Cultivated Sea Lettuce is a Multiorgan Protector from Oxidative and Inflammatory Stress by Enhancing the Endogenous Antioxidant Defense System

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Abstract

The health-promoting effects of seaweeds have been linked to antioxidant activity that may counteract cancer-causing oxidative-stress-induced damage and inflammation. Although antioxidant activity is commonly associated with direct radical scavenging activity, an alternative way to increase the antioxidant status of a cell is to enhance the endogenous (phase II) defense system consisting of cytoprotective antioxidant enzymes, including NAD(P)H:quinone oxidoreductase 1 (NQO1). These enzymes are transcriptionally regulated by the antioxidant response element (ARE) via the transcription factor Nrf2. Extracts derived from cultivated Ulva sp., a green alga regarded as a marine vegetable (sea lettuce), potently activated the Nrf2-ARE pathway in IMR-32 neuroblastoma and LNCaP prostate cancer cells. RNA interference studies showed that Nrf2 and phosphoinositide 3-kinase (PI3K) are essential for the phase II response in IMR-32 cells. Activity-enriched fractions induced Nrf2 nuclear translocation and target gene transcription, and boosted the cellular glutathione level and therefore antioxidant status. A single-dose gavage feeding of Ulva-derived fractions increased Nqo1 transcript levels in various organs. Nqo1 induction spiked in different tissues, depending on the specific chemical composition of each administered fraction. We purified and characterized four ARE inducers in this extract, including loliolide, isololiolide, a megastigmen, and a novel chlorinated unsaturated aldehyde. The ARE-active fractions attenuated lipopolysaccharide-induced iNOS and Cox2 gene expression in macrophagic RAW264.7 cells, decreasing nitric oxide (NO) and prostaglandin E2 (PGE2) production, respectively. Nqo1 activity and NO production were abrogated in nrf2−/− mouse embryonic fibroblasts, providing a direct link between the induction of phase II response and anti-inflammatory activity. Cancer Prev Res; 6(9); 989–99. ©2013 AACR.

Introduction

Marine algae (seaweeds) have been used as a food source and medicine for centuries (1). This includes green algae (Chlorophyta), red algae (Rhodophyta), and brown algae (Ochrophyta). Consumption of seaweed, which predominantly occurs in Japan, was found to be inversely related to various cancers, including colon, rectal, and stomach cancer (2, 3). Seaweed is a major part of the Okinawan food culture, and Okinawans have the longest life expectancy in the world and low disability rates (4). Numerous beneficial properties of algal extracts and constituents have been reported, however, usually only in a descriptive manner, without pinpointing specific bioactive components or invoking specific molecular pathways. Green algae of the genus Ulva, also known as sea lettuce, are among the most commonly consumed seaweeds. They reportedly have anti-inflammatory and antitumor properties and are implicated in cancer prevention and detoxification. For example, crude extracts of Ulva reticulata given to rats attenuated acetaminophen-induced hepatotoxicity by improving the hepatic antioxidant status (5). It has been postulated that Ulva extract protects the membrane from damage by toxic reactive metabolites produced by acetaminophen biotransformation (5). Ulva conglabata has neuroprotective and anti-inflammatory activity (6), whereas Ulva lactuca has antitumor and immunostimulating effects (7). The antioxidant activity of Ulva pertusa has been attributed to polysaccharides with high sulfate content (8). We recently described that U. lactuca can increase the cellular antioxidant status through an alternative mechanism, and attributed this activity in part to the presence of monounsaturated fatty acid constituents (9).

Although antioxidant activity is commonly associated with direct radical scavenging activity, an alternative way to increase the antioxidant status of a cell or body is to...
enhance the endogenous defense system consisting of anti-oxidant enzymes and detoxification enzymes, which presumably causes a more sustained, longer-lasting effect. Phase II and other antioxidant enzymes are commonly regulated by the antioxidant response element (ARE) on the transcriptional level (10). Increased expression of these enzymes correlates with a decrease in cellular damage by reactive oxygen species (ROS), which are implicated in inflammation and the pathogenesis of many age-related disorders, including cancer, neurodegeneration, and aging itself (11–13). In humans, the ARE regulates the expression of cytoprotective antioxidant enzymes [e.g., heme oxygenase-1 (HO-1), glutathione-S-transferases (GST), and NAD (P)H:quinone oxidoreductase 1 (NQO1)], which contribute to the endogenous defense against oxidative stress (10). The major transcription factor involved in the induction of phase II enzymes is nuclear factor erythroid-derived 2-related factor 2 (Nrf2), a Cap ‘n’ Collar (CNC) type basic region-leucine zipper (bZIP) transcription factor that, upon activation by ARE inducers, translocates to the nucleus, binds to the ARE sequence as a heterodimer with one of the small bZIP proteins, Mafs, and activates ARE-dependent genes. Nrf2 is negatively regulated by the cysteine-rich protein Keap1. Keap1 serves to sequester Nrf2 in the cytoplasm and interacts with Cul3-based E3 ubiquitin ligase to target Nrf2 for proteosomal degradation (14–16).

