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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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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 ultraviolet A (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, pharmacological 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 (MRP4), 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.

Keywords

azathioprine; Keap1; Nrf2; sulforaphane; UVA radiation

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), while cessation of immunosuppressive therapy leads to deceleration of skin carcinogenesis (3), but also to transplant rejection. A trend towards 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). Pre-malignant 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 patient within one 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) (1). Elegant studies conducted by Peter Karran and his colleagues have revealed that the combination of 6-thioguanine and ultraviolet A (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 pharmacological 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 three 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 small 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 modelled in the mouse, (ii) upregulation of the Keap1/Nrf2/ARE pathway affects this incorporation, and (iii) pharmacological 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 (heated-inactivated at 55°C for 90 min with 1% activated charcoal). Primary mouse embryonic fibroblasts (MEFs) were derived from day 13.5 embryos of wild-type or Nrf2-knockout C57BL/6 mice. MEFs grown in plastic culture dishes coated with 0.1% (w/v) gelatin, in Iscoves Modified Dulbecco's Medium (with L-

glutamine) supplemented with human recombinant epidermal growth factor (10 ng/mL), $1 \times$ insulin/transferrin/selenium and 10% (v/v) heat-inactivated FBS, all from Invitrogen, UK.

Animals and treatments

We used two 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 (*Keap1*^{flox/flox}) on the C57BL/6 genetic background (14), to which we refer as Keap1-knockdown (KD) mice. All animal experiments were performed 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. Wild-type and Keap1-knockdown C57BL/6 mice were from breeding colonies established at our facility. The animals were kept on a 12-h light/ 12-h dark cycle, 35% humidity, in individually ventilated cages, and were given free access to water and food (pelleted RM1 diet from SDS Ltd., Witham, Essex, UK). Stock solutions of azathioprine (Sigma-Aldrich Co., Poole, Dorset, UK) 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

Hepal1c17 (10^4 per well) and MEF cells (2×10^4 per well) were grown in 96-well plates for 24 h and then exposed to serial dilutions of inducers for either 48 h (Hepal1c17) or 24 h (MEFs). Cells were washed 3 times with Dulbecco's phosphate-buffered saline (DPBS) and lysed in 0.08% digitonin, 2 mM EDTA, pH 7.8. Enzyme activity of NQO1 was determined using menadione as a substrate (15, 16). For western blot analysis of MRP4, Hepal1c17 cells (2×10^5 per well) were grown for 24 h in 6-well plates. Cells were treated with 5 μM sulforaphane for further 24 h, washed 3 times with DPBS, and lysed in radioimmunoprecipitation assay (RIPA) buffer. Immunoblotting was performed using a rat monoclonal antibody (Abcam, Cambridge, UK) at a dilution of 1:200. The antibody against β -actin (1:5000 dilution, Abcam, Cambridge, UK) served as a loading control.

To determine enzyme activities in liver and skin, portions (~50 mg) of snap-frozen tissues were pulverized under liquid N₂. The resulting powder was resuspended in ice-cold 100 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl, 0.1 mM EDTA, and complete protease inhibitor cocktail (Roche, UK) at a dose of one tablet/10 ml buffer. This material was mechanically homogenized in an ice bath. The skin samples were additionally subjected to three freeze-thaw cycles. The resulting homogenates were subjected to two centrifugation steps at 4°C ($15,000 \times g$ for 10 min, followed by $100,000 \times g$ for 90 min). 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 GST with azathioprine (18) or 1-chloro-2,4-dinitrobenzene (CDNB) (19) as substrates, and for western blotting. The antibodies against GST A1 (1:5000 dilution), GST M1 (1:2000 dilution), and GST P1 (1:1000 dilution) were a gift from John D. Hayes (University of Dundee) (20). The antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000 dilution, Sigma-Aldrich Co., Poole, Dorset, UK) 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 performed on Perkin Elmer/Applied Biosystems Prism Model 7700 Sequence Detector instrument. The primers and probe used to measure mRNA for *Mrp4* have been described (21), and were synthesized by MWG-Biotech UK Ltd. (Milton Keynes, UK). For *Mrp5*, the primers and probe were purchased from Applied Biosystems, Mm01343621_m1. The TaqMan data for the mRNA species were normalized using β -actin (mouse ACTB, 4352933E) as an internal control.

