Highly Potent Activation of Nrf2 by Topical Tricyclic Bis(Cyano Enone): Implications for Protection against UV Radiation during Thiopurine Therapy

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Abstract

Chronic treatment with azathioprine, a highly effective anti-inflammatory and immunosuppressive agent, profoundly increases the risk for development of unusually aggressive cutaneous squamous cell carcinoma. Its ultimate metabolite, 6-thioguanine (6-TG) nucleotide, is incorporated in DNA of skin cells, and upon exposure to UVA radiation, causes oxidative stress, followed by damage of DNA and associated proteins. The acetylenic tricyclic bis(cyano enone) TBE-31 is a strong inhibitor of inflammation and a potent inducer of the Keap1/Nrf2/ARE pathway, which orchestrates the expression of a large network of cytoprotective genes. We now report that long-term (five days per week for four weeks) topical daily applications of small (200 nmol) quantities of TBE-31 cause a robust systemic induction of the Keap1/Nrf2/ARE pathway and decreases the 6-TG incorporation in DNA of skin, blood, and liver of azathioprine-treated mice, indicating extraordinary bioavailability and efficacy. In addition, TBE-31, at nanomolar concentrations, protects cells with 6-TG in their genomic DNA against oxidative stress caused by UVA radiation through induction of the Keap1/Nrf2/ARE pathway. At the same 6-TG DNA levels, Keap1-knockout cells, in which the pathway is constitutively upregulated, are highly resistant to UVA radiation–induced oxidative stress. The protective effects of both the Keap1-knockout genotype and TBE-31 are completely lost in the absence of transcription factor Nrf2. Our findings suggest that compounds of this kind are excellent candidates for mechanism-based chemoprotective agents against conditions in which oxidative stress and inflammation underlie disease pathogenesis. Moreover, their potential skin patch incorporation for transdermal delivery is an exciting possibility. Cancer Prev Res; 5(7); 973–81. ©2012 AACR.

Introduction

The Keap1/Nrf2/ARE pathway determines the ability of mammalian cells and organisms to adapt and survive conditions of oxidative, electrophilic, and inflammatory stress by regulating the expression of a network of more than 100 cytoprotective genes. Under basal conditions, this pathway does not operate at its maximal capacity but is highly inducible by various stress stimuli and also by small molecules, all of which have the ability to react with sulphydryl groups (1, 2). Inducers react with specific cysteine residues of the protein sensor Kelch-like ECH-associated protein 1 (Keap1) which loses its ability to target transcription factor NF-E2–related factor 2 (Nrf2) for ubiquitination and proteasomal degradation, resulting in stabilization of Nrf2, binding to the antioxidant response element (ARE) and transcriptional activation of cytoprotective genes, such as NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferases (GST), heme oxygenase 1, thioctic reductase, aldo-keto reductases (3–6). Inducers are also anti-inflammatory agents, and there is a linear correlation between these two biologic activities that spans 6 orders of magnitude of inducer concentrations (7, 8). Semisynthetic pentacyclic triterpenoids (9–11) and related synthetic tricyclic compounds (12–15) which contain Michael acceptors are the most potent inducers known to date, activating Nrf2 and inhibiting proinflammatory responses at sub- to low nanomolar concentrations (7, 16, 17). Furthermore, they show remarkable protective efficacy in a number of preclinical animal models of chronic disease, including carcinogenesis, cardiovascular disease, and neurodegeneration, and in early clinical trials (reviewed in refs. 15, 18, 19).
Among the tricyclic compounds, the acetylenic Tricyclic Bis(cyano Enone) TBE-31 [(±)-4bS,8aR,10aS)-10a-ethyl-4b,8,8-trimethyl-3,7-dioxo-3,4b,7,8,8a,9,10,10a-octahydrophenanthrene-2,6-dicarbonitrile; Fig. 1], bearing two highly reactive Michael acceptor groups within a rigid 3-ring system, is an extremely potent inductor (17).

