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Development of flexible-heteroarotinoids for kidney cancer

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Flex-Hets and metabolites were not mutagenic in the Ames test. In conclusion, SHetA2 regulates growth, differentiation, and apoptosis in kidney cancer cells through multiple molecular events downstream of nuclear factor-κB repression. Increasing the hydrophilicity of Flex-Hets does not attenuate the differential effect on cancer cells over normal cells, thus offering alternatives for improvement of therapeutic value. [Mol Cancer Ther 2009;8(5): 1227–38]

Abstract

Potential chemopreventive and therapeutic value of the lead Flexible Heteroarotinoid (Flex-Het), SHetA2, was indicated by growth inhibition of multiple cancer cell lines. The objective of this study was to evaluate the SHetA2 mechanism and *in vivo* activity in kidney cancer. SHetA2 induced apoptosis in the Caki-1 kidney cancer cell line through reduction of Bcl-2 protein and induction of PARP-1 and caspase 3 cleavages, whereas normal kidney epithelial cells exhibited resistance. Both normal and cancerous cells underwent G_1 arrest and loss of Cyclin D1. Tubule differentiation was induced in organotypic cultures and xenograft tumors in association with increases in E-Cadherin mRNA and protein expression. SHetA2 repressed activity of nuclear factor-κB, a transcription factor that regulates apoptosis, Bcl-2, growth, Cyclin D1, differentiation, and E-Cadherin in the opposite manner as SHetA2. Glutathione binding and generation of reactive oxygen species were not required for these activities. Oral SHetA2 inhibited growth in one of two renal cancer xenograft models without causing mortality or weight loss. Structure function analysis of related Flex-Hets for potential improvement of SHetA2 pharmaceutical properties showed that compounds with increased hydrophilicity slightly reduced the growth inhibition efficacy, but retained the differential effect on cancer over normal cells.

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Introduction

Kidney cancer represents ~3% of all human malignancies with a detection rate for new cases of 30,000 per year in the United States and 20,000 per year in the European Union (1). The majority of kidney cancers are renal cell carcinoma with an adenocarcinoma histology (2). Both the incidence and mortality of renal cell carcinoma have been steadily increasing since 1950 (2). The increased incidence can be partially explained by the implementation of new imaging techniques that have improved the capability to detect asymptomatic early stage cancers (2). The number of advanced cases, however, has also increased (2). Renal cancer has high mortality due to its high metastatic rate and its inherent resistance to standard chemotherapy and radiation therapy (3). Patients who are initially diagnosed with metastatic renal cancer have a 5-year survival rate of <10%, and renal cancer has an overall 5-year survival rate of 60% (4). Thus, there is a great need for novel pharmaceuticals to prevent and treat kidney cancer.

Flexible Heteroarotinoids (Flex-Hets) are a promising new class of anticancer compounds that regulate growth, differentiation, and apoptosis in multiple types of cancer cells (5). The SHetA2 compound was chosen as the lead Flex-Het, because it exerted the greatest growth inhibitory activities on cancer cells, while retaining the differential resistance in normal cells. All of the cancer cell lines in the National Cancer Institutes (NCI) 60 cell line human tumor panel were sensitive to the growth inhibitor activities of SHetA2 at micromolar concentrations (5). This broad range of activity was not due to generalized toxicity because normal and benign human ovarian, endometrial, and oral cultures were resistant to SHetA2-induced growth inhibition and apoptosis (6, 7). The mechanism of this differential apoptosis in cancerous over normal ovarian cells involves direct effects of Flex-Hets on mitochondria and the Bcl-2 family of proteins (7). Studies in animal models showed that SHetA2 inhibited growth of ovarian cancer cell lines without evidence of toxicity, and did not induce skin irritancy or teratogenicity (5, 8). Because of these encouraging results, SHetA2 was chosen for preclinical development as a cancer therapeutic agent in the NCI's Rapid Access to Intervention Development (RAID) program (Application 196, Compound NSC 726189) and now is being developed as a cancer

chemoprevention agent in the RAPID program. Potential application of SHetA2 for prevention and treatment of kidney cancer is indicated by the ability of this compound to inhibit growth of the 8 renal cancer cell lines (786-O, A498, ACHN, Caki-1, RXF 393, SN12 C, TK-10, U0-31) in the 60 cell line panel of NCI (5). The numbers of cells remaining at the end of the treatment period were fewer than the number of cells present at the initiation of treatment, indicating that cell death was induced. The objective of this study was to evaluate the potential antitumor activity and mechanism of action for SHetA2 against kidney cancer and to evaluate the efficacy of similar Flex-Hets that might provide improved the pharmaceutical properties.

