Oral Azathioprine Leads to Higher Incorporation of 6-Thioguanine in DNA of Skin than Liver: The Protective Role of the Keap1/Nrf2/ARE Pathway

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Abstract
Azathioprine is a widely used anti-inflammatory, immunosuppressive, and anticancer agent. However, chronic treatment with this drug is associated with a profoundly increased risk (in certain cases by more than 100-fold) of developing squamous cell carcinoma of the skin. Incorporation of its ultimate metabolite, thio-dGTP, in DNA results in partial substitution of guanine with 6-thioguanine which, combined with exposure to UVA radiation, creates a source of synergistic mutagenic damage to DNA. We now report that oral treatment with azathioprine leads to a much greater incorporation of 6-thioguanine in DNA of mouse skin than liver. These higher levels of 6-thioguanine, together with the fact that the skin is constantly exposed to UV radiation from the sun, may be responsible, at least in part, for the increased susceptibility of this organ to tumor development. Genetic upregulation of the Keap1/Nrf2/ARE pathway, a major cellular regulator of the expression of a network of cytoprotective genes, reduces the incorporation of 6-thioguanine in DNA of both skin and liver following treatment with azathioprine. Similarly, pharmacologic activation of the pathway by the potent inducer sulforaphane results in lower 6-thioguanine incorporation in DNA and protects 6-thioguanine–treated cells against oxidative stress following exposure to UVA radiation. Protection is accompanied by increased levels of glutathione and induction of multidrug resistance-associated protein 4, an organic anion efflux pump that also exports nucleoside monophosphate analogues. Our findings suggest that activation of the Keap1/Nrf2/ARE pathway could reduce the risk for skin cancer in patients receiving long-term azathioprine therapy. Cancer Prev Res; 4(10); 1665–74. ©2011 AACR.

Introduction
The thiopurines azathioprine, 6-mercaptopurine, and 6-thioguanine are highly effective anti-inflammatory, immunosuppressive, and anticancer agents. However, their long-term use is associated with increased risk of skin cancer (1). This is especially problematic for the population of solid organ transplant recipients, for whom the skin cancer risk is 100-fold greater than for the general population. The risk increases with duration of immunosuppression (2), whereas cessation of immunosuppressive therapy leads to deceleration of skin carcinogenesis (3), but also to transplant rejection. A trend toward an increase in risk for squamous cell carcinoma of the skin is also seen in patients receiving long-term azathioprine therapy for inflammatory bowel disease, and especially Crohn’s disease (4). Premalignant lesions are clinically treated with an array of therapies, including chemotherapy, topical immunomodulatory and anti-inflammatory agents, cryotherapy, surgical excision, and photodynamic therapy (5, 6). However, their use is only partially successful because the lesions are multiple, span large areas, and frequently relapse. More than 100 lesions may develop in a single paient within 1 year, with a high risk of metastasis (2). For these populations, skin cancer represents a major source of morbidity and mortality. Thus, detailed knowledge of the potential risk factors and development of new strategies for protection is urgently needed.

Azathioprine is a prodrug that is first metabolized to 6-mercaptopurine, which enters the purine salvage pathway and is ultimately converted to a thioguanine nucleotide and incorporated into DNA and RNA (Fig. 1; ref. 1). Elegant studies conducted by Peter Karran and his colleagues have revealed that the combination of 6-thioguanine and UVA radiation generates reactive oxygen intermediates (ROI) and is synergistically mutagenic in cells and that
treatment with azathioprine increases the skin photosensitivity to UVA radiation in humans (7). We have previously shown that pharmacologic induction of the Keap1/Nrf2/ARE pathway, a major regulator of the expression of a network of cytoprotective genes, protects cells against UVA-mediated generation of ROI and inhibits skin tumor development in SKH-1 hairless mice (8, 9). As its name suggests, this pathway has 3 essential components: (i) antioxidant response elements (ARE), specific sequences in the upstream regulatory regions of cytoprotective genes; (ii) Nrf2, a basic leucine zipper transcription factor responsible for both basal and inducible expression of cytoprotective genes; and (iii) Keap1, the sensor and chemical target for inducers. Under basal conditions, Keap1 forms a complex with Cul3 and binds Nrf2, thereby presenting Nrf2 for ubiquitination and proteasomal degradation. Keap1 has highly reactive cysteine residues that are chemically modified by inducers, resulting in conformational changes that abrogate its capacity to target Nrf2 for degradation; consequently, Nrf2 accumulates and translocates to the nucleus where, in heterodimeric combination with a different Maf protein, activates transcription of cytoprotective genes, including those that encode antioxidant and drug-metabolizing enzymes (10–12). Induction protects against toxicity and carcinogenicity; indeed, a number of small-molecule inducers of this pathway inhibit tumor development in various animal models (13). In this study, we asked whether: (i) incorporation of 6-thioguanine in DNA following oral azathioprine treatment could be modeled in the mouse, (ii) upregulation of the Keap1/Nrf2/ARE pathway affects this incorporation, and (iii) pharmacologic activation of this pathway could be used as a strategy for protection against oxidative stress generated by the combined action of 6-thioguanine and UVA radiation.

