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Abbreviations
ARE antioxidant responsive element
GST glutathione S transferases
Keap1 Kelch-like ECH-associated protein 1
NQO1 NAD(P)H quinone oxidoreductase 1
Nrf2 NF-E2-related factor 2
ROS reactive oxygen specie
Tbhq tert-butylhydroquinone
γ-GCS γ-glutamylcysteine synthetase
Abstract

Introduction

Materials and Methods

   Chemicals and cell cultures
   Establishment of a reporter cell line, and the luciferase reporter gene assay
   mRNA extraction and Real time RT-PCR
   Antibodies, immunoblot analysis, ubiquitination assay, and protein half-life measurement
   Transient transfection of siRNA, glutathione concentration, ROS detection, cell viability, and cell death detection

Results

   Identification of oridonin as an Nrf2 activator
   Oridonin activated the ARE-dependent response primarily through upregulation of the Nrf2 protein level
   Oridonin blocked Nrf2 ubiquitination whereas enhanced Keap1 ubiquitination.
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Abstract

BACKGROUND: Ground water contaminated with arsenic imposes a big challenge to human health worldwide. Using natural compounds to subvert the detrimental effects of arsenic represents an attractive strategy. The transcription factor, Nrf2, is a critical regulator of the cellular antioxidant response as well as xenobiotic metabolism. Recently, Activation of the Nrf2 signaling pathway has been reported to confer protection against arsenic-induced toxicity using a cell culture model.

OBJECTIVES: The goal of the present work was to identify a potent Nrf2 activator from plants as a chemopreventive compound and to demonstrate the efficacy of the compound in battling against arsenic-induced toxicity.

RESULTS: Oridonin activated the Nrf2 signaling pathway at a low subtoxic dose and was able to stabilize Nrf2 by blocking Nrf2 ubiquitination and degradation, leading to accumulation of the Nrf2 protein and activation of the Nrf2-dependent cytoprotective response. Pretreatment of UROtsa cells with 1.4 µM oridonin significantly enhanced the cellular redox capacity, reduced formation of reactive oxygen species (ROS), and improved survival of UROtsa cells following arsenic challenge.

CONCLUSIONS: We have identified oridonin as a novel class of Nrf2 activators and illustrated the mechanism by which the Nrf2 pathway is activated. Furthermore, we have demonstrated the feasibility of using natural compounds targeting Nrf2 as a therapeutic approach to protect humans from various environmental insults that we have to face every day.
Introduction

Arsenic is one of the major environmental pollutants. It exists in soil and minerals and readily enters the ground water system, contaminating drinking water. The concentration of arsenic in the ground water varies significantly in different geographic areas. Arsenic concentrations are highest in East Asia, including Bangladesh, West Bengal, India, and China (Kumagai and Sumi 2007; Smith et al. 2000; Tchounwou et al. 2003). Many efforts have been made in an attempt to reduce arsenic damage as exemplified by the guideline for arsenic in drinking water set by the World Health Organization and by local governments. Nevertheless, a large number of populations are still at risk of arsenic exposure and are suffering from arsenic-induced adverse effects, such as hypertension, arteriosclerosis, diabetes, hyperkeratosis, neuropathy, and cancer in the skin, liver, bladder, and lung (Kumagai and Sumi 2007; Smith et al. 2006; Steinmaus et al. 2000; Tseng 2002). Clearly, the best way to protect humans from arsenic-induced damage is to reduce arsenic intake. However, it is not always practical, because many people have no choice but to live off from drinking water and rice that are heavily contaminated with arsenic, as these are their only sources of food and water. Therefore, an alternative choice, of equal importance, is to subvert the detrimental effects of arsenic by modulating the body’s defense system.
Nrf2 is a critical transcription factor that regulates a cytoprotective response. Many of its downstream target genes are important in maintaining the cellular antioxidant response as well as xenobiotic metabolism. For example, γ-glutamylcysteine synthetase (γ-GCS) and the xCT cysteine antiporter are the key enzymes for synthesis of glutathione and maintenance of cellular redox homeostasis (Chan and Kwong 2000; Sasaki et al. 2002; Wild et al. 1999); Conjugating enzymes, such as glutathione S transferases (GSTs) and UDP-glucuronosyl transferase, facilitate the removal of toxic and carcinogenic chemicals by increasing their solubility and excretion (Kobayashi and Yamamoto 2006; Zhang 2006); Many transporters such as multidrug resistance proteins and p-glycoprotein are important in uptake and removal of xenobiotics (Hayashi et al. 2003; Maher et al. 2005; Vernhet et al. 2001; Xu et al. 2005). Activation of the Nrf2 signaling pathway is tightly regulated by Keap1 according to changes in the intracellular redox state when cells are exposed to exogenous stimuli. Under normal conditions, cells maintain low constitutive levels of Nrf2-target genes through constant ubiquitination and degradation of Nrf2, which is accomplished by the Keap1-dependent E3 ubiquitin ligase complex. Upon induction, Nrf2 is stabilized due to impaired Keap1-E3 ubiquitin ligase activity, resulting in activation of the Nrf2 signaling pathway (Cullinan et al. 2004; Furukawa and Xiong 2005; Kobayashi et al. 2004; Sun et al. 2007; Zhang et al. 2004). Chemopreventive compounds are able to activate the Nrf2-dependent adaptive response, thus confer protection against subsequent toxic or carcinogenic damage (Jeong et al. 2006; Yates and Kensler 2007). Intriguingly, in addition to
the beneficial antioxidants and many chemopreventive compounds, the Nrf2 signaling pathway can also be induced by many harmful chemicals such as arsenic, hydrogen peroxide, and even anticancer drugs including cisplatin (Aono et al. 2003; He et al. 2006; Massrieh et al. 2006; Pi et al. 2003; Purdom-Dickinson et al. 2007; Wang et al. 2006). This paradox may be explained by the balance between the induction of the Nrf2 defensive response and the toxic outcome elicited by a particular compound. The most attractive chemopreventive compounds are those that potentially induce the Nrf2-dependent defensive response without eliciting toxic effects, that is, those that tip the balance toward the Nrf2-dependent beneficial response. In accordance with this notion, many chemopreventive compounds, extracted from dietary sources or plants, have been shown to activate the Nrf2-dependent response at low doses and do not elicit detectable toxic effects. Nrf2 activators identified so far can be classified into categories including phenolic antioxidants (caffeic acid, epigallocatechin-3-gallate, butylated hydroxyanisole), dithiolethiones (oltipraz, 3H-1,2-dithiole-3-thione), isothiocyanates (sulforaphane), and triterpenoids (1-(2-cyano-3,12-dioxooleane-1,9[11]-dien-28-oyl)imidazole) (Yates and Kensler 2007; Zhang 2006). Recently, upregulation of the Nrf2-dependent defense response has proved to be beneficial in reducing arsenic-induced toxicity in a cell culture model (Wang et al. 2007). Stable knockdown of endogenous Nrf2 using Nrf2-shRNA, rendered cells more sensitive to arsenic-induced cell death. On the other hand, pretreatment with chemicals that activate Nrf2 enhanced cell resistance to arsenic-induced cell death. This study provides the framework of using natural
compounds to activate the Nrf2-dependent protective pathway to counteract arsenic-induced damage.