Nrf2 knockout mice show diminished detoxification capabilities (17), decreased responsiveness to chemoprotective agents (18), and enhanced susceptibility to oxidative stress-induced cell death (19). Conversely, Nrf2 overexpression protects from oxidative stress (19). NQO1-deficient individuals are at a considerably higher risk of developing leukemia following occupational exposure to benzene (20). The activation of the Nrf2-ARE pathway is a valid cancer-preventive strategy, and sulforaphane, a constituent of broccoli, is an example of a cancer-preventive natural product that acts through this mechanism (12). We hypothesized and preliminarily showed that some seaweeds are able to activate this signaling pathway and that some of the beneficial, particularly antioxidant, properties may be mediated through ARE activation as opposed to only direct scavenging properties (9).

ARE activation may be particularly relevant to prostate cancer (21). The most common hallmark in prostate cancer is the silencing of GST-π (GSTP1) due to DNA methylation, which is nearly universal (22–24). Because of the lack of GSTP1 expression in prostate cancer (regardless of grade or stage; refs. 22–24), induction of GSTs and other phase II enzymes through ARE activation is a promising prostate cancer-preventive strategy (25). Although prostate cancer is the second leading cause of cancer-related death in American men (26), prostate cancer is rarely diagnosed and contributes little to cancer mortality in Asia (27, 28). However, men migrating from Asia to the United States increase their risk, which remains elevated in their male descendants (29–32), likely attributable to lifestyle and dietary changes. Notably, diet in Asia largely includes seaweed, suggesting a possible connection between algae consumption and decreased prostate cancer risk. Many other diseases, including those with an inflammation component, are caused by excessive oxidative stress and may be prevented or interfered with via enhancing the cellular antioxidant status.

This study follows previous research that showed that extracts of marine algae can activate the Nrf2-ARE pathway, and that extracts of Ulva sp. were particularly active among a variety of seaweeds tested (9). We extracted large amounts of Ulva sp. available through cultivation to isolate individual compounds that are ARE inducers, tested enriched fractions in prostate cancer cells and other cellular models for their ability to activate the Nrf2-ARE pathway, and assessed downstream effects on inflammatory markers. We also correlated the cellular activity with in vivo activity in physiologically relevant settings to ultimately assess the chemopreventive potential of Ulva.

Materials and Methods

All chemicals and solvents used for extraction, fractionation, and isolation of compounds were purchased from Fisher Scientific unless specified. For chemicals, reagents, cells, culture media, RNA extraction, reverse transcription quantitative PCR (RT-qPCR), immunoblot analysis, and glutathione assays, see Supplementary Data for details.

ARE luciferase reporter assay in IMR-32 and LNCaP cells

ARE luciferase reporter plasmid (50 ng/well) and CAM- GFP (10 ng/well) plasmid for monitoring transfection efficiency were cotransfected into IMR-32 cells (3 × 10^4 cells/well) and LNCaP cells (2 × 10^4 cells/well) using FUGENE HD (Roche). Transfected cells were seeded in 96-well plates and incubated for 24 hours. Cells were treated with Ulva fractions, compounds, positive controls [tert-butyldihydroquinone (tBHQ) and sulforaphane at 10 μmol/L] and solvent control [1% dimethyl sulfoxide (DMSO)] and allowed to incubate for an additional 24 hours before luciferase activity was measured using BriteLite (PerkinElmer) luminescence detection reagent.

Inhibitor studies

IMR-32 cells were seeded in 6-well dishes (6 × 10^5 cells/well) 1 day before treatment. After pretreatment with the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (25 μmol/L) or the mitogen-activated protein/extracellular signal–regulated kinase 1 (MEK1) inhibitor PD98059 (50 μmol/L) for 30 minutes, cells were treated with fractions for 24 hours, and then proteins were harvested and subjected to immunoblot analysis.

RNA interference experiments

Non-targeting control siRNA and siGENOME SMART pool siRNA reagents targeting Nrf2 (mixture of four siRNAs) were obtained from Dharmacon. IMR-32 cells were seeded in 6-well dishes (3 × 10^5 cells/well) 24 hours before transfection. siRNAs were transfected with silencedFect at the effective dose of 50 nmol/L. Sixty hours after siRNA...
transfection, cells were treated with fractions or vehicle for 24 hours before total protein lysates were collected and subjected to immunoblot analysis.

**In vivo studies**

ARE-human placental alkaline phosphatase (hPAP) transgenic mice were as described previously (see Supplementary Data; ref. 33). Male transgenic mice (~35 g; n = 3) between 12 and 16 weeks of age were gavaged with a single dose (5 mg; ~140 mg/kg) of prioritized fractions NP3, NP4, NP5, and P4. Male control mice (n = 3) from both transgenic and nontransgenic (for hPAP activity) sets were fed with 200 µL of the vehicle (10% DMSO; 10% Cremophor in PBS) and various tissues were harvested 12 hours after feeding. Total RNA for RT-PCR was isolated using TRIzol reagent (Invitrogen) and protein for hPAP enzyme activity using freshly prepared TMNC buffer as described previously (33).

