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Article **Oncology**

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Triterpenoid electrophiles (avicins) activate the innate stress response by redox regulation of a gene battery

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Introduction

Many chronic human diseases associated with aging, such as cancer, are characterized by either an inadequate response or a hyperresponse to chronic stress. It is therefore important to search for pharmacological ways to regulate the stress response in clinical medicine. We recently discovered a family of triterpenoid electrophiles, called avicins, that contain an unusual side chain that contains two Michael reaction sites (1). Avicins have been shown to induce apoptosis by direct perturbation of the mitochondria (1) and to inhibit the PI3K/Akt-signaling pathway (2). Avicin's ability to suppress chemical-induced carcinogenesis by its anti-inflammatory, antimutagenic, and antioxidant properties (3) was supported by its inhibitory effect on NF-κB activation (4). The ability of DTT to block avicins' suppression of NF-κB activation suggested that these compounds might be regulating critical redox-sensitive thiol groups. We therefore asked whether avicins could regulate other redox-dependent transcription factors involved in stress responses.

We chose to study the module involving NF-E2–related factor 2 (Nrf2) and antioxidant response element (ARE), which is responsible for the transcriptional activation of numerous detoxifying enzymes and antioxidant proteins. One of the properties common to inducers of phase 2 enzymes is their ability to modify sulfhydryl groups by oxidation, reduction, or alkylation (5). The structure of avicins (1) includes α,β unsaturated carbonyl groups (two in the outer monoterpene chains and one at C28) that could react with nucleophiles, especially cysteine sulfhydryl groups, by a Michael-type addition.

In the present study, we show that avicins activate a battery of stress-induced proteins via the Nrf2 pathway in an epithelial cell line derived from a hepatic carcinoma called Hep G2. The implications of these findings were evaluated in vivo in mouse skin exposed to UV light. The protective effects of avicins on UVB-induced skin damage are reported here. In this paper, we suggest that avicins represent a new class of electrophilic metabolites capable of significantly enhancing innate

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Nonstandard abbreviations used: NF-E2–related factor 2 (Nrf2); antioxidant response element (ARE); avicin extracts (AE); heme oxygenase (HO); NADPH:quinone oxidoreductase 1 (NQO1); enhanced green fluorescence protein–Nrf2 plasmid (pEGFP-Nrf2); human ARE (hARE); glutathione-*S*-transferase (GST); glutathione peroxidase (GPx); glutathione (GSH); thioredoxin (TRX); TRX reductase (TRX_{red}); *tert*-butyl hydroquinone (tBHQ); 8-hydroxy-2′-deoxyguanosine (8-OH-dG); reactive oxygen species (ROS).

stress response, an ancient pathway to ensure organismal homeostasis against biotic and abiotic threats.

Methods

Cell lines. Human hepatoblastoma (Hep G2) cells obtained from the American Type Culture Collection (Rockville, Maryland, USA) were grown in α minimum essential medium supplemented with 10% FCS and 2 mM glutamine.

Avicins. The in vitro studies were carried out with avicin D, a single pure species (ref. 1 and Supplemental Figure 1, http://www.jci.org/cgi/content/full/113/1/65/DC1), purified from a crude mixture of triterpenoid saponins. Because of constraints of availability, all the in vivo studies were done using the mixture, which has been referred to as avicin extracts (AE).

Antibodies and plasmids. Anti-Nrf2 and anti–mutant p53 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Anti–heme oxygenase-1 (anti-HO-1), anti-ferritin, and anti–caspase 3 antibodies were purchased from Stressgen Biotechnologies Corp. (Victoria, British Columbia, Canada), from DAKO A/S (Glostrup, Denmark), and from Cell Signaling Technology Inc. (Beverly, Massachusetts, USA), respectively. Purified NADPH:quinone oxidoreductase 1 (NQO1) antibodies, enhanced green fluorescence protein–Nrf2 plasmid (pEGFP-Nrf2) Nrf2, and human ARE–LUC (hARE-LUC) constructs (6) were a gift from Anil Jaiswal (Department of Pharmacology, Baylor College of Medicine, Houston, Texas, USA).

Western blot analysis. Cellular proteins (50 μg) from Hep G2 cells treated with avicin D (2 μg/ml) were resolved on SDS PAGE. Proteins were transferred onto a nitrocellulose membrane and detected by chemiluminescence (ECL; Amersham Biosciences, Piscataway, New Jersey, USA). Intensities of the protein bands were quantified using NIH image software. Fold changes were calculated with respect to the control.

Transfection and nuclear localization of Nrf2. Hep G2 cells (0.03×10^6) grown on Lab-Tek II Chamber Slides (Nalge Nunc International, Naperville, Illinois, USA) were transfected with 0.2 μg of pEGFP-Nrf2 using the Effectene Transfection Reagent Kit (QIAGEN Inc., Valencia, California, USA) according to the manufacturer's protocol. Forty hours after transfection, fresh medium containing avicin D (2 μg/ml) was added to the chambers. Nuclear translocation of Nrf2 was observed by fluorescence microscopy (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany).

Transfection and ARE activation studies. Hep G2 cells were transfected with 0.5 μg of hARE-LUC plasmid and pRL-TK. The pRL-TK vector containing the herpes simplex virus thymidine kinase promoter region upstream of *Renilla* LUC was used as an internal control.

