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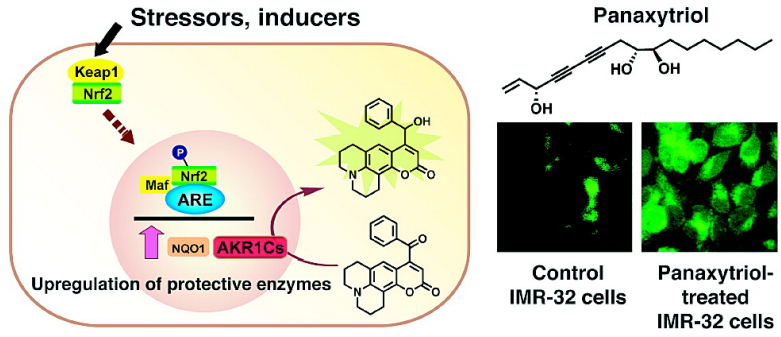

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Imaging Induction of Cytoprotective Enzymes in Intact Human Cells: Coumberone, a Metabolic Reporter for Human AKR1C Enzymes Reveals Activation by Panaxytriol, an Active Component of Red Ginseng

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Abstract: We here present an optical method for monitoring the activity of the inducible aldo-keto reductases AKR1C2 and AKR1C3 in living human cells. The induction of these enzymes is regulated by the antioxidant response element (ARE), as demonstrated in recent literature, which in turn is dependent on the transcription factor Nrf2. The activation of ARE leads to the transcription of a coalition of cytoprotective enzymes and thus represents an important target for the development of new therapies in the area of neurodegenerative diseases and cancer. Through the use of Coumberone, a metabolic fluorogenic probe, and isoform-selective inhibitors, the upregulation of cellular stress markers AKR1C2 and AKR1C3 can be quantitatively measured in the presence of ARE activator compounds, via either a fluorimetric assay or fluorescence microscopy imaging of intact cells. The method has both high sensitivity and broad dynamic range, as demonstrated by induction studies in three cell lines with dramatically different metabolic capabilities (transfected monkey kidney COS-1 cells, human neuroblastoma IMR-32 cells, and human liver HepG2 cells). We applied the new method to examine a number of neurotrophic natural products (spirotenuipesine A, spirotenuipesine B, scabronine G-methylester, and panaxytriol), and discovered that panaxytriol, an active component of red ginseng extracts, is a potent ARE inducer. The upregulation of AKR1C enzymes, induced by chemically homogeneous panaxytriol, was partially dependent on PKC and PI3K kinases as demonstrated by the application of selective inhibitors. This cellular mechanism may account for panaxytriol's neurotrophic, neuroprotective, and anticancer properties. The protective effects of ARE inducers against tumorgenesis and neurodegeneration fuel the growing interest in this area of research and the method described here will greatly enable these endeavors.

Introduction

Mammalian cells have evolved numerous mechanisms to detect and respond to a variety of challenges that perturb homeostasis and endanger cellular viability. The stress-inducing stimuli may be biological (e.g., pathological processes that lead to increased production of reactive oxygen species), chemical (drugs, pollutants, and toxins) or physical (UV radiation, pressure). Recent efforts have identified an important defense pathway that centers on the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) and its inhibitor Keap1 (Kelch ECH Associating Protein 1). Nrf2 is liberated by either alkylation or oxidation of Keap1 cysteine residues or by direct phosphorylation of Nrf2, which in turn leads to translocation of Nrf2 to the nucleus and activation of the antioxidant-responsive element (ARE) and increased expression of a large number of protective proteins (Figure 1A).[1]

ARE inducers have been known for decades to inhibit carcinogenesis (chemopreventive activity) and a number of small molecule inducers have been identified (e.g., *tert*-butylhydroquinone (*t*-BHQ) and sulforaphane).[2] Furthermore, it has been demonstrated that activation of the Nrf2/ARE pathway protects neurons against oxidative stress both in vitro and in vivo. For example, the size of brain lesions, caused by experimentally induced stroke, was significantly reduced by compounds that activate Nrf2.[3] Also, inhibition of mitochondrial complex II (by malonic acid or 3-nitropropionic acid) leads to metabolic stress that results in damage localized in the brain area known as the striatum and symptoms similar to those found in Huntington's disease. It was demonstrated that Nrf2-dependent ARE activation confers protection against this type of metabolic stress.[4]

