

# Bryonolic Acid Transcriptional Control of Anti-inflammatory and Antioxidant Genes in Macrophages in Vitro and in Vivo

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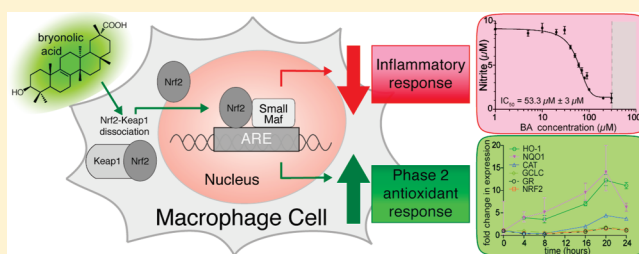
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**S** Supporting Information

**ABSTRACT:** Bryonolic acid (BA) (**1**) is a naturally occurring triterpenoid with pleiotropic properties. This study characterizes the mechanisms mediating the anti-inflammatory and antioxidant activities of BA and validates the utility of BA as a tool to explore the relationships between triterpenoid structure and activity. BA reduces the inflammatory mediator NO by suppressing the expression of the inflammatory enzyme inducible nitric oxide synthase (iNOS) in LPS-activated RAW 264.7 macrophage cells. In addition, BA robustly induces the antioxidant protein heme oxygenase-1 (HO-1) in vitro and in vivo in an Nrf2-dependent manner. Further analyses of Nrf2 target genes reveal selectivity for the timing and level of gene induction by BA in treated macrophages with distinct patterns for Nrf2-regulated antioxidant genes. Additionally, the distinct expression profile of BA on Nrf2 target genes relative to oleanolic acid suggests the importance of the triterpenoid scaffold in dictating the pleiotropic effects exerted by these molecules.



Triterpenoids are one of the most functionally and structurally diverse classes of secondary metabolites ubiquitous in the plant kingdom. Triterpenoids are cyclized from oxidosqualene to form approximately 200 chemically diverse triterpene skeletons. More than 20 000 triterpenoids have been documented, with new structures continually being identified and studied for their biological activity. In addition to the impressive skeletal diversity of these molecules, they also possess a variety of biological activities including anti-inflammatory, hepatoprotective, analgesic, antimicrobial, anti-mycotic, virostatic, immunomodulatory, and tonic effects.<sup>1</sup> One of the best-studied triterpenoids, oleanolic acid (OA) (**2**), served as a platform for the discovery of the semisynthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO).<sup>2,3</sup> CDDO and its methyl ester derivative, CDDO-Me, are potent anti-inflammatory<sup>3,4</sup> and chemopreventive agents<sup>5–8</sup> that have advanced to phase III clinical trials for renal sparing effects in diabetic nephropathy.<sup>9,10</sup>

We became interested in bryonolic acid (BA) (**1**) in part because of its unique chemical attributes within the triterpenoid family (namely, the unsaturated B–C ring fusion) and partly due to its interesting pleiotropic profile of biological activity. The activities reported for BA (**1**) include antiallergic properties, inhibition of homologous passive cutaneous anaphylaxis in rats, delayed hypersensitivity in mice,<sup>11,12</sup> antitumor activity,<sup>13</sup> and cytotoxicity toward various tumor cell lines.<sup>14,15</sup> Although reports have shown BA (**1**) to be a promising natural anti-inflammatory agent, the mechanism of

action mediating these effects has yet to be identified. We hypothesized that the previously observed BA (**1**) phenotypes could be explained by the activation of the transcription factor Nrf2.

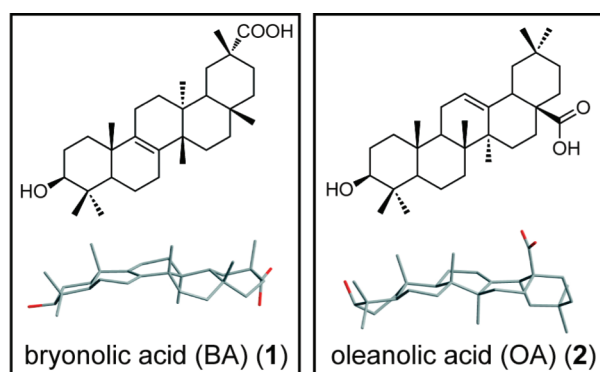
The NF-E2-related factor 2 (Nrf2) was first isolated as a DNA-binding protein to tandem repeats in the  $\beta$ -globin locus region and had been targeted for prevention of chemical carcinogenesis even before its complete characterization.<sup>16</sup> Nrf2 acts as an electrophilic and oxidative damage sensor and induces a battery of cytoprotective genes that detoxify reactive electrophiles and oxidants. Among the hundreds of genes regulated by Nrf2, the most studied include heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase, quinone 1 (NQO1), catalase (CAT), glutamate-cysteine ligase catalytic subunit (GCLC), and glutathione reductase (GR). Several classes of endogenous and exogenous ligands induce Nrf2, with triterpenoids being one of the most promising and clinically relevant examples. Upon induction, Nrf2 dissociates from Keap1, the principal cytoplasmic inhibitor of Nrf2 function.<sup>17</sup> Nrf2 subsequently escapes ubiquitination and proteosomal degradation, translocates to the nucleus, and effectively upregulates the expression of cytoprotective and antioxidant genes.<sup>18</sup>

Here we provide the first demonstration of the molecular mechanisms contributing to the anti-inflammatory/antiallergic

Received: October 10, 2011

Published: February 16, 2012

effects of BA (1). Through in vitro analysis of BA (1) activity in mouse macrophages and in vivo studies following systemic administration of BA (1) in mice, we show potent suppression of iNOS expression and a robust induction of HO-1 by BA (1) in an Nrf2-dependent manner. BA (1) induces other antioxidant and cytoprotective genes and triggers a unique expression profile for Nrf2 target genes when compared to a structurally similar triterpenoid, OA (2). The observed differential regulation by BA (1) of genes in this pathway is characterized by a rapid and more potent induction of HO-1 and NQO1, while other Nrf2 target genes such as CAT, GCLC, and GR respond with a more gradual and modest increase in expression. This newly discovered ability of BA to regulate expression of inflammatory and antioxidant enzymes validates the utility of BA (1) as a platform to explore the importance of the triterpenoid scaffold in defining the anti-inflammatory and chemopreventive properties of triterpenoids. In addition, BA (1) may potentially uncover unique mechanism(s) in regulating the inflammatory and antioxidant pathway in comparison to the oleanane triterpenoids due to its different regulation of antioxidant genes. More specifically, these studies set the stage for an effort combining the application of synthetic chemistry and chemical biology screens that has the potential to yield diverse triterpenoid structures with selective therapeutic properties.