We are interested in the development of potent inducers of the Keap1/Nrf2/ARE pathway as mechanism-based chemoprotective agents for reducing the risk for skin cancer that is associated with thiopurine therapies. Whereas the thiopurines (azathioprine, 6-mercaptopurine, and 6-thioguanine) are highly effective anti-inflammatory, immunosuppressive, and anticancer agents, their long-term use is associated with increased risk (in some cases by more than 100-fold) for the development of squamous cell carcinoma of the skin (20–23). Within an individual patient, the tumors are usually multiple, affect large areas of the skin, frequently relapse, and often metastasize, causing a very significant morbidity and even mortality (24). Although immunosuppression, in general, is a risk factor for skin cancer development, the use of one of the most commonly prescribed thiopurines, azathioprine, is causally related to increased sensitivity of the human skin to UVA radiation, which comprises more than 90% of the incident solar UV radiation. This direct relationship was recently shown by the observed reversal of UVA-mediated skin photosensitivity and DNA damage in renal transplant recipients by replacing azathioprine with mycophenolate mofetil (25). Nonetheless, the use of thiopurines in organ transplantation, as well as in the management of inflammatory and autoimmune diseases, is anticipated to continue, as they are highly effective and generally well tolerated (20). In addition, patients who have been on these drugs for many years and their doctors are reluctant to change the immunosuppressive therapy and potentially risk the transplant.

Azathioprine is a prodrug that undergoes a series of biotransformation steps to ultimately give 6-thioguanine (6-TG) nucleotide that is incorporated into DNA and RNA. DNA that contains 6-TG absorbs UVA radiation, generating reactive oxygen intermediates (ROI) and 6-TG photo-oxidation products (26, 27) that damage DNA and associated proteins (28–30). Stable covalent DNA–protein cross-links have been detected in cells with 6-TG in their DNA (31). Importantly, these cross-links involve a number of DNA repair proteins, such as the MSH2 mismatch repair protein and the XPA nucleotide excision protein, strongly suggesting that, in addition to being mutagenic, the combined action of 6-TG and UVA radiation may also compromise DNA repair (31). More than 25 years ago, Lennard and colleagues (32) reported an association between increased 6-TG nucleotide concentrations in erythrocytes with the occurrence of actinic keratoses and malignant skin tumors in renal transplant recipients treated with azathioprine. On the basis of these findings, the investigators suggested that skin cancer in these patients is caused by DNA damage in skin cells, which, in turn, is related to 6-TG incorporation and exposure to UV radiation. More recently, the use of azathioprine over a period of just 3 months was shown to increase the skin photosensitivity to UVA radiation in inflammatory bowel disease patients (28, 33). Lymphocytes and skin cells of patients undergoing therapies with azathioprine or 6-mercaptopurine contain 6-TG in their DNA at levels corresponding to approximately 0.02% of the total dG content (or ~100,000 DNA 6-TG per cell; refs. 28, 34, 35). We have observed a very similar level of 6-TG incorporation in skin DNA isolated from SKH-1 hairless mice that had been treated orally with azathioprine (36). In the same study, we also found that genetic upregulation of the Keap1/Nrf2/ARE pathway (by Keap1-knockdown) reduces the 6-TG DNA levels in murine skin and liver by approximately 30%. The main goal of the present investigation was to establish whether, similar to genetic upregulation, pharmacologic activation of this pathway by the potent inducer TBE-31 also affects the incorporation of 6-TG in skin DNA of azathioprine-treated mice. In addition, we tested the hypothesis that even at the same level of substitution of dG by 6-TG in genomic DNA, the Keap1/Nrf2/ARE pathway plays a critical role in determining the susceptibility of cells to UVA-mediated oxidative stress, and that protection by either genetic or pharmacologic activation of this pathway extends beyond its effects on the incorporation of 6-TG in DNA.

Materials and Methods

Materials

TBE-31 was synthesized as described previously (14, 15). All chemicals and cell culture reagents were of the highest purity available and were obtained from common commercial suppliers.

Cell cultures

Mouse embryonic fibroblasts (MEF), originally isolated from wild-type, Nrf2-knockout, Keap1-knockout, and Nrf2/Keap1-double knockout C57BL/6 mice, were cultured in plastic dishes coated for 30 minutes with 0.1% (w/v) gelatin before use. MEF cells were grown in Iscoves modified Dulbecco’s medium (with l-glutamine) supplemented with human recombinant EGF (10 ng/ml),...
1 × insulin/transferring/selenium, and 10% (v/v) heat-inactivated FBS, all from Invitrogen. Kera-308 murine keratinocytes, obtained from Cell Lines Service and used within 6 months after purchase, were grown in minimum essential medium (Eagle) supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) non-essential amino acids, and 2 mmol/L l-glutamine. Cell cultures were maintained in 5% CO2 at 37°C.