NQO1 activity. Hep G2 cells (2×10^6 to 3×10^6) were treated with avicin D (0.25–2 μg/ml) for 0–4 hours at 37°C, and cytosolic extracts were prepared. Dicoumarol-sensitive NQO1 activity was determined as described earlier (7).

Measurement of bilirubin levels and ferritin synthesis. Hep G2 cells were treated with avicin D in phenol red–free medium and lysed by the freeze-thaw method. Bilirubin levels were measured calorimetrically using a Bilirubin Total and Direct kit from Sigma-Aldrich (St. Louis, Missouri, USA).

Ferritin synthesis was studied by metabolic labeling in Hep G2 cells treated with avicin D (2 μg/ml). Cells were labeled with ^{35}S-methionine (125 μCi/ml) for 2 hours. Ferritin was immunoprecipitated from whole-cell extracts, and the antibody-protein complex was resolved on an SDS/12.5% polyacrylamide gel. The radioactive bands were visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, California, USA).

Assay for glutathione-S-transferase, glutathione peroxidase, and glutathione. Levels of glutathione-*S*-transferase (GST), glutathione peroxidase (GPx), and glutathione (GSH) in avicin D–treated cells were measured using *Oxis*Research assay kits (OXIS International Inc., Portland, Oregon, USA) according to the manufacturer's protocol.

Assay for thioredoxin reductase. Thioredoxin reductase (TRX_{red}) activity in lysates of avicin D–treated Hep G2 cells was estimated by measurement of auranofin-sensitive reduction of 5′,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to 5′-thio-2-nitrobenzoic acid (TNB) (8).

AE treatment and UV irradiation of animals. Seven-week-old SKH-1 (*hr/hr*) albino hairless female mice from Charles River Laboratories Inc. (Wilmington, Massachusetts, USA) were used for the study. AE dissolved in acetone was applied topically 15 minutes before or 15 minutes after each UV irradiation regimen. Using a UV apparatus (The Daavlin Co., Bryan, Ohio, USA) equipped with eight Westinghouse UV FS40 sunlamps (Atlantic Ultraviolet Corp. Hauppauge, New York, USA), mice were exposed to 100 mJ/cm^2 five times per week with 25% increases per week, until the dose reached 200 mJ/cm^2. All mice were treated and irradiated for 10 weeks and sacrificed 24 hours after the last UV dose to study intermediate-endpoint biomarkers of initiation and promotion.

Hyperplasia. The skin was prepared for histology and examined for sustained hyperplasia as previously described (9). Epidermal thickness was determined from at least 20 randomly selected sites per animal.

Immunohistochemistry. Skin sections prepared by standard procedures were incubated with primary antibody (anti–mutant p53 or anti-NQO1) followed by biotin-labeled goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, California, USA). Slides were stained with diaminobenzidine-chromogen and counterstained with aqueous hematoxylin.

Apoptosis. Apoptosis was detected and quantitated in paraffin-embedded skin sections, using rabbit polyclonal antibody (Asp175) against cleaved caspase 3 as described before (10).

Analysis of modified DNA bases. Frozen skin sections were analyzed for formation of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) as previously described (11).

Statistical evaluation. Single-tailed or two-tailed Student's *t* test was used to assess statistical significance of