## RESULTS AND DISCUSSION

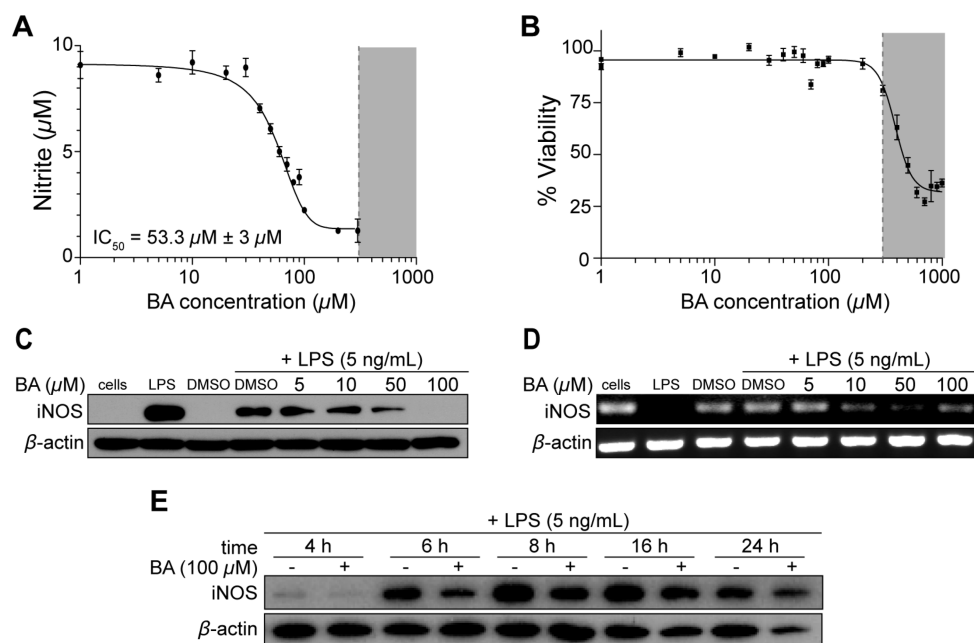
**Bryonolic Acid Decreases NO Levels and iNOS Expression in LPS-Activated RAW 264.7 Cells.** Initial studies of BA (1) have shown in vivo anti-inflammatory properties in rats and mice by inhibiting the allergic response.<sup>11,12,19</sup> However, the mechanisms mediating these effects have not been explored. In order to elucidate the anti-inflammatory effects of BA (1), we used an established in vitro model of LPS-activated RAW 264.7 leukemic mouse macrophage cells (RAW). Upon LPS activation of RAW cells, NO is produced, which spontaneously oxidizes to nitrite. In an initial experiment, LPS-activated RAW cells were treated with BA (1) and nitrite levels were measured from cell culture supernatants. Treatment with BA (1) reduced nitrite levels, demonstrating an  $IC_{50}$  value of  $53.3 \pm 3 \mu\text{M}$  after a 24 h treatment (Figure 1A). RAW cells remained viable in BA (1) concentrations as high as  $300 \mu\text{M}$  (gray shaded area), but cytotoxicity was apparent at higher concentrations, as measured by the MTT assay (Figure 1B). This decrease in viability may be attributed to higher DMSO exposure at this dose range (1.5% at a concentration of  $300 \mu\text{M}$  BA (1) from the maximum soluble stock of 20 mM). Due to the solubility of BA (1) and possible toxicity from

DMSO, the maximum concentration included in the calculated  $IC_{50}$  value and the subsequent cell culture experiments was  $100 \mu\text{M}$  at 0.5% DMSO.

To determine the mechanism through which BA (1) suppresses the production of NO following LPS exposure, we examined the expression profile of iNOS through immunoblot analysis in LPS-activated RAW cells. Treatment with  $50 \mu\text{M}$  BA (1) significantly reduced iNOS protein levels after a 24 h treatment compared to the DMSO control (Figure 1C). At  $100 \mu\text{M}$  BA (1), iNOS protein levels are no longer detectable. The iNOS mRNA levels in LPS-activated RAW cells were also reduced in the presence of 50 and  $100 \mu\text{M}$  BA (1) after a 24 h treatment (Figure 1D). Thus, the decrease of NO production is mediated by the suppression of iNOS expression, as shown by the decrease in both protein and mRNA levels in the presence of BA (1). To further assess this effect, RAW cells were treated at various time points with  $100 \mu\text{M}$  BA (1). LPS induced iNOS expression at 4 h, and BA (1) suppressed iNOS levels at this early time point (Figure 1E).

**Bryonolic Acid Induces Antioxidant Heme Oxygenase-1 Expression in RAW 264.7 Cells.** Previous data have shown robust induction of HO-1 by other triterpenoids.<sup>20,21</sup> Therefore, we next determined whether BA (1) might also induce HO-1. We observed a dose-dependent induction of HO-1 by BA (1), with HO-1 protein and mRNA levels induced at  $50 \mu\text{M}$  and  $100 \mu\text{M}$  after 24 h (Figure 2A and B) in LPS-activated RAW cells. Since LPS is known to induce HO-1, we next determined whether LPS is a requirement for HO-1 induction by BA (1). RAW cells were treated with BA (1) in the absence of LPS and probed for HO-1. As seen in Figure 2C, HO-1 was induced in the presence of 50 and  $100 \mu\text{M}$  BA (1) in the absence of LPS. HO-1 mRNA levels were induced in the presence of  $10 \mu\text{M}$  BA (1) with increasing expression at  $100 \mu\text{M}$  BA (1) (Figure 2D). To investigate the time course of HO-1 induction, RAW cells treated with  $100 \mu\text{M}$  BA (1) were harvested at different time points and probed for HO-1. BA (1) induced HO-1 protein levels as early as 6 h, and expression peaked approximately 24 h after treatment. The induction extends beyond 48 h and is diminished by 72 h (Figure 2E). This profile for HO-1 induction illustrates a long-term induction by BA (1) amounting to a total of approximately 66 h. HO-1 mRNA levels were also induced at 4 h with a 4.1-fold change compared to control at time = 0 (Figure 3). HO-1 mRNA levels continued to increase up to 20 h, at which time the peak induction demonstrated a 11.3-fold change in mRNA levels compared to control.

**Bryonolic Acid Induces Nrf2 Target Genes.** The induction of HO-1 by BA (1) led us to question whether BA (1) could also induce other phase 2 genes. Previous data showed an inverse correlation between the expression of the inflammatory gene iNOS and phase 2 genes in triterpenoid-treated cells.<sup>21</sup> Several phase 2 genes were probed including HO-1, NQO1, CAT, GR, and GCLC at various time points in BA (1)-treated RAW cells (Figure 3). Since Nrf2 also controls its own expression, we initially probed for Nrf2 mRNA levels. Nrf2 was induced 1.8-fold at 20 h in  $100 \mu\text{M}$  treated cells compared to control. Significant induction in expression for all of the genes occurred at the 20 h time point. NQO1 expression was significantly induced, demonstrating an 11.7-fold increase, similar in magnitude to the 11.3-fold increase in HO-1 mRNA levels. CAT was induced 1.7-fold at 16 h and almost doubled to 3.5-fold induction at 20 h. GCLC was induced 1.8-fold at 20 h and decreased to basal levels by 24 h. This pattern of induction



**Figure 1.** Bryonolic acid (BA) (1) decreases NO levels and inhibits iNOS expression in RAW 264.7 cells in a dose-dependent and time-dependent manner. RAW 264.7 cells were activated with 5 ng/mL LPS and treated with varying concentrations of BA for 24 h (A–D) or varying time points (E). (A) Nitrite levels were measured via Griess assay in LPS-activated cells treated with BA for 24 h. (B) RAW 264.7 cells were treated with varying concentrations of BA for 24 h, and the viability was measured by the MTT assay. (C) iNOS protein levels were quantified through immunoblot analysis in LPS-activated cells treated with varying concentrations of BA for 24 h. (D) iNOS mRNA levels were measured by RT-PCR. (E) iNOS protein levels were quantified through immunoblot analysis in LPS-activated cells treated with 100  $\mu\text{M}$  BA or DMSO control at various time points.

