HeLa (HPV 16+) human cervical cancer mouse model ($P < .004$). The TOV-112d human ovarian cancer mouse model had a statistically significant decrease in growth rate to both UPF ($P < .001$) and FVF ($P < .001$) after 30 days of supplementation.

Although duration of study was only short duration of 30 days, fucoidan extract modulation of immune markers was observed. Daily supplementation with FVF demonstrated a statistically significant improvement in both IL-6 ($P < .03$) and IgG ($P < .003$) and some nonsignificant increases in IFN-α/β and IFN-γ (Table 5). UPF also had some trend in improvement in the panel of immune markers evaluated that did not reach statistical significance.

Discussion

Prospective evaluation of the safety of the use of nutritional supplements in the oncology patient population both during and off treatment is a priority in the oncology health care team. However, there is limited clinical information available, hence utilizing systematic preclinical studies can help gain knowledge and perspective on each nutritional supplement. This systematic preclinical evaluation of 2 fucoidans, UPF or FVF, for use in oncology included evaluation of both phase I and phase II metabolism pathways to determine potential for drug/supplement interactions, in vitro single supplement and combination with chemotherapy growth inhibition evaluation in a panel of 15 human cancer cell lines and finally in vivo single agent studies were completed in 8 selected human cancer orthotopic models to evaluate impact on tumor growth as well as confirm immune modulation activity. The phase I and phase II metabolism studies demonstrated that UPF appears to have no potential for drug-supplement interactions, and FVF showed only limited potential of interactions with CYP450 and COMT pathways. Additional studies are warranted to evaluate potential for synergistic activity between fucoidan compounds and paclitaxel, tamoxifen, and additive activity with topotecan that was observed in the in vitro growth inhibition studies. All 8 animal models confirmed both UPF and FVF will not promote tumor growth and perhaps in few studies suggested may have some benefit to decrease tumor growth, likely

Table 4. Combination Regimen of Fucoidan and Chemotherapy Agents Growth Inhibition Activity[a].

Overall Interaction Index	Carboplatin	Doxorubicin	Gemcitabine	Paclitaxel	Topotecan	5-FU	Tamoxifen	Letrozole
Undaria fucoidan	Antagonistic	Antagonistic	Antagonistic	Synergistic	Additive	Antagonistic	Synergistic	Antagonistic
Cells achieving >10% increase in activity	SKOV3, Hep G2	None	HCC-38, CAL-27	SKOV3, TOV-11D, HeLa, SiHa, C-33A, HEC-1A, Ishikawa, HS294T, CAL-27, PC-3	SKOV3, HeLa, SiHa, C-33a	HeLa, SiHa, C-33a	SKOV3, TOV-11D, MCF-7, ZR-75	None
Fucus fucoidan								
Cells achieving >10% increase in activity	SKOV3, Hep G2, Hep-3B, SNU-387, HCC-38, HS249T	HCC-38, HS249T	SKOV3, HCC-38, HS249T, CAL-27	SKOV3, TOV-11D, HeLa, SiHa, C-33A, HEC-1A, Ishikawa, HS294T, CAL-27, PC-3	SKOV3, HeLa, SiHa, C-33a, HCC-38, HS249T	HeLa, SiHa, C-33a, Hep-3B, HCC-38	SKOV3, TOV-11D, MCF-7, ZR-75	None