As a result, the interest in the Nrf2/ARE pathway has been growing as a potential target for the development of new therapies for neurodegenerative diseases and cancer. Despite the importance of this system, there are no methods for continuous measurement and imaging of ARE-regulated protein expression and activity in living cells. The existing methods, most notably the colorimetric assay measuring the activity of

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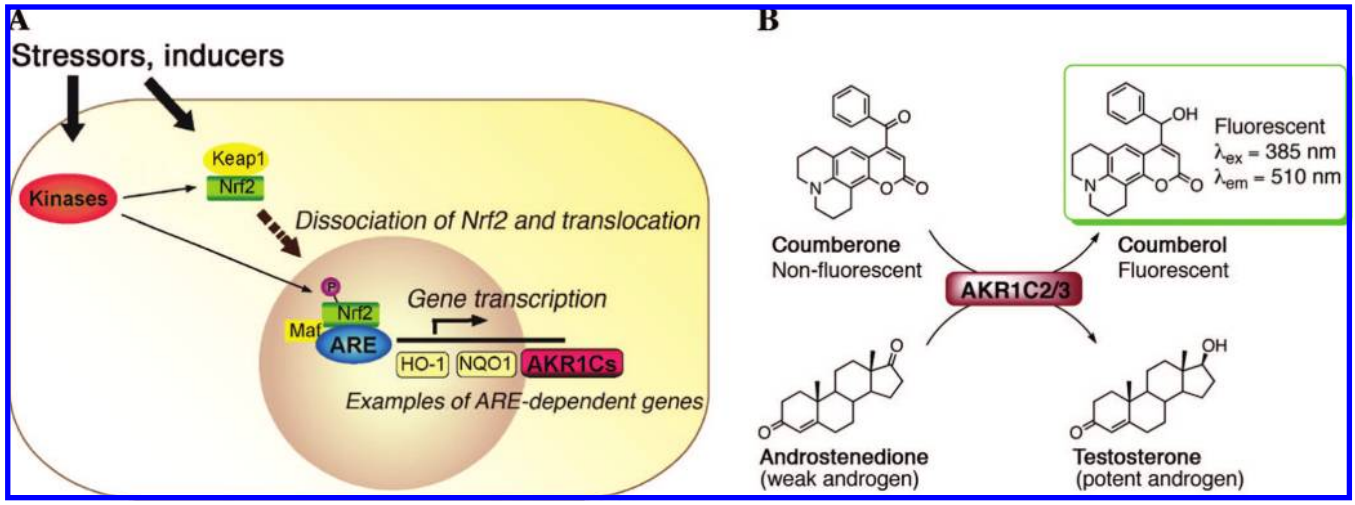

Figure 1. Human AKR1C enzymes are emerging cellular stress markers that indicate the activation of ARE (antioxidant-responsive element) and Coumberone is a synthetic fluorogenic substrate for isoforms AKR1C2 and AKR1C3. (A) The Nrf2/ARE signaling pathway regulates expression of cytoprotective enzymes including human AKR1C enzymes. Alkylation of Keap1 or phosphorylation of transcription factor Nrf2 leads to translocation of Nrf2 to the nucleus, which heterodimerizes with members of the small Maf family proteins and triggers the transcription of ARE-regulated genes. In human neuroblastoma IMR-32 cells, the expression of AKR1C3 is increased, along with established stress markers heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1), upon activation of ARE. (B) Coumberone is a synthetic reporter substrate for enzyme isoforms AKR1C2 and AKR1C3. It turns into the highly fluorescent alcohol, Coumberol, upon reduction of the carbonyl functional group, enabling direct imaging of AKR1C2 and AKR1C3 activity in cells.