Materials and Methods

Cell culture

All cell lines were maintained in 5% CO₂ at 37°C. Murine hepatoma Hepa1c1c7 cells (obtained from ATCC and used for fewer than 6 months after resuscitation) were grown in α-MEM supplemented with 10% FBS (heat-inactivated at 55°C for 90 minutes with 1% activated charcoal). Primary mouse embryonic fibroblasts (MEF) were derived from day 13.5 embryos of wild-type (WT) or Nrf2-knockout (KO) C57BL/6 mice. MEFs were grown in plastic culture dishes coated with 0.1% gelatin, in Iscoves Modified Dulbecco's Medium (with l-glutamine) supplemented with human recombinant epidermal growth factor (10 ng/mL), 1 x insulin/transferring/selenium and 10% (v/v) heat-inactivated FBS, all from Invitrogen, United Kingdom.

Animals and treatments

We used 2 strains of 8- to 12-week-old female mice: SKH-1 hairless and C57BL/6. The SKH-1 hairless mice are immunocompetent but have a defect in the hair cycle which results in permanent hair loss during adulthood. We also used C57BL/6 mice because of the availability of mice carrying a floxed allele of the Keap1 gene (Keap1flo/flo) on the C57BL/6 genetic background (14), to which we refer as Keap1-knockdown (KD) mice. All animal experiments were conducted in accordance with the regulations described in the UK Animals (Scientific Procedures) Act 1986. SKH-1 hairless mice were obtained from Charles River (Germany) and bred in our facility. WT and Keap1-KD C57BL/6 mice were from breeding colonies established at our facility. The animals 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 RM1 diet from SDS Ltd.). Stock solutions of azathioprine (Sigma-Aldrich Co.) were freshly prepared in 0.05 N NaOH and diluted 1:500 (v/v) into the drinking water. The water bottles with or without the drug were changed 3 times per week. To avoid light exposure of azathioprine, the bottles were kept wrapped in aluminium foil at all times. At the end of each treatment period, the animals were euthanized and their liver and dorsal skin harvested, flash frozen in liquid N₂, and stored at −80°C until analyses.

Biochemical analyses

Hepa1c1c7 (10⁵ per well) and MEF cells (2 x 10⁴ per well) were grown in 96-well plates for 24 hours and then

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Figure 1. Metabolism of azathioprine. Azathioprine is first converted to 6-mercaptopurine by thiolysis with GSH, which occurs nonenzymatically, and is also catalyzed by GSTs. 6-Mercaptopurine is then metabolized to give the nucleoside monophosphate thio-GMP in a series of enzymatic steps catalyzed by hypoxanthine-guanine phosphoribosyltransferase (HPRT), inosine monophosphate dehydrogenase (IMPDH) and guanine monophosphate synthetase (GMPS). Thio-GMP is a substrate for the sequential activities of deoxynucleoside kinases and reductase, ultimately leading to the formation of thio-dGTP which is incorporated into DNA. Excess thio-GMP is exported out of the cell by the action of the efflux pumps MRP4 and MRP5. Thio-dGTP is also formed from 6-thioguanine, which bypasses the initial conversion steps of azathioprine to give directly thio-GMP via the catalytic action of HPRT. Also shown are a series of catabolic reactions catalyzed by TPMT which inactivates 6-mercaptopurine, thio-IMP, thio-GMP, and 6-TG by S-methylation, and by xanthine oxidase (XO), which converts 6-mercaptopurine to 6-thiouric acid. The * in the structure of azathioprine indicates the electrophilic carbon which undergoes nucleophilic attack by the thiolate group of GSH.

Modified from Reference 1. Aza, Azathioprine; 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine.
exposed to serial dilutions of inducers for either 48 hours (Hepa1c1c7) or 24 hours (MEFs). Cells were washed 3 times with Dulbecco’s PBS (DPBS) and lysed in 0.08% digitonin, 2 mmol/L EDTA, pH 7.8. Enzyme activity of NQO1 was determined using menadione as a substrate (15, 16). For Western blot analysis of multidrug resistance-associated protein 4 (MRP4), Hepa1c1c7 cells (2 \(10^5\) per well) were grown for 24 hours in 6-well plates. Cells were treated with 5 \(\mu\)mol/L sulforaphane for further 24 hours, washed 3 times with DPBS, and lysed in radioimmunoprecipitation assay buffer. Immunoblotting was done using a rat monoclonal antibody (mAb; Abcam) at a dilution of 1:200. The antibody against \(\beta\)-actin (1:5,000 dilution; Abcam) served as a loading control.