**Animals and treatments**

All animal experiments were carried out in accordance with the regulations described in the U.K. Animals (Scientific Procedures) Act 1986. SKH-1 hairless mice, initially obtained from Charles River (Germany), were bred in our facility. The experimental animals (8- to 12-week-old, female) were kept on a 12-hour light/12-hour dark cycle, 35% humidity, in individually ventilated cages, and were given free access to water and food (pelleted or powdered RM1 diet from SDS Ltd.).

Stock solutions of azathioprine (Sigma-Aldrich Co.,) were freshly prepared in 0.05 N NaOH and diluted at a ratio of 1:500 (v/v) into the drinking water to a final concentration of 61.25 μg of azathioprine per mL of water. The water bottles were changed 3 times per week and were kept wrapped in aluminum foil at all times to avoid exposure to light. For oral administration of TBE-31, the compound was thoroughly mixed with powdered RM1 diet (27.6 mg of TBE-31 per kg of food, thus delivering 0.3 μmol of TBE-31 per mouse per day) and kept at 4°C. The mouse feeders were refilled daily with this mixture. Control RM1 diet was stored and delivered to the animals under identical conditions. For topical administration, 0.2 μmol of TBE-31 was applied in 200 μL of 80% acetone (v/v) over the entire back (dorsal skin) of the mouse, at 24-hour intervals, 5 days per week for 4 weeks. At the end of each experiment, the animals were euthanized and blood was drawn by cardiac puncture. Liver and skin were harvested, rapidly frozen in liquid N2, and stored at −80°C until analysis.

**Biochemical analyses**

Frozen tissue (~30 mg) was pulverized under liquid N2. The resulting powder was reconstituted in ice-cold 100 mmol/L potassium phosphate buffer, pH 7.4, containing 100 mmol/L KCl and 0.1 mmol/L EDTA. After mechanical homogenization in an ice bath, the resulting homogenates were subjected to centrifugation at 4°C (15,000 × g for 10 minutes). The supernatant fractions were used for determination of protein concentrations (37) and the enzyme activities of NQO1 with menadione as a substrate (38) and of GST with CDNB as a substrate (39). The same assay was used to determine the enzyme activities of NQO1 and GST in total cell lysates prepared in digitonin (0.8 g/L in 2 mmol/L L EDTA, pH 7.8).

**Quantitative reverse transcriptase PCR**

The primers and probe used to measure mRNA for MRP4 (40) were synthesized by MWG-Biotech UK Ltd. Total RNA from mouse liver and skin was extracted using RNeasy and RNeasy Fibrous Tissue Kit (Qiagen Ltd.), respectively. Total RNA (500 ng) was reverse transcribed into cDNA with Omniscript Reverse Transcription Kit (Qiagen Ltd.). Real-time PCR was carried out on Perkin Elmer/Applied Biosystems Prism Model 7700 Sequence Detector instrument. The TaqMan data for the mRNA species were normalized using β-actin (mouse ACTB, 435293E) as an internal control.

**Determination of 6-TG incorporation in DNA**

Frozen skin or liver tissue (~100 mg) was crushed in liquid N2, and DNA was extracted. Due to limited quantities of blood, DNA that was extracted from 2 to 3 blood samples was pulled together before the oxidation step, such that the final number of independently analyzed samples was 3 for each group. The levels of 6-TG in DNA were determined as described (28, 36): extracted DNA was oxidized with magnesium bis(monoperophosphophylate) MMPP in the dark for 30 minutes at room temperature and ethanol precipitated. Double-stranded DNA (120 μg) was denatured by heating at 90°C for 5 minutes, cooled on ice, and digested with 24U nuclease P1 (1 U/μL) for 1 hour at 50°C. After adjusting the pH to 8.0 with 20 μL of 1 mol/L Tris-Cl buffer (pH 8.0), the sample was incubated with alkaline phosphatase (2U) for 1 hour at 37°C. The resulting deoxynucleosides were separated by reverse phase high-performance liquid chromatography (HPLC) on Ascentis C18 column (Supelco, 250 mm x 4.6 mm, 5 μm) using Agilent 1100 system equipped with Agilent G1314A variable wavelength detector and Agilent G1321A fluorescence detector. A 30-mer single-stranded oligodeoxyribonucleotide (from Oligo Etc.), containing one 6-TG and four Gs, was used to construct the standard curves, following MMPP oxidation and nuclease P1/alkaline phosphatase digestions. Elution was with a gradient of 10 mmol/L KH2PO4 (pH 6.7) in methanol. The amount of GSO3dR was quantified by fluorescence (excitation 320 nm/emission 410 nm). After a 1:20 dilution, the dG content of the same sample was determined by absorbance at 260 nm.