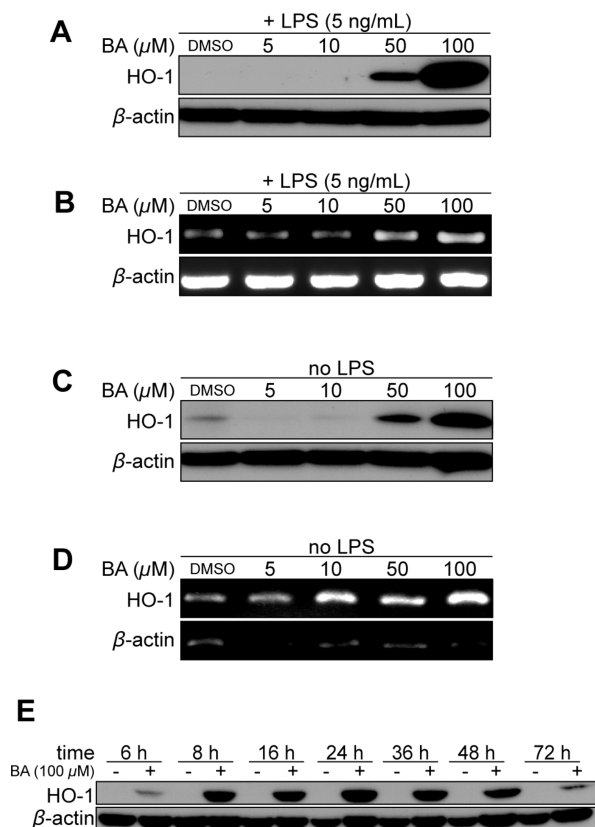
is also similar for GR, which was induced 1.5-fold at 20 h and decreased to basal levels by 24 h.

**Unique Dose–Response Profiles for Bryonolic Acid and Oleanolic Acid for NO Suppression and HO-1 Induction.** The numerous biological activities of triterpenoids led us to compare BA (1) with the well-studied and structurally similar triterpenoid OA (2). We compared the ability of OA (2) and BA (1) to suppress NO production in RAW cells. Nitrite levels decreased 85% from control in OA (2)-treated cells and 72% in BA (1)-treated cells (Figure 4A). Studies of OA (2) have led to the discovery of several potent synthetic triterpenoids, and selected OA (2) derivatives are now in late phase clinical trials.<sup>22,23</sup> However, when we compared the ability of both naturally occurring triterpenoids to induce HO-1, we found that BA (1) is considerably more effective than OA (2) (Figure 4B). At 50  $\mu\text{M}$ , there is greater HO-1 induction in BA (1)-treated cells compared to OA (2)-treated cells after 24 h. BA's (1) potency for inducing HO-1 is more apparent at the higher concentration of 100  $\mu\text{M}$ . In addition, we compared the potency of HO-1 induction by BA (1) with the structurally similar triterpenoids ursolic acid (UA) (3), betulinic acid (4), boswellic acid (5), and glycyrrhetic acid (6) (Figure S1, Supporting Information). We found that BA (1) induces HO-1 more potently compared to ursolic acid (3) and betulinic acid (4), but similar to boswellic acid (5) and glycyrrhetic acid (6) after 8 h of treatment. Interestingly, BA (1) is more potent at inducing HO-1 mRNA levels at the earlier 4 h time point compared to all of these triterpenoids (2–6). We noted that unlike BA (1) and OA (2), UA (3), betulinic acid (4), and glycyrrhetic acid (6) are all toxic to cells as early as 8 h after treatment.

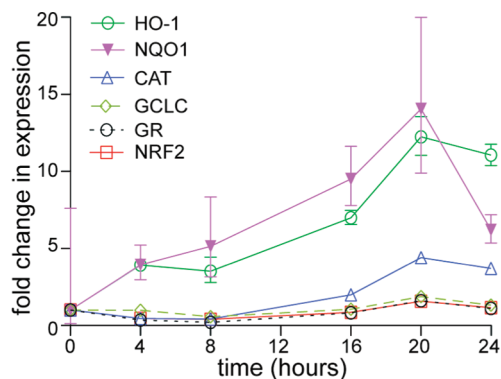
**Bryonolic Acid Induces Translocation of Nrf2 into the Nucleus.** The induction of HO-1 is regulated by the transcription factor Nrf2. In the presence of an inducer, Nrf2

is released from its cytoplasmic inhibitor Keap1 and is translocated into the nucleus, where it then binds to genes containing antioxidant response element (ARE) sites and induces transcription of the antioxidant phase 2 enzymes. In order to determine the mechanism of HO-1 induction by BA (1), we examined whether BA (1) is able to translocate Nrf2 into the nucleus. We treated RAW cells with 50 and 100  $\mu\text{M}$  BA (1) at various time points and probed both cytoplasmic and nuclear fractions for Nrf2. We observed that exposure to BA (1) decreased cytoplasmic Nrf2 in treated cells as early as 1 h (Figure S2, Supporting Information), and this decrease was more evident in the presence of 100  $\mu\text{M}$  BA (1). This reduction in cytoplasmic Nrf2 was more evident in BA (1)-treated cells from 1 to 4 h after treatment. Nrf2 accumulates in the nucleus in both BA (1)- and OA (2)-treated cells and remained nuclear throughout the time course, whereas no accumulation was observed in the control. These results show that BA (1) is more potent at inducing Nrf2 nuclear translocation when compared to OA (2).

**Differential Regulation of Nrf2 Target Genes by Bryonolic Acid.** We next determined whether the observed enhanced BA-induced (1) Nrf2 activation relative to OA (2) correlates with distinct expression profiles for Nrf2 target genes. Quantitative RT-PCR analyses revealed peak HO-1 induction in OA (2)-treated cells beginning 2–4 h after treatment, while peak HO-1 induction in BA-treated (1) cells occurred 16–20 h after treatment (Figure 5A). HO-1 expression levels in OA (2)-treated cells decreased to basal levels, while HO-1 expression levels in BA (1)-treated cells remained elevated at later time points as compared with OA (2). In addition, another prototypical target of Nrf2, NQO1, was induced in a similar manner to HO-1 (Figure 5B), while the expression profiles of CAT and GR were disparate in BA (1)- and OA (2)-treated cells. Whereas OA (2)-treated cells showed a peak induction

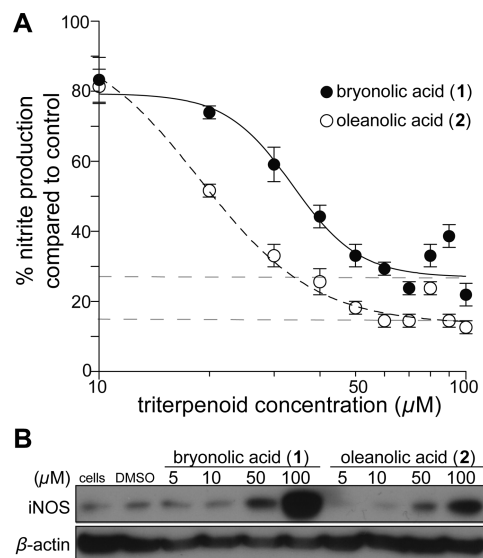


**Figure 2.** Bryonolic acid (BA) (1) induces HO-1 expression in RAW 264.7 cells in an LPS-independent manner. RAW 264.7 cells were activated either with 5 ng/mL LPS (A, B) or without (C, D) with varying concentrations of BA for 24 h (A–D) or varying time points (E). (A, C, and E) Immunoblot analysis of HO-1 protein levels. (B and D) mRNA level measurement of HO-1 by RT-PCR.