To determine enzyme activities in liver and skin, portions (~50 mg) of snap-frozen tissues were pulverized under liquid N\(_2\). The resulting powder was resuspended in ice-cold 100 mmol/L potassium phosphate buffer, pH 7.4, containing 100 mmol/L KCl, 0.1 mmol/L EDTA, and complete protease inhibitor cocktail (Roche) at a dose of 1 tablet per 10 ml buffer. This material was mechanically homogenized in an ice bath. The skin samples were additionally subjected to 3 freeze-thaw cycles. The resulting homogenates were subjected to 2 centrifugation steps at 4°C for 10 minutes, followed by 100,000 \(\times\) g for 90 minutes. The final 100,000 \(\times\) g supernatant fractions (cytosols) were used for determination of protein concentrations (17), enzyme activities of NQO1 with menadione as a substrate (15), and glutathione S-transferase (GST) with azathioprine (18) or 1-chloro-2,4-dinitrobenzene (CDNB; ref. 19) as substrates, and for Western blotting. The antibodies against GST A1 (1:5,000 dilution), GST M1 (1:2,000 dilution), and GST P1 (1:1,000 dilution) were a gift from John D. Hayes (University of Dundee, Dundee, Scotland; ref. 20). The antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5,000 dilution, Sigma-Aldrich Co.) was used as a loading control.

**Quantitative RT-PCR**

Total RNA from 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 done on Perkin Elmer/Applied Biosystems Prism Model 7700 Sequence Detector instrument. The primers and probe were designed to measure mRNA for Mrp4 (21) and were synthesized by MWG-Biotech UK Ltd. For Mrp5, the primers and probe were purchased from Applied Biosystems, Mm01343621_m1. The TaqMan data for the mRNA species were normalized using \(\beta\)-actin (mouse ACTB, 4352933E) as an internal control.

**Silencing of Mrp4**

Hepa1c1c7 cells (1.2 \(10^6\) per 10-cm dish) were transiently transfected with a mixture of 2 siRNAs, targeting Mrp4 (Silencer Select Predesigned siRNA (Ambion): s108960, antisense sequence AGCGGUJGAALICJUGCA- CGtg and s108962, antisense sequence AACGAIJUJAAA- UCCUJCCCGga). Silencer Select negative control 1 siRNA (Ambion) was used as a nontargeting control. siRNA oligos were transfected at final concentration of 10 nmol/L using siPORT NeoFX transfection reagent by reverse transfection method (Ambion). The medium was changed 18 hours after transfection with fresh medium containing 5 \(\mu\)mol/L sulforaphane or vehicle (0.1% acetonitrile). Six hours later, the medium was replaced with medium containing 1 or 2 \(\mu\)mol/L 6-thioguanine and 5 \(\mu\)mol/L sulforaphane. The levels of Mrp4 mRNA and 6-thioguanine in DNA were determined 48 hours posttransfection. Exposure to UVA radiation (see below) was also done at this time point.

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

Portions (~100 mg) of frozen skin/liver tissue were crushed in liquid N\(_2\). DNA was extracted, ethanol precipitated, exposed to magnesium bis(monoperoxyphthalate; MMPP) in the dark for 30 minutes at room temperature, and the oxidized DNA was ethanol precipitated. To denature double-stranded DNA, 120 \(\mu\)g of DNA in 70 \(\mu\)L of deionized water was heated to 90°C for 5 minutes and immediately transferred to ice, where it was kept for further 5 minutes. Denatured DNA was digested with 24U nuclease P1 (1U/\(\mu\)L) for 1 hour at 50°C. The sample pH was adjusted to 8.0 with 20 \(\mu\)L of 1 mol/L Tris-Cl buffer (pH 8.0), and deoxynucleosides were obtained following incubation with alkaline phosphatase (2U) for 1 hour at 37°C; these were separated by reverse phase high-performance liquid chromatography (HPLC) on Ascensit C18 column (Supelco, 250 mm \(\times\) 4.6 mm, 5 \(\mu\)m) as described (7), using Agilent 1100 system equipped with Agilent G1314A variable wavelength detector and Agilent G1321A fluorescence detector. A 30-mer single-stranded oligodeoxyribonucleotide, containing a single 6-thioguanine and 4 Gs, was used to construct the standard curves, following MMPP oxidation and nuclease P1/alkaline phosphatase digestion. The oligo was originally a kind gift from Peter Karran (Cancer Research UK) and thereafter obtained from Oligo Etc. For the analysis of G\(_{\text{SO}}\)dR, 90 \(\mu\)L (of a total volume of 110 \(\mu\)L) sample was injected. Five microliter of the same sample was added to 95 \(\mu\)L of deionized water and 90 \(\mu\)L of the diluted sample was injected for the analysis of dG. Elution was with a gradient of 10 mmol/L KH\(_2\)PO\(_4\) (pH 6.7) in methanol. G\(_{\text{SO}}\)dR was quantified by fluorescence (excitation 320 nm/emission 410 nm); dG was determined by absorbance at 260 nm.