Materials and Methods

Drugs and Cell Lines

Flex-Hets were synthesized as previously described (6, 9), dissolved in DMSO at 0.01 mol/L, stored in 50 μL aliquots at −20°C, and manipulated under subdued lighting to protect from photo-oxidation. Caki-1 was derived from a human skin metastatic lesion from a renal clear cell carcinoma. 786-O was derived from a human primary renal clear cell adenocarcinoma. Human kidney 2 (HK-2) is a proximal tubular cell line derived from normal kidney and immortalized with human papillomavirus 16 E6/E7 genes. RTC91696 cells (provided by Martin Turman, MD, University of Oklahoma Health Sciences Center, Oklahoma City, OK, at passage 3) are primary human cell culture derived from a normal kidney, which were cultured in MEMα media supplemented with epidermal growth factor (10 ng/mL); hydrocortisone (5 μg/mL); 6.35 μg/mL each of insulin, transferrin, and selenous acid; and 10% fetal bovine serum, penicillin, and streptomycin.

Growth Inhibition Assays

Cell lines were treated with 1 to 12.5 μmol/L of test compound or the same volume of DMSO solvent in triplicate. After 48 h, the CellTiter 96 AQueous Assay (Promega) was used to measure metabolically active cells. Growth indices were derived by dividing the average OD_{540} of each treatment by the average OD_{540} of control cultures. Prism 3.0 Software (GraphPad) was used to plot growth index versus concentration and to derive the efficacy and potency values using nonlinear regression analysis. Values for the cell lines were derived from plots of two to three independent experiments. Due to the limited number of cells and doublings before senescence, values for normal cell cultures were derived from plots of single experiments.

Apoptosis Assay

The Vybrant Apoptosis Assay kit #3 (Molecular Probes) was used to measure apoptosis and necrosis. After treatment, tissue culture medium from each culture was collected to harvest cells that had already lifted off the tissue culture plate and combined with adherent cells harvested by trypsinization. Cells were pelleted and incubated with Annexin-V-FITC and propidium iodide (PI) before evaluation by flow cytometry at an excitation wavelength of 488 nm and observation wavelengths of 530 and 575 nm.

Western Blot Analysis

The CytoBuster protein extraction reagent (Novagen) and protease inhibitor cocktail (Sigma) were used to extract proteins from treated cultures. Proteins (100 μg) were separated by SDS-polyacrylamide, and electroblotted onto nitrocellulose membranes, which were hybridized with mouse monoclonal antibodies Bcl-2 (100), Bcl-x_L(H5), Bax (2D2), Caspase 3, PARP-1 (Santa Cruz Biotechnology), or Cyclin-D1 (BD Biosciences). Horseradish peroxidase–conjugated secondary antibody and Western Blotting Luminol Reagent (Santa Cruz) were used for detection. Membranes were stripped and reprobed with mouse monoclonal β-actin (C4; Santa Cruz) or glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz).

Cell Cycle Analysis

Cultures incubated with DMSO solvent or SHetA2 using various dosages and treatment times were washed, harvested, and incubated in staining solution (PBS, 5% Triton X-100, 1 mg/mL RNase, and 1 mg/mL PI) for 3 h. FACSCalibur and Modfit Version 2.0 cell analysis software were used to evaluate the cell cycle profile.