**hPAP enzyme activity assay**

For alkaline phosphatase tissue activity, tissues were homogenized in TMNC [0.05 mol/L Tris, 0.005 mol/L MgCl2, 0.1 mol/L NaCl, 1% (CHAPS)] lysis buffer and refrozen at ~80°C. Endogenous phosphatases were heat-inactivated at 65°C for 30 minutes, and the samples were then incubated at room temperature in the presence of a chemiluminescent CSPD substrate (Tropix) for alkaline phosphatase. Activity was assessed by measuring the resulting luminescent signal representing relative hPAP activity according to the manufacturer’s instruction for Phospho-Light Reporter Gene Assay System (Applied Biosystems).

**Assay for induction of iNOS and Cox2 in macrophage cells**

RAW264.7 cells were seeded in 6-well plates for RNA experiments and treated with different concentrations of Ulva fractions for 1 hour. Cells were treated with 1 µg/mL of lipopolysaccharide (LPS) or 10 ng/mL of IFN-γ and incubated for 12 hours. Total RNA was extracted using RNeasy Mini Kit. PCR analyses were conducted on the aliquots of the cDNA preparations to detect iNOS, Cox2, Nqo1, and β-actin (internal standard) expression.

**RT-qPCR for Nrf2 and NQO1 expression in IMR-32 cells (purified compounds)**

IMR-32 cells (1 × 10⁶ cells/well) were seeded in 6-well plates 1 day before treatment. Cells were treated with variable concentrations of compounds for 12 hours. Total RNA was extracted and real-time PCR was conducted in triplicate using GAPDH expression as an internal control for normalization.

**PGE2 assay**

RAW264.7 cells were seeded in 96-well plates (4 × 10⁴ cells/well) 24 hours before treatment. Fractions or vehicle were applied for 1 hour before adding LPS (1 µg/mL), and incubated for further 24 hours. The supernatant was transferred to fresh collection tubes and used for the assay.

Amersham Prostaglandin E₂ Biotrak Enzyme immunoassay (EIA) system (GE Healthcare) was used for the assay and the manufacturer’s instructions were followed.

**NO assay**

RAW 264.7 cells (2 × 10⁴ cells/well), wild-type (10⁴ cells/well), Nrf2⁻/⁻ (10⁴ cells/well), and Keap1⁻/⁻ (5 × 10⁴ cells/well) mouse embryonic fibroblasts (MEF) were seeded in 96-well plates and pretreated for 1 hour with different concentrations of Ulva fractions or solvent control (1% EtOH), before adding LPS (1 µg/mL), IFN-γ (10 ng/mL), and/or TNF-α (10 ng/mL). Nitric oxide (NO) production in culture supernatant was assessed after 24 (RAW cells) or 20 hours (MEFs) by measuring nitrite concentration, an oxidative product of NO. Nitrite production was measured by mixing 50 µL of culture supernatant with 50 µL of Griess reagent (Promega), and absorbance was measured at 540 nm against a calibration curve generated for fresh sodium nitrite standard.

**Nqo1 activity assay**

Wild-type (8 × 10⁴ cells/well), Nrf2⁻/⁻ (8 × 10³ cells/well), and Keap1⁻/⁻ (4 × 10⁴ cells/well) fibroblasts were plated in 96-well plates, and 24 hours later, the cells were incubated with various concentrations of compound or solvent control for 40 hours. The activity was measured as described previously (34).

**Extraction of cultivated Ulva sp. and bioassay-guided isolation**

For detailed procedures for extraction, isolation, and characterization of compounds, see Supplementary Data. Material from cultured Ulva was freeze-dried (2.23 kg) and extracted with nonpolar (EtOAc, NP), polar organic (EtOH, P), and polar aqueous (1:1 EtOH/H₂O, W) solvents, as shown (Supplementary Scheme S1). Silica gel fractions (NP1–NP6) from the nonpolar EtOAc extract and the C₁₈ fractions from both EtOH (P1–P6) and 1:1 EtOH/H₂O (W1–W6) extracts were investigated for ARE activity to establish previously identified activity profiles. NP3 (424 mg), NP4 (129 mg), and NP5 (354 mg) from the nonpolar extract and P4 (420 mg) from the polar EtOH extract were prioritized on the basis of ARE activity. Each of the four prioritized fractions was chromatographed further using size exclusion chromatography (Sephadex LH20) and several sequences of reversed-phase high-performance liquid chromatography (HPLC) to give the following compounds.

**Leolithide (1).** Colorless amorphous solid; [α]D₂₀ = −52 (c 0.06, CHCl₃); ¹H NMR (400 MHz, CDCl₃) and ¹³C NMR (100 MHz, CDCl₃) spectra, see Supplementary Data; high-resolution electrospray ionization mass spectrometry (HRESIMS) m/z [M+Na]⁺ 219.0990 (caled. for C₁₁H₁₄NaO₃ 219.0997).

**Isololithide (2).** Colorless amorphous solid; [α]D₂₀ = +44 (c 0.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃) and ¹³C NMR (100 MHz, CDCl₃) spectra, see Supplementary Data; HRESIMS m/z [M+Na]⁺ 219.0988 (caled. for C₁₁H₁₄NaO₃ 219.0997).
3,5,6-Trihydroxy-7-megastigmen-9-one (3). Colorless amorphous solid; [α]D20 = –68 (c 0.09, MeOH); 1H NMR (400 MHz) and 2D NMR (600 MHz, CD3OD) data and spectra, see Supplementary Data; HRESIMS m/z [M+Na]+ 507.2936 (calcd. for C26H32NaO8, 507.3286 (calcd. for C26H32ClO8, 503.2936) and [M–H2O+Na]+ 247.1314 (calcd. for C14H20NaO3, 247.1310).