Silencing of *Mrp4*

Hepal1c7 cells (1.2×10^6 per 10-cm dish) were transiently transfected with a mixture of two small interfering RNAs (siRNA), targeting *Mrp4* (*Silencer*[®] Select Pre-designed siRNA (Ambion): s108960, antisense sequence AGCGGUGAAAUCUUGCACGtg and s108962, antisense sequence AACGAUUUAAAAUCCUCCCga). *Silencer*[®] Select negative control 1 siRNA (Ambion) was used as a non-targeting control. siRNA oligos were transfected at final concentration of 10 nM using siPORT[™] NeoFX[™] transfection reagent by reverse transfection method (Ambion). The medium was changed 18 h after transfection with fresh medium containing 5 μ M sulforaphane or vehicle (0.1% acetonitrile). Six hours later, the medium was replaced with medium containing 1 or 2 μ M 6-thioguanine and 5 μ M sulforaphane. The levels of *Mrp4* mRNA and 6-thioguanine in DNA were determined 48 h post-transfection. 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 N₂. DNA was extracted, ethanol-precipitated, exposed to magnesium bis(monoperoxyphthalate) (MMPP) in the dark for 30 min at room temperature, and the oxidized DNA was ethanol-precipitated. To denature double-stranded DNA, 120 μ g of DNA in 70 μ l of deionised water was heated to 90°C for 5 min and immediately transferred to ice, where it was kept for further 5 min. Denatured DNA was digested with 24U nuclease P1 (1U/ μ l) for 1 h at 50 °C. The sample pH was adjusted to 8.0 with 20 μ l of 1M Tris-Cl buffer (pH 8.0), and deoxynucleosides were obtained following incubation with alkaline phosphatase (2U) for 1 h at 37 °C; these were separated by reverse phase high-performance liquid chromatography (HPLC) on Ascentis[®] C18 column (Supelco, 250 mm $\times$ 4.6 mm, 5 μ 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-TG and four 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. (Wilsonville, OR, USA). For the analysis of G^{SO3}dR, 90 μ l (out of a total volume of 110 μ l) sample was injected. Five μ l of the same sample was added to 95 μ l of deionised water, and 90 μ l of the diluted sample was injected for the analysis of dG. Elution was with a gradient of 10 mM KH₂PO₄ (pH 6.7) in methanol. G^{SO3}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 reactive oxygen intermediates and reduced glutathione

Hepal1c7 cells (3.5×10^5 per well) were grown for 24 h in 6-well plates. They were thereafter treated with either 2 μ M 6-thioguanine, or co-treated with 2 μ M 6-thioguanine and 5 μ M *R,S*-sulforaphane (LKT Laboratories, St. Paul, MN) for further 24 h. The medium was removed, cells were washed 3 times with Hank's balanced salt solution (HBSS) and then incubated with 10 μ M 2',7'-dichlorodihydrofluorescein diacetate (Invitrogen Ltd., Paisley, UK) in 1.0 ml of HBSS for 30 min. The buffer containing the fluorescent probe was

removed, cells were washed 3 times with HBSS, and then exposed to UVA radiation in 1.0 ml of HBSS using UVA lamps with an emission spectrum of 320–420 nm. Any UVB component from the source (<0.05%) was filtered out by a 7-mm thick glass plate. The irradiance at the surface of the cells, at a distance of 300 mm from the lamps, was measured with a Waldmann UV meter calibrated to the source using a double-grating spectroradiometer (Bentham Instruments Ltd., Reading, UK). The time of exposure to deliver 1, 2, or 3 J/cm² was calculated. Sham-irradiated cells were kept alongside irradiated cells, but were wrapped in aluminium foil. Generation of ROI was quantified 1 h post-irradiation 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. Reduced glutathione was determined by incubating irradiated cells with 40 μ M monochlorobimane (mCB) for 1 h (22). Formation of the GS-mCB adduct was quantified 2 h post-irradiation with excitation at 390 nm and emission at 490 nm.

Statistical analysis

All values are means $\pm$ 1 S.D. The differences between groups were determined by Student's *t* test.

Results and Discussion

Azathioprine is a modest inducer of the Keap1/Nrf2/ARE pathway

Since we are interested in the development of small-molecule inducers of the Keap1/Nrf2/ARE pathway as potential pharmacological 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 glutathione (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 μ M (Fig. 2A, closed symbols). To establish whether induction is dependent on Nrf2, we used mouse embryonic fibroblasts (MEF) isolated from either wild-type (WT) or Nrf2-knockout (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), demonstrating 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 in order to avoid repeated intraperitoneal injections of the animals, we chose an oral route of administration. Since 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 paper. We found that incorporation of azathioprine (62.5 μ g/ml) in the drinking water of C57BL/6 mice for 3 weeks increased NQO1 activity in liver (by $\sim$ 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 modelled in the mouse. To this end, three 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 ~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, i.e., 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 two 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 to 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 (*Keap1*^{flox/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-knockdown (KD) mice. To confirm activation of the pathway, we determined the enzyme activity of NQO1: compared to wild-type, 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 glutathione-mediated thiolysis (Fig. 1) which involves a nucleophilic attack of the glutathione thiolate on the electrophilic 5'-carbon in the imidazole moiety of the prodrug, resulting in release of 6-mercaptopurine and glutathione-imidazole conjugate (24). Although this reaction occurs non-enzymatically, 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 wild-type *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 to wild-type, 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 non-enzymatic reaction constitutes more than 70% of the total activity in liver homogenates, and we were not able to detect any additional activity over the non-enzymatic conversion in skin samples. In contrast, using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, robust GST activity was observed in both liver and skin samples. The hepatic GST specific activities were 1063.0 ± 157.2 and 4905.8 ± 375.4 nmol/min/mg protein in cytosols from wild-type and Keap1-KD mice respectively. The corresponding activities in skin were 61.8 ± 6.2 and 121.3 ± 6.2 nmol/min/mg protein. Thus it appears 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 glutathione are essentially the same between the genotypes (14), indicating that *in vivo* the supply of glutathione could be limiting.