NAD(P)H:quinone oxidoreductase 1 (NQO1),[5] standard gene expression screens and Western blotting, are destructive and discontinuous. We here present an optical method for monitoring the ARE activation in living cells based on inducible human aldo-keto reductases AKR1C2 and AKR1C3 and the fluorogenic reporter substrate, **Coumberone** (Figure 1B).

Human hydroxysteroid dehydrogenases of the aldo-keto reductase (AKR) superfamily consist of four isoforms (AKR1C1−4) that play diverse roles in steroid metabolism and signaling by modulating steroid receptor occupancy.[6] Isoforms AKR1C1 and AKR1C2 have been shown to be part of the inducible cytoprotective enzymes regulated by the ARE.[7,8] In HepG2 cells, a proximal promoter containing the consensus ARE sequence has been identified; treatment with phase II inducers were found to cause nuclear translocation of Nrf2, followed by activation of ARE and induction of AKR1C1 and AKR1C2.[7b] A recent gene expression profile study indicated that AKR1C3, the isoform required in the biosynthesis of testosterone and prostaglandin PGF2α[6] is also inducible by *t*-BHQ (a strong activator of ARE) in human neuroblastoma IMR-32 cells.[9]

We have recently developed a fluorogenic substrate for AKR1C2, which we here term "Coumberone". The phenylketone is nonfluorescent while its reduction affords the highly fluorescent alcohol "Coumberol" (Figure 1B), allowing for direct measurement of the enzyme activity in cells.[10] In this work, we show that Coumberone is also an excellent substrate for AKR1C3 in cells, and that we can selectively read out the activity of either AKR1C2 or AKR1C3 through the use of isoform-selective inhibitors. Since the human AKR1Cs are part of the inducible, ARE-driven cytoprotective genes, Coumberone thus enables optical monitoring of ARE activation in a continuous and nondestructive manner. The application of this method led to the discovery of panaxytriol as a potent ARE inducer, which may account for its antitumor and neurotrophic biological activities.

Results

Selective Inhibition of AKR1C2 and AKR1C3 in Intact COS-1 Cells. While Coumberone exhibits 10-fold greater catalytic efficiency for AKR1C3 than AKR1C2 in vitro, it only modestly discriminates between the two enzymes in living cells. Since the expression levels and the inducibility of the two isoforms vary in different cell types, it was necessary to find isoform-selective inhibitors that could be used in conjunction with Coumberone to selectively report on induction of each isoform in intact cells. For this purpose, we chose COS-1 cells (African green monkey kidney cells) as a suitable model mammalian cell line.

We have reported that ursodeoxycholic acid is a potent inhibitor of AKR1C2 inside cells.[10] Here we show that it is also selective for AKR1C2 over other isoforms in cells, with more than 100-fold greater potency for AKR1C2 over AKR1C3 (apparent $IC_{50} = 0.14 \pm 0.02\ \mu M$ for AKR1C2; $IC_{50} > 30\ \mu M$ for AKR1C3, COS-1 cells, Figure 2A). As a result, the level of AKR1C3 induction can be measured selectively without interference from AKR1C2. With regard to AKR1C3 inhibition, indomethacin[11] shows high selectivity for AKR1C3 ($IC_{50} = 6.9 \pm 0.8\ \mu M$) over AKR1C2 ($IC_{50} > 100\ \mu M$, Figure 2B) in transfected COS-1 cells and enables the measurement of AKR1C2 activity changes in cells with high endogenous expression of AKR1C3 (e.g., human hepatoma HepG2 cells, see Discussion section). Importantly, Coumberone can thus be

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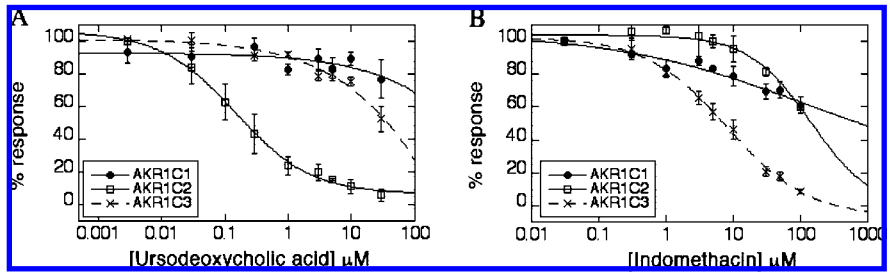