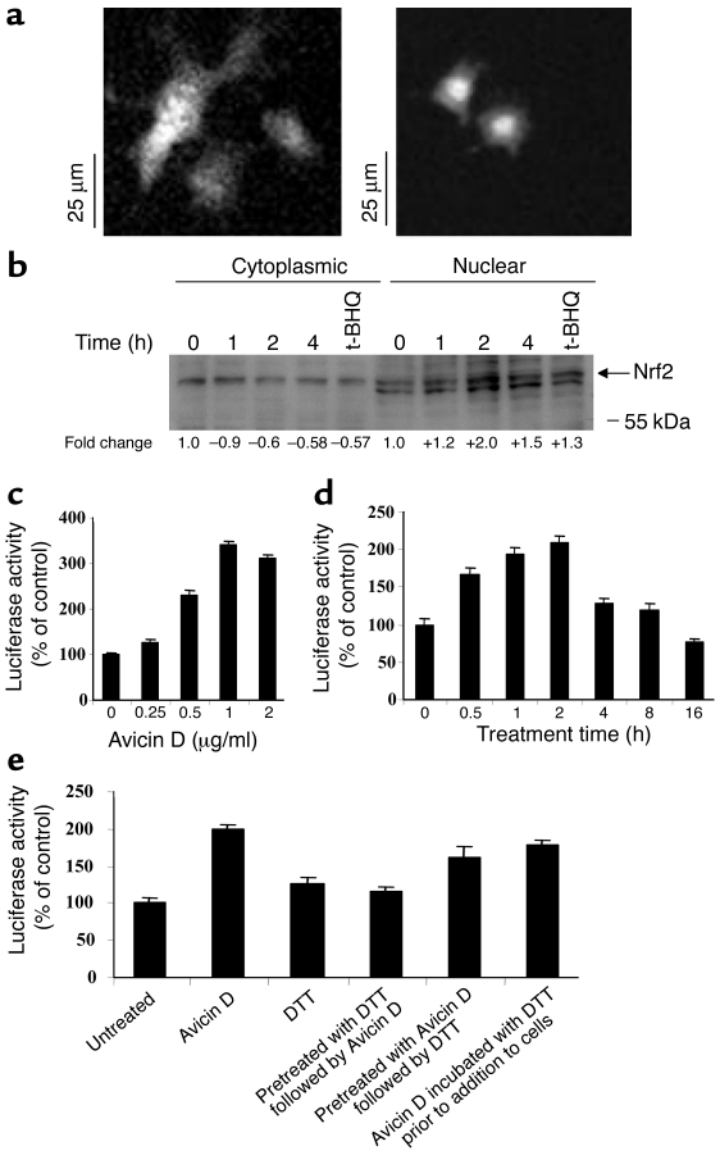

Figure 1
Nuclear localization of Nrf2 and activation of ARE-mediated gene expression. (**a**) Hep G2 cells transfected with EGFP-Nrf2 were either untreated or treated with avicin D (2 μg/ml) for 1 hour at 37°C. Nuclear localization of Nrf2 was studied by fluorescence microscopy (H&E staining; ×400). (**b**) Hep G2 cells were treated with avicin D (2 μg/ml) for 0–4 hours at 37°C. Western blot analysis of cytoplasmic and nuclear extracts was performed as described in Methods. Fifty micromolar t-BHQ for 4 hours was used as a positive control. (**c**–**e**) Hep G2 cells were transfected with NQO1-ARE-LUC plasmid, and LUC activity was measured as described in Methods. Dose response of ARE activation was studied using 0–2 μg/ml of avicin D for 2 hours at 37°C (**c**), and kinetics of ARE activation was studied using 2 μg/ml of avicin D for 0–16 hours at 37°C (**d**). (**e**) Effect of DTT (100 μM) on ARE activation was studied by application of DTT to cells for 2 hours, before, after, or along with avicin D (2 μg/ml).

difference between control and avicin-treated groups. A statistically significant difference was considered to be present at $P < 0.05$ (12).

Results

In vitro studies

Regulation of transcription. Nrf2, a member of the cap 'n' collar family of bZip transcription factors, is normally repressed through its cytoplasmic localization by binding to the cytoskeleton-associated protein Keap1 (13). Exposure of cells to inducers of phase 2 enzymes disrupts the Keap1-Nrf2 complex, allowing Nrf2 to translocate into the nucleus, where it binds to the ARE and activates transcription. Fluorescence microscopy of Hep G2 cells transfected with pEGFP-Nrf2 revealed the nuclear localization of Nrf2 in response to avicin D treatment (Figure 1a). Western blot analysis of the nuclear fractions of avicin D–treated cells showed a gradual increase in Nrf2 levels with time, while they declined concomitantly in the cytoplasm (Figure 1b). Avicin D–induced changes in the nuclear and cytoplasmic Nrf2 levels were comparable to those induced by *tert*-butyl hydroquinone (t-BHQ), an antioxidant known to induce phase 2 proteins such as NQO1 via activation of ARE (ref. 14; see Supplemental Figure 2a, http://www.jci.org/cgi/content/full/113/1/65/DC1, for loading control and molecular weight markers).

ARE, a *cis*-acting regulatory sequence identified in the promoter regions of several genes encoding phase 2 detoxification enzymes, binds to Nrf2 via a core sequence, 5′-TGACNNNGC-3′. Hep G2 cells transfected with hARE-LUC plasmid were exposed to avicin D, and changes in LUC activity were used as a measure of ARE activation. Figure 1c shows a dose-dependent increase of ARE-driven LUC activity with 1–2 μg/ml of avicin D inducing maximal activation. Avicin D also induced a time-dependent increase in LUC activity, starting at 30 minutes after treatment, peaking at 2 hours, and then gradually declining (Figure 1d).

The Keap1-Nrf2 complex is believed to serve as a cytoplasmic sensor of oxidative stress (13) based on the presence of highly reactive sulfhydryl groups in both these proteins (13, 15). To evaluate the role of cysteine residues in the action of avicins, we studied the effect of DTT on avicin D–induced ARE activation (Figure 1e). While DTT by itself induced a small increase in ARE activation, pretreatment of cells with DTT almost completely blocked the effect of avicin D. In contrast, DTT given after an avicin D treatment had minimal effect, suggesting the irreversibility of avicin D's action. Incubating avicin D with DTT before putting it on cells had no effect on the activation, indicating that DTT does not interact with avicin D but probably competes with it for binding to

cysteines that are critical for ARE activation. A recent report from Talalay's group has identified four cysteine residues that are key sensors regulating induction of phase 2 enzymes in the Keap1 molecule (16). Whether avicin D interacts with one or more of these cysteines will be the subject of our future studies.