Organotypic Cultures

A mixture of culture media, liquid collagen 1, and a single-cell suspension were solidified in tissue culture inserts as described (10). After 1 wk of growth, cultures were treated with 1 μmol/L SHetA2 or the same volume of DMSO solvent for 2 wk. Cultures were fixed in formalin, embedded, sectioned (5 μm), deparaffinized, and stained with H&E.

rtPCR

RNA was isolated from frozen cell pellets or tumors using the mini RNeasy kit and shredder (Qiagen). Triplicate PCR reactions were done using validated TaqMan primers (Applied Biosystems) specific for E-Cadherin and 18S genes. The $\Delta\Delta$ C_t method was used to normalize expression of the E-Cadherin test gene to the 18S housekeeping gene, and to determine the relative expression of the of E-Cadherin at each dosage or time point compared with the solvent only control cultures (11). Three independent experiments were done.

Nuclear Factor-κB Luciferase Reporter Assays

Cultures were cotransfected with 10 μg of DNA consisting of a 10:1 ratio of the nuclear factor-κB (NF-κB)–specific reporter plasmid, pNF-κB-luc (from Dr. Wei Qun Ding, University of Oklahoma Health Sciences Center) to evaluate NF-κB transcriptional activity and the pRL-TK (from Dr. A.L. Olson University of Oklahoma Health Sciences Center) as a transfection control using Metafectene Pro (Biontex). Twenty-four hours after transfection, the cells were trypsinized and replated onto 12-well plates. Twenty four hours after replating, cultures were treated with 10 μmol/L SHetA2 for various time points. In some instances, 20 ng/mL tumor necrosis factor α was added for the last 30 min of treatment. Lysates were prepared using Passive Lysis Buffer (Promega) and luciferase activity was measured using the Dual Luciferase Reporter Assay (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

Animal Xenografts

The *in vivo* efficacy of the test agent was evaluated in the human renal cell xenograft Caki-1. Briefly, the tumors were generated by s.c. injection of Caki-1 cells into the axillary region each of 100 athymic nude mice (nu/nuNCr, Animal Production Program, NCI-Frederick). These tumors were staged to 125 mg at which time the mice were randomized into the various treatment groups using the Study Director randomization program (StudyLog Systems, Inc.). NSC 726189 was formulated as a solution in 100% PEG 400. Drug solutions were prepared for dosing at 0.1 mL/10 grams body weight, and exact body weight dosing was used throughout the study. A group of 25 mice served as vehicle controls. NSC 726189 was administered at doses of 20, 40, and 60 mg/kg/dose administered once daily for 30 d). All mice were treated by oral gavage on a once daily for 30 d schedule. The tumor weight (in milligrams) was calculated from the length and width caliper measurements using the formula: [(tumor length × tumor width^2) / 2]. On days 1, 3, 7, 18, and 28 of dosing, fine needle aspirates were collected from 3 mice in each group (each mouse sampled only once) and flash frozen. Twenty-four hours after the last drug dose, all tumors were collected, divided into 2 pieces with 1 piece flash frozen and the second piece placed into 10% neutral buffered formalin. Percent growth inhibition in the drug treated tumors was compared with the vehicle-control treated animals.

Bacterial Mutation (Ames) Test

Dry powder of SHetA2 and SHetA3 compounds were sent to Charles River Laboratories Preclinical Services Montreal, Inc., where they were dissolved and evaluated for bacterial mutagenicity in the absence and presence of S9 rat liver microsomal fraction according to the following procedures. The bacteria were originally supplied by Moltxo. On the day before the assay, frozen aliquots of bacteria were thawed and cultured in Oxoid Nutrient Broth no. 2 containing ampicillin as a selective agent where appropriate to the late-log phase of growth. A 0.5-mL aliquot of S9 fraction (Phenobarbital/5,6-benzoflavone–induced male Sprague-Dawley rat liver fraction supplied by Moltox: +S9) or PBS 0.2 mol/L (pH7.4; -S9) was combined with 0.1 mL bacterial culture and 0.1 mL of SHetA2, SHetA3, or the positive control agent and incubated at 37°C for 30 min with shaking at 180 rpm. The sample then was mixed with molten top agar supplemented with 0.05 mmol/L biotin and minimal (0.05 mmol/L) histidine, and minimal (0.05 mmol/L) tryptophan, and overlaid on a minimal glucose plate (1.5% agar, Vogel-Bonner medium E, and 2% glucose). The plates were incubated at 37°C for 48 to 72 h and the numbers of revertant colonies then were counted.