Next, we treated Keap1-KD and wild-type mice with azathioprine for 3 weeks, and compared the two genotypes with respect to incorporation of 6-thioguanine in DNA. Interestingly, compared to 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 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 wild-type 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.

Pharmacological 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 two other possible Nrf2-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 ~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 RT-PCR showed that, compared to wild-type, 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 wild-type in both liver and skin of mutant mice.

MRP4 and MRP5 are organic anion transporters that also transport nucleoside monophosphate analogues, including thiopurine 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 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 thiopurine 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 pharmacological 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 h 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 to cells treated with 6-thioguanine alone, the levels of 6-thioguanine incorporation in DNA were lower in cells that were co-treated 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 two specific small interfering RNAs targeting distinct exons of the *Mrp4* gene. TaqMan RT-PCR performed 48 h post-transfection confirmed that, compared to cells transfected with non-targeting RNA, the mRNA levels of *Mrp4* were reduced by 70% in cells transfected with the siRNAs (Fig. 5B). In cells transfected with non-targeting 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 to cells transfected with non-targeting 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 demonstrate that, similarly to genetic upregulation of the Keap1/Nrf2/ARE pathway that leads to reduced 6-thioguanine DNA incorporation following azathioprine treatment in animals, pharmacological 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, in order to avoid any potential interference with the therapeutic efficacy of the drug.

Pharmacological 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 >10-fold, in cells that were treated with 2 μ M 6-thioguanine for 24 h prior to irradiation (Fig. 6A, black bars) compared to vehicle-treated cells (Fig. 6A, white bars). When cells were co-treated with 5 μ M sulforaphane and 2 μ M 6-thioguanine, and then exposed to UVA, there was a significant (~50%) reduction in ROI (Fig. 6A, grey bars) compared to cells treated with 6-thioguanine alone (Fig. 6A, black bars), demonstrating 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 non-targeting RNA (Fig. 6B). Nevertheless, the levels of ROI were significantly lower in sulforaphane-treated than in vehicle-treated cells, under both *Mrp4* siRNA and non-targeting 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 χ -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 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^{SO_2} and G^{SO_3} (45, 46). Recently, it was reported that G^{SO_2} 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 co-treated with sulforaphane and 6-thioguanine, using the cell permeable probe monochlorobimane (22). Exposure to UVA caused a dose-dependent GSH depletion (Fig. 6C). Treatment with sulforaphane increased GSH by ~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 to 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 >95% of the terrestrial ultraviolet 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. Pharmacological 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 glutathione 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 pharmacological 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.

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Figure 1. Metabolism of azathioprine

Azathioprine (Aza) is first converted to 6-mercaptopurine (6-MP) by thiolysis with glutathione (GSH) which occurs nonenzymatically, and is also catalyzed by the glutathione *S*-transferases (GST). 6-MP 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 (6-TG) 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 thiopurine *S*-methyltransferase (TPMT) which inactivates 6-MP, thio-IMP, thio-GMP, and 6-TG by *S*-methylation, and by xanthine oxidase (XO) which converts 6-MP to 6-thiouric acid. The * in the structure of azathioprine indicates the electrophilic carbon which undergoes nucleophilic attack by the thiolate group of glutathione (GSH). Modified from Reference 1.

Figure 2. Induction of NQO1 by azathioprine

Hepal1c7 cells (10^4 per well) (A) or mouse embryonic fibroblasts (2×10^4 per well) (B) were grown in 96-well plates for 24 h and then exposed to serial dilutions of the thiopurines. NQO1 enzyme activity and total protein concentrations were determined in cell lysates using menadione as a substrate. Results represent average values of 8 replicate wells. The standard deviations in each case were between 5-8 % of the values. (C) Two groups of C57BL/6 mice ($n = 5$ per group) received either water containing 62.5 $\mu\text{g/ml}$ of azathioprine or control water containing 0.0001 N NaOH for 3 weeks. At the end of the treatment period, the animals were euthanized, and their liver and skin were harvested. The specific activity of NQO1 was measured in cytosolic fractions of liver and skin. Means $\pm$ S.D. are shown. $p < 0.05$ (*).

