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Profiling patterns of glutathione reductase inhibition by the natural product illudin S and its acylfulvene analogues

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Abstract

Acylfulvenes (AFs) are a class of antitumor agents with favorable cytotoxic selectivity profiles compared to their natural product precursor, illudin S. Like many alkylating agents, illudin S and AFs readily react with thiol-containing small molecules such as cysteine, glutathione and cysteine-containing peptides; reduced cellular glutathione levels can affect illudin S toxicity. Glutathione reductase (GR) is a critical cellular anti-oxidant enzyme that regulates the intracellular ratio of reduced:oxidized glutathione. In this study, we found that acylfulvene analogues are GR inhibitors, and evaluated aspects of the drug-enzyme interactions as compared with the structurally related natural product illudin S and the known irreversible GR inhibitor, carmustine. Acylfulvene analogues exhibited concentration-dependent GR inhibitory activity with micromolar IC_{50} s; however, up to 2 mM illudin S did not inhibit GR activity. The absence of NADPH attenuates GR inhibition by AFs and the presence of glutathione disulfide (GSSG), the natural GR substrate, which binds to the enzyme active site, has a minimal effect in protecting GR from AFs. Furthermore, each compound can induce GR conformation changes independent of the presence of NADPH or GSSG. These results, together with gel filtration analysis results and mass spectrometry data indicate AF is a reversible inhibitor and HMAF an irreversible inhibitor that can form a bis-adduct with GR by reacting with active site cysteines. Finally in a cell-based assay, illudin S and HMAF were found to inhibit GR activity, but this inhibition was not associated with the reduction of GR levels in the cell. A model accounting for differences in mechanisms of GR inhibition by the series of compounds is discussed.

Keywords

glutathione reductase; illudin S; acylfulvene; enzyme inhibition; protein modification

Introduction

Fungal metabolism is a rich source of unique pharmacophore platforms, often exhibiting varying degrees of toxicity that may be detrimental to health or valuable for anti-infective or anticancer activity.^{1, 2} Unlike modern target-directed drug development strategies, the structural complexity and bottom-up characteristics associated with natural product bioactivity, often translates to compounds with the capacity to interact with multiple cellular targets.³ Understanding the range of contributing biochemical and chemical interactions and how they are interrelated may be a key for developing improved therapeutics or avoiding unwanted toxicities, and can also suggest new potential drug targets.

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Acylfulvenes (AFs) are a class of antitumor agents derived from the naturally occurring sesquiterpenoid toxin illudin S, isolated from the jack o' lantern mushroom (Figure 1).⁴⁻⁷ Although illudin S is extremely cytotoxic to cancer cells, it exhibits low selectivity towards malignant cells versus normal cells and thus a narrow therapeutic window.⁸ Semisynthetic analogues acylfulvene (AF) and hydroxymethylacylfulvene (HMAF) display improved therapeutic indices.^{9, 10} Cellular assays suggest that illudin S and AFs covalently bind to DNA, as well as RNA and protein (Scheme 1).^{11, 12} There is evidence that cytotoxicity is associated with bioactivation to a potent alkylating agent that reacts with DNA and interrupts DNA synthesis/repair (Scheme 1).^{11, 13-17} However, compared to conventional DNA alkylating agents, AFs display some unique activity profiles suggesting contributing interactions with other cellular targets and a distinct mechanism of action underlying cytotoxicity profiles.^{18, 19} AFs also alkylate cellular proteins and react with thiols such as cysteine, glutathione or cysteine-containing peptides under slightly acidic conditions.²⁰⁻²³ Protein modification and protein binding are general modes of drug toxicity.²⁴ Furthermore, for many cellular proteins such as the glutathione reductase and thioredoxin reductase systems, cysteine thiol groups are particularly important to defend against oxidative stress and regulate the activity of cellular signaling proteins. However, the influence of AFs on critical cellular redox-regulating enzymes and their potential contributions to the cytotoxicities of AFs are not understood.

Glutathione reductase (GR, EC 1.6.4.2) is a dimeric FAD-containing enzyme with a redox-active disulfide at its active site. The two cysteine residues are designated as the distal cysteine, which can interact with FAD domain, and proximal cysteine, which does not. GR catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) at the expense of NADPH.^{25, 26} The enzyme is responsible for maintaining a high intracellular ratio of GSH/GSSG, critical in defending against oxidative stress. GR inhibitors, such as isothiazol-3-one, can disrupt the GSH/GSSG balance and cellular reduction potentials.²⁷ While Glutathione-drug conjugate formation may serve as a cellular detoxification route, the process of glutathione depletion may also disrupt redox homeostasis.^{28, 29} Glutathione conjugation has been suggested to have a role in illudin S and AFs toxicology as a detoxification pathway: pre-treating cells with *N*-ethylmaleimide or D,L-buthionine (S, R)-sulfoxime, which readily reacts with GSH or inhibits GSH synthesis, respectively, enhances cell sensitivity toward illudin S, while 2-oxothiazolidine-4-carboxylic acid pretreatment, which elevates GSH, is protective.²⁹ However, these studies were carried out for illudin S, which readily reacts with GSH, while AFs essentially do not.^{21, 23} Furthermore, there are various examples of GR inhibition as a therapeutic strategy for antimalarial activity,³⁰ agents to decrease drug resistance,³¹ and anticancer activity.³² As a step toward mapping the overall biomolecular reactivity of AFs, and especially for understanding the potential role of interacting with redox-regulating enzymes on cytotoxic selectivities, we evaluated the influence of illudin S and AF on GR structure, activity, and cellular properties.

In this study, we characterize for the first time the reactivities of illudin S and AFs toward purified yeast GR, revealing chemical structure-based differences in inhibition potencies and physical modes of enzyme-drug interactions. The results of this study provide new information regarding how the cytotoxicities of illudin S and AF analogues may be mediated by influencing the cellular redox environment, and define chemical structure characteristics that differentiate reactivity profiles amongst this class of agents.

Experimental

Chemicals and reagents

Illudin S was provided by MGI Pharma (Bloomington, MN). Acylfulvene and HMAF were synthesized according to the published procedure with illudin S as starting material.^{6, 7} Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxidized glutathione

(GSSG) were purchased from EMD chemicals (Gibbstown, NJ). Tris base, EDTA, BSA, and Carmustine (BCNU, 1,3-bis(2-chloroethyl)-1-nitroso-urea) were obtained from Sigma Chemical (Milwaukee, WI). Baker's yeast GR used for fluorescence evaluation was obtained from Sigma Chemical and GR for other experiments was obtained from MP Biomedicals (Cleveland, OH). Recombinant rAOR was expressed and purified as published previously. 33 Dulbecco's modified Eagle's medium (DMEM) was purchased from Mediatech (Herndon, VA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Phosphate-buffered saline (PBS), 0.25% trypsin-EDTA, penicillin-streptomycin were obtained from Invitrogen (Carlsbad, CA). Tris-buffered saline was purchased from Biorad (Hercules, CA).