Here, we report the identification of a novel class of Nrf2 activators. Oridonin, also known as rubesecensin A, is a diterpenoid purified from the Chinese medicinal herb Rabdosia rubescens (RR). As one of the important traditional Chinese medicines, RR has been used by Chinese doctors to treat swelling of the throat, insect bites, snake bites, inflammation of the tonsils, and cancer of the esophagus, stomach, liver, prostate, and breast (Zhou et al. 2007). The active ingredients of RR are rubesecensin A (oridonin) and rubesecensin B. Currently the major research focus on oridonin is in its antiproliferation and antitumor activities. The anticancer activity of oridonin was thought to rely on its ability to inhibit cell growth, reduce angiogenesis, and enhance apoptosis (Chen et al. 2005; Ikezoe et al. 2003; Liu et al. 2006; Liu et al. 2004; Meade-Tollin et al. 2004; Zhang et al. 2004). Oridonin has been shown to inhibit cell growth and induce apoptotic cell death in many cancer cell lines, including leukemia (NB4, HL-60, HPB-ALL, Kasumi-1), glioblastoma (U118, U138), melanoma (A375-S2), cervical carcinoma (HeLa), ovarian carcinoma (A2780, PTX10), prostate carcinoma (LNCap, Du145, PC3), breast carcinoma (MCF-7, MDA-MB231), murine fibrosarcoma (L929), and non-small cell lung carcinoma (NCI-H520, NCI-H460, NCI-H1299) (Chen et al. 2005; Ikezoe et al. 2003; Liu et al. 2006; Liu et al. 2004; Zhang et al. 2004). The reported doses needed for growth inhibition and apoptosis varied significantly amongst different groups using different cell lines,
ranging from 0.5 µM (0.18 µg/ml) in Kasumi-1 cells to 56 µM (20.4 µg/ml) in HPB-ALL cells (Liu et al. 2006; Zhou et al. 2007). In addition, oridonin was also shown to enhance the efficacy of the cancer drug cisplatin in mouse sarcoma cells (Gao et al. 1993). Mechanistic studies have provided a molecular basis by which oridonin inhibits cell growth and induces apoptosis. Oridonin induced p21 expression resulting in cell cycle arrest in LNCaP and NCI-H520 cells (Ikezoe et al. 2003). Oridonin activated the caspase 3-dependent apoptotic pathway through upregulation of Bax and downregulation of Bcl-2 which promotes release of cytochrome c (Chen et al. 2005; Liu et al. 2006). Inhibition of telomerase activity was reported to be another mechanism that contributes to the anti-cancer function of oridonin (Liu et al. 2004). Since telomerase activity is absent in normal somatic cells, but is upregulated in cancer cells or tumor tissues, this allows oridonin to specifically target abnormal tissue. In addition, the total tyrosine kinase activity was also reduced in response to oridonin treatment (Li et al. 2007). In addition to cancer cell lines, the efficacy of oridonin in vivo was demonstrated in a colorectal carcinoma cell HT29-inoculated mouse model (Zhu et al. 2007). More significantly, a recent report has demonstrated that oridonin displayed a great antitumor activity specifically in acute myeloid leukemia with the t(8;21) translocation between AML1 and ETO genes using both cell culture and mouse models. Mechanistically, oridonin was shown to induce the caspase 3-dependent cleavage of the AML1-ETO fusion protein, leading to an accelerated apoptotic response (Zhou et al. 2007).
Here, we report a novel function of oridonin. It is identified as a novel class of Nrf2 activators. Similar to tert-butylhydroquinone (tBHQ), it inhibits ubiquitination and degradation of Nrf2, resulting in stabilization of Nrf2 and activation of the Nrf2 signaling pathway. Furthermore, the chemopreventive activity of oridonin was demonstrated using a previously established arsenic-UROtsa cell model. Pretreatment of UROtsa cells with 1.4 µM oridonin significantly enhanced the cellular redox capacity, reduced formation of reactive oxygen species (ROS), and improved survival of UROtsa cells following arsenic exposure.