Activation of a gene battery. Following its effects on Nrf2 and ARE, we next looked at the effects of avicin D on a battery of downstream gene products. NQO1, a flavoprotein induced in response to a variety of biotic and abiotic stresses, utilizes NADPH as an electron donor to catalyze the two-electron reduction and detoxification of quinones and their derivatives. Figure 2a reveals a dose-dependent increase of NQO1 expression in Hep G2 cells treated with avicin D for 2 hours. Induction of NQO1 achieved with 1–2 μg/ml (0.5–1 μM) of avicin D was comparable to that achieved with 100 μM of t-BHQ (2-hour treatment) (see Supplemental Figure 2b, http://www.jci.org/cgi/content/full/113/1/65/DC1, for loading control and molecular weight markers). To correlate expression with activity, we next assayed for dicoumarol-sensitive NQO1 activity in avicin D–treated Hep G2 cells using dichloroindophenol as an electron acceptor. Study of the kinetics of enzyme activity revealed an optimal induction of NQO1 activity between 2 and 4 hours of treatment with avicin D (Figure 2b). A dose-dependent increase in NQO1 activity was seen following a 2-hour treatment with avicin D. The increase in activity (2.6-fold) observed with 2 μg/ml of avicin D (Figure 2c) correlated well with the increase in expression (2.7-fold) seen under similar conditions (Figure 2a).

HO breaks down heme into equimolar amounts of biliverdin, carbon monoxide, and iron, all of which can be toxic. Mice lacking HO-1 are unable to modulate body iron stores and are susceptible to hepatic injury by iron (17). To study the effect of avicin D on HO-1, we first measured protein expression by Western blot analysis. A marked increase in HO-1 levels was observed at 8 and 16 hours after treatment (Figure 2d) with return to base line after 24 hours with avicin D (data not shown). The reason for the delayed induction of HO-1 (compared with the other ARE-regulated enzymes studied) is not clear. Besides ARE, the promoter region of the HO-1 gene contains consensus binding sites for NF-κB, activated protein-1, activated protein-2, IL-6 response element, and other transcription factors (18). Avicin-induced HO-1 levels could be dependent on the net effects of some or most of these transcription factors. It is also possible that the delay is at the step of protein synthesis and not transcription. Based on some reports that in stressed cells HO-1 is induced after the cellular thiols like GSH have been oxidized (19), it is possible that the delayed induction of HO-1 in avicin-treated cells could be a second phase of protection against oxidative stress. To determine whether increased HO-1 expression resulted in increased enzyme activity, we studied two of the downstream products of HO-1 activity, namely bilirubin and ferritin.

Bilirubin, the most abundant endogenous antioxidant in mammalian tissues, especially in human serum, is a potent scavenger of superoxide and peroxyl radicals (20). Therefore, it is exciting to observe that treatment of Hep G2 cells with avicin D resulted in a dramatic time-dependent increase in bilirubin levels (Figure 2e), which correlates with the striking increase in HO-1 activity. The induction of bilirubin could account for the antioxidant properties of avicins in lipid compartments of the cell.

Ferritin, an iron-storage protein, sequesters free iron and prevents its participation in reactive oxygen species

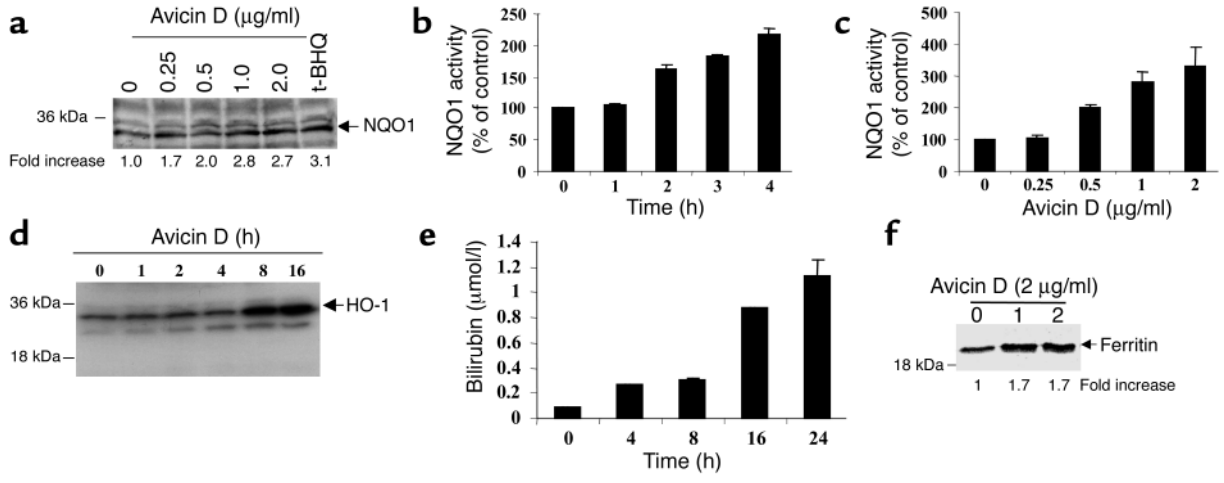

Figure 2
Effect of avicin D on NQO1 and HO-1 enzymes. (**a**) Expression of NQO-1 was studied in Hep G2 cells treated with 0–2 μg/ml of avicin D for 2 hours, by Western blot analysis. (**b** and **c**) Dicoumarol-sensitive NQO1 activity was assayed in Hep G2 cells treated with 0–2 μg/ml of avicin D for 2 hours (**b**) and 2 μg/ml of avicin D for 0–4 hours (**c**), as described in Methods. NQO1 activity was expressed in nanomoles of dichlorophenolindohenol (DCPIP) reduced per minute per milligram protein. (**d**) Hep G2 cells treated with avicin D (2 μg/ml) for 0–16 hours were analyzed for changes in levels of HO-1 by Western blot analysis. (**e**) Hep G2 cells treated with avicin D (2 μg/ml) for 0–24 hours were assayed for bilirubin levels as described in Methods. (**f**) Synthesis of ferritin was studied in Hep G2 cells treated with avicin D (2 μg/ml) for 16–24 hours.