Results

Differential Apoptosis Activity in Cancerous over Normal Cells

Sensitivity of kidney cancer cell lines and resistance of normal kidney epithelial tubule cells to Flex-Het induction of apoptosis was confirmed using flow cytometric analysis of Annexin-V-FITC and PI-stained cells. Representative histograms of the Caki-1 cancer cell line and RTC 91696 normal cultures treated with SHetA2 are shown in Fig. 1A. SHetA2

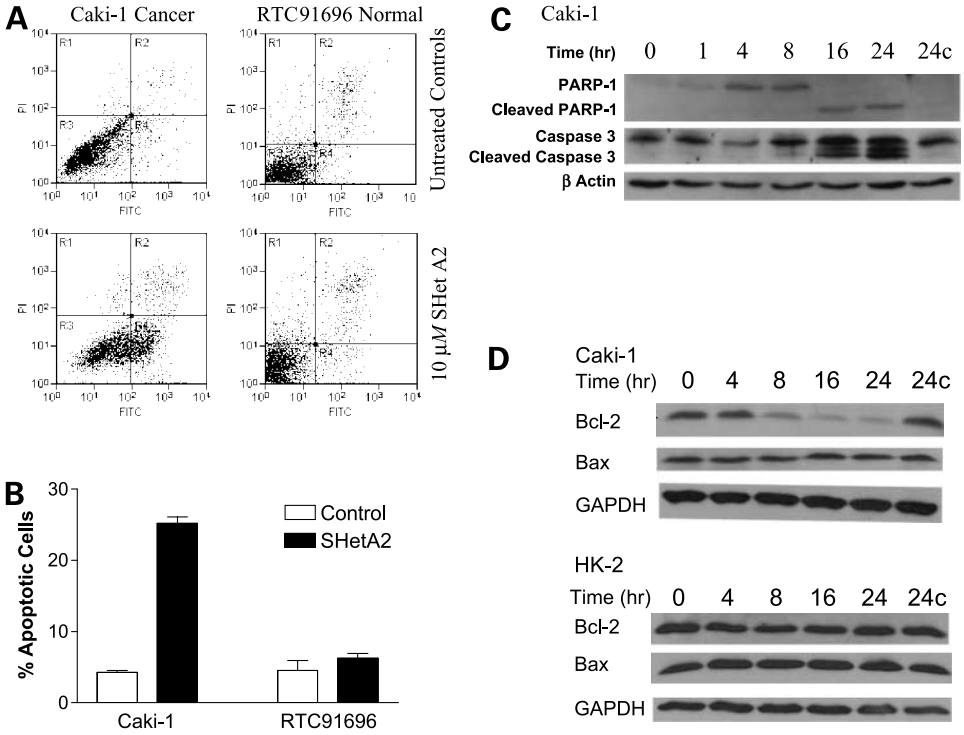


Figure 1. Differential induction of apoptosis in cancer over normal cells. Cultures were treated with 10 μmol/L SHetA2 for the indicated amount of time. **A,** cells were stained with Annexin-V and PI for flow cytometric evaluation of apoptosis. RTC91696 cells were used at passage 3 in parallel with the cytotoxicity assays in Table 1. **B,** the percentages of cells in the quadrant corresponding to apoptosis from four replicate experiments are presented. **C** and **D,** proteins from Caki-1 cells treated with 10 μmol/L SHetA2 for the indicated amount of time were extracted and evaluated by Western blot analysis. *24c,* treated with DMSO solvent only for 24 h.