**Materials and Methods**

**Chemicals and cell cultures.** Most chemicals, including sodium arsenite, tBHQ, and Hoechst 33258, were from Sigma Chemical Co. (St. Louis, MO). Rubescensin A (oridonin) was purchased from LKT laboratories, Inc (St. Paul, MN). Human MDA-MB-231 breast carcinoma cells were from ATCC (Manassas, VA) and cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 2 mM HEPES, and 6 ng/mL bovine insulin from Sigma Chemical Co. (St. Louis, MO). UROtsa cells were generously provided by Drs. Mary Ann and Donald Sens (University of North Dakota). UROtsa cells were grown in DMEM medium enriched with 5% FBS. All mammalian cells were incubated at 37 °C in a humidified incubator containing 5% CO₂.

**Establishment of a reporter cell line, and the luciferase reporter gene assay.** The luciferase plasmid, pGL4.22[luc2CP/Puro], was purchased from Promega (Madison, WI). A 39 bp ARE-containing sequence from the promoter
region of human NAD(P)H quinone oxidoreductase (NQO1) gene was inserted into the cloning site of the luciferase plasmid. The ARE-luciferase plasmid was transfected into MDA-MB-231 cells using Lipofectamine Plus from Invitrogen (Grand Island, NY), according to the manufacturer’s instructions. At 48 h post-transfection, cells were grown in medium containing 3 µg/ml puromycin for selection. Stable cell lines were considered established once all the cells in the negative control plate were killed. Stable cell lines were continuously grown in the MEM medium containing 3 µg/ml puromycin. For the reporter gene assay, the ARE-luciferase stable reporter cells were seeded the day before and treated with different doses of test compounds for 24 h. Cells were lysed in extraction buffer [0.1 M potassium phosphate and 1 mM dithiothreitol (DTT)] by freeze and thaw three times and luciferase activities were measured in an assay buffer (25 mM glycyglycine, 15 mM MgSO4, 500 µM ATP, 250 µM luciferin, and 250 µM CoA) using BioTek Synergy 2. The reporter gene assay was carried out in triplicate and mean ± SD was calculated. For the dual luciferase reporter gene assay, MDA-MB-231 cells were transfected with the same ARE-luciferase plasmid along with the renilla luciferase expression plasmid, pGL4.74[hRluc/TK] from Promega, (Madison, WI). At 24 h post-transfection, the transfected cells were treated with compounds for 24 h and both firefly and renilla luciferase activities were measured with the dual luciferase reporter assay system from Promega, (Madison, WI). Firefly luciferase activity was normalized to renilla luciferase activity. The experiment was carried out in triplicate and expressed as mean ± SD.
**mRNA extraction and Real time RT-PCR.** Total mRNA was extracted from cells using TRIZOL reagent (Invitrogen, Grand Island, NY) and equal amounts of RNA was reverse-transcribed to cDNA using Transcriptor First Strand cDNA synthesis Kit (Roche, Indianapolis, IN). The PCR condition, Taqman probes and primers for Nrf2, NQO1, heme oxygenase-1 (HO-1), and GAPDH were reported previously (Wang et al. 2007). Briefly, Taqman probes were from the universal probe library (Roche, Indianapolis, IN): hNrf2 (#70), hNQO1 (#87), hHO-1 (#25), and hGAPDH (#25). Primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA).