General considerations

GR, NADPH, GSSG, and bovine serum albumin (BSA) stock solutions were prepared in Tris-Cl buffer (50 mM, pH 7.2) containing EDTA (1 mM), i.e. TE buffer. All assays were carried out in TE buffer containing 0.3% (w/v) bovine serum albumin. Stock solutions of test compounds were prepared in DMSO, and aliquots of these solutions were added to the reaction mixture to yield a final DMSO concentration of 2% (v/v). GR activity was determined by measuring NADPH oxidation by monitoring change in UV absorbance at 340 nm with a Varian Cary 100 double-beam spectrophotometer.³⁴ All the measurements were performed in triplicates, and the data were presented as mean $\pm$ standard deviation. Fluorescence measurements were carried out on a Varian Cary spectrofluorometer at 90° in relation to the excitation source. Fluorescence was measured with excitation at 270 nm (bandpasses of 5 and 10 nm for excitation and emission respectively) and emission from 295 to 450 nm. HPLC analysis was carried out on an Agilent 1100 series instrument with diode array detector and autosampler. Analytes were eluted with a solvent gradient involving, initially, 10% acetonitrile in water, linearly increasing to 50% acetonitrile in water over a course of 30 min, at a flow rate of 1 mL/min. A Phenomenex Luna 5 μ m C₁₈(2) 100 Å 250 mm $\times$ 4.60 mm was used (Phenomenex, Torrance, CA).

Liquid chromatography-mass spectrometry (LC/MS) analyses were carried out on an Agilent 1100 capillary HPLC- iontrap mass spectrometer operated in positive ion mode; the HPLC was equipped with an autosampler. A Zorbax 300 SB-C3 column (150 mm $\times$ 0.5 mm, 5 μ m) was used. Analytes were eluted with a solvent gradient of 0.05% TFA in water (A) and 0.05% TFA in acetonitrile (B), at a flow rate of 15 μ L/min: initial conditions, 30:70 B:A, were held 3 min followed by a linear increase to 80:20 B:A over a course of 20 min. Spectra were obtained by full scan data acquisition performed within m/z 100-1500. Mass deconvolution was performed with the Agilent ion trap analysis software.

LC/MS/MS analysis of peptide mixtures was performed on an Agilent 1100 capillary HPLC in line with an Agilent 1100 iontrap mass spectrometer operated in positive ion mode. An Agilent Zorbax SB-C18 column (150 mm $\times$ 0.5 mm, 5 μ m) was used. Analytes were eluted with a gradient of solvent A (0.5% formic acid/0.01% TFA in water) and solvent B (0.5% formic acid/0.01% TFA in acetonitrile) at a flow rate of 15 μ L/min: initial conditions, 3:97 B:A, were held constant for 3 min, and then increased to 5:95 B:A in 7 min and held for 10 min followed by linear increase to 35:65 B:A over a course of 95 min, and finally to 75:25 B:A in 10 min.

Substrate screening

To determine whether test compounds were GR substrates, each compound (AF and HMAF, 400 μ M; Illudin S, 1 mM; reference blank, 2% DMSO) was combined individually with NADPH (200 μ M) and GR (2.5 μ M) in TE buffer with a final volume of 200 μ L and allowed to react at 37 °C for 2 h. The resulting solution was extracted with ethyl acetate (EtOAc, 200

μL) and centrifuged for 5 min (6000 g). The supernatant was collected and EtOAc was evaporated under a stream of N₂. The dried material was reconstituted in 100 μL DMSO and 50 μL was injected and analyzed with the HPLC method. As a positive control, the same procedure was carried out with AOR (2 μM) in place of GR.

Measurement of GR activity

GR inhibition assays were performed by combining NADPH (150 μM) and GR (5 nM) in TE buffer, total volume 200 μL in disposable acrylic cuvettes at 25 °C. GR was first treated with NADPH for 10 min before the addition of the test compound at the indicated concentration (AF, 62.5, 125, 250, 625, 750, 1000, 1250 μM; HMAF, 62.5, 125, 250, 625, 1250 μM; illudin S, 62.5, 125, 250, 625, 1250, 2000 μM) and further allowed to react for 30 min. GSSG (360 μL, 350 μM) and further allowed to react for 30 min. GSSG (360 μL, 350 μM) was then added and the decrease in absorbance at A₃₄₀ was monitored over 3 min. Measurements were performed in triplicate. IC₅₀ values were determined from a plot of relative activity v.s. compound concentration (Kaleidagraph). To evaluate the time-dependence of GR inhibition, GR was allowed to react with the test compounds (AF, 0, 500, 750, 1000 μM and 1250 μM; HMAF, 0, 125, 250, 500 and 1250 μM) in the same manner as described above and aliquots (200 μL) were taken at different time intervals (0, 2, 7, 13, 24, 30 min) and assayed as described above. To evaluate the effect of added substrate, i.e. GSSG on drug-mediated enzyme inhibition, GR was treated with test compounds in a total volume of 200 μL containing GSSG (250 μM or 1250 μM) for 30 min. Activity was determined by following A₃₄₀ upon addition of 360 μL TE buffer containing GSSG (350 μM) and NADPH (100 μM) in the manner described above. To evaluate the effect of NADPH on GR inhibition, GR was allowed to react with compounds in the absence of NADPH for 30 min, and activity was measured in the same way as described for evaluating the effect of GSSG.

To determine the reversibility of inhibition, GR was allowed to react with AFs (AF, 250, 625, 750, 1000, 1250 μM; HMAF, 62.5, 125, 250, 625, 1250 μM) as described. After the 30 min reaction period, unbound compound was removed by gel-filtration with a size-exclusion micro bio-spin P6 pre-packed column according to the manufacturer's protocols. Briefly, the column was placed in 2 mL centrifuge tube to drain the excess packing buffer by gravity and the drained buffer was drained. The column was placed back into the tube and centrifuged to remove the packing buffer (2 min, 1000 g). The column was placed in a clean centrifuge tube, and the reaction solution was loaded into two columns (100 μL/each) and the column was centrifuges for 4 min at 1000 g. The resulting solution was combined, and the activity was determined following the procedure described above.

Protein adduct analysis

GR (250 μg, 5 nmol) was allowed to react with AFs (1.25 mM) in a total volume of 1 mL TE buffer containing NADPH (1 mM) for 3 h at 25 °C. An aliquot (1 μL) of reaction solution was withdrawn and diluted to 200 μL; enzyme activity was determined as described above for measurement of GR activity. At 3 h, 50% GR activity was inhibited by HMAF, and 30% by AF. An Amicon ultra-4 centrifugal filter device (30,000 NMWL, Millipore, MA) was used to concentrate GR and remove unbound compound. GR was reconstituted in 1 mL TE buffer and re-treated in the same manner. After another 3 h incubation, GR activity was diminished 100% by HMAF, and 50% by AF. Modified GR was again concentrated with an Amicon filter, and unbound compound was further removed with a size exclusion micro Bio-spin P6 pre-packed column (6,000 NWML, Biorad, Hercules, CA). GR (250 μg, 5 nmol) was allowed to react with carmustine (0.25 mM) in a total volume of 1 mL TE buffer containing NADPH (1 mM) for 3 h at 25 °C. An Amicon ultra-4 centrifugal filter and micro bio-spin P6 pre-packed column were used to concentrate GR and remove unbound compound. The resulting solution was dried on a Speedvac concentrator (Savant, Waltham, MA). GR (20 μg in 8 μL TE buffer) was analyzed

by LC/MS using method described in the general consideration. Both native and modified GR proteins eluted as a single peak with retention time 12.5 min.