[a]Comprehensive synergistic activity was seen in both fucoidans combined with paclitaxel and tamoxifen. Additive activity was seen in combination with topotecan. Letrozole combined with fucoidans displayed no antagonistic activity.
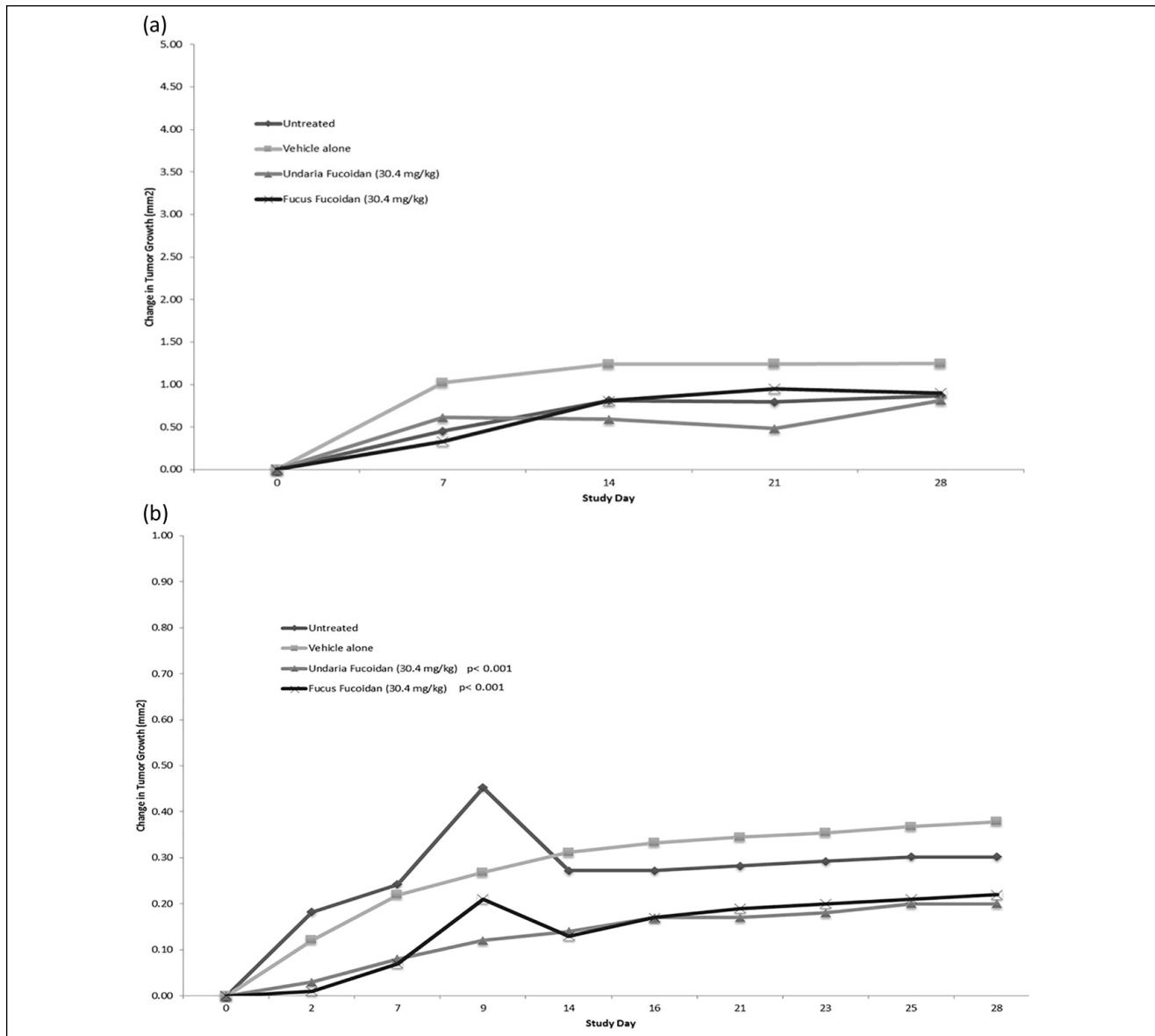
Figure 2. (A) Summary of the impact of fucoidan on rate of tumor growth in SKOV$_3$ human ovarian cancer mouse model. No significant change in tumor size in all groups. Vehicle alone showed the most change in tumor size within the SKOV$_3$ group. (B) Summary of impact of fucoidan on rate for tumor growth in TOV-112D human ovarian cancer mouse model. UPF and FVF showed a decrease in tumor growth compared to the untreated and vehicle groups.

through immune modulation effects. The short-term exposure of only 30 days in the animal studies did confirm in vivo immune modulation with both UPF and FVF.[5]

This was first study to complete a comprehensive evaluation of both phase I and phase II hepatic metabolism of UPF and FVF to define any potential for drug/supplement interactions. While there was synergistic activity with tamoxifen and paclitaxel in breast cancer cell lines in other studies, this was first study to also observe this in vitro synergistic activity in also 6 other cancers including ovarian, endometrial, cervical, prostate, hepatic carcinoma, and prostate cancers.[21,22]