8-Chloro-6,7-dihydroxy-deca-2,4-dienal (4). Colorless oil; [α]D20 = –188 (c 0.0008, MeOH); 1H and 2D NMR (500 MHz, CD3OD) data and spectra, see Table 1 and Supplementary Data; HRESIMS m/z [M+Cl]– 253.0404, 255.0376 (ratio 9:6; calcd. for C10H15Cl2O3, 253.0398; C10H15Cl2O3, 255.0369).

### Results

**Extract from cultivated Ulva sp. is a potent ARE activator in cellular in vitro systems**

We previously sampled a variety of field-collected seaweeds from the Florida coastline and, using an ARE-luciferase reporter gene assay, found that *Ulva lactuca* is a particularly potent activator of the ARE. These *Ulva lactuca* field collections yielded monounsaturated fatty acid type compounds as representative ARE-active components (9). Large-scale cultivation of *Ulva sp.* now enabled us to rigorously evaluate the biological activity of the resulting extracts after carrying out a bioassay-guided fractionation. We used two cell lines for our initial assessment of ARE activity: (i) IMR-32 human neuroblastoma cells, a commonly used cellular model of oxidative stress, which we have previously used to identify ARE activators (9, 35, 36), and (ii) androgen-sensitive (LNCaP) prostate cancer cells, as a common genetic feature of prostate cancer is the silencing of a GST gene (ARE-regulated; refs. 22–24). Prostate cancer may be prevented most suitably by compounds acting through an antioxidant-type mechanism; however, the cancer-preventive agent sulforaphane shows diminished responsiveness in androgen-insensitive cell lines (25).

The freeze-dried sample was successively extracted with EtOAc and EtOH, followed by silica gel or C18 reversed-phase chromatography, respectively (Fig. 1A), to yield several activity-enriched fractions, suggesting the presence of multiple bioactive components. Further chemical profiling and purification indeed confirmed that the ARE activity is attributed to an array of metabolites, prompting us to initially characterize the biological activities of the chromatography fractions rather than individual components and to also capture potential synergistic effects. The EtOAc (nonpolar) extract yielded the most active fractions, designated NP3, NP4, and NP5 (Fig. 1A), in the reporter assay (Fig. 1B). NP3 and NP4 activated the reporter 25- and 11-fold, respectively, at 100 μg/mL and showed toxicity at higher concentrations (100 μg/mL) in the IMR-32 cells. NP5 and the EtOH (polar) extract-derived P4 fraction (Fig. 1A) were highly active at 100 μg/mL, with 23- and 36-fold activity, respectively (Fig. 1B). These activities correlated with the effects observed on the transcription of endogenous NQO1, an ARE-regulated target gene, as determined by quantitative PCR (qPCR) after reverse transcription. The four active fractions induced NQO1 expression up to 8- to 18-fold (Fig. 1C). Transcript levels of NRF2 were at most marginally increased in NP4, NP5, and P4 (1.8- to 2.5-fold) and to a slightly greater extent in fraction NP3 (4.2-fold; Fig. 1D). Overall, these data suggest that Nrf2 transcription factor activation on the protein level rather than gene expression level must have been largely responsible for the potent NQO1 induction. The reporter activation and induction of NQO1 transcription were not cell-type specific, as similar results were obtained in LNCaP cells (Fig. 1E–G), although different fractions were more effective in different reporter cell types.

### Table 1. NMR (500 MHz) data for 8-chloro-6,7-dihydroxy-deca-2,4-dienal (4) in CD3OD

<table>
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<th>J in Hz (in Hz)</th>
<th>δH</th>
<th>COSY</th>
<th>HMBC</th>
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<tr>
<td>1</td>
<td>9.53, d (6.0)</td>
<td>196.4, CH</td>
<td>H-2</td>
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<td>2</td>
<td>6.15, dd (15.0, 8.0)</td>
<td>132.6, CH</td>
<td>H-1, H-3</td>
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<td>3</td>
<td>7.33, dd (15.0, 11.0)</td>
<td>154.4, CH</td>
<td>H-2, H-4</td>
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<tr>
<td>4</td>
<td>6.67, dddd (15.2, 11.0, 1.8)</td>
<td>129.5, CH</td>
<td>H-3, H-5</td>
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<tr>
<td>5</td>
<td>6.47, dd (15.2, 5.2)</td>
<td>147.6, CH</td>
<td>H-4, H-6</td>
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<tr>
<td>6</td>
<td>4.66, dd (5.2, 2.0, 1.8)</td>
<td>71.9, CH</td>
<td>H-5, H-7</td>
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<tr>
<td>7</td>
<td>3.54, dd (8.8, 2.0)</td>
<td>77.6, CH</td>
<td>H-6, H-8</td>
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<tr>
<td>8</td>
<td>4.01, dd (8.8, 2.8)</td>
<td>64.6, CH</td>
<td>H-7, H-9</td>
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<td>9a</td>
<td>2.10, dddd (14.3, 7.2, 2.8)</td>
<td>27.9, CH2</td>
<td>H-8, H-9, H-10</td>
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<td>9b</td>
<td>1.70, dddd (14.3, 7.2, 1.8)</td>
<td>10.6, CH3</td>
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<td>C-8</td>
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<td>7-OH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.11, d (7.7)</td>
<td>7.62, CH</td>
<td>H-7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Observed in DMSO-d6.