Protein digestion and modified peptide analysis

Adducted protein samples obtained as described above (~200 µg, 4 nmol) were reconstituted in 20 µL guanidine-HCl (8 M, pH 7.2) containing 50 mM dithiothreitol (DTT) and heated at 95 °C for 15 min. Iodoacetamide (IAA, 5 µL, 0.5 M in TE buffer) was added and allowed to react for 30 min at 25 °C. Ammonium bicarbonate buffer (600 µL, pH 8), followed by trypsin (10 µg, ~0.43 nmol) was added. Proteolytic digestion was allowed to occur at 37 °C for 24 h. Equal amount of the resulting mixtures were placed into two centrifuge tubes, and dried on a Speedvac concentrator and reconstituted in 200 µL of solvent A (0.5% formic acid/0.01% TFA in water). Modified peptides were analyzed by the general LC/MS/MS method.

Fluorescence analysis of GR

To evaluate conformational changes of GR in the presence of test compounds, GR (2 µM) was treated with test compounds in a total volume of 0.5 mL TE buffer at indicated conditions for 30 min at 25 °C. Changes in intrinsic fluorescence were evaluated by using the general method. To evaluate the effect of test compounds on GR, GR was treated with either AF or HMAF (0.01, 0.04, 0.2, 1 mM) or illudin S (0.02, 0.1, 0.4, 2 mM). To evaluate the effect of test compound on GR in the presence of GSSG, GR was treated with test compounds in the presence of GSSG (400 µM). To evaluate the effect of test compounds on NADPH-reduced GR, GR was pre-reduced by NADPH (100 µM) for 10 min followed by the compound treatment. GR was also pre-treated with both NADPH (100 µM) and GSSG (400 µM) for 10 min prior to addition of the test compounds.

Cell culture and cellular GR determination

Hela cells were maintained as monolayers in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified, 5% CO₂ atmosphere at 37 °C. Hela cells were subcultured in the medium described above for three days to reach 80% confluence (100 mm² plate, 2×10⁶ cells/plate). Cells were treated with test compounds diluted with medium (0.1% final concentration of DMSO) for two or ten hours. After the treatment period cells were washed with PBS twice. Cells were collected as follows: 2 mL of 0.25% trypsin-EDTA was added and the cells were incubated for 5 min at 25 °C, and then cells were scratched off and divided evenly into three centrifuge tube (1.5 mL), and centrifuged (5 min, 1000g) followed by removal of the supernatant. Cells were resuspended in 0.2 mL of lysis buffer (50 mM Tris-HCl, pH 7.5; 1 mM EDTA; 0.1% Triton X-100; 1 mM phenylmethanesulfonyl fluoride; 1 mM benzamide; 1.4 µM pepstatin A; and 2.0 µM leupeptin) and sonicated at 4 °C (5 s bursts). The resulting cell lysate was centrifuged for 10 min (8000 g, 4 °C), and the supernatant was withdrawn for analysis. Cell cytosol containing 50 µg protein as determined by the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL) was incubated with 325 µL of 1 mM GSSG in TE buffer at 25 °C for 10 min. NADPH (150 µL, 1 mM) was added, and the rate of NADPH consumption was monitoring changes in absorbance at 340 nm for 5 min at 25 °C.

Cell cytosol containing 25 µg protein was analyzed by 4-12% SDS-PAGE according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) and transferred onto a membrane (PVDF, Invitrogen, Carlsbad, CA) for 1 h at 33 V, 4 °C. Membranes were blocked with 5% (w/v) nonfat milk powder in tris-buffered saline (TBS)/Tween 20 (0.05%) overnight at 4 °C. The membrane was incubated with primary anti-GR (ABR, Rockford, IL) and anti-actin (Invitrogen, Carlsbad, CA) antibodies diluted 2000 times in TBS/Tween 20 (5 mL) for 1 h. The membrane was washed three times with TBS/Tween 20 (5 mL, 5 min) and subject to incubation with 5 mL of secondary conjugated antibody (goat anti-rabbit IgG horseradish peroxidase, Biorad, Hercules, CA,

1:2000 dilution in TBS/Tween 20,) for 1 h. After four 5 min washes with TBS/Tween 20 (5 mL/each), enhanced chemiluminescence was measured with a western blotting analysis system (Pierce, Rockford, IL).

Results

Concentration and time-dependent inhibition of GR

To test the hypothesis that illudin S and AFs may inhibit GR and to compare their potencies as GR inhibitors, various concentrations of the test compounds were incubated with GR in the presence of NADPH for 30 min. Loss of enzyme activity was observed in a concentration-dependent manner for AFs (Figure 2A). Calculated IC₅₀ values are 216 μM for HMAF and 871 μM for AF; 2 mM illudin S did not inhibit GR. As a positive control, under the same conditions, we measured a 71 μM IC₅₀ for carmustine, which is consistent with published data (Figure S1).³⁵ On the basis of these data, four concentrations of AFs were selected to study the time-dependence of the observed inhibition. GR was incubated with varying HMAF concentrations for 30 min (Figure 2B and 2C), and [HMAF] is much higher than [GR] at time zero. The enzyme activity decreased with time, characteristic of irreversible inhibition, and inhibitory parameters (K_i and k_{inact} , Scheme 2) were determined.³⁶ Thus, the linear correlation derived from a plot of the reciprocal of apparent inhibition rate constants (K_{app}) versus the reciprocal of HMAF concentration (Figure 2C inset) yields the inactivation rate constant k_{inact} (0.2 min⁻¹) and inhibitory constant K_i (180 μM) on the basis of eq. 1.

$$\frac{1}{K_{app}} = \frac{1}{k_{inact}} + \frac{K_i}{k_{inact}} \frac{1}{[HMAF]} \quad \text{eq. 1}$$

These data further confirm that GR inhibition by HMAF is irreversible; however, data from the same experiment in the case of AF suggest rapid initial activity loss, then establishment of equilibrium. Such behavior does not fit the same kinetic model,³⁶ indicating differences in the GR inhibition mechanisms for AF vs. HMAF.

GR Substrate screening

To verify that there was no GR-mediated conversion of illudin S and AFs to their major cytosolic metabolites, we compared the released products of reactions involving test compounds and GR with those from AOR, a cytosolic enzyme with well-characterized AF-bioactivating capacity (Scheme 1).^{13, 37-39} In AOR-catalyzed reduction of AF and HMAF with NADPH as a cofactor, one major metabolite was observed by HPLC analysis of the extracted reaction mixture, and this product had the same retention time and UV spectra as the synthetic standards M_A and M_H (Figure 1)^{38, 39}. Under the experimental conditions, no metabolites were observed to be formed from the reaction of AOR/NADPH with illudin S.^{37, 40} Finally, no M_A, M_H, or M_I was observed when illudin S and AFs were tested as possible substrates for GR-mediated conversion at constant NADPH concentration and in the absence of GSSG (Figure 3).

Gel-filtration of inhibited GR

To determine whether GR inhibition by AFs is reversible, gel-filtration studies were conducted in which GR was allowed to react with AFs in the same way as described for studies carried out to determine the concentration-dependence of inhibition. Inactivated GR was passed through a size exclusion micro Bio-spin P6 gel-filtration column (NWML 6000) to remove non-covalently bound compounds. The results of this process were that the GR activity inhibited by AF was recovered, but not that inhibited by HMAF (Figure 4). These results indicate that AF is a reversible inhibitor and HMAF is an irreversible inhibitor.