**Exposure to UVA radiation: Determination of ROIs**

MEFs (150,000 per well) and Kera-308 keratinocytes (250,000 per well) were grown in 6-well plates. Cells were then treated with 50 nmol/L TBE-31 for 18 to 24 hours, after which they were exposed to 0.5 μmol/L 6-TG in the absence of TBE-31 for a further 24 hours. The growth medium was removed, cells were washed 3 times with Hank’s balanced salt solution (HBSS), and incubated with 10 μmol/L 2',7'-dichlorodihydrofluorescein diacetate (Invitrogen Ltd) in 1.0 mL of HBSS for 30 minutes. The buffer containing the fluorescent probe was removed, and the cells were washed 3 times with HBSS. Exposure to UVA radiation (3 J/cm2) was in 1.0 mL of HBSS using lamps with an emission spectrum of 320 to 420 nm. The UVB portion of the radiation source (<0.05%) was filtered out by use of a 7-mm thick glass plate. The irradiance at the surface of the cells, at a distance of 300 mm from the lamp, was measured with a Waldmann UV meter calibrated to the source using a double-grating...
spectroradiometer (Bentham Instruments Ltd). Sham-irradiated cells were kept alongside irradiated cells but were wrapped in aluminum foil. The generation of ROI was quantified 15 minutes postirradiation by the fluorescence intensity of the oxidized probe using a microtiter plate reader (SpectraMax M2, Molecular Devices) with excitation at 485 nm and emission at 530 nm.

Statistical analysis

All values are means ± 1 SD. The differences between groups were determined by Student t test.

Results and Discussion

Topical application of TBE-31 potently induces cytoprotective proteins in mouse skin and liver and reduces the incorporation of 6-TG in DNA

Incorporation of 6-TG in skin DNA is a critical factor that contributes to the increased skin photosensitivity and cutaneous cancer risk of patients treated with azathioprine (28, 32, 33). Compared with their wild-type counterparts, Keap1-knockdown mice, in which the Keap1/Nrf2/ARE pathway is constitutively upregulated, have 30% lower levels of 6-TG in their DNA (36). As TBE-31 is a very potent inducer of the Keap1/Nrf2/ARE pathway both in vitro and in vivo (16, 17), we hypothesized that, similar to genetic upregulation of the pathway, pharmacologic induction by TBE-31 may affect the levels of 6-TG in DNA of wild-type mice that are receiving azathioprine. To test this hypothesis, 2 groups of SKH-1 hairless mice were treated with azathioprine in the drinking water for 3 weeks. One week before and throughout the period of azathioprine treatment, one group of mice received topically (on the dorsal skin) daily 200 nmol applications of TBE-31, 5 days a week, for 4 weeks. The control animals received vehicle (80% acetone, v/v) treatment. To estimate the magnitude of induction of the Keap1/Nrf2/ARE pathway in dorsal skin, the site of TBE-31 application, we determined the enzyme activities of the Nrf2-target gene products NQO1 and GST and quantified the mRNA levels of multidrug resistance-associated protein 4 (MRP4/ABCC4). Compared with dorsal skin of control animals, the NQO1 and GST activities were 8-fold (P < 0.0001) and 2-fold (P < 0.0001) higher, respectively, and the MRP4 mRNA levels were increased by 1.3-fold (P = 0.0002), in dorsal skin of mice that received TBE-31 (Fig. 2A–C). Surprisingly, NQO1, GST, and MRP4 in ventral skin were also upregulated and essentially to the same degree as in dorsal skin, by 7.2-fold (P < 0.0001), 2-fold (P < 0.0001), and 1.4-fold (P = 0.005), respectively. This finding was surprising as the compound was only applied to the dorsal

![Figure 2](image-url)