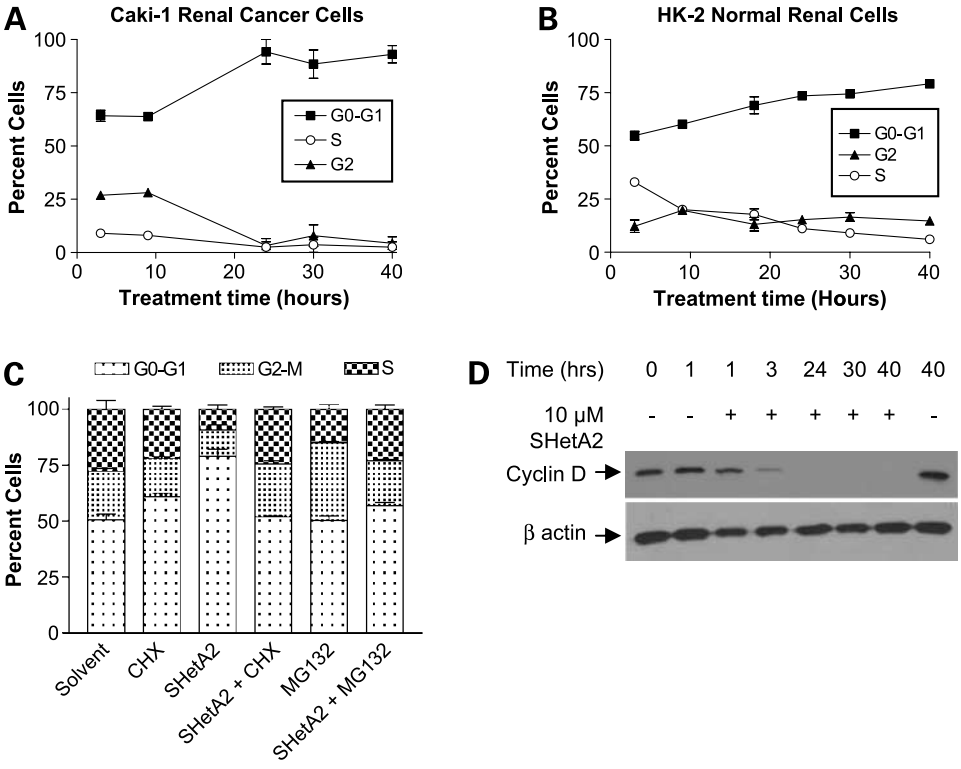


Figure 2. G_1 cell cycle arrest in both cancer and normal cells. Flow cytometric analysis of cell cycle distribution of Caki-1 cells (**A**) or HK-2 normal kidney cells (**B**) treated with 10 μmol/L SHetA2 (**C**) or the same volume of solvent for 24 h before fixation and PI staining. Cyclocheximide (*CHX*) and a proteasome inhibitor (*MG132*) inhibited the SHetA2-induced G_1 cell cycle arrest (**C**). Western blot analysis showed that Cyclin D1 protein levels are decreased in association with the cell cycle arrest (**D**).

treatment increased the percentage of Caki-1 cancer cells represented in the lower right hand quadrant of the histogram corresponding to apoptosis. In contrast, the majority of cells in normal renal cultures treated with SHetA2 remained in the lower left-hand quadrant of the histogram corresponding to living cells. Quantification of repeated experiments confirmed induction of apoptosis in cancer but not normal cultures (Fig. 1B). To validate the SHetA2-induced apoptosis in cancer cells at the molecular level, two well-characterized apoptosis proteins that are activated by cleavage, PARP-1, and Caspase 3 were evaluated. Western blot analysis of protein extracts from Caki-1 cultures showed that SHetA2 treatment induced cleavage of PARP-1 and Caspase 3 within 16 hours of treatment (Fig. 1C). The Bcl-2 antiapoptotic protein was decreased in cancer cells but not normal cells, whereas the Bax proapoptotic protein was not decreased in either cell type (Fig. 1D). The maximum 72-hour treatment time and 12.5-μmol/L concentration did not induce apoptosis in normal cells. Higher concentrations were not evaluated because they would not be clinically achievable with SHetA2.