<sup>b</sup>Assigned from HSQC experiment.
In both cell lines, the magnitudes of activation rivaled those of the corresponding positive controls tBHQ and sulforaphane (Fig. 1B–G).

Next, we tested whether NQO1 protein levels paralleled the increased transcript levels. We chose the IMR-32 cell line, which gave a more robust induction of NQO1 transcription. Again, all four fractions strongly elevated NQO1 protein levels as measured by immunoblot analysis, and the optimal concentrations were sample-dependent (Fig. 2A). We found that nuclear extracts derived from all treated cells were highly enriched in Nrf2 (Fig. 2B), suggesting that the fractions caused Nrf2 nuclear translocation. To determine whether the increase in NQO1 protein relied on Nrf2, we depleted cells of Nrf2 via RNA interference using previously validated NRF2-specific siRNAs (9, 36) and then treated with fractions at the active concentrations. Expectedly, we found that none of the Ulva fractions was able to promote NQO1 levels in siNRF2-treated IMR-32 cells (Fig. 2C), indicating that Nrf2 is the essential transcription factor mediating NQO1 induction by the Ulva fractions. Furthermore, pretreatment with a PI3K inhibitor prevented NQO1 induction, whereas a MEK1 inhibitor had only marginal effects (Fig. 2D), indicating that the activity of the fractions in

Sea Lettuce Protects from Oxidative Stress by ARE Activation

Figure 1. Extraction and fractionation of cultivated Ulva sp. and assessment of ARE-related transcriptional effects in neuroblastoma and prostate cancer cells. A, cultivated Ulva was extracted successively with EtOAc and EtOH and extracts were subjected to chromatography. Fractions indicated in red showed greatest ARE activity and were used for more detailed studies. B–D, dose-dependent ARE activities (normalized to vehicle control) of prioritized fractions in IMR-32 neuroblastoma cells. tBHQ (10 μmol/L) served as a positive control. B, reporter assay. IMR-32 cells were transfected with ARE-luciferase reporter and treated 24 hours later with nonpolar and polar fractions for another 24 hours. Reporter activation was measured by luminescence (n = 3 ± SD). C and D, Effects on endogenous NQO1 (C) and NRF2 (D) transcript levels as measured by RT-qPCR. IMR-32 cells were treated with fractions for 12 hours, total RNA was isolated and reverse-transcribed to cDNA that was then subjected to TaqMan analysis (n = 3). GAPDH expression was used as an internal control for normalization. E–G, dose-dependent ARE activities (normalized to vehicle control) of prioritized fractions in LNCaP prostate cancer cells as carried out for IMR-32 cells (B–D, respectively). Sulforaphane (SF; 10 μmol/L) served as a positive control.
this cell type relies on a functional PI3K signaling pathway, but only to a much lesser extent on mitogen-activated protein kinase (MAPK) signaling, as observed for field-collected *U. lactuca* (9). The fractions did not seem to activate PI3K signaling, as they did not pronouncedly increase the phosphorylation status of Akt (downstream of PI3K) and GSK-3β (downstream of Akt); the latter is a key regulator of Nrf2 stability and downstream effector of Nrf2 inducers (37). PI3K inhibitor LY29002 effectively abrogated Akt and GSK-3β phosphorylation (Fig. 2D), potentially decreasing Nrf2 stability. In addition, PI3K has also recently been implicated in Nrf2 translation (35), augmenting the PI3K contribution to net Nrf2 levels. We then tested the effects of the fractions at their non-toxic active concentration on levels of the major small-molecule antioxidant, glutathione, in a time-dependent manner. We observed a 10% to 30% drop of glutathione levels after 2 hours (Fig. 2E), possibly due to transient disruption of the cellular redox status. Upon longer incubation times (16 and 24 hours), we measured a strong increase of glutathione levels of up to 220% to 440%, depending on the fraction, consistent with ARE activation as glutathione biosynthetic genes are regulated by the Nrf2-ARE pathway (9).