- **hNrf2**: forward (acacggtccacagctcac) and reverse (tgtaaatcaaatccatgtcctg);
- **hNQO1**: forward (atgtatgacaaaggacccttc) and reverse (tcccttgcaagagagtacatgg);
- **hHO-1**: forward (aactttcagaagggccaggt) and reverse (ctgggctctccttgttgc);
- **hGAPDH**: forward (ctgacttcaacagcgacacc) and reverse (tgctgtagccaaattcgttgt).

The real-time PCR condition was as follows: one cycle of initial denaturation (95°C for 10 min), 40 cycles of amplification (95°C for 10 sec and 60°C for 20 sec), and a cooling period (50°C for 5 sec). The data presented are relative mRNA levels normalized to GAPDH and the value from the untreated cells was set as 1. Triplicate samples were used to get mean ± SD.

**Antibodies, immunoblot analysis, ubiquitination assay, and protein half-life measurement.** The antibodies for Nrf2, Keap1, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were lysed in a sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% Glycerol, 100 mM DTT, 0.1% bromophenol blue). Following sonication, cell lysates were electrophoresed
through a SDS-polyacrylamide gel and subjected to immunoblot analysis. For
detection of the ubiquitinated Nrf2 in vivo, cells were transfected with expression
vectors for HA-ubiquitin, Keap1, and Gal4-Neh2 (the N-terminal domain of Nrf2
containing the ubiquitin conjugating sites). The transfected cells were either left
untreated or treated with chemicals along with 10 µM MG132 (Sigma Chemical
Co., Saint Louis, MO) for 4 hours. Cells were lysed by boiling in a buffer
containing 2% SDS, 150 mM NaCl, 10 mM Tris-HCl and 1 mM DTT. These
lysates were then diluted five-fold in buffer lacking SDS and incubated with anti-
Nrf2 or anti-Keap1 antibodies. Immunoprecipitated proteins were analyzed by
immunoblot with antibodies directed against the HA epitope (Zhang and Hannink
2003). To detect endogenous Nrf2 ubiquitination, the UROtsa cells were treated
with 10 µM MG 132 and lysed and diluted in the same way. Nrf2 was
immunoprecipitated with an anti-Nrf2 antibody and subjected to immunoblot
analysis with an anti-ubiquitin antibody (Sigma Chemical Co., Saint Louis, MO).
To measure the Nrf2 half-life, cells were either left untreated or treated with
oridonin for 4 h. 50 µM cycloheximide was added to block protein synthesis. Total
cell lysates were collected at different time points and subjected to immunoblot
analysis with an anti-Nrf2 antibody. The relative intensity of bands was quantified
by the ChemiDoc CRS gel documentation system and Quantity One software
from BioRad (Hercules, CA).

Transient transfection of siRNA, glutathione concentration, ROS detection,
cell viability, and cell death detection. The Nrf2-siRNA and the control siRNA
were purchased from Qiagen (Valencia, CA). Transient transfection of siRNA was performed using HiPerFect Transfection Reagent according to the manufacturer’s protocol (Qiagen, Valencia, CA). Intracellular glutathione concentration was measured using the QuantiChrom glutathione assay kit from BioAssay Systems. All the procedures were followed according to the manufacturer’s instructions. For detection of ROS, cells were pretreated with 1.4 µM oridonin for 24 h, followed by As(III) treatment or As(III) plus oridonin cotreatment for another 24 h. ROS levels were measured using dichlorofluorescein (Sigma Chemical Co., 10 µg/ml final concentration) and flow cytometry. Cell viability was measured by MTT [3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (Wang et al. 2007) and colony formation assays. Colony formation assay was performed in 35 mm plates with 200 UROtsa cells. Attached cells were left untreated or treated with oridonin for 24 h, followed by treatment with different doses of As(III) for another 48 h. Following exposure, medium was replaced with fresh medium and cells were incubated for 12-14 days. The cells were then fixed and stained with crystal violet (0.5% in 95% ethanol). Colonies in each plate were counted. For detection of apoptotic cell death, two different methods were used: (i) Annexin V-FITC apoptosis detection (Sigma Chemical Co.), (ii) Hoechst staining (1 µg/ml) for detection of the condensed nuclei. All experiments were conducted in triplicate and expressed as mean ± SD. The statistical significance was determined by the student t-test and labeled in the figure with asterisks (*p<0.05, **p<0.01).
Results

Identification of oridonin as an Nrf2 activator. Using the stable ARE luciferase reporter cell line, derived from the MDA-MB-231 cells, combined with a 96 well high-throughput screening system established in our laboratory, we identified a novel Nrf2 activator that belongs to the class of diterpenoids (Figure 1A). The MDA-MB-231 cell line was used to show Nrf2 activation for a couple reasons: (1) MDA-MB-231 cells can be easily transfected. (2) The Nrf2 pathway is most sensitive in this cell line in response to Nrf2-inducers. Oridonin induced transcription of the ARE-dependent luciferase gene in a dose dependent manner in the stable cell line (Figure 1B). To confirm oridonin activation of Nrf2 using the high-throughput screening method, a dual luciferase reporter gene assay was also performed in which a renilla luciferase gene is included as an internal control for transfection efficiency and for toxicity induced during oridonin exposure. Consistent with the data obtained from the high-throughput screening, oridonin induced the ARE-dependent luciferase activity in a dose dependent manner (Figure 1C). Slight induction (1.5 fold) was observed at as low as 1.4 µM and reached maximum induction (11.3 fold) at 14 µM. There was no obvious toxicity at 14 µM, as judged by cell morphology and renilla luciferase activity.