**Figure 3.** Bryonolic acid (BA) (1) induces Nrf2 and its target genes. RAW 264.7 cells were treated with BA at different time points. qRT-PCR was performed probing for Nrf2 and Nrf2 target genes including catalase (CAT), glutamate-cysteine ligase catalytic subunit (GCLC), glutathione reductase (GR), heme oxygenase-1 (HO-1), and NAD(P)H dehydrogenase quinone 1 (NQO1) at varying time points in cells treated with 100 μM BA.

followed by a steep decline in gene expression, BA (1)-treated cells demonstrated only a gradual increase in expression of these genes over the entire time course (Figure 3C and E). This differential regulation exerted by BA (1) does not affect non-Nrf2 regulated genes, β-actin, or GAPDH (Figure S3, Supporting Information). Taken together, these data show that subtle structural differences between triterpenoids with a



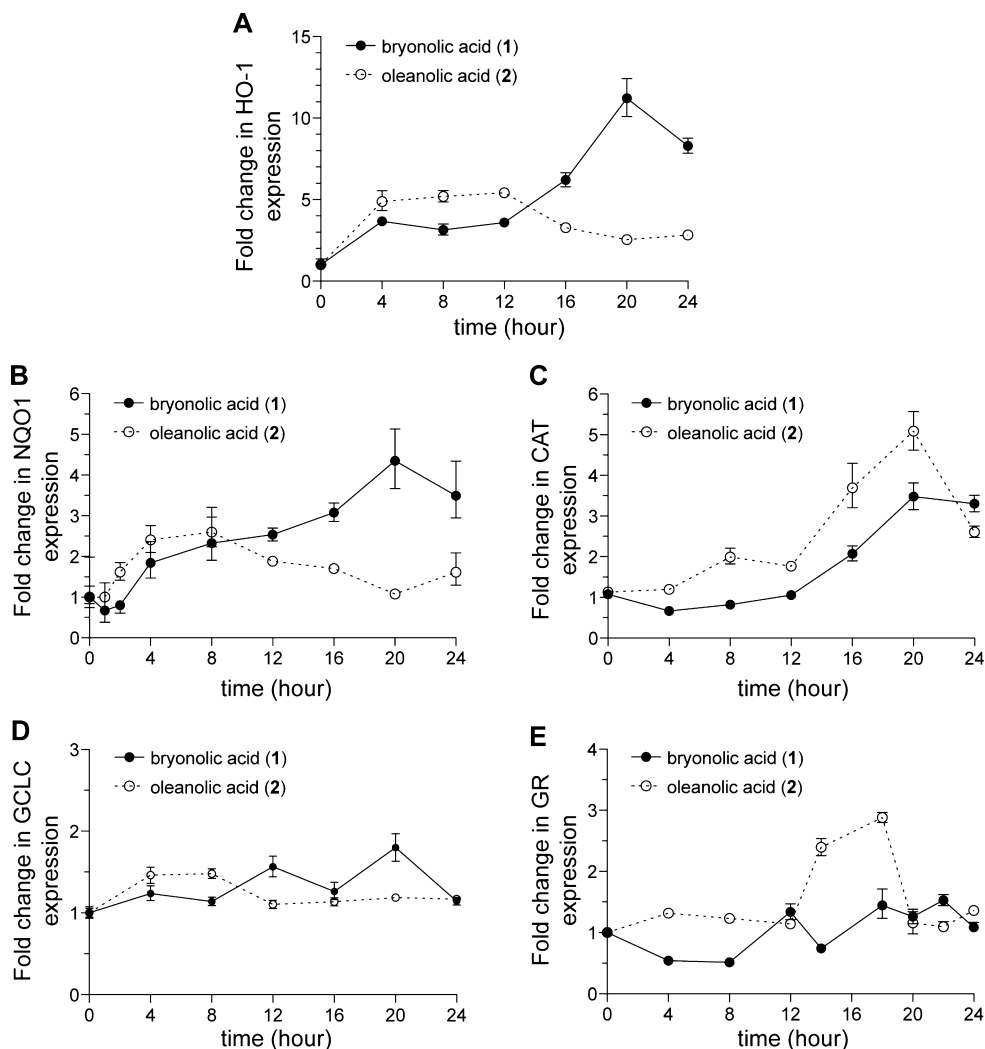
**Figure 4.** Activity comparison of bryonolic acid (BA) (1) versus oleanolic acid (OA) (2) at reducing nitrite levels and inducing HO-1. RAW 264.7 cells were treated with various concentrations of BA (1) or OA (2) for 24 h. (A) Nitrite levels were measured via the Griess assay. (B) Immunoblot analysis of HO-1 in the whole cell lysates of triterpenoid-treated cells.

similar carbocyclic skeleton affect the ability of these small molecules to modulate expression of the Nrf2 target genes in a different manner.

**Bryonolic Acid Activity in Primary Macrophage Is Dependent on the Nrf2-Keap1 Pathway.** In order to demonstrate the requirement for Nrf2 in the BA induction (1) of HO-1, we performed a series of experiments in primary peritoneal macrophages and probed for HO-1. Consistent with the observed activity in the RAW cell line, BA (1) induced HO-1 expression in a dose-dependent manner in primary peritoneal macrophages, and it did so with greater potency (induction observed at 10 μM BA (1) in primary macrophages in comparison with 50 μM in the RAW cells; Figure 6A). However, when we treated primary macrophages from Nrf2-deficient mice (Nrf2<sup>-/-</sup>), BA (1) no longer induced HO-1. Thus, the induction of HO-1 by BA (1) is dependent on the Nrf2-Keap1 pathway.

**Bryonolic Acid Induces HO-1 in Vivo in an Nrf2-Dependent Manner.** In order to determine whether this demonstrated capacity of BA (1) to induce HO-1 can be exerted following systemic exposure to BA (1) in vivo, wild-type mice were treated with 500 mg/kg BA (1) by intraperitoneal (ip) injection and sacrificed 8 h after treatment. Mouse livers were then harvested, as hepatocytes have been reported to exhibit highly inducible HO-1 expression.<sup>24,25</sup> Mouse livers that had been homogenized and probed for HO-1 after 8 h of BA (1) treatment showed a significant induction of HO-1, as determined by immunoblot analysis (Figure 6B). The same experiment performed in Nrf2<sup>-/-</sup> mice similarly demonstrated that in the absence of an intact Nrf2-Keap1 pathway, BA (1) was unable to induce HO-1 in vivo. Taken together, these data show that BA (1) potently induces HO-1 in a manner dependent on the Nrf2-Keap1 pathway.

Our goal in this study was to understand the molecular mechanism of the anti-inflammatory activity of BA (1) and to validate the use of BA (1) as a platform for studies designed to explore the relationship of structure to the pleiotropic effects of



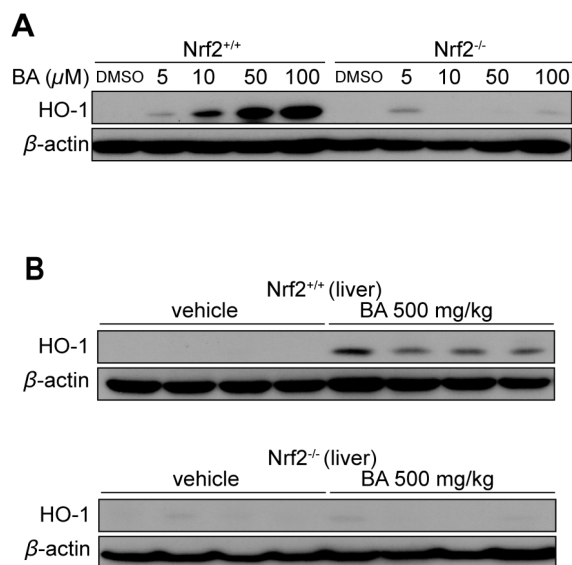
**Figure 5.** Bryonolic acid (BA) (1) differentially induces Nrf2 target genes compared with oleanolic acid (OA) (2). RAW 264.7 cells were treated with 100  $\mu$ M BA (1) or OA (2) at different time points. qRT-PCR was performed to measure HO-1 (A), NQO1 (B), CAT (C), GCLC (D), and GR (E) expression at various time points.

triterpenoids. This is the first definitive report demonstrating a molecular mechanism through which BA (1) exerts potent anti-inflammatory activity, by reducing NO levels via suppression of iNOS expression as shown in LPS-activated macrophages, in a dose- and time-dependent manner. We previously reported that BA (1) induces HO-1 in vitro in LPS-activated RAW cells<sup>26</sup> and herein report the induction of HO-1 expression in a dose-dependent and time-dependent manner independent of LPS. In addition, we show that BA (1) induces HO-1 in an Nrf2-dependent manner in primary mouse macrophages and in hepatocytes in vivo following systemic administration of BA (1).