Effect of GSSG on GR inhibition

To probe the nature of the AF-GR interaction with respect to the proximity of binding at or near the active site where the natural substrate GSSG interacts, a competitive inhibition assay was carried out in the presence of GSSG. A moderate protective effect against inhibition by HMAF was observed, which did not depend on GSSG concentration (Figure 5B). Thus, when GSSG concentration was equal to or less than the highest drug concentration (1250 μ M), no protection towards GR inhibition was observed, suggesting that HMAF may be more competitive for the active site. However, the presence of GSSG did not protect GR from inhibition by AF (Figure 5A). These results suggest that AF may bind to the GR at an allosteric site, but HMAF binds to the active site as well as other binding sites. In contrast, 250 μ M GSSG protected GR from inhibition by carmustine (Figure S2), suggesting that carmustine only binds to GR active site.

Effect of NADPH on the inhibition of GR by AFs

NADPH reduces the disulfide bridge at the GR active site to generate two free cysteine thiols that in turn reduce GSSG to GSH through disulfide/dithiol exchange. To determine whether the reduced form of GR is required for GR inhibition by AFs, NADPH was omitted from the incubation solution and added only at the point of measuring GR activity (a requirement of the spectroscopic assay). In this study, GR inhibition by AFs was observed to be mildly attenuated by lack of pre-reduction. Thus, GR inhibition by 1000 μ M or 1250 μ M AF was reduced by 30%; GR inhibition by 625 μ M or 1250 μ M HMAF was reduced by 45% (Figure 6). In contrast, GR pre-reduction is absolutely required for GR inhibition by carmustine (Figure S3), suggesting that AFs may interact with portions of GR other than the active site, in a process that may contribute in part to enzyme inactivation.

Drug-induced intrinsic GR fluorescence quenching

Data obtained from the experiments described indicate that GR inhibition depends only mildly on active site protection by GSSG and pre-reduction by NADPH. We were therefore interested in determining whether drug binding induced any conformational changes in the enzyme and such changes therefore were probed by analyzing intrinsic GR fluorescence. Illudin S and AFs are inherently non-fluorescent molecules, and the buffer system does not have an appreciable effect on the intrinsic fluorescence of GR unless NADPH, which has fluorescence emission peak at 460 nm and causes reduction on GR intrinsic fluorescence, is present. As shown in Figure 7, all of the compounds tested caused significant decreases in GR fluorescence. Peak splitting in the 300-360 nm spectral region was observed regardless of whether GSSG or NADPH were present which may be associated with GR unfolding.^{41, 42} Thus, at micromolar concentrations, illudin S and AFs can completely eliminate the intrinsic GR fluorescence, suggesting extensive binding interactions of these small molecules with GR, and that GR inhibition by AFs is at least partially due to a drug-induced GR conformational change. Carmustine can also induce a 50% decrease in GR fluorescence intensity, which is observed at 1 mM drug concentration, and does not cause the peak shift and splitting characteristic of illudin and AF-mediated fluorescence changes (Figure S3).

MS analysis of a whole protein adduct

On the basis of gel-filtration data, we established that GR inhibition by HMAF is irreversible, while for AF it is reversible. Therefore, LC/MS was used to obtain information regarding the chemical nature of HMAF-GR. In the control sample, i.e. enzyme treated with an equal amount of DMSO, a peak with m/z 51499 corresponding to the GR monomer was observed (Figure 8), which is consistent with published data in which the GR monomer m/z 51488, as well as the truncated monomer m/z 51719, was observed, with no GR homodimer detected.⁴³ A higher GR concentration (5 μ M) was used for LC/MS sample preparation, such that GR incubated

with 1 mM AFs in the presence of NADPH at a total volume of 1 mL for three hours at 25 °C, was not completely inactivated. GR was concentrated and unbound drug was removed with an Amicon Ultra-4 centrifuge filter (NWML 30,000). The concentrated enzyme was reconstituted and treated by the same procedure for three more hours, which resulted in total loss of GR activity by HMAF and 50% loss by AF. Filtered samples were analyzed by LC/MS, and the results are presented in Figure 8. The AF-treated GR was unchanged compared to the control, consistent with the expected lack of covalent adduction. After reaction with HMAF, however, the parent GR was completely absent, and a new peak with an m/z 51992, corresponding to the bis-adduct (GR monomer + 2×246 Da), was observed. In contrast, mono-adduct was observed for carmustine-treated GR (Figure S4), which is consistent with X-ray data previously obtained for carmustine-GR crystals.⁴⁴

LC/MS/MS analysis of GR active site peptide modified by HMAF

The whole protein analysis data presented above indicates the formation of a bis-adduct between GR and HMAF. Further insight regarding identities of HMAF-modified amino acids was obtained by LC/MS/MS analysis of proteolytically digested GR. Modified and control samples were treated with trypsin after DTT reduction and IAA alkylation to block cysteine disulfide formation.⁴⁵ For control GR, the observed full scan ion m/z 716.6 corresponds to the doubly charged active site peptide ALGGTC⁴²VNVGC⁴⁷VPK (calculated MW: 1430.2 Da, Figure 9A). When subjected to collision-induced dissociation (CID) in the ion trap mass spectrometer, the masses of y and b series ions⁴⁶ were in agreement with theoretical values for this peptide with IAA modified cysteines (Figure 9A). The full scan ion m/z 716.6 was not observed in the LC/MS/MS spectrum of HMAF-treated GR, and instead a doubly charged peptide peak ion m/z 905.4 $[M + 2H]^{2+}$ was observed. This mass corresponds to modification of the active-site peptide modified with two equivalents of HMAF (calculated MW: 1808.8 Da, Figure 9B). This fragmentation signal is weak, and not all diagnostic y and b ions were detected. The fragments (MH^+ m/z 1318.6 and $MH^+ - H_2O$ m/z 1301.5) represents the peptide with HMAF off, suggesting that the modification is not stable under ionization conditions. Inherent in the peptide sequence, the calculated $b_6^* - b_9^*$ ion masses are the same as $y_5^* - y_8^*$ (Table S1), therefore, annotation of these peaks represents one or the other, or both, fragments. The unique ions b_{11}^* (m/z 1467.4), y_9^* (m/z 1410.5) and a_{13}^* (m/z 1635.6), which represent mass increases of m/z 2×246 were observed, strongly suggesting that adducts are formed by alkylation of both cysteines.

Cellular GR inhibition

Human cervical cancer cells (Hela) were used to determine whether the test compounds influence cellular GR activity and GR protein levels. Due to the cytotoxicity of these compounds, concentrations causing less than 20% cell death were selected to treat cells. After exposure to equitoxic doses of illudin S or AFs for 2 h and 12 h respectively, cells were collected and cellular GR activities were measured. As shown in Figure 10A, a 2 h treatment did not cause any significant inhibitory effect on cellular GR activity by illudin S or AFs, but 20-40% inhibition of GR activity was observed for carmustine treatment. Cellular GR activity was inhibited about 40% after 12 h exposure to 4 μ M HMAF and 70% from 5 μ M carmustine (Figure 10B). Thus, the relative magnitudes of cellular GR inhibition by HMAF or carmustine comparably correlate with their inhibition potencies in the cell-free system, while AF did not inhibit cellular GR activity. Interestingly, illudin S (12 h) inhibited GR activity by 50%, but in a concentration-independent manner. Western blotting analysis did not suggest any reduction in cellular GR protein levels for AFs or illudin S (Figure 11), however carmustine slightly reduces GR levels and a new band was observed with a molecular weight close to the GR dimer. The new band may be the crosslink adduct formed between carmustine and two GR monomers, or between carmustine, GR, and some other cellular protein with a similar molecular weight.