Oridonin activated the ARE-dependent response primarily through upregulation of the Nrf2 protein level. Previous studies have demonstrated that the ARE-dependent reporter gene activity correlated very well with the
protein level of Nrf2. Therefore, the same cell lysates from the dual luciferase reporter gene assay were used for immunoblot analysis for detection of Nrf2, Keap1 and β-actin. While the Keap1 levels remained constant, oridonin enhanced the levels of Nrf2 protein in a dose dependent manner with the highest induction at 14 µM (Figure 2A). During the reporter gene assay, it should be noted that any doses higher than 14 µM caused marked toxicity as indicated by an increased number of rounded and floating cells. A large body of literature indicates that oridonin's antitumor activity relies on its ability to inhibit cell growth and to induce cell death. Since Nrf2 regulates a cellular survival response, it is envisioned that treatment with high doses of oridonin may inhibit Nrf2, allowing cells to undergo cell death. Therefore, Nrf2 protein levels in response to high doses of oridonin were tested. Following treatment of MDA-MB-231 cells with different doses of oridonin for 24 h, all cells including floating cells were collected. Equal amounts of proteins were subjected to immunoblot analysis with Nrf2, Keap1 and β-actin antibodies. Interestingly, at doses higher than 28 µM, Nrf2 protein levels decreased in a dose-dependent manner, while the expression of Keap1 or β-actin had no significant change (Figure 2B, lanes 7-9). Previously, it has been demonstrated that Nrf2 activators, including tBHQ, induce the Nrf2 signaling pathway primarily through stabilization of the Nrf2 protein, rather than upregulation of its mRNA. Next, mRNA expression of Nrf2 and its target genes, NQO1 and HO-1, in response to oridonin was measured using real time RT-PCR. Nrf2 mRNA increased slightly in a dose dependent manner in response to oridonin, whereas tBHQ had no effect (Figure 2C, upper panel). As expected,
mRNA of \textit{NQO1} or \textit{HO-1} was induced significantly by oridonin in a dose dependent manner (Figure 2C, middle and lower panels). These results demonstrate that oridonin is able to induce the Nrf2 signaling pathway mainly through upregulation of Nrf2 at the protein level.