**UV irradiation of cells, determination of ROI, and reduced glutathione**

Hepa1c1c7 cells (3.5 \(10^5\) per well) were grown for 24 hours in 6-well plates. They were thereafter treated with either 2 \(\mu\)mol/L 6-thioguanine, or cotreated with 2 \(\mu\)mol/L 6-thioguanine and 5 \(\mu\)mol/L sulforaphane (KLT Laboratories) for further 24 hours. The medium was removed, cells were washed 3 times with HBSS, and then incubated with 10 \(\mu\)mol/L 2',7'-dichlorodihydrofluorescein diacetate...
groups were determined by Student's t test. Because we are interested in the development of small-molecule inducers of the Keap1/Nrf2/ARE pathway as potential pharmacologic agents for reducing the risk for skin cancer in populations receiving long-term azathioprine therapy, it was important to determine first whether azathioprine itself might affect this pathway. Inducers have a characteristic chemical signature, reactivity with sulfhydryl groups (23). Azathioprine has an electrophilic 5'-carbon in its imidazole moiety (Fig. 1), which reacts with the cysteine sulfhydryl of GSH (24, 25). We therefore considered that azathioprine might be an inducer. To test this possibility, we used a quantitative bioassay that evaluates the enzyme activity of NAD(P)H:quinone oxidoreductase 1 (NQO1), a prototypic Nrf2 target gene (15, 16).

As expected, 6-mercaptopurine (Fig. 2A, open symbols) and 6-thioguanine (not shown), neither of which has direct sulfhydryl reactivity, are essentially inactive. In contrast, azathioprine is a modest inducer with a CD (Concentration that Doubles the NQO1 enzyme activity) value of 6.3 μmol/L (Fig. 2A, closed symbols). To establish whether induction is dependent on Nrf2, we used MEFs isolated from either WT or Nrf2-KO mice. Whereas there was a dose-dependent upregulation of NQO1 in WT MEF, the enzyme levels did not change when Nrf2-KO MEF were exposed to azathioprine (Fig. 2B), showing the essential role of Nrf2 for the mechanism of induction.

The finding that azathioprine is an inducer in cells prompted us to examine whether it was also able to affect the Keap1/Nrf2/ARE pathway in vivo. To our knowledge, azathioprine has been previously administered to animals only intraperitoneally; however because it is an oral drug in humans, and to avoid repeated intraperitoneal injections of the animals, we chose an oral route of administration. Because the initial metabolism of azathioprine occurs predominantly in the liver (25), and our long-term goal is to develop protective strategies against skin carcinogenesis under conditions of azathioprine use, we chose the liver and the skin as the organs of interest in all studies described in this article. We found that incorporation of azathioprine (62.5 μg/mL) in the drinking water of C57BL/6 mice for 3 weeks increased NQO1 activity in liver (by ~30%, P = 0.04), but not in skin (P = 0.25) (Fig. 2C). Thus at this dose, azathioprine has only a modest inducer activity in liver. Nevertheless, it is interesting that this commonly prescribed drug has the ability to upregulate the expression of cytoprotective genes in vivo.
Oral azathioprine treatment leads to incorporation of 6-thioguanine in DNA of mouse skin and liver