Figure 2. TBE-31 potently induces the Keap1/Nrf2/ARE pathway in mouse skin and liver. Groups of SKH-1 hairless mice (n = 7) received daily doses of TBE-31 (black bars) either topically (200 nmol in 200 μL of 80% acetone, v/v) to their dorsal skin (A–C) or orally (300 nmol per 3 grams of powdered RM1 diet) in their food (D–F) for 4 weeks. The corresponding control groups (white bars) received either 80% acetone (v/v) or identical powdered RM1 diet without TBE-31. One week within the treatment period, the animals were given azathioprine at a concentration of 62.5 μg/mL in the drinking water for a further 3 weeks. The specific activities of NQO1 (Treated/Control) were measured in tissue homogenates of skin and liver. Means ± SD are shown. C and F, the amount of mRNA for MRP4 was analyzed by quantitative reverse transcriptase PCR, using b-actin mRNA as the internal control. In each group, the mRNA from the individual mice was measured separately, in triplicate. Data represent means ± SD and are expressed as treated to control ratios. * P < 0.0001; ** P < 0.001; *** P < 0.01.
skin of the mice. It indicated that TBE-31, when administered under this treatment schedule, may have systemic effects. To address this possibility, we determined the levels of NQO1, GST, and MRP4 in liver and found that they were all highly upregulated by 4.7-fold \((P < 0.0001)\), 2.8-fold \((P < 0.0001)\), and 3.3-fold \((P = 0.007)\), respectively. Remarkably, these effects were comparable with the systemic effects of dietary TBE-31 which upregulated hepatic NQO1, GST, and MRP4 by 2.2-fold \((P < 0.0001)\), 3-fold \((P < 0.0001)\), and 3-fold \((P = 0.0006)\), respectively (Fig. 2D–F). In samples of dorsal and ventral skin, the corresponding increases in NQO1 and GST activities by dietary TBE-31 were 2.7-fold \((P < 0.0001)\) and 1.5-fold \((P < 0.0001)\), whereas the MRP4 levels were unchanged. Of note, in our previous experience (unpublished), such systemic effects on any of these systems were not observed when sulforaphane, another potent Nrf2 activator, was topically administered under similar experimental conditions.

MRP4 participates in the export of nucleoside monophosphate analogs \((41–43)\), and its inducible gene expression is known to be regulated by Nrf2 \((40, 44, 45)\). Furthermore, silencing of mrp4 increases the 6-TG incorporation in DNA of Hepa1c1c7 cells treated with this thiopurine \((36)\). As TBE-31 caused an upregulation of the MRP4 mRNA levels, we next evaluated the effect of topical treatment with TBE-31 on the incorporation of 6-TG in DNA isolated from skin and liver of azathioprine-treated mice. As expected, the 6-TG levels in skin DNA were much higher than the corresponding levels in liver (Fig. 3), consistent with our previous observations \((36)\). Compared with control animals, the levels of 6-TG in DNA isolated from ventral skin of TBE-31-treated animals were reduced by 30\% \((P = 0.023)\). Incorporation of 6-TG in liver DNA was also affected by TBE-31 and to the same extent as it was in ventral skin \((30\%, P = 0.006)\). Most notably, the largest effect was on dorsal skin, the site of TBE-31 application, where the DNA 6-TG levels were reduced by 50\% \((P < 0.0001)\). We then determined the incorporation of 6-TG in DNA isolated from blood. In the control group, the levels of 6-TG in blood DNA were similar to the 6-TG levels in skin DNA, in close agreement with studies in human subjects \((28)\). In resemblance with the effect of TBE-31 on 6-TG incorporation in DNA isolated from skin, the 6-TG DNA levels in blood of mice that had been treated topically with TBE-31 were approximately 50\% lower \((P = 0.01)\) than those in blood of control animals. Notably, the effect of TBE-31 on 6-TG incorporation in DNA of dorsal skin is larger than the local effect on MRP4, implying that, in addition to MRP4, there are other (presently unidentified) contributing factors. Most importantly, because of the direct link between 6-TG incorporation in DNA and increased skin photosensitivity to UVA radiation \((25, 28, 33)\), the ability of TBE-31 to decrease the levels of 6-TG in skin DNA is expected to lower one of the main risk factors for skin cancer development associated with thiopurine therapies.