Immune modulation and potential mechanisms for anticancer activity of the fucoidan compounds has been observed in multiple preclinical studies throughout the literature. For example, Senthilkumar and colleagues recently published a summary review of all the current proposed mechanisms of brown seaweed fucoidans activity against cancer cell growth, immune modulation being cited as mechanism associated with UPF, which was also observed in this study.[23] Immune modulation in preliminary human studies has also been confirmed.[13] Based on the findings from this study, the next steps will be to complete preclinical screening to identify the
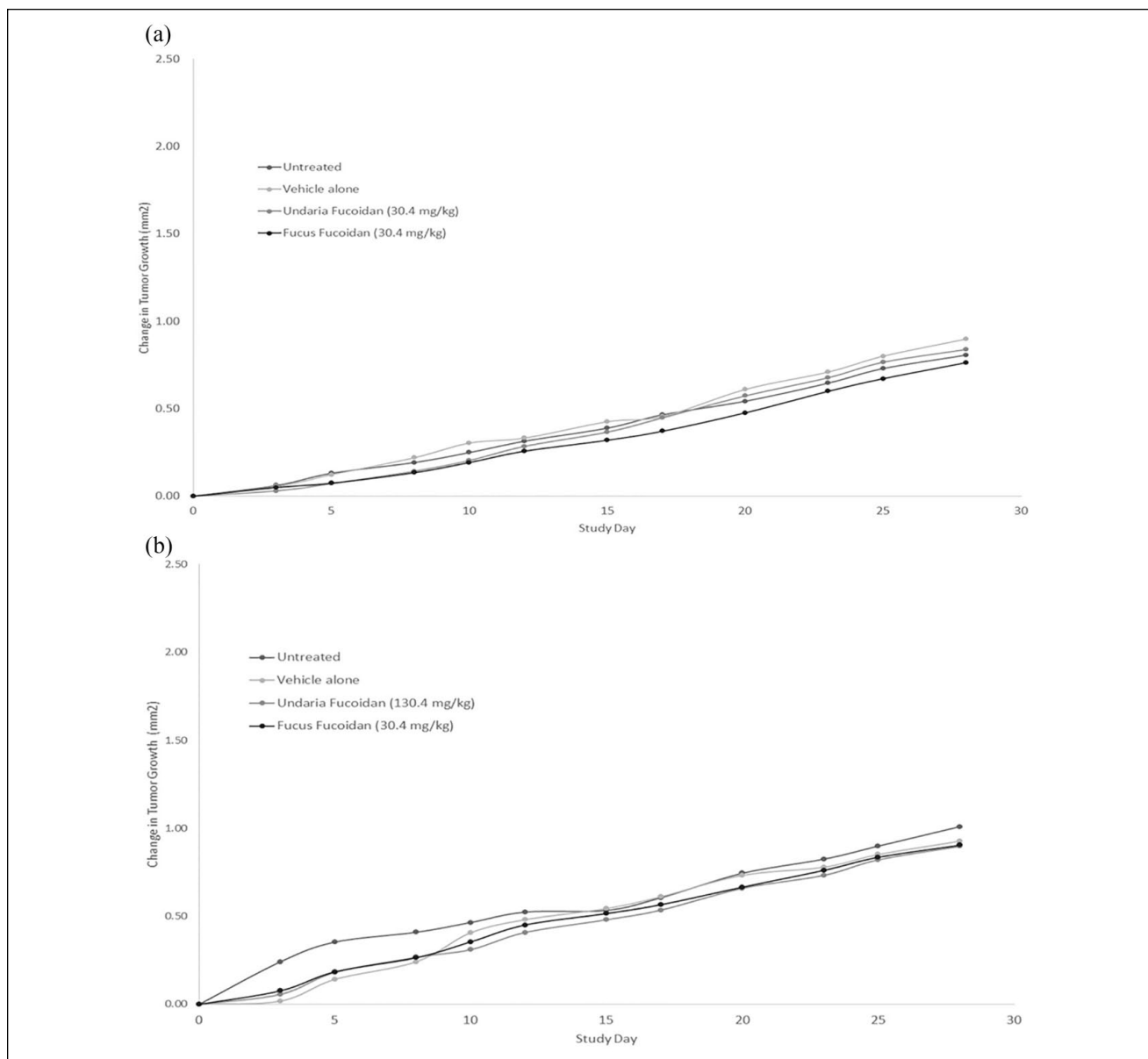
Figure 3. (A) Single agent fucoidan in MCF-7 human breast cancer mouse model. Both fucoidans showed no significant change in tumor growth. (B) Single agent fucoidan in ZR-75 human breast cancer mouse model. Both fucoidans showed no significant change in tumor growth.