\textbf{Oridonin blocked Nrf2 ubiquitination whereas enhanced Keap1 ubiquitination.} tBHQ enhances the Nrf2 protein level by interfering with the Keap1-dependent ubiquitin conjugation process. Therefore, the ability of oridonin in modulating Nrf2 ubiquitination was tested. For this assay, Gal4-Neh2, a model fusion protein previously used for the Nrf2 ubiquitination test, was used (Zhang and Hannink 2003). Similar to tBHQ, oridonin suppressed Nrf2 ubiquitination (Figure 3A, left Gal4-Neh2 panel). Furthermore, it was previously shown that tBHQ caused a shift of ubiquitination from the substrate Nrf2 to the substrate adaptor Keap1 (Zhang et al. 2005). Similar to tBHQ, oridonin treatment was also effective in enhancing ubiquitination of Keap1 (Figure 3A, left Keap1 panel). These results demonstrate that oridonin is able to induce a shift of ubiquitination from Nrf2 to Keap1. One of the major roles for ubiquitin conjugation onto a protein is to target the protein for 26S proteasome-mediated degradation. Next, the half-life of Nrf2 in the absence or presence of oridonin was measured. Half-life of the endogenous Nrf2 protein in MDA-MB-231 cells was 19 min, while treatment with oridonin increased half-life to 51 min (Figure 3B, left panel). Taken together, these results indicate that oridonin activates the Nrf2 pathway by inhibiting ubiquitination and degradation of Nrf2, leading to an increase in Nrf2 protein level and activation of the Nrf2-dependent response.
Efficacy of oridonin in protecting against As(III)-induced toxicity. To test the feasibility of using oridonin as a chemopreventive compound to elicit the Nrf2-mediated protective response to defend against environmental insults, the UROtsa cell line, an established model system for arsenic toxicity, was used. First, activation of the Nrf2 pathway by oridonin was determined in this cell line. Ubiquitination of endogenous Nrf2 in UROtsa cells was blocked by oridonin or tBHQ treatment (Figure 3A, right panel). Consistent with a decrease in ubiquitination of Nrf2 in response to oridonin, the half life of Nrf2 was increased from 10 min in the untreated condition to 16 min in response to oridonin treatment (Figure 3B, right panel). Next, the oridonin dose range that induces the Nrf2 protein was determined in UROtsa cells. Compared to MDA-MB-231 cells, UROtsa cells had a narrow range of Nrf2 induction, from 1.4 µM to 14 µM (Figure 4A, lanes 3-6, compare with Figure 2B, lanes 2-7). At doses higher than 14 µM, toxicity was observed and induction of Nrf2 was decreased (Figure 4A, lanes 7-9). At dose of 56 or 112 µg/ml, there was a decrease even in the level of β-actin due to cytotoxicity (Figure 4A, lane 8 and lane 9). Nevertheless, reduction of the Nrf2 protein was significantly more substantial, indicating that reduction of Nrf2 at high doses may not solely be due to reduced cell number. Based on this result, a low dose (0.5 µg/ml) was chosen for the protection assays. One of the major functions of Nrf2 is to regulate an antioxidant response by upregulating intracellular antioxidants and genes such as GCS and the xCT cysteine antiporter that encode key enzymes in the synthesis of glutathione. To confirm activation of the Nrf2-dependent response by 1.4 µM oridonin, the intracellular
glutathione level in the oridonin treated cells was compared to that in the untreated cells. Oridonin treatment resulted in a significant increase in the glutathione level (Figure 4B). Thus, oridonin is able to augment the cellular redox capacity, which is the key mechanism in suppressing oxidative stress-induced damage by environmental insults. In the protection assays, sodium arsenite [As(III)] was used to treat UROtsa cells. The ability of oridonin in alleviating As(III)-induced reactive oxygen species (ROS) was measured (Figure 4C). 30 µM As(III) treatment for 24 h increased the level of ROS significantly while 5.6 µM oridonin itself had no effect. Pretreatment of cells with several doses of oridonin for 24 h and further cotreatment with As(III) for an additional 24 h resulted in a significant reduction of the ROS levels, especially with 5.6 µM oridonin. These data clearly demonstrate the efficacy of oridonin in suppressing oxidative stress imposed by As(III) exposure. Finally, the effectiveness of oridonin in protecting cells from acute cell death in response to As(III) was assessed. UROtsa cells were left untreated or pretreated with 1.4 µM oridonin. Following a 24 h pretreatment period, several doses of As(III) were added to both groups and incubated for an additional 48 h before measuring total cell death using both MTT and colony formation assays. Pretreatment followed by cotreatment with oridonin significantly improved cell survival as judged by the MTT assay (Figure 4D, top panel) and the colony formation assay (Figure 4D, bottom panel). To confirm that the protection against As(III)-induced cell death is attributed to the activation of Nrf2 by oridonin, the MTT assay was performed in UROtsa cells that were treated with Nrf2-siRNA for 48 h. The immunoblot
analysis confirmed the effectiveness of Nrf2-siRNA in reducing Nrf2 expression (Figure D, middle panel). Convincingly, inhibition of Nrf2 expression in UROtsa cells reverted the MTT curve, i.e., oridonin lost its protection against As(III) toxicity, rather, it aggravated the As(III)-induced cell death (Figure D, middle panel). This result demonstrates that the oridonin-mediated protection requires activation of the Nrf2 pathway. Next, apoptotic cell death was quantified using Annexin V-FITC/flow cytometry. 30 µM As(III) treatment for 48 h increased the percentage of apoptotic cells whereas pretreatment followed by cotreatment with 1.4 µM oridonin reduced apoptotic cell death to a level comparable to the untreated cells (Figure 4E, upper panels). 1.4 µM oridonin itself did not increase apoptotic cell death (data not shown). Next, Hoechst staining was used for detection of condensed chromosomes in the apoptotic cells. 30 µM As(III) increased the number of positive-stained cells, while pretreatment followed by cotreatment with oridonin markedly reduced the number of apoptotic cells (Figure 4E, lower panel). Together, these results demonstrate that a low dose of oridonin is able to protect cells from As(III)-induced damage as illustrated by reduced ROS and increased survival in response to As(III).

Discussion

The pivotal role of Nrf2 in chemoprevention has clearly been shown in Nrf2 null mice. These mice express lower basal levels of the Nrf2 target genes, such as NQO1, GST, GCS, UDP-glucuronosyltransferase, glutathione peroxidase-2, and HO-1 (Chan and Kwong 2000; Cho et al. 2002; Hayes et al. 2000; Kwak et al.
As a consequence, these mice are more susceptible to toxic and carcinogenic challenges such as butylated hydroxytoluene, benzo[a]pyrene, diesel exhaust, aflatoxin B₁, N-nitrosobutyl (4-hydroxybutyl) amino, pentachlorophenol, acetaminophen, ovalbumin, cigarette smoke, and 4-vinyl cyclohexene diepoxide (Aoki et al. 2001; Chan et al. 2001; Chan and Kan 1999; Enomoto et al. 2001; Hu et al. 2006; Iida et al. 2004; Iizuka et al. 2005; Ramos-Gomez et al. 2001; Rangasamy et al. 2005; Umemura et al. 2006). These results provide the basis for chemopreventive intervention targeting the Nrf2 signaling pathway. Many previously identified naturally occurring compounds, including sulforaphane, epigallocatechin-3-gallate, caffeic acid phenethyl ester, and curcumin have proved to be Nrf2 activators, which further implies the importance of Nrf2 in chemoprevention (Jeong et al. 2006; Nishinaka et al. 2007; Zhang 2006). Identification, validation, and optimization of new Nrf2 activators are essential for the development of effective dietary supplements or therapeutic drugs that can be used to boost the Nrf2-dependent adaptive system to protect humans from various environmental insults.