Figure 2. Determination of IC_{50} values for ursodeoxycholic acid and indomethacin in intact transfected COS-1 cells using Coumberone. COS-1 cells were seeded in 6-well dishes and transfected with pcDNA3-AKR1C1, pcDNA3-AKR1C2, or pcDNA3-AKR1C3. Twenty-four hours later, a cocktail of inhibitor-Coumberone (in DMSO) was added into the wells and the fluorescence growth measured over time. Concentration of Coumberone: 5 μM. (A) Ursodeoxycholic acid has apparent IC_{50} values of 0.14 $\pm$ 0.02 μM for AKR1C2 and >30 μM for AKR1C1 and AKR1C3. (B) Indomethacin has apparent IC_{50} values of 6.9 $\pm$ 0.8 μM for AKR1C3 and >100 μM for AKR1C1 and AKR1C2. Error bars are derived from three independent experiments on separate days. Each independent experiment was run in triplicate.

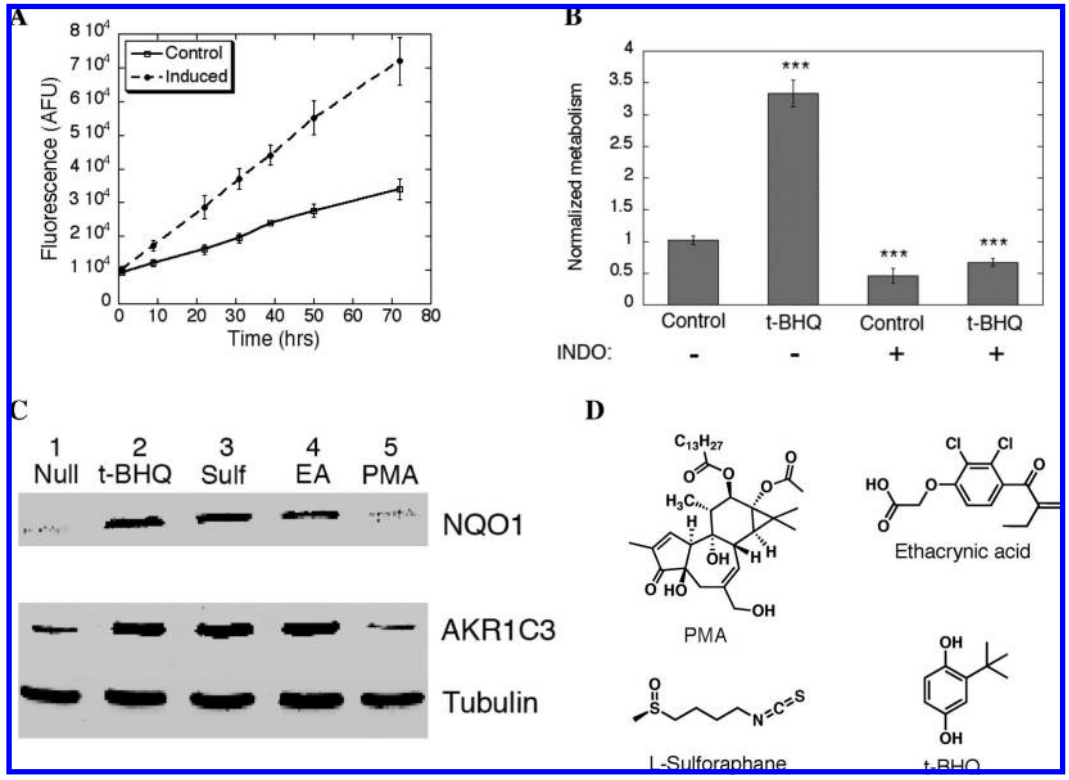

Figure 3. Selective measurement of AKR1C3 induction in human neuroblastoma IMR-32 cells. (A) Metabolism of Coumberone in IMR-32 cells when treated with DMSO vehicle (control) and *tert*-butylhydroquinone (*t*-BHQ) as monitored by fluorescence increase of the experimental media over time. (B) Normalized metabolism rate in IMR-32 cells under various conditions. The metabolism rate is defined as the fluorescence growth per hour. It is normalized to the metabolic rate under control conditions (background metabolism). Coumberone: 5 μM; INDO: 70 μM indomethacin (selective AKR1C3 inhibitor); Control: DMSO vehicle; *t*-BHQ: 10 μM for 24 h; ***: extremely significant ($P < 0.0005$) when compared to control cells, $n = 5$. (C) AKR1C3 protein expression in response to ARE inducers in IMR-32 cells as determined by Western blotting. The AKR1C3 expression changes correlate with those of NAD(P)H:quinone oxidoreductase 1 (NQO1), an established member of phase II detoxifying enzymes. Lane 1: DMSO vehicle; 2: *t*-BHQ (10 μM for 24 h); 3: sulforaphane (5 μM for 48 h); 4: ethacrynic acid (20 μM for 48 h); 5: phorbol 12-myristate 13-acetate (50 nM for 24 h). (D) Chemical structures of ARE inducers used in this study.

used for the selective optical readout of each isoform, either AKR1C2 or AKR1C3, by simple application of a selective inhibitor.