These data, together with the observed induction of antioxidant genes, provide a mechanism to explain how BA (1) exerts the previously reported anti-allergic and anti-inflammatory properties observed in preclinical models in mice and rats.<sup>11,12</sup> More importantly, the observed effects of BA (1) on the production of iNOS and HO-1 are consistent with the reported role of these molecules in allergy and inflammation as highlighted in several studies. For example, iNOS expression is significantly increased after an allergen challenge in a preclinical anaphylaxis mouse model<sup>27</sup> and is highly expressed in several forms of dermatoses in humans.<sup>28</sup>

Furthermore, induction of HO-1 inhibits allergic inflammation in mice<sup>29</sup> and in humans,<sup>30</sup> and it is intriguing that the anti-allergic properties of several molecules have been attributed to an effect on HO-1 expression or activity.<sup>31,32</sup> Although the anti-inflammatory activity of HO-1 is established, further studies are required to determine how HO-1 contributes to the anti-inflammatory activity of BA (1).<sup>33–35</sup>

Surprisingly, BA (1) exhibits a unique expression profile compared with OA (2), inducing the characteristic robust HO-1 expression while yielding a different expression profile of the Nrf2 target genes from the structurally similar OA (2). In comparison to other structurally similar triterpenoids, ursolic acid (3), betulinic acid (4), boswellic acid (5), and glycyrrhetic acid (6), BA (1) induces HO-1 mRNA at the earlier time point (Figure S1, Supporting Information). In the absence of Nrf2, BA (1) failed to induce HO-1, suggesting that BA (1) acts primarily through this pathway. This is further corroborated by an analysis of Nrf2 nuclear translocation in which we observed a marked decrease in cytoplasmic Nrf2 and an increase in nuclear accumulation of Nrf2 in BA (1)-treated cells. The chemopreventive and chemotherapeutic effects of the oleanane-derived semisynthetic triterpenoids (CDDO and CDDO derivatives) have been attributed to the potent



**Figure 6.** Bryonolic acid (BA) (1) induces HO-1 in primary peritoneal macrophages and liver and is dependent on the Nrf2 pathway. (A) Primary peritoneal macrophages harvested from Nrf2 wild-type or Nrf2 knockout (C57BL/6J background) mice were treated with varying concentrations of BA (1) for 48 h. Immunoblot analysis of HO-1 protein levels in BA (1)-treated primary macrophages. (B) Immunoblot analysis of HO-1 protein levels in liver tissues of Nrf2 wild-type (upper panel) or Nrf2 knockout (lower panel) mice treated with 500 mg/kg BA or vehicle for 8 h by ip.

induction of HO-1- and the Nrf2-dependent genes.<sup>24,36–43</sup> The synthetic effort leading to the discovery of CDDO originated from improvement upon the weak anti-inflammatory activity of OA (2). Since BA (1) is more potent in comparison with OA (2) at inducing HO-1, combined with the extended gradual induction of other Nrf2-dependent genes, we can anticipate BA (1) to be an excellent platform for the development of potent anti-inflammatory and chemopreventive agents.

While one might expect that targeting the Nrf2 pathway with any triterpenoid would result in similar upregulation of antioxidant and cytoprotective genes,<sup>25,44,45</sup> our studies indicate that the minimal structural differences between the BA (1) and OA (2) triterpenoids influence their capacity to modulate a similar set of target genes. There are multiple interpretations for the observed HO-1 phenotype and the unique expression profile of BA (1). One possibility is that BA (1) is targeting a pathway different from Nrf2, which results in the difference of gene expression between BA (1) and OA (2). However, the mechanistic studies in which we explored BA (1) effects in primary mouse macrophages *in vitro* and in hepatocytes *in vivo* show that BA (1) failed to induce HO-1 without an intact Nrf2 pathway. These data support the conclusion that the observed BA (1) phenotype is mediated through the Nrf2 pathway. A second interpretation is that the primary target of BA (1) is Nrf2, but there exists structural specificity that dictates the differential regulation of Nrf2 target genes. There is precedence for this interpretation, as several ligands including OA (2) have been shown to bind to the farnesoid x receptor (FXR) and selectively modulate the expression of specific target genes important for bile acid regulation.<sup>46</sup> Our results suggest that BA (1) may be acting in a similar fashion while targeting the Nrf2 pathway. In addition, although Nrf2 is a key transcription factor for the induction of antioxidant genes, several studies have shown differences in the regulation of these genes. Small Maf

accessory proteins that heterodimerize with Nrf2 have been shown to play a role in the differential regulation of HO-1 and NQO1.<sup>47,48</sup> However, further studies are required to identify the cognate binding partner of BA (1) and to determine the molecular underpinnings of the observed expression profile.

It has been suggested that inducers of the phase 2 response are also suppressors of inflammation. Triterpenoids have been shown to coordinately regulate markers of inflammation and phase 2 response and that inducer potency is a reliable predictor of anti-inflammatory activity.<sup>21,49</sup> However, it is interesting to note that this is not the case for the observed BA (1) phenotype, where HO-1 induction is more potent and suppression of iNOS is weaker in BA (1)-treated cells compared with OA (2). Further mechanistic studies on BA (1) will hopefully uncover novel regulation of both the phase 2 and inflammatory pathway by triterpenoids.

Translationally, the capacity of BA (1) to induce HO-1 and other target genes could be leveraged through the use of BA (1) as a platform for developing novel selective modulators of Nrf2-dependent gene expression. By fully exploring the triterpenoid scaffold, there is potential to unlock the full potential of triterpenoids as selective inflammatory regulators. Such an effort could lead to the design of synthetic triterpenoids that may modulate expression of specific genes in a selected disease context. For example, the HO-1 phenotype observed *in vivo* has clear translational implications in the context of malaria, caused by infection of the *Plasmodium* genus of parasites. Many of the clinical manifestations of infection by *Plasmodium* are directly linked to the hemolysis of red blood cells and release of hemoglobin and the effects of hemoglobin degradation products. Hemoglobin released from red blood cells is oxidized by reactive oxygen species (ROS)<sup>50</sup> during inflammation, resulting in free heme, which serves as an amino acid source for the parasite.<sup>51,52</sup> HO-1 plays an important role in modulating the inflammatory response by breaking down the deleterious heme and produces anti-inflammatory byproducts, effectively protecting the host from developing cerebral and noncerebral forms of malaria.<sup>53,54</sup> The importance of this mechanism is supported by the observation that *Plasmodium* infection leads to rapid hepatic failure and lethality in mice with a targeted disruption of the HO-1 gene.<sup>55</sup> Thus, host survival in this context is dependent on the capacity to upregulate the HO-1 enzyme.<sup>56</sup> Ultimately, further studies will set a foundation for in-depth analyses of the triterpenoid scaffold and how this may be manipulated to generate potent and selective modulators of inflammation.