Oridonin represents a novel class of Nrf2 activators that has not previously been demonstrated. Mechanistic studies presented here indicate that oridonin induced the Nrf2-dependent response primarily by enhancing the Nrf2 protein level. The increase in the Nrf2 protein level in response to oridonin is attributed mainly to the stabilization of Nrf2 with minor contribution from increased mRNA. Similar to tBHQ, oridonin is able to block ubiquitination and degradation of Nrf2, resulting in
the prolonged half life of Nrf2. Furthermore, we have demonstrated the effectiveness of a low dose of oridonin (1.4 µM) in eliciting the Nrf2-dependent cytoprotective response in an As(III)-toxicity model. Low doses of oridonin are able to enhance the cellular reducing capacity by significantly elevating the reduced glutathione level, thus inhibiting the formation of ROS, resulting in increased survival in response to As(III) exposure. Furthermore, glutathione is able to conjugate arsenic to facilitate arsenic excretion, therefore reducing As(III) toxicity (Shinkai et al. 2006). In addition to GCS that modulates intracellular glutathione levels, other Nrf2 downstream genes, including GST, UDP-glucuronosyl transferase and multidrug resistance proteins, also contribute to the Nrf2-mediated protection against arsenic toxicity (Hayashi et al. 2003; Kobayashi and Yamamoto 2006; Maher et al. 2005; Vernhet et al. 2001; Xu et al. 2005; Zhang 2006). It is worth it to mention, that although the current study only shows the protection of oridonin against acute As(III)-toxicity, it certainly can be applied to other toxic and carcinogenic chemicals since oridonin induces the well characterized Nrf2-dependent defensive response.

This cell-based study provides the evidence that oridonin can be used at a low dose as a chemopreventive compound that specifically targets Nrf2. Further studies on the chemopreventive activity of oridonin in animal models are needed. If oridonin is shown to have great chemopreventive potential, then it has a great economic advantage since it can easily be extracted from Rabdosia rubescens (RR), “the Chinese grass”. In addition, identification of diterpenoids as a new
class of Nrf2 activators will broaden the choice for new chemopreventive compounds. Moreover, the diterpenoid structure can serve as a scaffold for the development of chemopreventive drugs. Identification of naturally occurring diterpenoids or synthetic optimization of the diterpenoid, oridonin, that potently and specifically induce the Nrf2 signaling pathway will greatly improve the efficacy of chemopreventive drugs and decrease side effects, which will have a profound impact on human health.

High doses of oridonin have been shown to have anticancer activity by causing cell cycle arrest, inhibiting proliferation, and inducing the apoptotic cell death. The dose range needed for oridonin to exhibit anticancer activities in these studies conducted by different laboratories with a variety of cancer cell lines, is very broad with 100 fold differences. Although this may be partially due to the purity of oridonin used among groups, it largely indicates a difference in sensitivity of cancer cells to the oridonin-induced apoptotic response. In this study, the effect of oridonin in inducing the Nrf2 protein level was assessed in two different cell lines, the breast carcinoma MDA-MB-231 and an immortalized but nontransformed bladder urothelium UROtsa. UROtsa cells showed a narrower window of Nrf2 induction in response to different doses of oridonin. It is very interesting to note that oridonin induced the Nrf2 protein and the reporter gene activity in a dose dependent manner to a certain point, then, the Nrf2 protein level and the reporter gene activity dropped suddenly. The dose where an initial decrease was observed in the Nrf2 protein in MDA-MB-231 cells or in UROtsa
cells is 56 µM or 28 µM respectively. This decrease in Nrf2 protein level in response to high doses of oridonin is not solely due to cell toxicity because Keap1 or β-actin levels only decreased slightly. Based on the important role of Nrf2 in cell survival, It is conceivable that Nrf2 has to be repressed prior to the execution of cell death. In support of this notion, Nrf2 has been reported as a substrate of caspase 3 (Ohtsubo et al. 1999). The cleavage sites in Nrf2 for caspase 3 have been identified. Based on the Nrf2 induction profile, it is remarkable that oridonin functions as a chemopreventive compound at low doses by activating the Nrf2 cytoprotective pathway, while at high doses, it activates apoptotic cell death and concomitantly inhibits the Nrf2-dependent survival pathway. Further studies are needed in understanding molecular events that cause the switch between life and death.