Selective Measurement of AKR1C3 Induction in Human Neuroblastoma IMR-32 Cells. To validate the real-time measurement of ARE-dependent enzyme induction in human cells, we first examined the induction of AKR1C3 with *t*-BHQ in neuroblastoma IMR-32 cells, which has previously been demonstrated on a transcriptional level.[9] Human neuroblastoma IMR-32 is an established cell line used as a model for human neurons. Through the measurement of fluorescence growth of the media over time in a 96-well plate, we found that Coumberone metabolism was indeed increased when these cells were incubated with the known ARE activator *t*-BHQ (Figure 3A). Similar induction was observed with other inducers, namely ethacrynic acid and sulforaphane (Figure S1, Supporting Information), affording a 3- to 4-fold increase in the rate of fluorescence growth for all examined compounds. Addition of indomethacin (selective AKR1C3 inhibitor) significantly suppressed the increase in probe metabolism (Figure 3B), suggesting that *t*-BHQ mainly induces expression of AKR1C3 isoform. This finding was further substantiated by measuring the inhibition

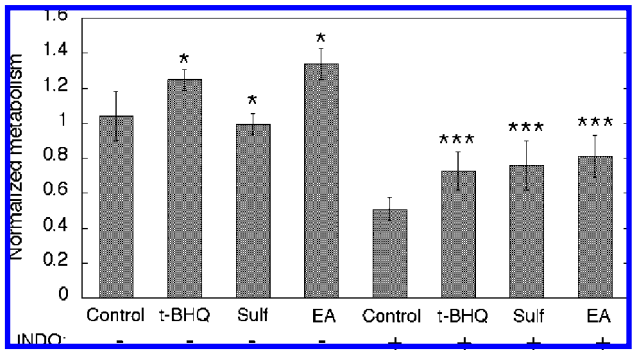

Figure 4. Selective measurement of AKR1C2 induction in human hepatoma HepG2 cells. Normalized metabolism rate in HepG2 cells under different conditions. Coumberone: 10 μM; INDO: 80 μM indomethacin; Control: DMSO vehicle; *t*-BHQ: 40 μM for 24 h; Sulf: 10 μM for 48 h; EA: 40 μM for 48 h; *: not statistically significant ($P > 0.05$); ***: extremely statistically significant ($P < 0.0005$) when compared to control cells, $n = 8$.

curve for indomethacin in IMR-32 cells (Figure S2, Supporting Information) and by Western blotting of the cell homogenates obtained after the completion of the fluorimetric measurements (Figure 3C). *Furthermore, the protein expression of AKR1C3 correlated with that of NQO1 (Figure 3*C), the current standard for measuring the activation of the ARE system, *validating AKR1C3 as a useful marker of cytoprotective enzyme induction.*

Thus, Coumberone allows for the real-time, practical measurement of AKR1C3 induction in living cells. Our method is highly sensitive, due to the amplification of the signal via catalytic turnover, enabling measurements in neuroblastoma IMR-32 cells that have very low expression levels of AKR1C protein (in comparison to highly active liver cells; see below).