Incorporation of 6-thioguanine has been documented in lymphocytes and skin of patients undergoing systemic treatment with azathioprine or 6-mercaptopurine (7, 26, 27). We were interested to find out whether this phenomenon could be modeled in the mouse. To this end, 3 groups of SKH-1 hairless mice were treated with different doses of azathioprine in the drinking water for 4 weeks. A fourth group that received water, containing the same amount of dilute NaOH (final concentration of 0.0001 N) in which the stock solutions of azathioprine were prepared, served as the control. As expected, no 6-thioguanine was detected in DNA from skin or liver of mice from the control group. In contrast, analysis of DNA from skin and liver of animals that received azathioprine revealed a dose-dependent incorporation of 6-thioguanine in both organs (Fig. 3A). Remarkably, the DNA 6-thioguanine levels were much higher (~4-fold) in skin than in liver. The skin samples from animals that were treated with the 62.5 µg/mL dose of azathioprine contained 6-thioguanine representing approximately 0.02% substitution of DNA guanine. Notably, the same extent of substitution has been reported for DNA isolated from skin of human subjects that had been treated with azathioprine (7). Analysis of skin and liver DNA from animals that received azathioprine for different lengths of time, that is, 2, 3, or 4 weeks, showed similar levels of 6-thioguanine at these time points and further confirmed that 6-thioguanine incorporation is consistently higher in skin than in liver (Fig. 3B). The difference in 6-thioguanine incorporation between the 2 organs was maintained even after much longer treatment periods; thus, azathioprine administration for 14 weeks at a dose of either 31.3 µg/mL or 62.5 µg/mL resulted in 3-fold higher levels of 6-thioguanine in skin DNA than in liver (not shown). This finding is surprising in view of the fact that the levels of 6-mercaptopurine following azathioprine treatment are much higher (~6-fold) in rodent liver than in plasma (25). The exact reasons for the higher degree of 6-thioguanine incorporation in skin than in liver DNA are not known. Both higher detoxification capacity of liver in comparison with skin and faster rate of tissue renewal in skin than in liver might be contributing factors. Importantly however, the high levels of 6-thioguanine in skin DNA, together with the fact that the skin is continuously exposed to solar UVA radiation, suggest a possible explanation why the skin of azathioprine-treated patients is particularly vulnerable to UVA-induced erythema and the development of skin cancer.

Genetic upregulation of the Keap1/Nrf2/ARE pathway results in decreased incorporation of 6-thioguanine into DNA of mice treated with azathioprine

Mice carrying a floxed allele of the Keap1 gene (Keap1flox/flox) have reduced expression of Keap1 and, consequently, increased expression of Nrf2 target genes (14). Thus these animals represent an ideal in vivo genetic model for constitutively activated Keap1/Nrf2/ARE pathway, and we refer to them as Keap1-KD mice. To confirm activation of the pathway, we determined the enzyme activity of NQO1: compared with WT, it was higher in Keap1-KD mice, by 3- and 5-fold, in skin and liver, respectively (not shown).

The first step in the activation of azathioprine is a GSH-mediated thiolysis (Fig. 1) which involves a nucleophilic attack of the GSH thiolate on the electrophilic 5'-carbon in the imidazole moiety of the prodrug, resulting in release of 6-mercaptopurine and GS–imidazole conjugate (24). Although this reaction occurs nonenzymatically, it is also catalyzed by class Alpha and class Mu GST isozymes (18, 24, 28–30). The human GST A1-1, GST A2-2, and GST M1-1 are especially efficient catalysts (18), and patients with a WT GSTM1 genotype have an increased probability for adverse reactions during azathioprine treatment (31). Because the liver is the organ responsible for generating circulating 6-mercaptopurine following azathioprine administration (24), we evaluated the protein levels of hepatic GST A1/2, GST M1, and GST P1 isoforms, and found that they were all markedly increased in Keap1-KD mice (Fig. 4A). We then examined the GST activity using azathioprine as a substrate. Compared with WT, the enzymatic formation of 6-mercaptopurine was elevated by 1.6-fold in livers of Keap1-KD mice (Fig. 4B), from 0.78 ± 0.02 to 1.29 ± 0.02 nmol/min/mg protein. Notably, these activities are modest and suggest that in the mouse, a large portion of the 6-mercaptopurine is derived independently of the GSTs. Indeed, under these assay conditions, the nonenzymatic reaction constitutes more than 70% of the total activity in liver homogenates, and we were not able to detect any additional activity over the nonenzymatic

![Figure 3](image-url)
conversion in skin samples. In contrast, using CDNB as a substrate, robust GST activity was observed in both liver and skin samples. The hepatic GST specific activities were 1,063.0 \pm 157.2 and 4,905.8 \pm 375.4 nmol/min/mg protein in cytosols from WT and Keap1-KD mice, respectively. The corresponding activities in skin were 61.8 \pm 6.2 and 121.3 \pm 6.2 nmol/min/mg protein. Thus it seems that, in contrast to the human enzymes, azathioprine is not a very good substrate for their murine counterparts. In addition, even though the GST protein levels are higher in livers of Keap1-KD mice, the levels of GSH are essentially the same between the genotypes (14), indicating that in vivo the supply of GSH could be limiting.