Discussion

DNA alkylation is thought to play a major role in illudin S and AF toxicity, however at equitoxic concentrations, the incorporation of AFs into genomic DNA in tumor cells is similar with that of illudin S, suggesting other cellular reactivity factors may play a role in distinguishing the improved therapeutic index of AFs versus illudin S.^{11, 12} For example, reductase-mediated bioactivation has been shown to contribute in part to dictating differences in cytotoxicity profiles for AFs.⁴⁷ Further, the extreme toxicity of illudin S has been attributed to its reactivity towards thiols like GSH.²⁹ By analogy, it has been hypothesized that illudin S can react with thiol-containing enzymes, which may be expected to contribute to cytotoxicity.²⁹ Compared with illudin S, AFs are much less reactive towards small-molecule thiols.^{6, 21} However, no studies have been carried out to test whether illudin S reacts with thiol-containing enzymes differently than AFs, in order to address the potential role of this process in their toxicity.⁴⁸ On the basis of data obtained in the present study, a proposed model accounting for differences in reactivity profiles of illudin S and AFs toward the critical thiol-containing GR, compared with the known GR inhibitor carmustine, is illustrated in Scheme 4.

In the current study, yeast GR was selected as a model to profile relative reactivities of illudin S and its AF analogues toward enzymes containing critical thiols. GR is the key enzyme involved in cellular redox regulation. Yeast GR is a homodimeric flavoenzyme that shares considerable sequence homology with human and *E. coli* GR. Human and yeast GR exhibit ~68% similarity with almost identical FAD-binding domain sequences that contain the critical redox-active dithiol.^{49, 50} The dimeric forms of human and *E. coli* GR are important for catalysis, and the monomers are not enzymatically active.⁵¹⁻⁵² Mechanistic studies of dimer dissociation and unfolding of yeast GR, induced by acid and pressure⁴² or by a denaturing agent (i.e. guanidine hydrochloride),⁴¹ were associated with changes in GR intrinsic fluorescence. In addition, unfolding can release more accessible cysteines compared to native GR.

Monitoring changes in intrinsic protein fluorescence is an approach to evaluate GR conformational changes with respect to the unfolding and possible dissociation of the GR homodimer.^{41, 53} Not all reported GR inhibitors can induce fluorescence changes, but for examples such as endogenous aldehydes, isocyanates, and trehalose, capacity to change GR intrinsic fluorescence correlates with GR binding and conformational changes.⁵⁴⁻⁵⁶ In this study, we found that each test compound quenched GR intrinsic fluorescence to different extents. Micromolar illudin S and AFs significantly quench GR intrinsic fluorescence regardless of the presence or absence of GSSG and NADPH (Figure 7). Carmustine, however, does not affect GR fluorescence at micromolar concentration and quenches 50% GR fluorescence at 1 mM drug concentration (Figure S3). Together, these data suggest that the interactions between GR and illudin S or AFs are more extensive, i.e. involve physical interactions that may manifest in fluorescence change, than for carmustine, which has been reported to react with one cysteine residue at the GR active site.⁴⁴ There are 12 tyrosine, 4 tryptophan, and 13 phenylalanine residues on the solvent-accessible surface of GR. The observed fluorescence quenching for the interactions of AFs or illudin S with GR may reflect direct interaction of drug with these residues, and it is possible that that quenching GR intrinsic fluorescence may or may not be associated with conformational changes.^{57, 58} The peak splitting that causes the red or blue-shifts in the protein intrinsic fluorescence spectra may also reflect possible GR conformational change induced by the drugs. This hypothesis is also supported by the present observation that the GSSG-bound form of GR can still be inhibited by AFs (Figure 5). Without NADPH present, GR can also be inhibited by AFs, and this profile is similar to the reported inhibitor *O*-phthalaldehyde, which reacts with non-essential residues (Figure 6).⁵⁹ In contrast, NADPH is required for carmustine-mediated inhibition, and GSSG can completely block it (Figure S2). Although illudin S induces similar fluorescence changes,

it does not inhibit enzyme activity (up to 2 mM illudin S was tested). The lack of planarity and additional substituents in the cyclopentane ring of illudin S compared to AFs may be contributing factors, i.e. steric hinderances, leading to the diminished reactivity of illudin S toward the enzyme active site vs. small-molecule thiols.

Results of this study indicate that AF is a reversible GR inhibitor and HMAF is an irreversible inhibitor. Mass spectrometry analysis of whole GR protein modified by HMAF, or carmustine as a positive control, reveals that HMAF forms a bis-adduct (Figure 8) and carmustine forms a mono-adduct with GR (Figure S4). The presence of four-fold higher levels of BSA in addition to GR (BSA contains 35 cysteine residues) does not interfere with the formation of the observed GR-HMAF adduct, suggesting that the alkylation process, under conditions in which enzyme inhibition is observed, is selective for the GR active site and not a result of indiscriminant reactions with cysteine residues in the protein. When BSA was allowed to react with HMAF for an extended period of time (two days) at room temperature, a mono-adduct was observed that increased the protein mass by m/z 228 (data not shown). This mass change is consistent with the direct displacement of the primary hydroxyl group by the cysteine thiol.

The purified GR used in this study contains four cysteine residues (refer to yeast GR sequence accession number BAA07109), and therefore studies were carried out to address whether there is any molecular specificity in the covalent interaction of HMAF with GR. Information regarding the drug-GR covalent adducts was obtained by LC-ESI-MS analysis of modified whole protein. We utilized LC-ESI-MS/MS and matrix-assisted laser desorption/ionization mass spectrometry (MALDI) to identify the modification sites. In such analyses, chemical modifications may significantly modulate peptide ionization efficiencies.⁶⁰ By MALDI, none of the intact or modified active site peptides (IAA- or HMAF) was detected, possibly due to the overall high hydrophobicity, since ESI tends to favor the ionization of hydrophobic peptides compared to MALDI.⁶¹ However, the peptides containing the other two cysteines other than the active site cysteines were detected by MALDI and neither of them were modified after HMAF treatment, suggesting that the GR alkylation by HMAF may not occur at these two cysteines. By LC-ESI-MS/MS, fragments of control IAA-alkylated peptides exhibited a strong spectral signature in which most y and b fragment ions were detected (Figure 9); however, for the free active site sequence, the corresponding peptide peak (m/z 659.4) was not observed. The lack of a peak corresponding to the free peptide may be due to disulfide formation, or that IAA-alkylation may enhance ionization efficiencies of the active site peptides. By contrast, no IAA alkylated active site peptide (m/z 716.6) was observed in HMAF-treated GR digestion mixtures, indicating active site sequence modification by HMAF. While certain complexities in the mass fragmentation spectra of the peptide observed after reaction with HMAF make some fragments hard to address, key y and b ions were detected that are consistent with the modification of each active site cysteine by HMAF (Scheme 3).