Selective Measurement of AKR1C2 Induction in Human Hepatoma HepG2 Cells. To extend the applicability of our method, we investigated the human hepatoma HepG2 cell line. HepG2 cells are often used in stress-response studies and in contrast to IMR-32 cells, exhibit dramatically higher Coumberone metabolism due to high constitutive levels of AKR1C2 and AKR1C3 enzymes. The high metabolism background masks the enzyme induction, affording only small increases in probe metabolic conversion and poor contrast between the control and treated cells (Figure 4). We therefore used indomethacin to silence AKR1C3 activity, which in turn enabled selective readout of AKR1C2 induction: a significant improvement in the contrast between control and inducer-treated cells was observed (Figure 4). These results highlight the broad range and applicability of the visualization method described here, which allows the examination of AKR1C induction in cells of different origins with different inducible isoforms (AKR1C3 in IMR-32 cells and AKR1C2 in HepG2 cells) and vastly different metabolic background.

Examination of Neurotrophic Natural Products for AKR1Cs Upregulation; Panaxytriol is a Novel Inducer. Having developed and validated the new optical method for AKR1C induction in different cells, we next examined a number of natural products as potential ARE activating compounds. These substances, which include spirotenuipesines A and B,[12] scabronine G-methylester[13] and panaxytriol,[14] all exhibit neurotrophic activity (Figure 5A). We hypothesized that some of these compounds, particularly spirotenuipesine B and scabronine G-methylester, may be ARE inducers, due to the presence of electrophilic functional groups (epoxide and α,β-unsaturated aldehyde), which may undergo alkylation reaction with the cysteine residues of the Keap1 sensor protein.

Surprisingly, in IMR-32 cells, these compounds were inactive, while panaxytriol showed a robust increase in Coumberone metabolism as compared to the vehicle control ($\sim$2-fold, Figure 5B). Metabolic inhibition with indomethacin and Western blot analysis confirmed that panaxytriol induces expression of AKR1C3 in IMR-32 cells (Figure S3, Supporting Information) in a dose-responsive manner (Figure 5C and also Figure S4, Supporting Information). We also showed that panaxytriol induces AKR1C2 isoform in HepG2 cells (Figure S5, Supporting Information).

It has been demonstrated on a gene expression level that ARE activation in IMR-32 cells, induced by *t*-BHQ, is dependent on kinases PI3K and PKC.[9,15] In harmony with this finding, Coumberone metabolism in *t*-BHQ-induced cells was partially reduced in the presence of PI3K inhibitor LY294002 as well as PKC inhibitor Ro-31-8220 (Figure S6, Supporting Information), showing the responsiveness of our probe to different levels of enzyme induction. Further studies also showed that panaxytriol-induced expression of AKR1C3 was attenuated by incubation with these kinase inhibitors (Figure 5D). These results support the proposal that panaxytriol increases AKR1C3 expression in IMR-32 cells via a similar mechanism as *t*-BHQ, relying in part on PI3K and PKC kinases.

Panaxytriol is an active component of red ginseng (the steamed and dried root of *Panax ginseng* C. A. Meyer), popular folk medicine with worldwide use. Panaxytriol has been shown to be cytotoxic in a range of tumor cells in vitro and the synthetically prepared panaxytriol inhibited tumor proliferation in vivo.[14] Inhibition of mitochondrial respiration has been proposed as a potential mechanism underlying the tumorstatic activity.[16] Interestingly, panaxytriol also shows neurotrophic activity, as demonstrated by the induction of neurite growth in PC-12 cells.[14,17] It was also reported that the lipophilic fraction of ginseng extracts, which contains panaxytriol and related compounds, promotes survival of rat cortical neurons and this neurotrophic effect is dependent on PKC.[17] Our study demonstrates that the chemically homogeneous panaxytriol is a potent inducer of AKR1C enzymes in human cells, indicating its ARE activation activity, which in turn is dependent on PKC and PI3K kinases. This mechanism of action is likely to contribute to panaxytriol's neurotrophic and neuroprotective activity as well as carcinostatic activity.