## ■ EXPERIMENTAL SECTION

**Materials.** C57BL/6J mice (wild-type) were purchased from Jackson Laboratory (Bar Harbor, ME, USA), and the Nrf2-knockout (Nrf2<sup>-/-</sup>) mice on C57BL/6J background were purchased from RIKEN BioResource Center (Tsukuba, Japan). BA (1) was isolated from the roots of *Cucurbita pepo* L. as previously reported,<sup>26</sup> and OA (2) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Stocks were made fresh in DMSO. Ursolic acid (3), betulinic acid (4), boswellic acid (5), glycyrrhetic acid (6), DMSO, Cremophore EL, and LPS from *E. coli* were purchased from Sigma (St. Louis, MO, USA). All the primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and secondary antibodies from Southern Biotech (Birmingham, AL, USA). DMEM and RPMI media were purchased from GIBCO (Grand Island, NY, USA) and then supplemented with low endotoxin FBS (<0.06 EU), obtained from Thermo Scientific (Logan, UT, USA). The Trypan blue, penicillin/streptomycin, Griess assay reagents, TRIZol reagent, PureLink RNA

Mini Kit, SuperScript One-Step RT-PCR, SuperScript III First-Strand Synthesis System for RT-PCR, RIPA buffer, 0.2  $\mu$ m PVDF membrane, Novex 4–20% tris glycine gels, and running and transfer buffers were all purchased from Invitrogen (Grand Island, NY, USA). The protease cocktail inhibitor tablet was purchased from Roche (Indianapolis, IN, USA), and MTT cell proliferation assay kit from ATCC (Manassas, VA, USA). TaqMan Fast Universal PCR Master Mix was obtained from Applied Biosystems (Foster City, CA, USA). Thioglycollate for primary macrophage stimulation was purchased from Becton Dickinson (Sparks, MD, USA) and ECL Plus from Amersham (Buckinghamshire, UK). PBS was obtained from Cellgro by Mediatech, Inc. (Manassas, VA, USA). Autoradiography film was purchased from MidSci (St. Louis, MO, USA).

**Cell Culture.** The leukemic mouse macrophage cells (RAW 264.7) were obtained as a gift from Dr. Michael Sporn (Dartmouth College, NH, USA), cultured in DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin, and kept in culture at 37 °C in a 5% CO<sub>2</sub> environment. Cells were kept in culture for no longer than a month and routinely checked for LPS responsiveness every few passages via detection of NO production measured using the Griess assay.

**NO Measurement.** RAW 264.7 cells were plated ( $1 \times 10^5$  cells/well) in a 96-well plate and allowed to attach for several hours before activation with 5 ng/mL LPS. LPS-activated cells were treated with varying concentrations of BA (1) (1–1000  $\mu$ M) for 24 h. NO levels were measured via Griess assay using 100  $\mu$ L of Griess reagent with 100  $\mu$ L of cell culture supernatant. Absorbance was read at 550 nm using the Sunrise plate reader by TECAN (Mannedorf, Switzerland). IC<sub>50</sub> values were calculated by fitting a nonlinear sigmoidal variant dose curve to the data using Prism 5.0 software by Graphpad Inc.

**Toxicity Measurement.** The MTT cell proliferation assay kit was used according to the manufacturer's specifications to measure toxicity of BA (1)-treated RAW 264.7 cells after 24 h. The Trypan blue exclusion test was used in Figure S3 (Supporting Information).

**RAW 264.7 Cell Treatment.** RAW 264.7 cells were plated at  $4 \times 10^6$  cells/60 mm plate and allowed to attach for 2 h. Cells were activated with 5 ng/mL LPS and immediately treated with varying concentrations of BA (1) (maintaining 0.5% DMSO), BA (1) alone without LPS activation, or DMSO control. Cells were harvested for immunoblotting analysis after a 24 h treatment.

**Primary Peritoneal Macrophage Isolation and Treatment.** Prior to primary peritoneal macrophage harvest, 6–8-week-old C57BL/6J and Nrf2<sup>-/-</sup> (C57BL/6J background) mice were injected with 2 mL of 4% thioglycollate via ip. Primary peritoneal macrophages were collected with PBS, plated in 60 mm plates ( $3 \times 10^6$  cells/plate) in RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin, and allowed to attach for 2–3 h. Prior to LPS activation and BA (1) treatment, cells were washed with PBS 3 or 4 times to remove nonmacrophage cells. Cells were treated with varying concentrations of BA (1) (5, 10, 50, or 100  $\mu$ M) or DMSO control. Cells were harvested for immunoblotting analysis after a 48 h treatment.

**Immunoblotting Analysis.** Cells and homogenized tissues were lysed with RIPA buffer containing protease inhibitors. Lysates were probed for HO-1 and iNOS using a 1:1000 primary antibody dilution for RAW 264.7 cells and primary macrophage cells and a 1:500 HO-1 antibody dilution for tissue lysates. Secondary antibodies (1:5000 dilution) were detected using ECL plus with autoradiography.

**RT-PCR Analysis.** Total RNA from the treated cells was extracted and purified using TRIZol reagent. cDNA was synthesized and PCR reactions were performed using Superscript One-Step RT-PCR. Primer sequences for RT-PCR analysis for iNOS and HO-1<sup>57</sup> and CAT, GCLC, GR, NQO1, and  $\beta$ -actin were adopted from a previous publication.<sup>58</sup> The conditions were used accordingly: 55 °C for 30 min for reverse transcription, 94 °C for 2 min for predenaturation, followed by 30 cycles of 94 °C for 30 s for denaturing, 55 °C for 30 s for annealing, and 72 °C for 1 min for extension, followed by one cycle of 72 °C for 10 min for final extension.

**Quantitative Real-Time PCR Analysis.** Total RNA from treated cells was isolated with the PureLink RNA Mini Kit and converted to

cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR. TaqMan Fast Universal PCR Master Mix was used for real-time RT-PCR with mouse-specific Taqman gene expression assay from Applied Biosystems. The TaqMan PCR primers and probes used were as follows: iNOS (Mm01309902\_m1), HO-1 (Mm00516007\_m1), NQO1 (Mm00500821\_m1), catalase (Mm00437992\_m1), GCLC (Mm00802655\_m1), GR (Mm00833903\_m1), and 18S rRNA (Hs99999901\_s1) as a control. Amplification was performed using the 7500 Fast Real-Time PCR system and the 7500 Fast System SDS Software-Sequence Detection software version 1.3.1.21 by Applied Biosystems. The assay used for the study was the relative quantification assay ( $\Delta\Delta$ Ct) using the Run mode Fast 7500 profile (95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s).

**In Vivo BA Administration.** Both the C57BL/6J and the Nrf2<sup>-/-</sup> mice were treated with either a single dose of 500 mg/kg BA (1) (dissolved in 80% PBS/10% DMSO/10% Cremophore) or vehicle control administered by ip injection. Mice were sacrificed, and liver tissue was harvested 8 h following BA (1) administration. All experiments were performed in accordance with an approved protocol by the Institutional Animal Care and Use Committee at Case Western Reserve University.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Comparative analysis of HO-1 induction by triterpenoids (Figure S1), immunoblot analysis of cytoplasmic and nuclear Nrf2 in bryonolic acid- and oleanolic acid-treated RAW 264.7 cells (Figure S2), positive and negative controls for BA (1)-treated RAW 264.7 cells (Figure S3), and selective regulation of Nrf2 target genes by BA (1) (Figure S4) are presented. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

The work was supported by grants from the NIH, R01CA157735 and R03CA132168, and Reuter Foundation to G.P.T. and J.J.T., Landon Foundation INNOVATOR award from AACR to G.P.T., and an NIH fellowship, F31CA134211, to T.N.G.