References


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Figure legends

Figure 1. (A) Structure of the diterpenoid oridonin. (B) Identification of oridonin as an Nrf2 activator using a high-throughput screening system. The stable MDA-MB-231 cells expressing ARE-luciferase were seeded in 96 well plates. Cells were grown to 90% confluence and treated with the indicated doses of oridonin for 24 h before analysis of the luciferase activity. (C) MDA-MB-231 cells were cotransfected with a plasmid containing an ARE-luciferase reporter gene and a plasmid encoding renilla luciferase driven by the herpes simplex virus thymidine kinase promoter. The transfected cells were treated with the indicated doses of oridonin for 24 h prior to measurement of firefly and renilla luciferase activities in cell lysates. All luciferase reporter gene assays were run in triplicate and expressed as mean ± SD.

Figure 2. (A) An aliquot of cell lysates from the dual luciferase reporter gene assay was used for immunoblot analysis for detection of Nrf2, Keap1, and β-actin. (B) MDA-MB-231 cells were treated with the indicated doses of oridonin for 24 h. Total cell lysates were subjected to immunoblot analysis with anti-Nrf2, anti-Keap1, and anti-β-actin antibodies. (C) mRNA from similarly treated cells was extracted and reverse transcribed into cDNA prior to real-time PCR analysis for detection of Nrf2, NQO1, and HO-1.

Figure 3. (A) MDA-MB-231 cells were cotransfected with expression vectors for HA-ubiquitin, a Gal4-Neh2 fusion protein, and Keap1. The transfected cells were
left untreated or treated with 8.4 μM oridonin or 100 μM tBHQ for 4 h, along with 10 μM MG132. Cells were lysed in 2% SDS and immediately heated. Anti-Gal4 or anti-Keap1 immunoprecipitates were analyzed by immunoblot with anti-HA antibodies for detection of the ubiquitin conjugated Neh2 or Keap1 (right panel). Ubiquitination of endogenous Nrf2 was assessed in UROtsa cells treated with DMSO, 8.4 μM oridonin or 100 μM tBHQ for 4 h, along with 10 μM MG132. Nrf2 was immunoprecipitated with an anti-Nrf2 antibody and ubiquitinated Nrf2 was detected with an anti-ubiquitin antibody (left panel). (B) MDA-MB-231 cells (left panel) and UROtsa cells (right panel) were either left untreated or treated with 8.4 μM oridonin for 4h. Cycloheximide (50 μM) was added to block protein synthesis. Cells were lysed at the indicated time points and cell lysates were subjected for immunoblot analysis with anti-Nrf2 and anti-β-actin antibodies. The intensity of the bands was quantified using Quantity One software.

Figure 4. (A) UROtsa cells were treated with the indicated doses of oridonin for 24 h. Cell lysates were collected and subjected to immunoblot analysis with anti-Nrf2, anti-Keap1, and anti-β-actin antibodies. (B) Intracellular glutathione concentrations in UROtsa cells untreated or treated with 1.4 μM oridonin were measured using the QuantiChrom glutathione assay kit. The experiment was conducted in triplicate and expressed as mean ± SD. ** p<0.01. (C) UROtsa cells were left untreated or pretreated with several doses of oridonin for 24 h. Then the non-pretreated cells or the pretreated cells were further treated with As(III) or As(III) plus oridonin respectively for another 24 h, followed by measurement of
ROS by dichlorofluorescein/flow cytometry. The experiment was run in triplicate and mean ± SD was calculated. ** p<0.01. (D) UROtsa cells were left untreated or pretreated with 1.4 µM oridonin for 24 h. Cells were then treated with the indicated doses of As(III) in the absence (solid curve) or presence of 1.4 µM oridonin (dashed curve) for another 48 h. Cell survival was measured by the MTT assay (top panel). UROtsa cells were transfected with control siRNA or Nrf2-siRNA for 48 h. Nrf2 protein levels were assessed by immunoblot analysis with an anti-Nrf2 antibody to confirm knockdown of Nrf2 expression (small panel). The Nrf2-siRNA transfected cells at 48 h post-transfection were used for the MTT assay as described (middle panel). Two-hundred cells in 35 mm plates were pretreated and cotreated in the same way as the MTT assay. Cell survival was measured by the colony formation assay (bottom panel). All the experiments were run in triplicate and mean ± SD was calculated. * p<0.05. (E) UROtsa cells were pretreated and cotreated as described in the MTT assay. Apoptotic cell death was detected using Annexin V-FITC and flow cytometry method. The experiment was run in triplicate and mean ± SD was calculated. ** p<0.01. (upper panel). UROtsa cells growing on cover slides were pretreated and cotreated in the same way. Apoptotic cells were visualized by condensed nuclei using Hoechst staining. Scale bars represent 25µm. The experiment was repeated and similar results were obtained.