SHetA2 Induces G_1 Cell Cycle Arrest and Cyclin D1 Degradation

Because apoptosis did not contribute to the growth inhibition in normal kidney cultures, it was theorized that the mechanism of growth inhibition in normal cultures was caused by cell cycle arrest. To test this, flow cytometric analysis of PI-stained cells was used to measure the effects of SHetA2 on the cell cycle profiles of normal and cancerous kidney cultures. SHetA2 increased the percentages of cells in the G_0-G_1 phase of the cell cycle in both the Caki-1 cancer and normal HK-2 cell lines (Fig. 2A-B). Prevention of this G_1 arrest by cycloheximide and a proteasomal inhibitor, MG132, indicated that the mechanism of G_1 arrest requires protein synthesis and a functional proteasomal degradation pathway (Fig. 2C). To explore the mechanism of cell cycle arrest, the effects of treatment on Cyclin D1, a major regulator of G_1-S progression, was evaluated. Expression of Cyclin D1 is tightly controlled during the cell cycle, with decreased expression during S phase progression. Decreased expression of Cyclin D1 was observed by Western blot analysis within 1 hour of SHetA2 treatment (Fig. 2D).

Role of Glutathione Binding in SHetA2 Regulation of Growth and Apoptosis

Studies done by the NCI RAID program showed that SHetA2 binds to glutathione *in vitro* and *in vivo* (12). If glutathione binding is involved in the SHetA2 mechanisms of G_1 arrest and apoptosis, then treatment of cells with excess glutathione and a glutathione precursor (NAC) should be able to attenuate or prevent the induction of these end points. Simultaneous treatment of Caki-1 cells with glutathione or NAC prevented G_1 arrest in Caki-1 cancer cells (Fig. 3A) but not in normal HK-2 cells (Fig. 3B). Glutathione and NAC did not alter SHetA2-induced G_1 arrest in two ovarian cancer cell lines evaluated (data not shown). Apoptosis in Caki-1 cancer cells was not altered by glutathione or NAC (Fig. 3C).

Differentiation

Another activity that adds promise to the potential of SHetA2 as an anticancer agent is differentiation induction.

At concentrations below 3 μmol/L, SHetA2 and other heteroarotinoids induced glandular differentiation and reversed the cancerous phenotype in ovarian organotypic cultures (10). Induction of a more differentiated phenotype and tubule formation was observed in two independent animal kidney cancer xenograft models (Fig. 4A-H). E-Cadherin was implicated in the mechanism of differentiation through previous microarray analysis and the known role of this molecule in cell-cell adhesion, communication, and tissue structure (13). Real-time rtPCR analysis of RNA isolated from tumors of animals treated with SHetA2 showed a slight increase in E-Cadherin mRNA compared with untreated tumors (Fig. 4I). A time course analysis of SHetA2 effects on E-Cadherin expression in SHetA2-treated Caki-1 cultures revealed two phases of induction (Fig. 4J). Upregulation was observed within 1 hour and the rate of increase in steady-state mRNA levels continued until 4 hours of treatment, when the induction leveled off at 20-fold. Another induction occurred at 16 hours with an increasing rise in steady-state mRNA levels that resulted in the observed 60-fold induction at 24 hours of treatment. Increased expression of E-Cadherin protein over time was also observed in SHetA2 treated Caki-1 cancer and HK-2 normal kidney cultures (Fig. 4K).

Inhibition of NF-κB

Repression of the NF-κB transcription factor was implicated in the mechanism of SHetA2 because this transcription factor regulates growth, differentiation, apoptosis, and the specific SHetA2-regulated genes in the opposite manner as SHetA2 (14). Real-time rtPCR showed that SHetA2 does not regulate NF-κB at the mRNA expression level (data not shown). The NF-κB transcriptional activity was induced by tumor necrosis factor α and was significantly inhibited by SHetA2 (Fig. 5A). In the absence of tumor necrosis factor α, NF-κB activity was barely detectable. A time course of NF-κB repression was done to evaluate if SHetA2 regulation of this transcription factor occurs through the canonical pathway involving Iκκβ- and NEMO-dependent degradation of IκB that takes place within minutes as opposed to the noncanonical pathway that involves Iκκα-dependent processing of p100 to the p52 NF-κB subunit and takes several hours (15). The observed inhibition of NF-κB activity within 30 minutes of treatment (Fig. 5B) suggests that the mechanism is occurring through the canonical pathway.