## ■ REFERENCES

- (1) Dzubak, P.; Hajduch, M.; Vydra, D.; Hustova, A.; Kvasnica, M.; Biedermann, D.; Markova, L.; Urban, M.; Sarek, J. *Nat. Prod. Rep.* **2006**, *23*, 394–411.
- (2) Honda, T.; Rounds, B. V.; Gribble, G. W.; Suh, N.; Wang, Y.; Sporn, M. B. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2711–2714.
- (3) Suh, N.; Honda, T.; Finlay, H. J.; Barchowsky, A.; Williams, C.; Benoit, N. E.; Xie, Q. W.; Nathan, C.; Gribble, G. W.; Sporn, M. B. *Cancer Res.* **1998**, *58*, 717–723.
- (4) Honda, T.; Rounds, B. V.; Bore, L.; Finlay, H. J.; Favaloro, F. G. Jr.; Suh, N.; Wang, Y.; Sporn, M. B.; Gribble, G. W. *J. Med. Chem.* **2000**, *43*, 4233–4246.
- (5) Suh, N.; Wang, Y.; Honda, T.; Gribble, G. W.; Dmitrovsky, E.; Hickey, W. F.; Maue, R. A.; Place, A. E.; Porter, D. M.; Spinella, M. J.; Williams, C. R.; Wu, G.; Dannenberg, A. J.; Flanders, K. C.; Letterio, J. J.; Mangelsdorf, D. J.; Nathan, C. F.; Nguyen, L.; Porter, W. W.; Ren, R. F.; Roberts, A. B.; Roche, N. S.; Subbaramaiah, K.; Sporn, M. B. *Cancer Res.* **1999**, *59*, 336–341.