Imaging AKR1C Induction in Intact Cells. The fluorimetric method for monitoring AKR1C2 and AKR1C3 induction, described above, led to identification of panaxytriol as a new inducer and allowed for quantitative characterization of its inducing potency. The method is operationally simple and readily applicable to high-throughput formats. Importantly, induction of AKR1C can also be monitored in individual living

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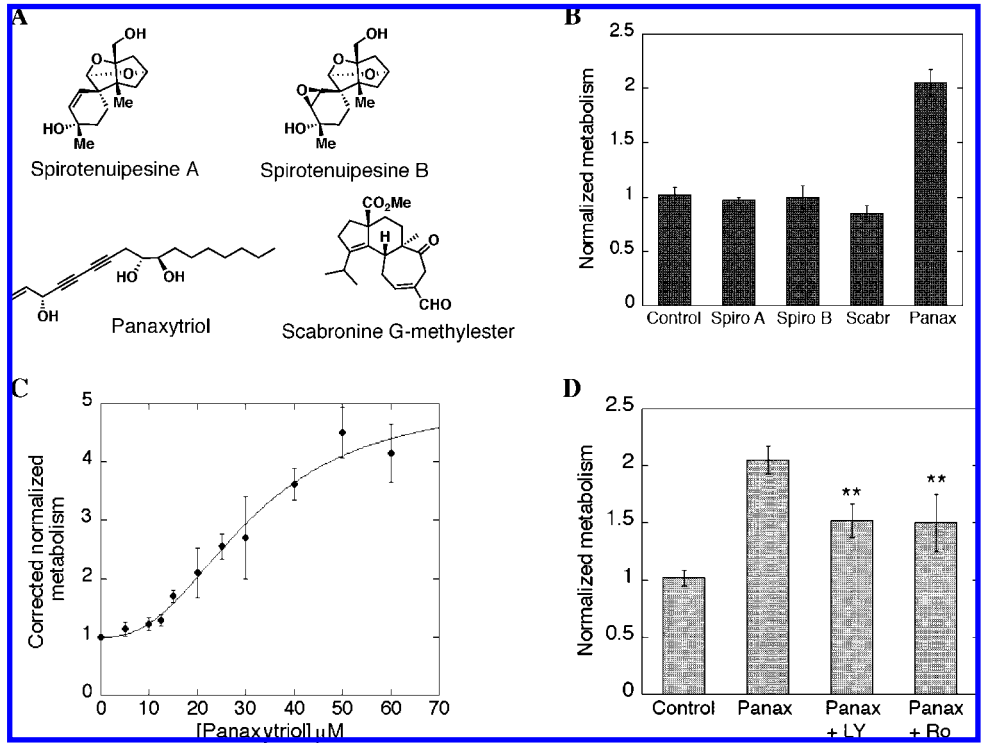

Figure 5. Examination of neurotrophic natural products reveals panaxytriol as a novel inducer of AKR1Cs. (A) Chemical structures of spirotenuipesine A, spirotenuipesine B, panaxytriol, and scabronine G-methyl ester. (B) Standardized Coumberone metabolism in IMR-32 cells. Coumberone: 5 μM; Control: DMSO vehicle; Spiro A: 1 μM spirotenuipesine A for 48 h; Spiro B: 1 μM spirotenuipesine B for 48 h; Scabr: 40 μM scabronine G-methyl ester for 24 h; Panax: 15 μM panaxytriol for 24 h; error bars are derived from 2 independent experiments on separate days. Each independent experiment consisted of 12 wells in a 96-well plate. (C) The dose-response curve for panaxytriol. Average corrected EC_{50} value for panaxytriol is 31.2 $\pm$ 4.9 μM. Average corrected EC_{50} values were calculated by dividing the normalized metabolism values for each independent experiment by the fraction of viable cells for that same experiment (see Figure S4 of the Supporting Information). Error bars are derived from three independent measurements. (D) Inhibition of PI3K and PKC kinases attenuates the panaxytriol-induced upregulation of AKR1C3. Control: DMSO vehicle; Panax: 15 μM panaxytriol for 24 h; Panax + LY: 15 μM panaxytriol and 12.5 μM LY294002 (PI3K inhibitor) ($P = 0.0066$); Panax + Ro: 15 μM panaxytriol and 1 μM Ro-31-8220 (PKC inhibitor) ($P = 0.0019$); **: very statistically significant, $n = 5$ when compared to panaxytriol-treated cells (Panax).