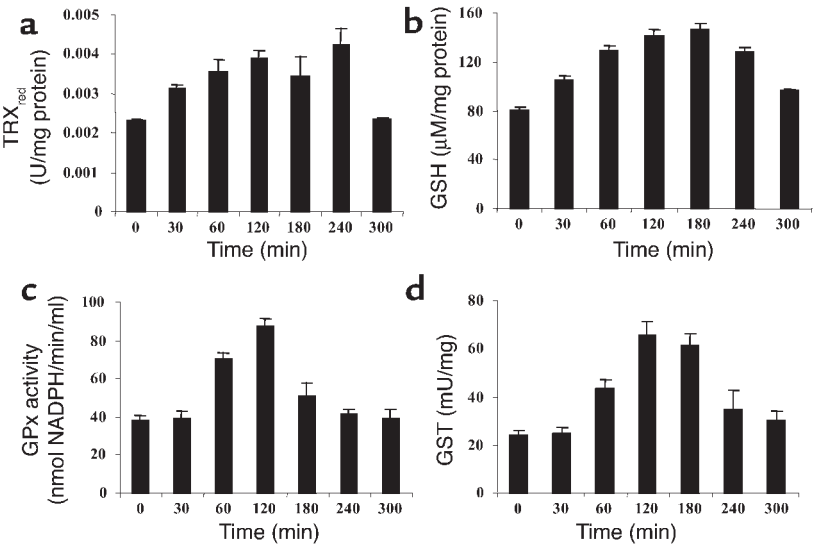

Figure 3
Effect of avicin D on TRX$_{red}$ (**a**), GSH (**b**), GPx (**c**), and GST levels (**d**). Hep G2 cells were treated with avicin D (2 μg/ml) for 0–300 minutes, and cells were lysed by the freeze-thaw method. Levels or activity of the different proteins were measured as described in Methods. The results shown for each of the proteins are representative of three different assays.

(ROS) generation and thereby maintains cellular iron homeostasis. Oxidant stress activates multiple ferritin-regulation pathways (21), one of which is an ARE-mediated mechanism (22). As shown in Figure 2f, a twofold increase in ferritin synthesis was seen in Hep G2 cells following a 16-hour treatment with avicin D, which is consistent with the time of HO-1 activation. Thus, regulation of ferritin by avicins could contribute to their anti-inflammatory action.

The intracellular redox state is regulated by the contribution of different molecules, such as TRX, GSH/GSSG), and NADPH. High ratios of reduced to oxidized forms of TRX and GSH are maintained by the activities of TRX$_{red}$ and GSH reductase, respectively. Figure 3a shows the effect of avicin D on auranofin-sensitive TRX$_{red}$ activity, which increases within 30 minutes of treatment. GSH plays a key role in regulating intracellular redox as well as in detoxifying the cell of various potentially harmful molecules. It acts as a cofactor for GPx, a selenium-dependent enzyme known to be the predominant mechanism for reduction of H_2O_2 to water and of lipid peroxides to alcohol, and known to detoxify peroxynitrite. GSH renders a variety of electrophilic compounds less toxic by forming conjugates with them. These reactions require the action of GSTs, a ubiquitous family of detoxification enzymes. Figure 3b shows a time-dependent increase in the levels of GSH in avicin D–treated Hep G2 cells, which begin to decline after 3 hours of treatment. The modest increase and then decline in GSH levels could be accounted for by the constant utilization of GSH within the cell, as well as the possibility that avicin can bind to the cysteine in GSH, thereby quenching it. This is consistent with the increased activities of GPx and GST seen in these cells following treatment with avicin D (Figure 3, c and d). In confirmation of the increase in GPx and GST activities, we observed a decrease in lipid peroxidation both in avicin-treated cells and in a cell-free system (Supplemental Figure 3, http://www.jci.org/cgi/content/full/113/1/65/DC1). Cells in tissue culture are believed to be under a much higher oxidative stress than cells in vivo (23). The ability of avicins to reduce levels of cellular H_2O_2 as well as lipid peroxides under these conditions might explain the short half-life of these detoxification proteins.