- (6) Deeb, D.; Gao, X.; Dulchavsky, S. A.; Gautam, S. C. *J. Exp. Ther. Oncol.* **2008**, *7*, 31–39.
- (7) Sogno, I.; Vannini, N.; Lorusso, G.; Cammarota, R.; Noonan, D. M.; Generoso, L.; Sporn, M. B.; Albini, A. *Recent Results Cancer Res.* **2009**, *181*, 209–212.
- (8) Kim, E. H.; Deng, C.; Sporn, M. B.; Royce, D. B.; Risingsong, R.; Williams, C. R.; Liby, K. T. *Cancer Prev. Res.* **2012**, *5*, 89–97.
- (9) Petronelli, A.; Pelosi, E.; Santoro, S.; Saule, E.; Cerio, A. M.; Mariani, G.; Labbaye, C.; Testa, U. *Leuk. Res.* **2011**, *35*, 534–544.
- (10) Pergola, P. E.; Raskin, P.; Toto, R. D.; Meyer, C. J.; Huff, J. W.; Grossman, E. B.; Krauth, M.; Ruiz, S.; Audhya, P.; Christ-Schmidt, H.; Wittes, J.; Warnock, D. G. *N. Engl. J. Med.* **2011**, *365*, 327–336.
- (11) Tanaka, S.; Uno, C.; Akimoto, M.; Tabata, M.; Honda, C.; Kamisako, W. *Planta Med.* **1991**, *57*, 527–530.
- (12) Tabata, M.; Tanaka, S.; Cho, H. J.; Uno, C.; Shimakura, J.; Ito, M.; Kamisako, W.; Honda, C. *J. Nat. Prod.* **1993**, *56*, 165–174.
- (13) Kondo, T.; Inoue, M.; Mizukami, H.; Ogihara, Y. *Biol. Pharm. Bull.* **1995**, *18*, 726–729.
- (14) Takeda, T.; Kondo, T.; Mizukami, H.; Ogihara, Y. *Chem. Pharm. Bull.* **1994**, *42*, 730–732.
- (15) Kongtun, S.; Jirachariyakul, W.; Kummalue, T.; Tan-ariya, P.; Kunnachak, S.; Frahm, A. W. *Planta Med.* **2009**, *75*, 839–842.
- (16) Moi, P.; Chan, K.; Asunis, I.; Cao, A.; Kan, Y. W. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 9926–9930.
- (17) Itoh, K.; Wakabayashi, N.; Katoh, Y.; Ishii, T.; O'Connor, T.; Yamamoto, M. *Genes Cells* **2003**, *8*, 379–391.
- (18) Itoh, K.; Tong, K. I.; Yamamoto, M. *Free Radical Biol. Med.* **2004**, *36*, 1208–1213.
- (19) Rios, J. L.; Bas, E.; Recio, M. C. *Curr. Med. Chem.: Anti-Inflammatory Anti-Allergy Agents* **2005**, *4*, 65–80.
- (20) Thimmulappa, R. K.; Scollick, C.; Traore, K.; Yates, M.; Trush, M. A.; Liby, K. T.; Sporn, M. B.; Yamamoto, M.; Kensler, T. W.; Biswal, S. *Biochem. Biophys. Res. Commun.* **2006**, *351*, 883–889.
- (21) Dinkova-Kostova, A. T.; Liby, K. T.; Stephenson, K. K.; Holtzclaw, W. D.; Gao, X.; Suh, N.; Williams, C.; Risingsong, R.; Honda, T.; Gribble, G. W.; Sporn, M. B.; Talalay, P. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 4584–4589.
- (22) Koschmieder, S.; D'Alo, F.; Radomska, H.; Schoneich, C.; Chang, J. S.; Konopleva, M.; Kobayashi, S.; Levantini, E.; Suh, N.; Di Ruscio, A.; Voso, M. T.; Watt, J. C.; Santhanam, R.; Sargin, B.; Kantarjian, H.; Andreeff, M.; Sporn, M. B.; Perrotti, D.; Berdel, W. E.; Muller-Tidow, C.; Serve, H.; Tenen, D. G. *Blood* **2007**, *110*, 3695–3705.
- (23) Tsao, T.; Kornblau, S.; Safe, S.; Watt, J. C.; Ruvolo, V.; Chen, W.; Qiu, Y.; Coombes, K. R.; Ju, Z.; Abdelrahim, M.; Schober, W.; Ling, X.; Kardassis, D.; Meyer, C.; Schimmer, A.; Kantarjian, H.; Andreeff, M.; Konopleva, M. *Cancer Res.* **2010**, *70*, 4949–4960.
- (24) Reisman, S. A.; Buckley, D. B.; Tanaka, Y.; Klaassen, C. D. *Toxicol. Appl. Pharmacol.* **2009**, *236*, 109–114.
- (25) Reisman, S. A.; Aleksunes, L. M.; Klaassen, C. D. *Biochem. Pharmacol.* **2009**, *77*, 1273–1282.
- (26) Barker, E. C.; Gathbonton-Schwager, T. N.; Han, Y.; Clay, J. E.; Letterio, J. J.; Tochtrop, G. P. *J. Nat. Prod.* **2010**, *73*, 1064–1068.
- (27) Sade, K.; Schwartz, I. F.; Etkin, S.; Schwartzberg, S.; Levo, Y.; Kivity, S. *J. Invest. Allergol. Clin. Immunol.* **2007**, *17*, 379–385.
- (28) Rowe, A.; Farrell, A. M.; Bunker, C. B. *Br. J. Dermatol.* **1997**, *136*, 18–23.
- (29) Listopad, J.; Asadullah, K.; Sievers, C.; Ritter, T.; Meisel, C.; Sabat, R.; Docke, W. D. *Exp. Dermatol.* **2007**, *16*, 661–670.
- (30) Kirino, M.; Kirino, Y.; Takeno, M.; Nagashima, Y.; Takahashi, K.; Kobayashi, M.; Murakami, S.; Hirasawa, T.; Ueda, A.; Aihara, M.; Ikezawa, Z.; Ishigatsubo, Y. *J. Allergy Clin. Immunol.* **2008**, *122*, 290–297.
- (31) Matsushima, M.; Takagi, K.; Ogawa, M.; Hirose, E.; Ota, Y.; Abe, F.; Baba, K.; Hasegawa, T.; Hasegawa, Y.; Kawabe, T. *Inflammation Res.* **2009**, *58*, 705–715.
- (32) Matsushima, H.; Tanaka, H.; Mizumoto, N.; Takashima, A. *Blood* **2009**, *114*, 64–73.
- (33) Kim, H. P.; Wang, X.; Zhang, J.; Suh, G. Y.; Benjamin, I. J.; Rytter, S. W.; Choi, A. M. *J. Immunol.* **2005**, *175*, 2622–2629.
- (34) Otterbein, L. E.; Bach, F. H.; Alam, J.; Soares, M.; Tao, Lu, H.; Wysk, M.; Davis, R. J.; Flavell, R. A.; Choi, A. M. *Nat. Med. (N. Y., NY, U. S.)* **2000**, *6*, 422–428.
- (35) Rocuts, F.; Ma, Y.; Zhang, X.; Gao, W.; Yue, Y.; Vartanian, T.; Wang, H. *J. Immunol.* **2010**, *185*, 2134–2139.
- (36) Thimmulappa, R. K.; Scollick, C.; Traore, K.; Yates, M.; Trush, M. A.; Liby, K. T.; Sporn, M. B.; Yamamoto, M.; Kensler, T. W.; Biswal, S. *Biochem. Biophys. Res. Commun.* **2006**, *351*, 883–889.
- (37) Thimmulappa, R. K.; Fuchs, R. J.; Malhotra, D.; Scollick, C.; Traore, K.; Bream, J. H.; Trush, M. A.; Liby, K. T.; Sporn, M. B.; Kensler, T. W.; Biswal, S. *Antioxid. Redox Signaling* **2007**, *9*, 1963–1970.
- (38) Ferguson, H. E.; Thatcher, T. H.; Olsen, K. C.; Garcia-Bates, T. M.; Baglole, C. J.; Kottmann, R. M.; Strong, E. R.; Phipps, R. P.; Sime, P. J. *Am. J. Physiol.: Lung Cell. Mol. Physiol.* **2009**, *297*, L912–L919.
- (39) Ichikawa, T.; Li, J.; Meyer, C. J.; Janicki, J. S.; Hannink, M.; Cui, T. *PLoS One* **2009**, *4*, e8391.
- (40) Aleksunes, L. M.; Goedken, M. J.; Rockwell, C. E.; Thomale, J.; Manautou, J. E.; Klaassen, C. D. *J. Pharmacol. Exp. Ther.* **2010**, *335*, 2–12.
- (41) Auletta, J. J.; Alabran, J. L.; Kim, B. G.; Meyer, C. J.; Letterio, J. J. *Interferon Cytokine Res.* **2010**, *30*, 497–508.
- (42) Stack, C.; Ho, D.; Wille, E.; Calingasan, N. Y.; Williams, C.; Liby, K.; Sporn, M.; Dumont, M.; Beal, M. F. *Free Radicals Biol. Med.* **2010**, *49*, 147–158.
- (43) Neymotin, A.; Calingasan, N. Y.; Wille, E.; Naseri, N.; Petri, S.; Damiano, M.; Liby, K. T.; Risingsong, R.; Sporn, M.; Beal, M. F.; Kiaei, M. *Free Radicals Biol. Med.* **2011**, *51*, 88–96.
- (44) Yates, M. S.; Tauchi, M.; Katsuoka, F.; Flanders, K. C.; Liby, K. T.; Honda, T.; Gribble, G. W.; Johnson, D. A.; Johnson, J. A.; Burton, N. C.; Guilarte, T. R.; Yamamoto, M.; Sporn, M. B.; Kensler, T. W. *Mol. Cancer Ther.* **2007**, *6*, 154–162.
- (45) Wang, X.; Ye, X. L.; Liu, R.; Chen, H. L.; Bai, H.; Liang, X.; Zhang, X. D.; Wang, Z.; Li, W. L.; Hai, C. X. *Chem.-Biol. Interact.* **2010**, *184*, 328–337.
- (46) Liu, W.; Wong, C. *Phytother. Res.* **2010**, *24*, 369–373.
- (47) Yeligar, S. M.; Machida, K.; Kalra, V. K. *J. Biol. Chem.* **2010**, *285*, 35359–35373.
- (48) Sun, J.; Hoshino, H.; Takaku, K.; Nakajima, O.; Muto, A.; Suzuki, H.; Tashiro, S.; Takahashi, S.; Shibahara, S.; Alam, J.; Taketo, M. M.; Yamamoto, M.; Igarashi, K. *EMBO J.* **2002**, *21*, 5216–5224.
- (49) Liu, H.; Dinkova-Kostova, A. T.; Talalay, P. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 15926–15931.
- (50) Francis, S. E.; Sullivan, D. J. Jr.; Goldberg, D. E. *Annu. Rev. Microbiol.* **1997**, *51*, 97–123.
- (51) Goldberg, D. E.; Slater, A. F.; Cerami, A.; Henderson, G. B. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87*, 2931–2935.
- (52) Sherman, I. W.; Ruble, J. A.; Tanigoshi, L. *Mil. Med.* **1969**, *134*, 954–961.
- (53) Ferreira, A.; Marguti, I.; Bechmann, I.; Jeney, V.; Chora, A.; Palha, N. R.; Rebelo, S.; Henri, A.; Beuzard, Y.; Soares, M. P. *Cell* **2011**, *145*, 398–409.
- (54) Pamplona, A.; Ferreira, A.; Balla, J.; Jeney, V.; Balla, G.; Epiphany, S.; Chora, A.; Rodrigues, C. D.; Gregoire, I. P.; Cunha-Rodrigues, M.; Portugal, S.; Soares, M. P.; Mota, M. M. *Nat. Med. (N. Y., NY, U. S.)* **2007**, *13*, 703–710.
- (55) Seixas, E.; Gozzelino, R.; Chora, A.; Ferreira, A.; Silva, G.; Larsen, R.; Rebelo, S.; Penido, C.; Smith, N. R.; Coutinho, A.; Soares, M. P. *Natl. Acad. Sci. U. S. A.* **2009**, *106*, 15837–15842.
- (56) Ferreira, A.; Balla, J.; Jeney, V.; Balla, G.; Soares, M. P. *J. Mol. Med. (Heidelberg, Ger.)* **2008**, *86*, 1097–1111.
- (57) Khor, T. O.; Huang, M. T.; Kwon, K. H.; Chan, J. Y.; Reddy, B. S.; Kong, A. N. *Cancer Res.* **2006**, *66*, 11580–11584.
- (58) Zhu, H.; Jia, Z.; Zhang, L.; Yamamoto, M.; Misra, H. P.; Trush, M. A.; Li, Y. *Exp. Biol. Med. (Maywood, NJ, U. S.)* **2008**, *233*, 463–474.