**Figure 3.** Topical application of TBE-31 reduces the incorporation of 6-thioguanine in DNA of mouse skin, liver, and blood. Two groups of SKH-1 hairless mice \((n = 7)\) received daily topical applications to their dorsal skin of either TBE-31 \((200 \text{ nmol in } 200 \mu \text{L, black bars})\) or vehicle \((80\% \text{ acetone, v/v, white bars})\) for 4 weeks. One week within the treatment period, both groups of animals were given azathioprine at a concentration of 62.5 \(\mu \text{g/mL}\) in the drinking water for another 3 weeks. At the end of the experiment, the animals were euthanized, blood was collected by cardiac puncture, and liver and skin were harvested. The incorporation of 6-thioguanine in DNA was quantified by the fluorescence of guanine sulfonate deoxyriboside (GSO3dR) following DNA extraction, oxidation, digestion, and HPLC separation. Means ± SD are shown. *, \(P < 0.0001\); **, \(P < 0.01\); ***, \(P < 0.05\).
resistant to 6-TG sensitization, and the ROI levels in these cells were only slightly higher (by 35%) than the corresponding levels of ROI in UVA-irradiated cells that had not been treated with 6-TG (Fig. 5A). The protective effect of the Keap1-knockout genotype was completely abolished in the absence of Nrf2, and in Keap1/Nrf2-double knockout cells, the degree of sensitization by the 6-TG treatment was even greater (8-fold) than in their wild-type counterparts. These results showed that the constitutive upregulation of Nrf2-dependent cytoprotective proteins caused by the absence of Keap1 enhances resistance, and conversely, their down-regulation because of the absence of Nrf2 increases susceptibility to the damaging effect of the combination of 6-TG and UVA radiation.

We have previously reported that TBE-31, at nanomolar concentrations, protects cells against peroxynitrite toxicity and showed the essential requirement for Nrf2 for protection (46). We next asked whether TBE-31 could protect cells with 6-TG in their genomic DNA against oxidative stress caused by UVA radiation. When wild-type MEFs were exposed to TBE-31 for 18 hours before treatment with 6-TG and then irradiated 24 hours later, there was a significant reduction (by 40%) in the formation of ROI in comparison with cells that were not exposed to the protective agent (Fig. 5A). In DNA of TBE-treated cells, the 6-TG incorporation was slightly lower (by 17%) than in control cells (Fig. 4B, black bars), perhaps making a small contribution to, but not fully accounting for the protective effect. Notably, because

Figure 4. Exposure to 6-TG of cultured cells leads to a dose-dependent incorporation of 6-TG nucleotide in DNA. A, wild-type MEFs (150,000 per well) were grown in 10-cm dishes for 24 hours. The medium was changed, and the cells were exposed to different concentrations of 6-thioguanine for a further 24 hours. Data represent average values of 2 to 3 independent experiments. B, MEFs were plated on 10-cm dishes. Twenty-four hours later, they were treated with either vehicle (0.1% acetonitrile, white bars) or 0.05 µmol/L TBE-31 (black bars) for 24 hours. The medium was removed, and the cells were then exposed to 6-thioguanine, in the absence of TBE-31, for a further 24 hours. The incorporation of 6-thioguanine in DNA was quantified by the fluorescence of guanine sulfonate deoxyriboside (GSO3dR) following DNA extraction, oxidation, digestion, and HPLC separation. Means ± SD are shown (n = 3). Results are representative of 3 independent experiments. WT, wild-type; K0, Keap1-knockout, K0N0, Keap1/Nrf2-double knockout cells. *, P < 0.001.

Figure 5. TBE-31 protects UVA-irradiated 6-TG–treated MEFs against the generation of ROI via induction of the Keap1/Nrf2/ARE pathway. MEFs (150,000 per well) were plated on 6-well plates. Six hours later, they were treated with either vehicle (0.1% acetonitrile, white bars) or 50 nmol/L TBE-31 (black bars) for 18 hours. The medium was removed, and the cells were then exposed to either vehicle (0.00005 N NaOH) or 0.5 µmol/L 6-TG for a further 24 hours. After loading with 2',7'-dichlorodihydrofluorescein diacetate, the cells were washed with HBSS and exposed to UVA (3 J/cm²) in 1.0 mL of HBSS. A, ROIs generated by the UV radiation were quantified by the fluorescence intensity of the probe 30 minutes postirradiation. B, the enzyme activity of NQO1 was measured in lysates prepared from sham-irradiated control cells. Data are expressed as ratio of wild-type control cells. Means ± SD are shown (n = 3). Results are representative of 3 independent experiments. WT, wild-type; K0, Keap1-knockout, K0N0, Keap1/Nrf2-double knockout cells. *, P < 0.001.
TBE-31 had been removed from the cell culture medium 24 hours before irradiation, it is very unlikely to have had a direct antioxidant effect. The more probable cause for its protective effect is its indirect antioxidant activity, that is, the upregulation of the Keap1/Nrf2/ARE pathway. This conclusion is further supported by the fact that, in sharp contrast to wild-type cells, TBE-31 did not show protective effect in any of the mutant cell lines and even led to higher ROI formation in Nrf2-deficient (K0N0) cells (Fig. 5A, black bars), most likely due to the low glutathione levels in the these cells (47).