**Enriched extracts from cultivated *Ulva* sp. activate ARE-driven gene transcription in vivo**

To test whether the *in vitro* ARE activity translates into *in vivo* activity, and thereby whether the mixtures are sufficiently bioavailable and/or have a low enough clearance rate and, by extrapolation, whether *Ulva* sp. consumption can lead to activation of the endogenous ARE-regulated defense system, we treated mice with the bioactive fractions by oral gavage. We used transgenic mice developed by Johnson and colleagues (33) in which the ARE sequence of the rat *Nqo1* enhancer region has been linked to hPAP reporter gene, a model that is suitable to evaluate enzymatic and immunohistochemical responses. These mice were previously used to test the effectiveness of triterpenoids as inducers of the Nrf2-ARE pathway *in vivo* (38). Analogously, we fed prioritized concentrated fractions to male mice (single dose of 140 mg/kg; *n* = 3), harvested tissues 12 hours later and, to assess hPAP activity, focused on two tissues with (i) detoxification responsibilities (liver), and (ii) a potentially high degree of oxidative activity (lung).

were collected, separated by SDS-PAGE, and subjected to Western blot analysis. D, effect of pharmacologic kinase inhibitors on natural products–induced NQO1 protein expression. Cells were pretreated with PI3K inhibitor LY294002 (25 µmol/L) or MEK1 inhibitor PD98059 (50 µmol/L) for 30 minutes and then exposed for 24 hours to active fractions of the indicated *Ulva* fractions (see A). Total protein was collected and subjected to SDS-PAGE followed by Western blot analysis to monitor NQO1 levels and phosphorylation status of Akt and GSK-3β. E, time-dependent effects on glutathione levels. Cells were treated for the indicated times with the active concentration of Ulva fractions (see A), cells were harvested and analyzed for total glutathione (GSH and GSSG), which was measured using the Glutathione Assay Kit (Sigma). Results are represented as mean ± SD (*n* = 3).

Figure 2. Downstream responses and mechanism of ARE-controlled gene expression of active fractions in IMR-32 cells. A, effect on NQO1 protein levels. Cells were treated with various concentrations of NP3, NP4, NP5, and P4 for 24 hours, total protein lysates prepared, resolved by SDS-PAGE, and subjected to Western blot analysis. β-Actin served as loading control. Toxic concentrations. The most active nontoxic concentration from this analysis for each fraction was used for subsequent studies (NP3, 10 µg/mL; NP4, 32 µg/mL; NP5, 32 µg/mL; P4: 100 µg/mL). B, analysis of Nrf2 nuclear translocation and stabilization by Western blot analysis using previously established active fraction concentrations. Nuclear and cytoplasmic extracts were prepared with the NE-PER Reagent Kit (Pierce) 24 hours after treatment, resolved by SDS-PAGE, and the Western blots were probed with Nrf2 antibody, OCT1 and β-tubulin levels served as loading controls for nuclear and cytoplasmic extracts, respectively. C, cells were transfected with siRNAs targeting *Nrf2* or with nontargeting control siRNAs (50 nmol/L). Upon 60-hour incubation, cells were treated with the indicated fractions at active concentrations (see A) for another 24 hours before total protein lysates were collected, separated by SDS-PAGE, and subjected to Western blot analysis. E, time-dependent effects on glutathione levels. Cells were treated for the indicated times with the active concentration of Ulva fractions (see A), cells were harvested and analyzed for total glutathione (GSH and GSSG), which was measured using the Glutathione Assay Kit (Sigma). Results are represented as mean ± SD (*n* = 3).
In both cases, we observed increased activity by the treatments, up to 3.2-fold in liver and up to 3.9-fold in lung (Fig. 3A), suggesting that the active Ulva components are sufficiently bioavailable based on the measured functional response.

These encouraging results from the reporter assay prompted us to analyze in detail the endogenous Nqo1 expression by RT-qPCR relative to controls, as a measure of the true antioxidant enzyme status potential in the tissues. We also extended the analysis to additional tissues. All of the prioritized fractions derived from cultured Ulva showed in vivo activity, but the upregulation of Nqo1 spiked in different tissues, as roughly represented in a heatmap for averages of all mice for active fractions (Fig. 3B). These data suggest that different organs may be targeted with distinctive fractions to achieve protective effects. This is likely due to different chemistry and bioavailability of different components, which remains to be investigated.

Isolation, structure determination, and activity of representative ARE inducers from cultivated Ulva sp.

As pointed out earlier, there seemed to be a wide array of ARE activators in the samples. In an effort to deconvolute the complex mixtures present in NP3, NP4, NP5, and P4, we subjected the fractions to a series of size exclusion chromatography using Sephadex LH20, and subsequently to several sequences of reversed-phase HPLC. We identified that NP3, the most active fraction of the four, comprised several small molecules of interest (Fig. 4A). More specifically, the later eluting fractions 7 and 8 from LH20 chromatography (1:1 DCM/Methanol) were identified as mixtures of small molecules, potentially electrophiles with α,β-unsaturated carbonyl moieties. Additional LH20 chromatography (eluent: 2:5:1 hexanes/DCM/Methanol) and subsequent reversed-phase HPLC provided the known compounds loliolide (1), isololiolide (2), 3,5,6-trihydroxy-7-megastigmen-9-one (3; refs. 39–42), along with a new aldehyde, 8-chloro-6,7-dihydroxy-deca-2,4-dienal (4; Fig. 4A). On the basis of 1H NMR and High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) profiles, we concluded that the remaining LH20 fractions mostly consisted of fatty acid glycerides. The structures of all known compounds (1–3) were determined using NMR and mass spectrometry experiments and by comparison of data to literature (40–42).