In vivo studies

Epidermal thickness. Mice exposed to UVB for 10 weeks show increased levels of hyperplasia and hyperkeratosis, with focal patches of dysplasia (Figure 4a). As shown in Figure 4b, pretreatment of skin with AE resulted in a significant decrease in hyperplasia. Application of AE after UVB irradiation was less effective in reducing hyperplasia (data not shown). These results were confirmed by BrdU staining (Supplemental Table 1, http://www.jci.org/cgi/content/full/113/1/65/DC1). SKH-1 mice treated for 2 weeks with AE alone showed no changes in epidermal thickness, cell proliferation, and apoptosis. This confirms our earlier observation in SENCAR mice, in which a 4-week treatment with AE alone had no effect on epidermal thickness or dermal cellularity (3).

p53 mutations. The p53 protein plays a crucial role in the cellular response to UVB-induced DNA damage. The basal and suprabasal layers of the epidermis from mice that had been exposed to UVB for 10 weeks showed intense p53 immunoreactivity (Figure 4c) in the form of microscopic clusters of preneoplastic cells overexpressing mutant p53. Pretreatment with AE resulted in a significant decrease in the number of mutant p53–positive cells in these clusters (Figure 4d and Supplemental Table 1, http://www.jci.org/cgi/content/full/113/1/65/DC1). These results suggest that AE could possibly reverse the detrimental process of skin carcinogenesis by inhibiting UV-induced p53 mutations by one or more of the following mechanisms: (a) removal of damaged cells, (b) suppression of oxidative stress, and (c) repair of DNA damage.

Apoptosis. Based on the decrease in p53 mutations, we evaluated the effect of AE on apoptosis of skin cells. Compared with the untreated, UVB-exposed skin (Figure 4e), skin pretreated with AE prior to UVB irradiation showed an increased number of apoptotic cells (Figure 4f and Supplemental Table 1, http://www.jci.org/cgi/content/full/113/1/65/DC1).

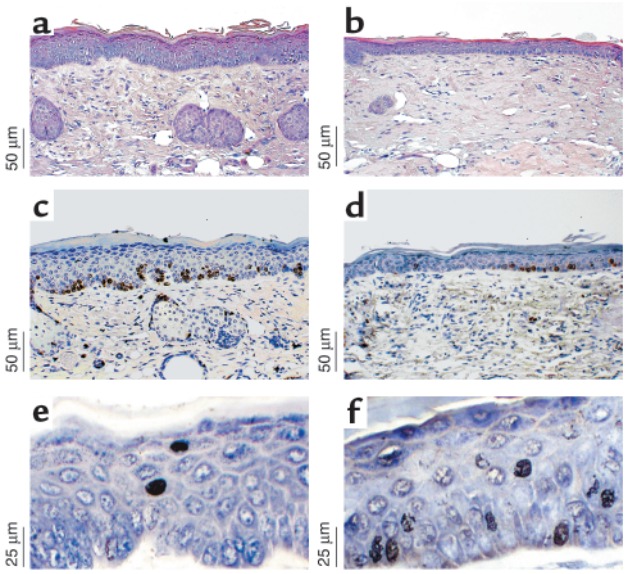

Figure 4
Representative microphotographs showing the effect of AE on epidermal hyperplasia (**a** and **b**; H&E staining; ×200), p53 mutations (**c** and **d**; H&E staining; ×200), and apoptosis (**e** and **f**; H&E staining; ×400) in SKH-1 hairless mouse skin. Mice treated with acetone (**a**, **c**, and **e**) or 0.4 mg of AE (**b**, **d**, and **f**) were irradiated with UVB as described in Methods.

Oxidative damage and immunohistochemistry of phase 2 enzymes. Free radicals are known to cause DNA damage via strand breakage and/or formation of modified bases like 8-OH-dG. UVB treatment led to an approximately fourfold increase in the ratio of 8-OH-dG per 100,000 pmol dG (10^5dG) (pmol/pmol) in DNA of control mice (Figure 5). Application of AE to the skins immediately after UVB exposure or before UV exposure also decreased the 8-OH-dG levels significantly (Figure 5a).

Based on the in vitro results from Hep G2 cells, we studied the effect of AE on NQO1 expression in vivo. AE-treated skin showed a twofold increase in NQO1 staining (Figure 5b). While exposure to UV increased the NQO1 expression by about 8%, pretreatment with AE prior to UV exposure resulted in a 15.7% increase in NQO1 staining (Figure 5b). Similar effects were seen on HO-1 expression in these skin samples (data not shown). These results indicate that the induction of phase 2 detoxification proteins by AE could contribute to its protective effects seen in vivo.

Discussion

In her Nobel lecture two decades ago, Barbara McClintock discussed the innate ability of genomes to restructure when faced with external or internal threats (stress) (24). As organisms age, the ability to withstand chronic cellular stress diminishes, affecting virtually all organ systems, leading to a myriad of human diseases including cancer. Recent work has linked chronic cell injury and cellular repair with inadequate stress response, telomere shortening, gene mutations, genomic instability, and finally cancer (25). Of potential relevance is the evidence that stem cells demonstrate features of cells under stress (26). Identification of molecules that can prevent dysfunctioning of the redox machinery or repair the damage caused by it is therefore becoming important in clinical medicine (27). Transcription factors that regulate expression of antioxidant proteins and enzymes that regenerate them (28) form one such class of molecules. Often these sensor proteins contain critical cysteines (thiol groups) that modify the location of signaling molecules or transcription factors (28). Thus the importance of redox regulation of cysteines in oxidative and nitrosative stress is being increasingly recognized in clinical medicine (28).