Next, we treated Keap1-KD and WT mice with azathioprine for 3 weeks and compared the 2 genotypes with respect to incorporation of 6-thioguanine in DNA. Interestingly, compared with SKH-1 hairless mice that received an identical dose of azathioprine for the same length of time, C57BL/6 animals contained higher DNA levels of 6-thioguanine; by 4- and 2-fold in liver and skin, respectively. This difference most likely reflects the low mRNA and enzyme activity levels of thiopurine S-methyltransferase (TPMT) in the C57BL/6 mouse strain (32, 33); TPMT being the enzyme that catalyzes the S-methylation of a number of intermediates in the azathioprine metabolic pathway (Fig. 1). More importantly, and in close agreement

Figure 4. Compared with WT, Keap1-KD mice have higher expression of all major classes of GSTs, MRP4, and MRP5, and lower 6-thioguanine DNA levels. A, Western blots in which aliquots from liver cytosols (100,000 \times g supernatant fractions) from each animal were resolved by SDS-PAGE, transferred to immobilon-P, and probed with specific antibodies against GSTA1, GSTM1, and GSTP1. Equal loading was confirmed by probing the blots with an antibody against GAPDH. B, the specific activity of GST (with azathioprine as a substrate) was measured in homogenate supernatants of liver of WT (n = 5) and Keap1-KD (n = 4) mice. Means \pm SD are shown. *, P < 0.01. C, groups of C57BL/6 WT (black bars) or Keap1-KD (white bars) mice (n = 5 per group) received 62.5 \mu g/mL of azathioprine in the drinking water for 3 weeks. The incorporation of 6-TG in DNA of skin and liver was quantified by the fluorescence of guanine sulfonate deoxyriboside (GSO3dR) following DNA extraction, oxidation, digestion, and HPLC separation. Means \pm SD are shown. *, P < 0.05. D, Mrp4 and Mrp5 mRNAs are upregulated in liver and skin of Keap1-KD mice. The amount of mRNA for Mrp4 and Mrp5 was analysed by quantitative RT-PCR, using \beta-actin mRNA as an internal control. In each group, the mRNA from 4 individual mice was measured separately, in triplicate. Data represent means \pm SD and are expressed as ratio of WT. *, P < 0.05; **, P < 0.001. 6-TG, 6-thioguanine.
with the SKH-1 hairless mice data, 6-thioguanine incorporation was much higher in DNA of skin than of liver of C57BL/6 mice, independently of the Keap1 genotype (Fig. 4C). The levels of 6-thioguanine in DNA were significantly different between the genotypes in liver ($P = 0.001$), and were approaching significance in skin ($P = 0.07$). Unexpectedly however, instead of being higher, the DNA 6-thioguanine levels were 30% lower in both skin and liver of Keap1-KD mice than in WT animals, suggesting that in addition to the GSTs and overriding the effect of the GSTs, other factors regulated by the Keap1/Nrf2/ARE pathway contribute to the azathioprine-dependent incorporation of 6-thioguanine into DNA.

**Pharmacologic upregulation of MRP4 correlates with lower 6-thioguanine incorporation in DNA of cells treated with 6-thioguanine**

The multidrug resistance-associated proteins 4 and 5 (MRP4/ABCC4 and MRP5/ABCC5) represent 2 other nonf2-dependent factors that could determine the extent of incorporation of 6-thioguanine in DNA (Fig. 1). Liver-specific genetic deletion of Keap1 dramatically increases the mRNA levels of Mrp4 and Mrp5 by approximately 80- and 40-fold, respectively (34); profound increases in the protein levels of Mrp4 have been also reported in livers of Keap1-KD mice (21). TaqMan reverse transcriptase PCR (RT-PCR) showed that, compared with WT, the levels of mRNA for Mrp4 in Keap1-KD mice were increased by 8.3- and 3.6-fold in liver and skin, respectively (Fig. 4D). The Mrp5 mRNA levels were 2-fold higher than WT in both liver and skin of mutant mice.

MRP4 and MRP5 are organic anion transporters that also transport nucleoside monophosphate analogues, including thiourine monophosphates (35, 36). Overexpression of MRP4 in cells leads to resistance to 6-mercaptopurine and 6-thioguanine (37). Conversely, Mrp4 deficiency in mice results in accumulation of 6-thioguanine nucleoside and 6-thioguanine monophosphate in bone marrow and also in increased hematopoietic toxicity (38). A single-nucleotide polymorphism (G2269A) in the human MRP4, leading to impaired membrane localization of the protein, has been recently associated with increased thiourine sensitivity in Japanese patients with inflammatory bowel disease (39). We therefore considered that the reduced incorporation of 6-thioguanine in DNA of Keap1-KD mice could be due to high MRP4 levels, which will facilitate the export of the 6-thioguanine nucleotide metabolite of azathioprine.