The HRMS analysis of 4 showed a distinctive isotopic cluster for [M+Cl]+ at m/z 253.0404/255.0376 consistent with a molecular formula C10H15ClO3 (calcd. 253.0398/255.0369). The 1H NMR spectrum of 4 indicated the...
existence of an aldehyde function δ 9.53 (1H, d, J ≈ 8.0 Hz), in addition to four signals characteristic for conjugated olefinic protons at δ 6.15 (1H, dd, J = 15.0 and 8.0 Hz), δ 7.33 (1H, dd, J = 15.0 and 11.0 Hz), δ 6.67 (1H, ddd, J = 15.2, 11.0 and 1.8 Hz), and δ 6.47 (1H, dd, J = 15.2 and 5.2 Hz). The latter were attributed to H-2, H-3, H-4, and H-5 of a linear system. The correlation spectroscopy (COSY) experiment firmly established a single spin system from H-1 to H-10. In the absence of a direct carbon experiment due to insufficient sample, the carbon shifts were assigned with the aid of edited-heteronuclear single-quantum correlation spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC) experiments (Table 1). The data revealed compound 4 to have an oxylipin structure. In addition to the aldehyde carbonyl (δ_C 196.4) and olefinic carbons (δ_C 132.6, 154.4, 129.5, and 147.6), three additional low-field resonances were assigned at δ_C 71.9 (C-6) and 77.6 (C-7) for two oxymethines, and at δ_C 64.6 (C-8) indicative of bearing a chlorine atom. The connectivity from C-1 to C-10 was further established by HMBC experiment. Two additional resonances at 5.21 (1H, d, J = 6.2 Hz) and 5.11 (1H, d, J = 7.7 Hz) in the 1H NMR spectrum acquired in DMSO-d6 supported the presence of exchangeable protons that were assigned to the hydroxy groups at C-6 and C-7, respectively. Therefore, the planar structure for 4 was determined as 8-chloro-6,7-dihydroxy-deca-2,4-dienal and reported herein as an unprecedented oxylipin analog (Fig. 4A).

Metabolites 1–4 were investigated for ARE activity by evaluating the activation of NQO1 translocation in IMR-32 cells in a dose-dependent manner (Fig. 4B). The oxylipin showed the most pronounced activity among the purified metabolites (6.4-fold at 20 μg/mL) comparable to tBHQ (5.9-fold) under identical conditions. At the same time, all purified compounds had no or only marginal effects on NRF2 transcript levels. However, the purification from the activity-enriched fractions did not increase the potency, suggesting that the combination with other active and/or matrix components may contribute to the overall activity. 1H NMR and HPLC-DAD profiling of the chromatographic fractions from NP4, NP5, and P4 indicated complex mixtures of fatty acid glycerides as the major constituents in addition to the compounds discussed earlier and trace amounts of fatty acids. We previously isolated and characterized representative monounsaturated fatty acids and a corresponding amide derivative from field-collected Ulva lactuca as ARE activators, which seemed to be more dominant in that species (9).

Anti-inflammatory activity of fractions derived from cultivated Ulva sp.

We then decided to measure downstream physiologic effects as a result of phase II and antioxidant enzyme upregulation in a disease-relevant context. Because there is evidence that links the induction of phase II enzymes with protection from inflammatory stress (11, 43), we used the RAW 264.7 macrophage cell line to correlate ARE activation to LPS- and IFN-γ-stimulated iNOS and Cox2 transcription. All fractions were able to abrogate LPS-induced expression of iNOS and Cox2, two proinflammatory target genes, almost completely at higher concentrations (iNOS; Fig. 5A) or partially (Cox2; Fig. 5B). We then measured the products of iNOS and Cox2 enzyme action, that is, NO and prostaglandin E2 (PGE2), respectively. All fractions strongly reduced NO production in a dose-dependent manner (Fig. 5C). Similarly, by using ELISA, we determined that these fractions also partially decreased PGE2 levels (Fig. 5D), paralleling the RT-qPCR results. The knockdown was inversely correlated with Nqo1 transcript levels, which were strongly elevated in RAW264.7 cells in a dose-dependent manner (Fig. 5E), suggesting that ARE activation is a relevant mechanism by which the Ulva fractions exert their anti-inflammatory effect. Furthermore, NP3 seemed to be most potent in both the knockdown of iNOS and Cox2 and the induction of Nqo1 mRNA levels, consistent with the data obtained in IMR-32 and LNCaP cells. To determine whether the anti-inflammatory activity is stimulus-dependent, we found that three of the fractions (NP3, NP4, and NP5) also inhibited IFN-γ–induced NO production, whereas the weakest ARE activator, P4, did not have a substantial effect (Fig. 5F). These data suggest that the anti-inflammatory effect of Ulva fractions is largely stimulus-independent, yet there are some notable differences that may be attributed to the different chemical composition of the fractions. We then used the most potent and chemically most characterized fraction (NP3) to test for Nqo1 activity and NO synthesis inhibition in isogenic MEF cells as previously described (43). Fraction NP3 induced Nqo1 activity only in wild-type MEFs but not in Nrf2 knockout (Nrf2−/−) and Keap1-knockout (Keap1−/−) cells (Fig. 5G). When MEFs were stimulated with IFN-γ and TNF-α, fraction NP3 reduced NO levels only in wild-type MEFs but not Nrf2−/− MEFS up to 10 μg/mL (Fig. 5H). We cannot exclude the possibility of non-Nrf2–mediated anti-inflammatory effects at higher concentration as previously observed for other ARE activators (44), as the apparent reduction of NO in Nrf2−/− MEFs at 32 μg/mL overlapped with the toxicity (Fig. 5H). In summary, the anti-inflammatory effects of the investigated Ulva fraction seem to be mediated by an Nrf2/ARE–dependent mechanism.