In this paper we have described the ability of avicins to regulate the Nrf2-ARE system and induce a battery of genes that restore redox balance within the cell. The two electrophilic Michael addition sites (α,β unsaturated carbonyl groups) on the side chain of the avicin molecules are probably responsible for its binding to one or more critical cysteines, most likely on the Keap1 molecule, to allow for Nrf2 to translocate to the nucleus and activate the ARE. This speculation is consistent with the ability of avicins to activate OxyR (unpublished data), a bacterial transcription factor whose activity is regulated by cysteines (29) and has functions similar to those of Nrf2. Mass spectrometric studies

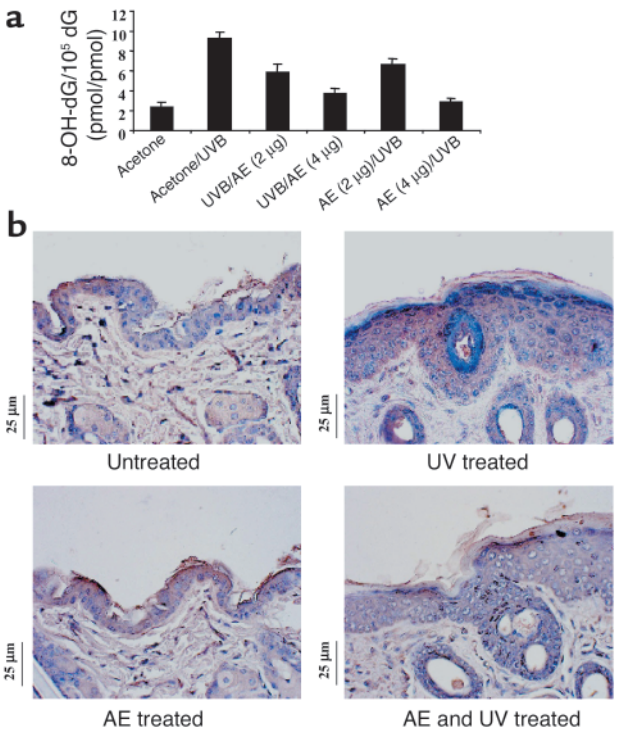

Figure 5
(**a**) Effect of AE on 8-OH-dG formation. Skin sections from mice treated with AE after or before exposure to UVB were analyzed for 8-OH-dG levels as described in Methods. (**b**) Effect of AE on expression of NQO1 in mouse skin. Mice treated with acetone (control) or AE (0.4 mg) were exposed to UV. Immunohistochemistry of NQO1 (H&E staining; ×400) was done as described in Methods.

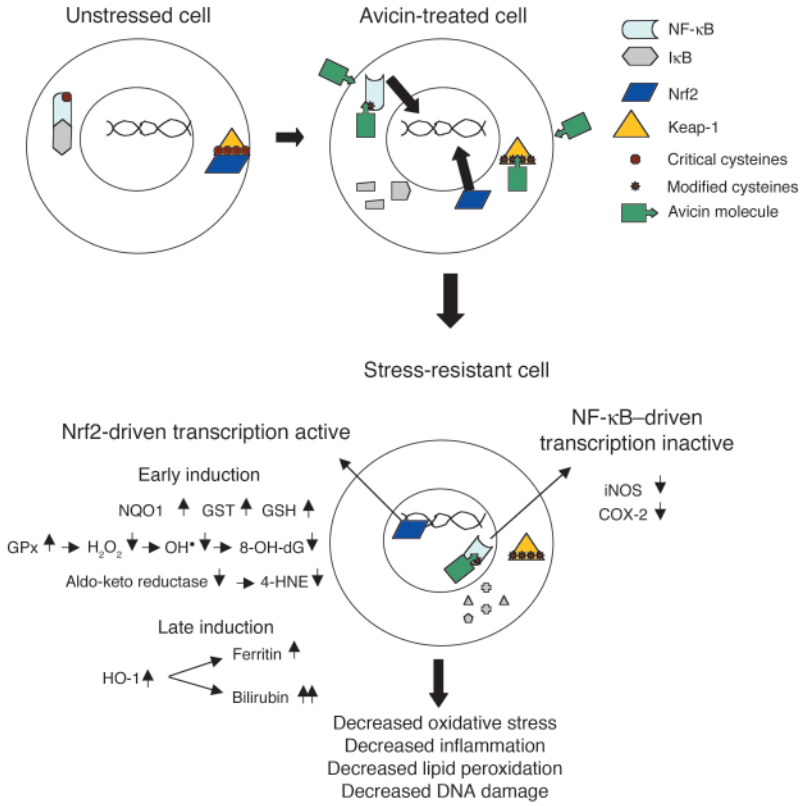

Figure 6
A proposed model of avicin-induced enhancement of the innate stress response. By redox regulation of thiols, avicins have opposing effects on NF-κB–mediated and Nrf2-mediated transcription, resulting in downregulation of proinflammatory components of the innate immune response and upregulation of the intrinsic stress response. The early and late induction of Nrf2-regulated proteins enables a prolonged protective effect. Avicins' proapoptotic response to stress, reported earlier, is functionally separate but linked to the enhancement of innate stress response. 4-HNE, 4-hydroxy-2-nonenal. OH•, hydroxyl radical.

with avicins and target proteins are underway to define the precise chemical changes. Alternative non–redox-dependent mechanisms of activation of Nrf2 (30) are also being studied.