To test this possibility, we used a cell culture model in which murine hepatoma (Hepa1c1c7) cells were exposed to 6-thioguanine, a commonly used surrogate for azathioprine, which bypasses the metabolic biotransformation steps of this drug. The isothiocyanate sulforaphane served as a pharmacologic inducer (40, 41). Evaluation of the enzyme activity of NQO1 confirmed activation of the pathway, and the levels of NQO1 were increased by 4.5-fold 24 hours after sulforaphane treatment (not shown). In addition, Western blot analysis revealed that exposure to sulforaphane led to a robust upregulation of Mrp4 (Fig. 5A). Importantly, compared with cells treated with 6-thioguanine alone, the levels of 6-thioguanine incorporation in DNA were lower in cells that were cotreated with sulforaphane and 6-thioguanine (see below). To assess directly the involvement of the transporter in the sulforaphane-mediated reduction of 6-thioguanine incorporation in DNA, we utilized a transient gene silencing approach with 2 specific siRNAs targeting distinct exons of the Mrp4 gene. TaqMan RT-PCR done 48 hours posttransfection confirmed that, compared with cells transfected with nontargeting RNA, the mRNA levels of Mrp4 were reduced by 70% in cells transfected with the siRNAs (Fig. 5B). In cells transfected with nontargeting RNA, sulforaphane treatment resulted in a 3-fold upregulation of Mrp4 mRNA (Fig. 5B), in agreement with the Western blot data (Fig. 5A). Gene silencing of Mrp4 correlated well with the extent of 6-thioguanine incorporation in DNA. Thus, compared with cells transfected with nontargeting RNA, the 6-thioguanine DNA levels were increased by 2-fold in cells transfected with the siRNAs (Fig. 5C). In cells transfected with control oligos, sulforaphane treatment resulted in 50% reduction of the 6-thioguanine incorporation in DNA, and the effect of sulforaphane was attenuated (to 30%) in siRNA-transfected cells. Taken together, these results show that, similar to genetic upregulation of the Keap1/Nrf2/ARE pathway that leads to reduced 6-thioguanine DNA incorporation following azathioprine...
treatment in animals, pharmacologic activation by sulforaphane also lowers the levels of 6-thioguanine in DNA in cells. Furthermore, because in these experiments: (i) we used 6-thioguanine, and not azathioprine, thus bypassing the GSH/GST-mediated bioactivation step, and (ii) the effect of sulforaphane was diminished under conditions of Mrp4 gene silencing, we conclude that upregulation of Mrp4 represents an important factor contributing to the reduced 6-thioguanine DNA incorporation caused by sulforaphane treatment. The finding that sulforaphane (and similarly, any other bioavailable potent inducer of the Keap1/Nrf2/ARE pathway) can reduce the incorporation of 6-thioguanine in DNA following a thiopurine treatment has important practical implications. It underscores the critical necessity for a very careful design of dosing regimens, routes, and means of administration of the protective agent to avoid any potential interference with the therapeutic efficacy of the drug.

**Pharmacologic induction of the Keap1/Nrf2/ARE pathway protects against oxidative stress caused by the combined action of UVA radiation and 6-thioguanine**

One of the immediate consequences of exposure of 6-thioguanine–treated cells to UVA radiation is robust generation of ROI, which cause mutagenic DNA damage (7). When Hepa1c1c7 cells were exposed to UVA radiation, there was a dose-dependent production of ROI (Fig. 6A, white bars), in agreement with previous studies with human and murine keratinocytes (8). Remarkably, the ROI levels increased profoundly, by more than 10-fold, in cells cotreated with 5 μmol/L sulforaphane and 2 μmol/L 6-thioguanine and then exposed to UVA, there was a significant (~50%) reduction in ROI (Fig. 6A, grey bars) compared with cells treated with 6-thioguanine alone (Fig. 6A, black bars), showing the protective effect of sulforaphane. Because under identical conditions sulforaphane caused Mrp4 upregulation correlating with reduced 6-thioguanine DNA levels, its protective effect could be partly due to increased metabolite export, resulting in lower thiopurine incorporation in DNA. To test this possibility, we examined the effect of sulforaphane on the UVA-induced ROI formation under conditions of Mrp4 gene silencing. Of note, in this experiment the sensitization by 6-thioguanine was apparently reduced, possibly due to the transfection conditions. The protective effect of sulforaphane was slightly diminished in Mrp4 siRNA-transfected cells in comparison with cells transfected with the nontargeting RNA (Fig. 6B). Nevertheless, the levels of ROI were significantly lower in sulforaphane-treated than in vehicle-treated cells, under both Mrp4 siRNA and nontargeting RNA conditions. Because sulforaphane has no direct antioxidant properties, this result suggested that, in addition to reduction of the 6-thioguanine incorporation in DNA, protection by sulforaphane against UVA-induced oxidative stress could be mediated by its "indirect antioxidant effects," namely induction of the Keap1/Nrf2/ARE pathway (42), and the possible role of GSH was next examined.