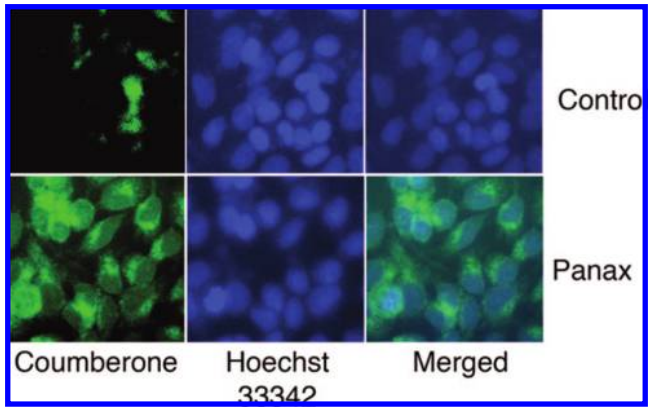

Figure 6. Imaging AKR1C induction via fluorescence microscopy in IMR-32 cells. Control: DMSO vehicle; Panax: 20 μM panaxytriol for 24 h. Pictures were taken 24 h after Coumberone (5 μM) was added. One hour before imaging, Hoechst 33342 (250 nM) was added to stain the nuclei.

cells via fluorescence microscopy (Figure 6). Coumberone enables real-time imaging of AKR1C induction in a variety of cells, including those with low metabolic activities (IMR-32 cells). Thus, many different fluorescence detection formats can be used, depending on the model system of interest.

Conclusions and Discussion

In conclusion, we have developed a new optical method for monitoring the induction of AKR1C2 and AKR1C3 enzymes in living cells. It is now well established that AKR1Cs are part of the inducible battery of cytoprotective genes regulated by the ARE cis-element and, therefore, the new method described above is suitable for discovery and examination of new ARE activators. In the two cells lines used in this study, human neuroblastoma IMR-32 and human liver HepG2 cells, it has been shown that the ARE-regulated transcription is dependent on the activation of the Nrf2 transcription factor. Thus, in cells where the Nrf2-ARE-AKR1C causal link has been established, this new method may be used for indication of Nrf2 activation in intact cells and in real-time. The method is operationally simple, requiring only the addition of Coumberone to living cells and measuring fluorescence increase over time via fluorimetry or fluorescence microscopy. Metabolic probe Coumberone, applied together with appropriate inhibitors, allows for selective readout of either AKR1C2 or AKR1C3 isoform, both of which are part of the inducible set of protective enzymes. The method has both high sensitivity (due to the amplification of the signal) and broad dynamic range, as demonstrated by induction studies in different cell lines with dramatically different metabolic capabilities (transfected monkey kidney COS-1 cells, human neuroblastoma IMR-32, and human liver HepG2 cells). Using this method, we have discovered that panaxytriol is an AKR1C inducer, which may account for the dual function of panaxytriol as a carcinostatic and neurotrophic agent. The protective effects of ARE inducers against tumorgenesis and neurodegeneration fuel the growing interest in this

area of research and the method described here will greatly enable these endeavors.

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Supporting Information Available: Enzymology assay, cellular studies, and fluorescence microscopy imaging. This material is available free of charge via the Internet at http://pubs.acs.org.

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