On the basis of these results, we propose a possible model describing differences in patterns of GR interactions by small molecules comprised of the following steps: 1. Drug-enzyme binding (E:Drug); 2. Induction of a conformational change in GR (E*), reflected by a change in intrinsic fluorescence; 3. Enzyme inactivation, i.e. measured by loss of GSSG-reducing capacity; 4. Chemical reaction between the activated GR-drug complex and conversion to covalent adducts. In this process, illudin S only proceeds to the first stage and induces conformational changes that are not associated with activity loss. AF reacts with GR reversibly, and the binding of AF to GR is probably locating at sites other than the active site since the presence of GSSG does not protect GR activity. The interaction of GR with HMAF is more complicated. Initially as a reversible inhibitor, HMAF competes with GSSG for the active site, but also has other binding sites, consistent with our observation that GSSG can only partially block inhibition. Further, the absence of NADPH does not diminish GR inhibition, and this process may induce unfolding to expose the proximal cysteine. As an irreversible inhibitor,

HMAF can proceed through all steps shown in scheme 4 and react with NADPH-reduced GR, leading to reactions with both cysteines at the active site and forming a bis-adduct. Finally, in comparison, carmustine may not induce conformational changes that facilitate the exposure of the proximal cysteine, and thus forms a mono-adduct at the distal cysteine.

To evaluate the influence of the test compounds on cellular GR at equitoxic concentrations, human cervical cancer cells (Hela), which are generally responsive towards AFs, were treated with test compounds at equitoxic concentrations that maintain 80% cell survival.¹⁸ The data obtained indicates that AF does not inhibit cellular GR, which is consistent with the cell-free observations since the weak and reversible GR inhibition may be attenuated by other species in the context of the cellular environment. HMAF and carmustine both inhibit cellular GR with relative potencies that mirror their relative *in vitro* IC₅₀, suggesting that covalent modification contributes in a similar manner for these agents to the observed cellular enzyme inhibition. Interestingly, illudin S was observed to inhibit cellular GR activity in a concentration-independent manner. This observation is difficult to explain, and may be related to illudin activation to a chemically reactive and GR-interactive species by cellular reductase or by GSH conjugation (Figure 7).^{29, 40} Further studies are needed to reconcile the observed differences and elucidate the potential role of drug metabolism in modulating GR inhibition potency; however, preliminary studies in cell free systems have not been informative because of potential confounding protein-protein interactions between isolated reductase enzymes (data not shown).

Cellular thiol-based redox regulation is tightly controlled by antioxidant enzymes such as GR, as well as thioredoxin reductase, thioredoxin and glutathione S-peroxidase.^{62, 63} The current study is the first step towards mapping illudin S and AFs reactivities towards these enzymes and potential cellular consequences, which will provide useful information regarding the mechanisms of small molecule cytotoxicity, and facilitate the design and synthesis of more potent antitumor derivatives. Furthermore, this study links the antitumor profiles of these cytotoxins with their interaction profiles for a representative enzyme containing key cysteine residues.

Conclusions

We have evaluated mechanisms of GR inhibition by the natural product illudin S and its AF analogues, and compared their activities with the known GR inhibitor carmustine. On the basis of results of experiments devised to evaluate enzyme inhibition, protein intrinsic fluorescence changes, reversibility of drug-enzyme interactions, covalent modification of whole proteins, and proteomic mapping of peptide covalent modification, a descriptive model accounting for structure-based differences in the reactivities of these molecules is proposed. Each of the test molecules exhibits some degree of interaction with the enzyme, however, illudin S does not inhibit GR, AF is a reversible inhibitor, and HMAF is an irreversible inhibitor that covalently modifies the protein at active site cysteine residues. In the complex environment of the cell, HMAF and illudin S inhibit GR, but do not reduce GR protein levels. Overall, the reactivities of illudin S and AF analogues toward GR are opposite what might be expected on the basis of reactivity towards thiol-containing molecules, emphasizing the importance of balancing chemical reactivity and molecular recognition in dictating biochemical responses. Furthermore, the minor impacts on cellular GR activity and expression suggest further studies needed to map reactivity profiles for illudin S and AFs toward additional cellular targets to better understand relative mechanisms of cytotoxicity. The data obtained in this study may be important in further improving alkylating agents as anticancer drugs, as we gain more information regarding the role of covalent protein modification in cytotoxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Zhou X, Gong Z, Su Y, Lin J, Tang K. J. Pharm. Pharmacol 2009;61:279–291. [PubMed: 19222900]
2. Saleem M, Ali MS, Hussain S, Jabbar A, Ashraf M, Lee YS. Nat. Prod. Rep 2007;24:1142–1152. [PubMed: 17898901]
3. Lam KS. Trends Microbiol 2007;15:279–289. [PubMed: 17433686]
4. Anchel M, Hervey A, Robbins WJ. Proc. Natl. Acad. Sci. U. S. A 1950;36:300–305. [PubMed: 15417544]
5. MCMORRIS TC, ANCHEL M. J. Am. Chem. Soc 1965;87:1594–1600. [PubMed: 14302677]
6. McMorris TC, Kelner MJ, Wang W, Diaz MA, Estes LA, Taetle R. Experientia 1996;52:75–80. [PubMed: 8575564]
7. McMorris TC, Kelner MJ, Wang W, Yu J, Estes LA, Taetle R. J. Nat. Prod 1996;59:896–899. [PubMed: 8864242]
8. Kelner MJ, McMorris TC, Taetle R. J. Natl. Cancer Inst 1990;82:1562–1565. [PubMed: 2402017]
9. Kelner MJ, McMorris TC, Estes L, Wang W, Samson KM, Taetle R. Invest. New Drugs 1996;14:161–167. [PubMed: 8913837]
10. MacDonald JR, Muscoplat CC, Dexter DL, Mangold GL, Chen SF, Kelner MJ, McMorris TC, Von Hoff DD. Cancer Res 1997;57:279–283. [PubMed: 9000568]
11. Kelner MJ, McMorris TC, Montoya MA, Estes L, Uglik SF, Rutherford M, Samson KM, Bagnell RD, Taetle R. Cancer Chemother. Pharmacol 1998;41:237–242. [PubMed: 9443641]
12. Kelner MJ, McMorris TC, Montoya MA, Estes L, Uglik SF, Rutherford M, Samson KM, Bagnell RD, Taetle R. Cancer Chemother. Pharmacol 1999;44:235–240. [PubMed: 10453725]
13. Dick RA, Yu X, Kensler TW. Clin. Cancer Res 2004;10:1492–1499. [PubMed: 14977853]
14. Gong J, Vaidyanathan VG, Yu X, Kensler TW, Peterson LA, Sturla SJ. J. Am. Chem. Soc 2007;129:2101–2111. [PubMed: 17256933]
15. Jaspers NG, Raams A, Kelner MJ, Ng JM, Yamashita YM, Takeda S, McMorris TC, Hoeijmakers JH. DNA Repair (Amst) 2002;1:1027–1038. [PubMed: 12531012]
16. Koepfel F, Poindessous V, Lazar V, Raymond E, Sarasin A, Larsen AK. Clin. Cancer Res 2004;10:5604–5613. [PubMed: 15328203]
17. Amato RJ, Perez C, Pagliaro L. Invest. New Drugs 2002;20:413–417. [PubMed: 12448659]
18. Poindessous V, Koepfel F, Raymond E, Comisso M, Waters SJ, Larsen AK. Clin. Cancer Res 2003;9:2817–2825. [PubMed: 12855662]
19. Hidalgo M, Izbicka E, Eckhardt SG, MacDonald JR, Cerna C, Gomez L, Rowinsky EK, Weitman SD, Von Hoff DD. Anticancer Drugs 1999;10:837–844. [PubMed: 10587294]
20. Herzig MC, Arnett B, MacDonald JR, Woynarowski JM. Biochem. Pharmacol 1999;58:217–225. [PubMed: 10423161]
21. McMorris TC, Yu J. Tetrahedron 1997;53:14579, 14580–14590.
22. McMorris TC, Yu J, Ngo HT, Wang H, Kelner MJ. J. Med. Chem 2000;43:3577–3580. [PubMed: 11000013]
23. Tanaka K, Inoue T, Tezuka Y, Kikuchi T. Chem. Pharm. Bull. (Tokyo) 1996;44:273–279. [PubMed: 8998835]
24. Liebler DC. Chem. Res. Toxicol 2008;21:117–128. [PubMed: 18052106]