It is well established that exposure to sulforaphane causes elevation in cellular GSH due to the Nrf2-dependent transcriptional upregulation of γ-glutamate-cysteine ligase (43), the enzyme that catalyses the rate-limiting step in the GSH biosynthesis, and of γ-CT (44), the core subunit of the cystine/glutamate membrane transporter, which is responsible for the uptake of cystine that in turn is reduced to

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**Figure 6.** Protection against UVA radiation–induced oxidative stress by sulforaphane in Hepa1c1c7 cells. A, cells (3.5 × 10⁵ per well) were plated on 6-well plates. After 24 hours, they were treated with either vehicle (0.1% acetonitrile, white bars), 2 μmol/L 6-thioguanine (black bars), or cotreated with 2 μmol/L 6-thioguanine and 5 μmol/L sulforaphane (grey bars) for further 24 hours. Cells were then washed with HBSS and exposed to UVA (1, 2, or 3 J/cm²) in 1.0 mL of HBSS. ROI generated by the UV radiation were quantified by the fluorescent probe 2,7′-dichlorodihydrofluorescein and fluorescence intensity was measured 1 hour postirradiation. B, cells (1.75 × 10⁶ per well in 6-well plates) were transiently transfected with a mixture of 2 siRNAs targeting Mrp4, or with a nontargeting control oligo. The medium was changed 18 hours after transfection with either medium containing 0.1% acetonitrile (black bars) or 5 μmol/L sulforaphane (grey bars). Six hours later, the medium was changed, and the cells were treated with 6-thioguanine, in the presence of sulforaphane, for further 24 hours. Cells were prepared and exposed to UVA (3 J/cm²) as described under panel A. C, cells were seeded, treated, and exposed to UVA radiation as described under panel A. Reduced GSH was quantified using MCB and the fluorescence intensity of the GS–mCB adduct was determined 2 hours postirradiation. For all panels, means ± SD are shown (n = 3). 6-TG, 6-thioguanine; SF, sulforaphane.
cysteine and used as a precursor for the biosynthesis of GSH. It is also known that UVA radiation mediates the oxidation of free 6-thioguanine, or 6-thioguanine–containing DNA, to G\(^{SO2}\) and G\(^{SO3}\) (45, 46). Recently, it was reported that G\(^{SO2}\) forms an addition product with GSH (47). We therefore examined the levels of GSH following UVA radiation in cells that had been either treated with 6-thioguanine alone, or cotreated with sulforaphane and 6-thioguanine, using the cell permeable probe mCB (22). Exposure to UVA caused a dose-dependent GSH depletion (Fig. 6C). Treatment with sulforaphane increased GSH by approximately 30%. Although reduction in GSH still occurred as a consequence of radiation exposure independently of treatment with sulforaphane, at all doses of UVA the levels of GSH were higher in sulforaphane-treated cells. Even those sulforaphane-treated cells that had been exposed to the highest dose of UVA had GSH levels comparable with sham-irradiated cells that had not been treated with the protective agent. These findings suggest that, in addition to MRP4 upregulation, the increase in GSH also contributes to the protective effect of sulforaphane and highlight the multiple layers of protection provided by induction of the Keap1/Nrf2/ARE pathway.

**Conclusions**

The new finding that oral treatment with azathioprine results in much higher incorporation of 6-thioguanine in DNA in skin than in liver, together with the known facts that (i) UVA comprises more than 95% of the terrestrial UV radiation (48) and (ii) the combination of azathioprine and UVA radiation creates synergistic mutagenic DNA damage (7), provides one possible explanation for the high incidence of skin cancer in individuals receiving long-term azathioprine therapy. Genetic upregulation of the Keap1/Nrf2/ARE pathway reduces the incorporation of 6-thioguanine in DNA in both skin and liver following treatment with azathioprine. Pharmacologic activation by the potent inducer sulforaphane protects against oxidative stress generated by the combined action of 6-thioguanine and UVA. Protection correlates with increased levels of GSH and MRP4. Treatment with sulforaphane decreases the 6-thioguanine incorporation in DNA. Although this effect is attenuated by MRP4 gene silencing, protection against oxidative stress still occurs, emphasizing the indirect antioxidant properties of sulforaphane mediated through induction of the Keap1/Nrf2/ARE pathway. Taken together, these findings suggest that whereas systemic treatment with a pharmacologic activator of the Keap1/Nrf2/ARE pathway may potentially interfere with the therapeutic efficacy of azathioprine, the development of a topical formulation with such an activator warrants further research.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Oral Azathioprine Leads to Higher Incorporation of 6-Thioguanine in DNA of Skin than Liver: The Protective Role of the Keap1/Nrf2/ARE Pathway

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