optimal combinations with chemotherapy and then proceed to clinical setting for prospective clinical trials. While it is unlikely to have any drug/supplement interactions based on the data from metabolism studies presented in this study as well as limited bioavailability observed by Irhimeh and colleagues, traditional pharmacokinetic/safety studies should be completed prior to proceeding to larger phase II studies for defining efficacy and role of combinations with fucoidans. In an initial small study by Ikeguchi and colleagues that evaluated combination of fucoidan with 5-fluorouracil/leucovorin plus oxaliplatin (FOLFOX) or 5-fluorouracil/leucovorin plus

irinotecan (FOLFRI), the authors reported less fatigue, ability to have increased duration of treatment, and longer survival in the 10 patients that received fucoidan with chemotherapy compared to the 10 patients that had chemotherapy alone.[24] While not statistically significant, this study supports that there is merit in continuing to evaluate and define the role of supplementation with fucoidans to optimize chemotherapy outcomes. A pharmacokinetic/safety study evaluating FOLFOX and paclitaxel/carboplatin in combination with fucoidan 500 mg twice a day is already underway as well.
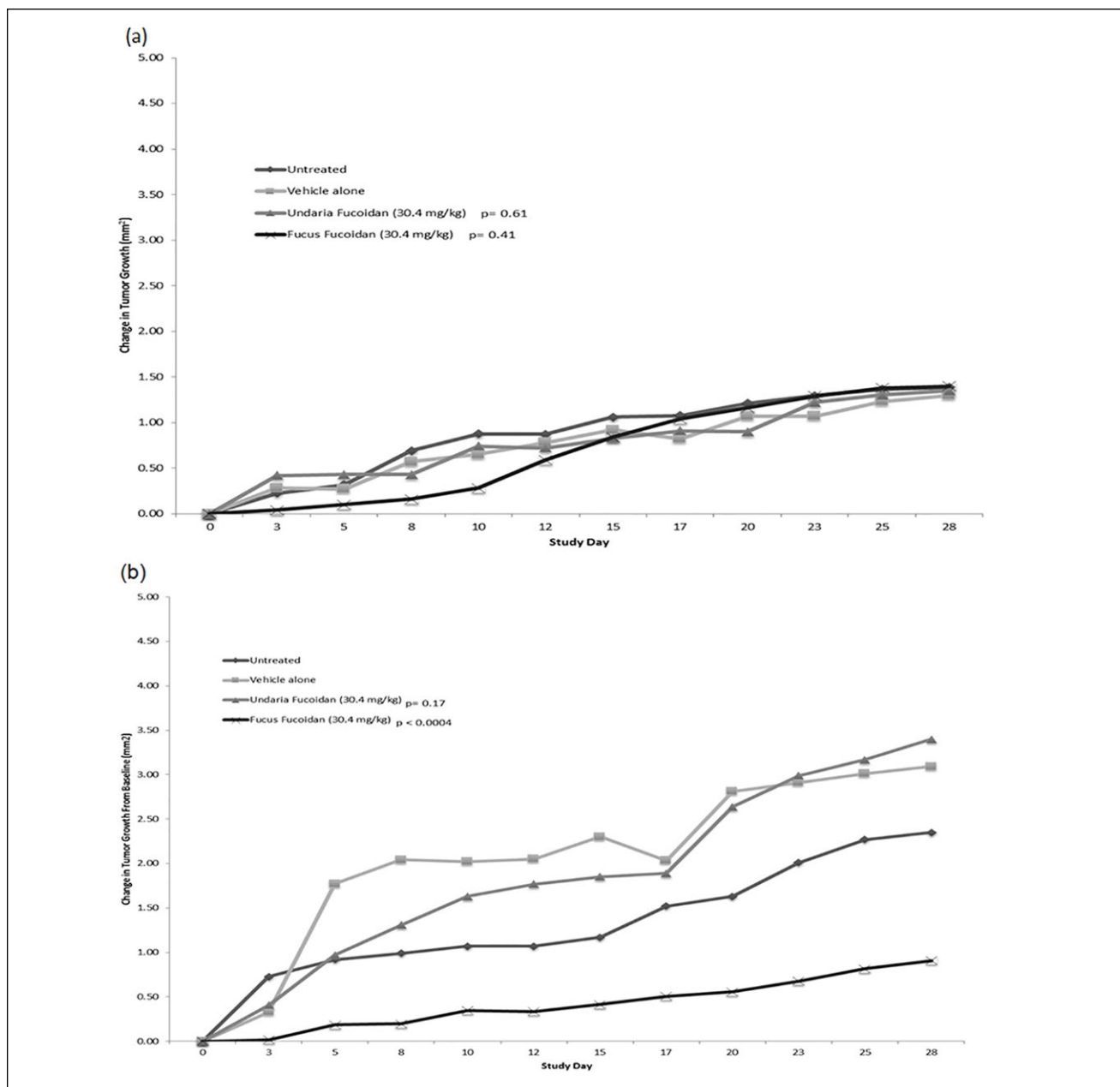
Figure 4. (A) Summary of impact of fucoidan on rate for tumor growth in SiHa human cervical cancer mouse model. Both fucoidans showed no significant change in tumor growth. (B) Summary of impact of fucoidan on rate for tumor growth in HeLa human cervical cancer mouse model. FVF demonstrated a decrease in tumor growth. UPF showed tumor growth comparable to vehicle group.