25. Massey V, Williams CH Jr. *J. Biol. Chem* 1965;240:4470–4480. [PubMed: 4378936]
26. Sweet WL, Blanchard JS. *Biochemistry* 1991;30:8702–8709. [PubMed: 1888731]
27. Arning J, Dringen R, Schmidt M, Thiessen A, Stolte S, Matzke M, Bottin-Weber U, Caesar-Geertz B, Jastorff B, Ranke J. *Toxicology* 2008;246:203–212. [PubMed: 18313196]
28. Paci A, Rezai K, Deroussent A, De Valeriola D, Re M, Weill S, Cvitkovic E, Kahatt C, Shah A, Waters S, Weems G, Vassal G, Lokiec F. *Drug Metab. Dispos* 2006;34:1918–1926. [PubMed: 16896064]
29. McMorris TC, Kelner MJ, Wang W, Moon S, Taetle R. *Chem. Res. Toxicol* 1990;3:574–579. [PubMed: 2103329]
30. Deharo E, Barkan D, Krugliak M, Golenser J, Ginsburg H. *Biochem. Pharmacol* 2003;66:809–817. [PubMed: 12948862]
31. Vanhoefer U, Yin MB, Harstrick A, Seeber S, Rustum YM. *Biochem. Pharmacol* 1997;53:801–809. [PubMed: 9113101]
32. Bauer H, Fritz-Wolf K, Winzer A, Kuhner S, Little S, Yardley V, Vezin H, Palfey B, Schirmer RH, Davioud-Charvet E. *J. Am. Chem. Soc* 2006;128:10784–10794. [PubMed: 16910673]
33. Dick RA, Kwak MK, Sutter TR, Kensler TW. *J. Biol. Chem* 2001;276:40803–40810. [PubMed: 11524419]
34. Massey V, Williams CH Jr. *J. Biol. Chem* 1965;240:4470–4480. [PubMed: 4378936]
35. Witte AB, Anestal K, Jerremalm E, Ehrsson H, Arner ES. *Free Radic. Biol. Med* 2005;39:696–703. [PubMed: 16085187]
36. KITZ R, WILSON IB. *J. Biol. Chem* 1962;237:3245–3249. [PubMed: 14033211]
37. Tanaka K, Inoue T, Kadota S, Kikuchi T. *Xenobiotica* 1990;20:671–681. [PubMed: 2238702]
38. McMorris TC, Elayadi AN, Yu J, Hu Y, Kelner MJ. *Drug Metab. Dispos* 1999;27:983–985. [PubMed: 10460795]
39. McMorris TC, Elayadi AN, Yu J, Kelner MJ. *Biochem. Pharmacol* 1999;57:83–88. [PubMed: 9920288]
40. Tanaka K, Inoue T, Kadota S, Kikuchi T. *Xenobiotica* 1992;22:33–39. [PubMed: 1377439]
41. Louzada PR, Sebollela A, Scaramello ME, Ferreira ST. *Biophysical journal* 2003;85:3255, 3256–3261. [PubMed: 14581225]
42. Morais ACS, Chapeaurouge A, Ferreira ST. *The international journal of biochemistry & cell biology* 2005;37:1890–1899.
43. Seefeldt T, Zhao Y, Chen W, Raza AS, Carlson L, Herman J, Stoebner A, Hanson S, Foll R, Guan X. *J. Biol. Chem* 2009;284:2729–2737. [PubMed: 19049979]
44. Karplus PA, Krauth-Siegel RL, Schirmer RH, Schulz GE. *Eur. J. Biochem* 1988;171:193–198. [PubMed: 3338461]
45. Sechi S, Chait BT. *Anal. Chem* 1998;70:5150–5158. [PubMed: 9868912]
46. Johnson RS, Martin SA, Biemann K, Stults JT, Watson JT. *Analytical Chemistry* 1987;59:2621, 2622–2625. [PubMed: 3688448]
47. Gong J, Neels JF, Yu X, Kensler TW, Peterson LA, Sturla SJ. *J. Med. Chem* 2006;49:2593–2599. (DOI:10.1021/jm051104t [doi]). [PubMed: 16610802]
48. Dennehy MK, Richards KA, Wernke GR, Shyr Y, Liebler DC. *Chem. Res. Toxicol* 2006;19:20–29. (DOI:10.1021/tx050312l). [PubMed: 16411652]
49. Gisela KE, SCHIRMER RH, Renate UG. *European journal of biochemistry / FEBS* 1981;120:407, 408–419. [PubMed: 7032915]
50. Jones ET, Williams CH Jr. *J. Biol. Chem* 1975;250:3779–3784. [PubMed: 1092678]
51. Arscott LD, Drake DM, Williams CH Jr. *Biochemistry* 1989;28:3591–3598. [PubMed: 2663073]
52. Arscott LD, Drake DM, Williams CH Jr. *Nature* 1978;273:120–124. [PubMed: 25387]
53. Rendon JL, Mendoza-Hernandez G. *Arch. Biochem. Biophys* 2001;387:265–272. [PubMed: 11370850]
54. Sebollela A, Louzada PR, Sola-Penna M, Sarone-Williams V, Coelho-Sampaio T, Ferreira ST. *Int. J. Biochem. Cell Biol* 2004;36:900–908. [PubMed: 15006642]

55. Vander Jagt DL, Hunsaker LA, Vander Jagt TJ, Gomez MS, Gonzales DM, Deck LM, Royer RE. *Biochem. Pharmacol* 1997;53:1133–1140. [PubMed: 9175718]
56. Baylor KJ, Shine CM, Heffron JJ, McIntosh J. *Biochem. Soc. Trans* 1997;25:47S. [PubMed: 9056945]
57. Karplus PA, Schulz GE. *J. Mol. Biol* 1987;195:701–729. [PubMed: 3656429]
58. Mittl PR, Schulz GE. *Protein Sci* 1994;3:799–809. [PubMed: 8061609]
59. Pandey A, Katiyar SS. *J. Enzyme Inhib* 1996;11:141–149. [PubMed: 9204403]
60. Dongre AR, Jones AL, Somogyi A, Wysocki VH. *J. Am. Chem. Soc* 1996;118:8365, 8366–8374.
61. Stapels MD, Barofsky DF. *Anal. Chem* 2004;76:5423–5430. [PubMed: 15362902]
62. Jaeger T, Flohe L. *Biofactors* 2006;27:109–120. [PubMed: 17012768]
63. Berndt C, Lillig CH, Holmgren A. *Am. J. Physiol. Heart Circ. Physiol* 2007;292:H1227–36. [PubMed: 17172268]

Scheme 1.