Evaluation of the enzyme activity of the prototypic Nrf2-dependent cytoprotective protein NQO1 confirmed induction of the pathway in wild-type cells: the levels of NQO1 were increased by 4-fold (Fig. 5B) at the time when the cells were irradiated. As expected, the NQO1 activity was very high (more than 10 times wild type) in Keap1-knockout MEFs, whereas the activity of this enzyme was low and not inducible in Nrf2/Kepap1-double knockout cells. Of note, although TBE-31 had no significant additional effect on the NQO1 activity in Keap1-knockout cells in the absence of 6-TG, a slight (20%) upregulation was observed in 6-TG-treated cells. The powerful protective effect of TBE-31 was also clearly evident in 6-TG-treated and UVA-irradiated keratinocytes (Kera-308), and there was a highly significant reduction (by 60%) in the formation of ROI in TBE-31–treated keratinocytes in comparison with cells that were not exposed to the protective agent (Fig. 6A). Similar to the responses in wild-type MEFs, the activities of the prototypic Nrf2-dependent enzymes NQO1 and GST were induced by 6.5- and 2-fold, respectively, when keratinocytes were exposed to 50 nmol/L TBE-31 (Fig. 6B and C).

Taken together, the results from these experiments showed that: (i) TBE-31, at nanomolar concentrations, protects against oxidative stress caused by the synergistic action of 6-TG and UVA radiation, and (ii) the protective effect of TBE-31 is due to activation of the Keap1/Nrf2/ARE pathway.

**Conclusions**

At nanomolar concentrations, the potent inducer TBE-31 protects cells that contain 6-TG in their genomic DNA against oxidative stress caused by UVA radiation by upregulating the Keap1/Nrf2/ARE pathway. In addition, small daily doses of TBE-31 reduce the incorporation of 6-TG in the skin of mice that are being treated with azathioprine. Thus, this acetylenic tricyclic compound lowers two of the risk factors that contribute to the development of skin carcinogenesis associated with thiopurine therapies. The challenges ahead are to be able to optimize the dosing regimens and develop a suitable delivery vehicle to achieve only local skin-specific effects, avoiding any possibility for interference with the therapeutic efficacy of the concurrent treatments. In preliminary experiments, we have found that this could be achieved by topically applying lower and intermittent (e.g., twice a week) rather than chronic doses. In a broader context, the remarkable potency and bioavailability of TBE-31 suggest that its potential protective role should be further explored in animal models of chronic diseases with known oxidative stress component contributing to their pathogenesis. Furthermore, transdermal drug delivery offers numerous advantages over oral administration, such as reduced first-pass drug degradation, lower liver toxicity, and fewer adverse events (48). However, mainly because of the efficient barrier properties of the skin which have evolved to minimize water loss and exclude entry of environmental toxins into the body, there are currently only 11 approved drugs for which this delivery route is possible (48). Thus, our findings offer the exciting possibility for the development of TBE-31 as a pharmaceutical agent that could potentially be delivered in a transdermal patch.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: S. Kalra, T. Honda, A.T. Dinkova-Kostova
Development of methodology: S. Kalra, A.T. Dinkova-Kostova
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Kalra, E.V. Knatko, M. Yamamoto, A.T. Dinkova-Kostova
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Kalra, E.V. Knatko, A.T. Dinkova-Kostova
Writing, review, and/or revision of the manuscript: E.V. Knatko, T. Honda, A.T. Dinkova-Kostova
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Zhang, T. Honda, A.T. Dinkova-Kostova
Study supervision: A.T. Dinkova-Kostova

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