Figure 3. Incorporation of 6-thioguanine in DNA of skin (black bars) and liver (white bars) following oral administration of azathioprine to SKH-1 hairless mice
Groups of SKH-1 hairless mice ($n = 3$ per group) received the indicated daily doses of azathioprine in the drinking water for 4 weeks (A) or azathioprine at a concentration of 125 $\mu\text{g/ml}$ of water for either 2, 3, or 4 weeks (B). At the end of each treatment period, the animals were euthanized, and their liver and skin were harvested. The incorporation of 6-thioguanine (6-TG) in DNA was quantified by the fluorescence of guanine sulfonate deoxyriboside ($\text{G}^{\text{SO}_3}\text{dR}$) following DNA extraction, oxidation, digestion, and HPLC separation. Means $\pm$ S.D. are shown. $p < 0.001$ (*).

Figure 4. Compared to wild-type, Keap1-knockdown 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 wild-type (WT, $n = 5$) and Keap1-knockdown (KD, $n = 4$) mice. Means $\pm$ S.D. are shown. $p < 0.01$ (*). (C) Groups of C57BL/6 wild-type (black bars) or Keap1-knockdown (white bars) mice ($n = 5$ per group) received $62.5 \mu\text{g/ml}$ of azathioprine in the drinking water for 3 weeks. The incorporation of 6-thioguanine (6-TG) in DNA of skin and liver was quantified by the fluorescence of guanine sulfonate deoxyriboside ($\text{G}^{\text{SO}_3\text{dR}}$) following DNA extraction, oxidation, digestion, and HPLC separation. Means $\pm$ S.D. are shown. $p < 0.05$ (*). (D) *Mrp4* and *Mrp5* mRNAs are upregulated in liver and skin of Keap1-knockdown mice. The amount of mRNA for *Mrp4* and *Mrp5* was analysed by quantitative RT-PCR, using β -actin mRNA as an internal control. In each group, the mRNA from 4 individual mice was measured separately, in triplicate. Data represent means $\pm$ S.D. and are expressed as ratio of WT. $p < 0.05$ (*). $p < 0.001$ (**).

Figure 5. Treatment with sulforaphane induces MRP4 and reduces the incorporation of 6-thioguanine in DNA of Hepa1c1c7 cells

(A) Cells (2×10^5 per well) were grown in 6-well plates for 24 h, treated with either vehicle (0.1% acetonitrile, AcN) or 5 μ M sulforaphane (SF) for a further 24 h, then washed with Dulbecco's phosphate-buffered saline, and lysed. Cell lysates were resolved by SDS/PAGE, transferred to immobilon-P, and probed with a monoclonal antibody against MRP4. Equal loading was confirmed by probing the blot with an antibody against β -actin. (B and C) Cells (1.2×10^6 per 10-cm dish) were transiently transfected with a mixture of two siRNAs targeting *Mrp4*, or with a non-targeting control oligo. The medium was changed 18 h after transfection with either medium containing 5 μ M sulforaphane (SF) or vehicle (AcN). (B) The amount of mRNA for *Mrp4* was analysed by quantitative RT-PCR, using β -actin mRNA as an internal control 48 h post-transfection. (C) Six hours following exposure to SF, cells were co-treated with 1 μ M 6-thioguanine (6-TG) and 5 μ M SF or vehicle for further 24 h. The incorporation of 6-TG in DNA was quantified by the fluorescence of guanine sulfonate deoxyriboside ($G^{SO_3}dR$) following DNA extraction, oxidation, digestion, and HPLC separation. Means $\pm$ S.D. are shown ($n = 3$).

Figure 6. Protection against UVA radiation-induced oxidative stress by sulforaphane in Hepa1c1c7 cells

(A) Cells (3.5×10^5 per well) were plated on 6-well plates. After 24 h hours, they were treated with either vehicle (0.1% acetonitrile, white bars), 2 μM 6-thioguanine (6-TG, black bars), or co-treated with 2 μM 6-thioguanine and 5 μM sulforaphane (SF, grey bars) for further 24 h. Cells were then washed with Hank's buffered saline solution (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 h post-irradiation. (B) Cells (1.75×10^5 per well in 6-well plates) were transiently transfected with a mixture of two siRNAs targeting *Mrp4*, or with a non-targeting control oligo. The medium was changed 18 h after transfection with either medium containing 0.1% acetonitrile (black bars) or 5 μM SF (grey bars). Six hours later, the medium was changed, and the cells were treated with 6-thioguanine, in the presence of SF, for further 24 h. 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 glutathione (GSH) was quantified using monochlorobimane and the fluorescence intensity of the GS-monochlorobimane adduct was determined 2 h post-irradiation. For all panels, means $\pm$ S.D. are shown ($n = 3$).