The strengths of this study is that it was systematic preclinical evaluation with in vitro, ex vivo, and in vivo studies evaluating fucoidans, UPF and FVF. Evaluating both phase I (cytochrome P450) metabolism and also the phase II pathways (GST, UGT, QOR, and COMT) provided more complete information on potential for drug interactions. Completing the in vitro screening in a diverse panel of human cancers allowed the consistency additive/synergistic activity with paclitaxel, tamoxifen, and topotecan to

be demonstrated. Finally, conducting 8 in vivo human cancer orthotropic mouse models demonstrated reproducibility and reliability of data that showed fucoidans do not promote tumor growth rate and perhaps its immune modulation may contribute to inhibition of tumor growth but that data will need to be confirmed in future human studies. A definite limitation of this study was the concentrations of fucoidans selected for the metabolism studies were well above what is clinically achievable based on

Table 5. Expression of Immunomodulatory Markers in Single Agent Animal Studies[a].

	Mean IL-6 (pg/mL)	Mean IgG (μg/mL)	Mean INF-α/β (pg/mL)	Mean INF-γ (pg/mL)
Baseline	0	421.3	143	83.3
Treatment				
Week 1	0.2	264.2	152.3	90.0
Week 4	48.0	663.7	148.0	99.0
Fucoidan *Undaria*	$P = .31$	$P < .03$	$P = .37$	$P = .06$
Week 1	7.4	1048.2	139.7	93.0
Week 4	47.6	1389.2	168.7	115.3
Fucoidan *Fucus*	$P < .03$	$P < .03$	$P = .31$	$P = .07$
Week 1	17.5	1441.3	144.7	90.7
Week 4	310.2	1682.1	169.7	170.0

[a]Week 1 UPF had 7-fold greater expression and Week 1 FVF had approximately 18-fold greater expression of mean IL-6 than No Treatment group Week 1. UPF had comparable levels of IG-6 as No Treatment on Week 4; however, FVF Week 4 group had 6- to 7-fold greater expression of IG-6 than the other groups. Week 4 UPF demonstrated nearly 50% increase in mean IgG markers compared to Week 4 No Treatment Group. Week 1 UPF and FVF had nearly 5 times greater mean IgG than the No Treatment Group. IFN-α/β had no significant differences at either time point between the untreated and treated fucoidans.

preliminary pharmacokinetic studies in healthy volunteers.[13] There were no metabolism pathway mediated interactions observed at the lowest 14 μg/mL concentration of fucoidans, which is still above plasma concentrations observed in the human pharmacokinetic study where the maximum concentration achieved (c_{max}) was 2 μg/mL.[13] Perhaps higher concentrations might be achievable in animal models with smaller total volume or in the human gut for a short term (2-4 hours) during transit through gastrointestinal tract, but there are currently no data available on concentrations achieved in human gut after oral administration.

Conclusion

This comprehensive preclinical evaluation of 2 different types of fucoidans for use in oncology demonstrated overall that both UPF and FVF appear to have limited potential for any drug interactions at clinically achievable concentrations. In the panel of human cancer cell lines evaluated, fucoidans did not have inherent cytotoxicity activity alone but may have some additive or synergistic activity with chemotherapy that needs further evaluation. Confirmatory animal studies are ongoing to evaluate potential with combination of selected chemotherapy agents. Finally, both UPF and FVF did not negatively affect tumor growth in animal studies and some potential benefit was observed in these studies. Similar to other preclinical studies, immune modulation was also observed after short-term exposure to UPF and FVF. In conclusion, after evaluating multiple aspects in this series of preclinical studies, fucoidans overall appear to be safe to proceed to be evaluated in clinical studies to determine benefit of fucoidans alone or in combination with chemotherapy in oncology patients.

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