Discussion

ARE activation is linked to cancer prevention and is a novel therapeutic approach for numerous oxidative stress-mediated diseases (10–13). In particular, natural products present in terrestrial cruciferous vegetables have been extensively investigated, but were usually found to have low stability and bioavailability. In alternative drug discovery efforts, high-throughput screening campaigns have yielded novel synthetic ARE activators (35). Reata Pharmaceutical’s bardoxolone methyl (RTA 402) is an ARE activator with anti-inflammatory properties, which has reached phase III clinical trials for patients with chronic kidney disease and type II diabetes following promising phase II study results, and is most advanced toward translation to the bedside (45). Our interest in exploring marine natural products for drug discovery and the amount of descriptive literature

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about potential benefits of algae consumption, combined with epidemiologic evidence about low cancer rates in the major geographic areas of seaweed consumption, led us to investigate various marine algae for their ability to activate the ARE. Species of *Ulva* can be easily cultivated and are already consumed by humans. Further investigation is warranted to focus on characterization and development of cultivated *Ulva* as a protective natural remedy against cancer, inflammation, and other oxidative stress-mediated chronic diseases.

Induction of the Keap1/Nrf2-ARE pathway is due to a multitude of extract components, some of which we have structurally characterized here. Loliolide (1) and isololiolide (2) are well represented in several species of algae belonging to the genera *Undaria*, *Padina*, *Cystophora*, *Dictyota*, and *Ulva* (41, 46). The origin of loliolide has been extensively discussed and concluded to be either a photooxidation or degradation product of algal carotenoids such as fucoxanthin (47); in fact, in certain other preparations of *Ulva*, we encountered fucoxanthin, which was recently shown to exert ARE activity (48). Loliolide is a phytotoxic compound displaying inhibition of algae growth, germination, ant-repellent, and immunosuppressive properties (46). The immunosuppressive properties of this monoterpene were attributed to disruption of T lymphocyte function (40). The norisoprenoid megastigmen (3) was previously reported from *U. lactuca* (42), although no biological importance had been linked to this compound. A common feature of ARE inducers extends to their ability to modify sulfhydryl groups, by oxidation, reduction, or alkylation (49). The structural features of compounds 1–4 are consistent with Michael acceptors, which are prominent among the classes of inducers of cytoprotective enzymes (50). Compounds 1–4 are all electrophiles with \( \alpha, \beta \)-unsaturated carbonyl moieties that can act as Michael acceptors, which could...
alkylate Keap1, thereby modifying the Keap1–Nrf2 complex and leading to Nrf2 nuclear translocation and target gene induction. We previously identified 7(E)-9-keto-octadec-7-enoic acid, 7(E)-9-keto-hexadec-7-enoic acid, and 7(E)-9-keto-octadec-7-enamide as ARE-active components in field-collected Ulva lactuca (9), all three of which contain the same reactive functionality to alkylate Cys residues in a similar fashion. These and/or similar unsaturated fatty acid components may also have contributed to the overall activity of the cultivated Ulva sp.

Although we have been able to enrich the extract through bioassay-guided fractionation, further purification of individual components did not lead to activity improvement, suggesting that several agents may have either additive, synergistic, and/or potentiating effects; this remains to be rigorously tested. The importance of matrix components was also shown in our single-dose in vivo study, which indicated differential activation in different tissues. The diversity and dissemination of active and other (possibly by themselves) inactive compounds may affect the net ARE activation, presumably due to modulation of bioavailability, clearance rate, and absorption profile. Expectedly, the mice did not show any adverse effects, and in ongoing future work, we will investigate the effects of chronic administration on various organs. Interestingly, field-collected Ulva lactuca extracts enriched in monounsaturated fatty acids showed preferential cardioprotective properties (9). In the future, one may envision that specific organs or tissues could be targeted with tailored natural product preparations, depending on the disease condition to be prevented or treated. Ulva lactuca and other species of Ulva can be easily cultivated on a large scale, and thus may be a renewable source for effective cancer chemoprevention and to prevent and/or relieve other oxidative stress-mediated disease conditions. Furthermore, secondary metabolite production of this green algae may be manipulated through environmental conditions to ultimately increase the chemopreventive potential of the resulting extracts. The different therapeutic properties reported for different Ulva species may be explained by the differences in chemical composition (5–7), but many activities may also be at least partially attributed to ARE activation.

Disclosure of Potential Conflicts of Interest
R. Ratnayake and V.J. Paul have ownership interest in a patent application. H. Luesch is Chief Scientific Officer at Oceanea Pharmaceuticals, Inc. and has ownership interest in a patent application filed. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Ratnayake, Y. Liu, V.J. Paul
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Ratnayake, Y. Liu, V.J. Paul, H. Luesch
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References


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