The enhancement of the Nrf2-regulated gene battery could have important clinical ramifications for various forms of cancer and a multitude of other diseases that are characterized by an inadequate stress response (31). For example, individuals with polymorphism in the NQO1 gene have been shown to be at an increased risk of developing leukemias and other cancers (32). Bilirubin is known to be protective against physiological jaundice and oxygen radical–mediated injury (33). A recently published case study of a child deficient in HO (34) is illustrative of its role as an anti-inflammatory stress protein. HO-1 has also been implicated in a variety of cytoprotective roles, including protection against liver and pulmonary injury, acetaminophen toxicity, complement-dependent inflammatory response, and elimination of haem (35). Studies using knockout mice as well as mice overexpressing GPx have confirmed its antioxidant role in vivo (36). Decrease in activity of GST due to hypermethylation has been implicated in several malignancies, particularly prostate cancer (37). Interestingly, in postmitotic cells, enhancement of glial Nrf2 protects neurons from oxidative stress (38).

The ability of avicins to induce higher levels of both HO-1 and bilirubin, compared with GSH could be a novel and important finding. Excess GSH can suppress the stress response, in particular HO-1 (39). Thus the delayed and greater induction of HO-1 and downstream bilirubin probably tilts the balance in favor of anti-inflammation and immune suppression. This notion is supported by avicin's ability to suppress the proinflammatory transcription factor NF-κB. Other compounds with α,β unsaturated carbonyl groups (40) show similar effects on the NF-κB and Nrf2 systems. Thus, a new concept is emerging that indicates to us that compounds with such signature components (α,β unsaturated carbonyl groups), by upregulating the innate stress response and downregulating components of the proinflammatory innate immune response (2–4), can reduce inflammation and oxidative damage (see model, Figure 6). These compounds also induce apoptosis of damaged cells by mechanisms that are closely associated with, but independent of, thiol regulation (refs. 1, 41; and our unpublished observations). This makes them potential therapeutic agents for reducing many forms of cellular stress.

Stress responses are often cross-protective. Thus the activation of Nrf2 should protect against a variety of biotic and abiotic stresses. To study the in vivo protective effects of avicins, we chose UVB light, an ancient stressor, a complete carcinogen, and the major cause of skin cancers. Avicins delivered topically, either before or immediately after exposure to UVB in SKH-1 mice, significantly suppressed skin damage. With regards to initiation, avicins were unable to diminish the UVB-induced base dimerization (data not shown), indicating the absence of a sunscreen effect. On the other hand, avicins induced a dramatic decrease in the generation of ROS as measured by levels of 8-OH-dG, which is a chemical signature of hydroxyl radical attack on DNA. Expression of phase 2 proteins like NQO1 and HO-1 was also enhanced in vivo. Since generation of ROS has been directly associated with DNA damage such as p53 mutagenesis (42), the significant suppression of p53 mutations probably occurs in part as a result of the antioxidant effects of avicins. The proapoptotic property of avicins could also account for the observed decrease in p53 mutations. The ability to

remove damaged cells by apoptosis (1), an additional important form of stress response in multicellular organisms, suggests novel intervention strategies for premalignant and malignant skin and other diseases.

One of the important pathophysiological consequences of chronic UV exposure is depletion of antioxidants such as GSH, which is known to sensitize the skin to damage (43). Thus the ability of avicins to increase GST and TRX_{red}, which recycles ascorbic acid as well as other reducing substances (44), further supports the UV-protectant effects of avicins. Decrease in lipid peroxidation and increase in the levels of lipid-soluble antioxidants like bilirubin and in scavengers of lipid peroxides such as GSH and GST also protect the cells from UV-induced membrane damage.

The five-ring triterpene structures called hopanoids are ancient and have been identified as the main membrane-lipid support in a number of prokaryotes that evolved before the rise in atmospheric oxygen, when synthesis of sterols became possible (45). Hopanoids have a fascinating property of preventing the inward leakage of protons (46), which could contribute to a decrease in oxidative stress. The evolution of the side chain containing the two reactive electrophile sites converted the triterpene to a hybrid signaling molecule and probably first occurred in plants. Like ROS and reactive nitrogen species, reactive electrophilic metabolites such as avicins evolved as cell-signaling molecules with submolecular (atomic) specificity (47). We speculate that the avicins may regulate redox balance in plants too, based on the avicins' identification in critical organs necessary for overall fitness, namely the seedpods, germinating seeds, and roots (2, 48). Like certain phenolic compounds known to prevent cellular damage (49), the avicins are induced when *Acacia victoriae* is under stress (48). The variety of defense molecules induced by avicins play critical roles in plant defense against oxidant stress (50).

In conclusion, avicins represent a new class of metabolites that regulate cellular redox balance through DNA-binding sensor proteins and that appear capable of modulating stress-associated disease phenotypes that are thermodynamically imbalanced (51). Based on the properties of avicins reported here and previously, we speculate that they mimic many of the effects of calorie restriction (49). The increased understanding of avicins' effects on inflammatory tissue, including UV-exposed skin, offers exciting opportunities for disease modulation (52, 53, 54). It is hoped that the avicins, having moved from the Australian desert to the laboratory, will successfully find application at the bedside.

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