Proposed mechanisms of biomolecular alkylation by AFs

Scheme 2.

Kinetic model for HMAF-induced GR inactivation. HMAF:GR is the noncovalent complex of HMAF with enzyme and HMAF-GR represents the inactivated alkylated enzyme.

Figure 1.
Structure of illudin S, acylfulvene analogues AF and HMAF, and their major metabolite, and carmustine (BCNU).

Figure 2.

Inhibition of GR by AF, HMAF and illudin S. A, concentration-dependent inhibition of GR. GR (5 nM) was incubated with test compounds in the presence of NADPH (150 μM) for 30 min at 25 °C (AF (◆), 62.5, 125, 250, 625, 750, 1000, 1250 μM; HMAF (●) and Illudin S (▲), 62.5, 125, 250, 625, 1250 μM. B, time-dependent inhibition of GR by AF at 0 (●), 500 μM (◆), 750 μM (▲), 1000 μM (■), and 1250 μM (▼). C, time-dependent inhibition of GR by HMAF at 0 (●), 125 μM (■), 250 μM (▲), 500 μM (◆), and 1250 μM (▼). GR (5 nM) was incubated with AFs, aliquots were withdrawn and assayed for residual GR activity at different time intervals. The slopes of the lines representing apparent rate constants of inhibition (K_{app}) were determined by linear regression analysis. The data were derived from a representative of two independent experiments. The inset is the Kitz and Wilson replotting of GR inhibition by HMAF: double reciprocal plot of K_{app} vs HMAF concentration [I]. The K_i and k_{inact} values were determined to be 180 μM and 0.2 min⁻¹ based on the following formula: $1/K_{app} = 1/k_{inact} + (K_i/k_{inact}) \times 1/[I]$

Figure 3.

Substrate screening analysis for the potential conversion of illudin S and AFs to reduced metabolites by GR. Test compounds (AF, 400 μ M; HMAF, 400 μ M; illudin S, 1mM) were allowed to react with AOR (2 μ M) or GR (2.5 μ M) in TE buffer containing NADPH (200 μ M) for 2 h at 37 $^{\circ}$ C, followed by EtOAc extraction and HPLC analysis. Control samples were incubated in buffer containing NADPH (200 μ M) and extracted in the same manner. The peak at 5 min observed in each sample is DMSO. A. AF (26 min), M_A (21 min); A1. AF control; A2. AF-AOR; A3. AF-GR; B. HMAF (17 min), M_H (15 min); B1. HMAF control; B2. HMAF-AOR; B3. HMAF-GR; C. illudin S (13 min); C1. illudin S control; C2. illudin S-AOR; C3. illudin S-GR.

Figure 4.

Gel-filtration analysis of AF and HMAF-inactivated GR. GR (5 nM) was incubated with test compounds (AF(●), 250, 625, 750, 1000, 1250 μM; HMAF(◆), 62.5, 125, 250, 625, 1250 μM) in the presence of NADPH (150 μM) in TE buffer for 30 min at 25 °C. Unbound compound was removed by micro bio-spin P6 pre-packed size exclusion columns (Biorad, Hercules, CA) with NWML 6000.

Figure 5.

Effect of GSSG on the inhibition of GR by AFs. GR (5 nM) was incubated with AFs (A. AF, 250, 500, 750, 1000, 1250 μM; B. HMAF, 62.5, 125, 250, 625, 1250 μM) for 30 min in the presence of different concentration of GSSG [(●) 0, (◆) 250, (▲) 1250 μM] for 30 min, and then activity was measured.

Figure 6.

Effect of NADPH on GR Inhibition by AFs. GR (5 nM) was incubated with AFs (A. AF, 250, 500, 750, 1000, 1250 μM; B. HMAF, 62.5, 125, 250, 625, 1250 μM) in the presence (●) or absence (◆) of NADPH for 30 min at 25 °C, and then activity was measured.

Figure 7.

GR fluorescence spectrum changes in the presence of varying concentration of illudin S and AFs. Illudin S (20, 100, 400, 2000 μM) and AFs (10, 40, 200, 1000 μM) were incubated with GR (2 μM) in a total volume of 500 μL TE buffer for 30 min at 25 $^{\circ}\text{C}$ under different conditions: A. in the absence of NADPH; B. in the presence of NADPH (100 μM); C. in the absence of NADPH and in the presence of GSSG (400 μM); D. in the presence of NADPH (100 μM) and GSSG (400 μM).

Figure 8.

LC/MS spectra derived from GR samples. GR (5 nmol) was allowed to react with AFs (1.25 mM) in TE buffer containing NADPH (1 mM). Modified GR was then concentrated and unbound compound removed before LC/MS analysis.

Scheme 3.

Proposed chemical mechanism of GR inhibition by HMAF.

Figure 9.

LC/MS/MS analysis of the GR active-site peptide. A. peptide from control GR with cysteine residues modified by iodoacetamide (m/z 716.6 [M+2H]²⁺), B. peptide from GR-HMAF adduct (m/z 905.4 [M+2H]²⁺). Other minor ions that appear, but are not labeled in the figure include: m/z 299.2 (b₄), 326.2 (y₃-NH₃), 944.1 (b₈⁺, y₇⁺-H₂O), 1015.3 (a₈⁺-H₂O), and 1301.5 (MH⁺-NH₃).

Figure 10.

Inhibition of GR in HeLa cells treated with illudin S, AFs and carmustine at equitoxic concentrations. HeLa cells were exposed to test compounds (AF 0.2, 1.0, 4.0 μM ; HMAF: 0.2, 1.0, 4.0 μM ; illudin S: 0.02, 0.10, 0.40 μM ; carmustine: 5, 20 μM) for 2 h (A) and 12 h (B), then cellular GR activity was measured. Each bar represents the mean and standard deviation of the results from four experiments. Asterisks represent significant difference relative to controls: * $p < 0.05$, ** $p < 0.01$.

Figure 11.

Influence on cellular GR protein levels after treatment by illudin S, AFs and carmustine. HeLa cells were exposed to test compounds (AF 0.2, 1.0, 4.0 μ M; HMAF: 0.2, 1.0, 4.0 μ M; illudin S: 0.02, 0.10, 0.40 μ M; carmustine: 20 μ M) for 2 h (A) and 12 h (B) respectively. Cellular GR was measured by western blotting, and 25 μ g cell lysate protein corresponding to each treatment was analyzed by western blotting.

Scheme 4.

Proposed patterns of GR inhibition by illudin S, AFs, and carmustine. Each agent appears to alter the properties and activity of the enzyme to different extents, related to binding, conformational changes, and degree of alkylation.