In vivo Growth Inhibition of SHetA2 on Caki-1 Renal Cancer Xenografts

In studies conducted by the NCI's RAID program, Caki-1 renal cancer xenografts exhibited the greatest sensitivity to the lead compound, SHetA2. Oral administration of SHetA2 at 20 and 40 mg/kg/day for 30 days induced a trend toward reduced xenograft tumor sizes that was not statistically significant (Fig. 6). The higher dose of 60 mg/kg/day induced significant growth inhibition over treatment days 13 through 23, with maximal growth inhibition of 36% achieved on day 34, which was the 23rd day of treatment (Fig. 6A). The degree of growth inhibition was no longer significant at the time of animal sacrifice that occurred 1 day after treatment was terminated, which may be due to the reduced numbers of animals remaining at this time (18 in the untreated control group and 7 in the treated group). There were 4 tumor-related deaths in the untreated

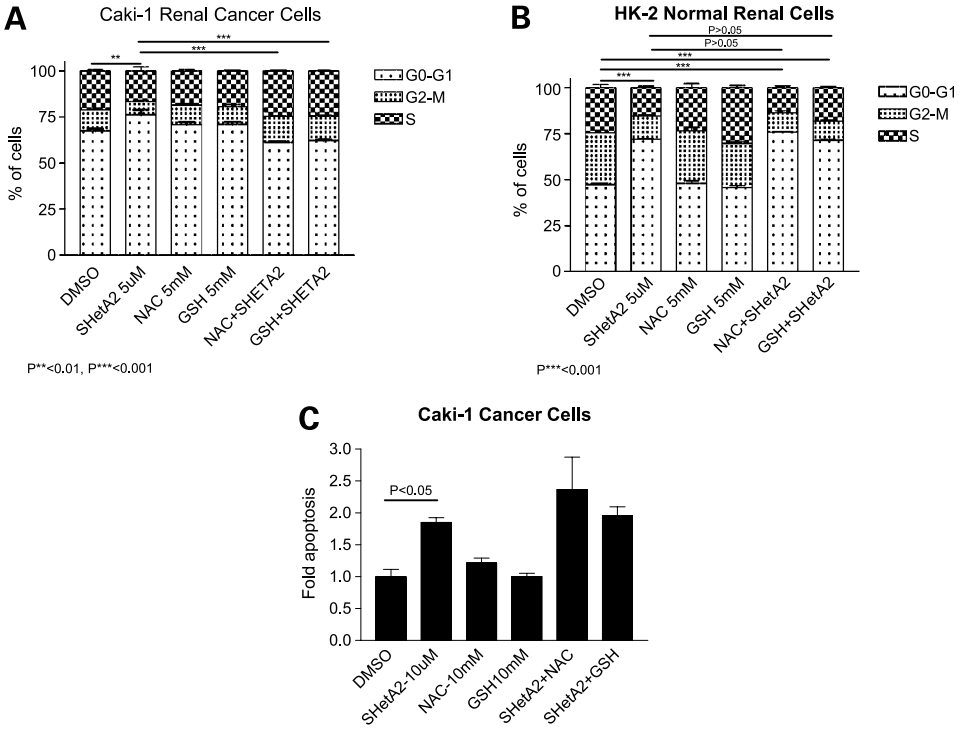


Figure 3. Glutathione and NAC in apoptosis and G_1 arrest. Flow cytometric analysis of PI-stained cells showed that reduced glutathione (*GSH*; 5 mmol/L) and NAC (5 mmol/L) could prevent G_1 arrest in Caki-1 cancer cells (**A**) but not HK-2 normal cells (**B**) treated with 5 μmol/L SHetA2 for 24 h. **C,** flow cytometric analysis showed that apoptosis was not inhibited by the reduced glutathione (10 mmol/L) or NAC (10 mmol/L) in Caki-1 cells treated with 10 μmol/L SHetA2 for 20 h. *Columns,* mean and standard error of a representative experiment of two independent experiments done in quadruplicate; *bars,* SE. *P* values represent significant differences in the indicated columns determined by posttests using One-Way ANOVA analysis.

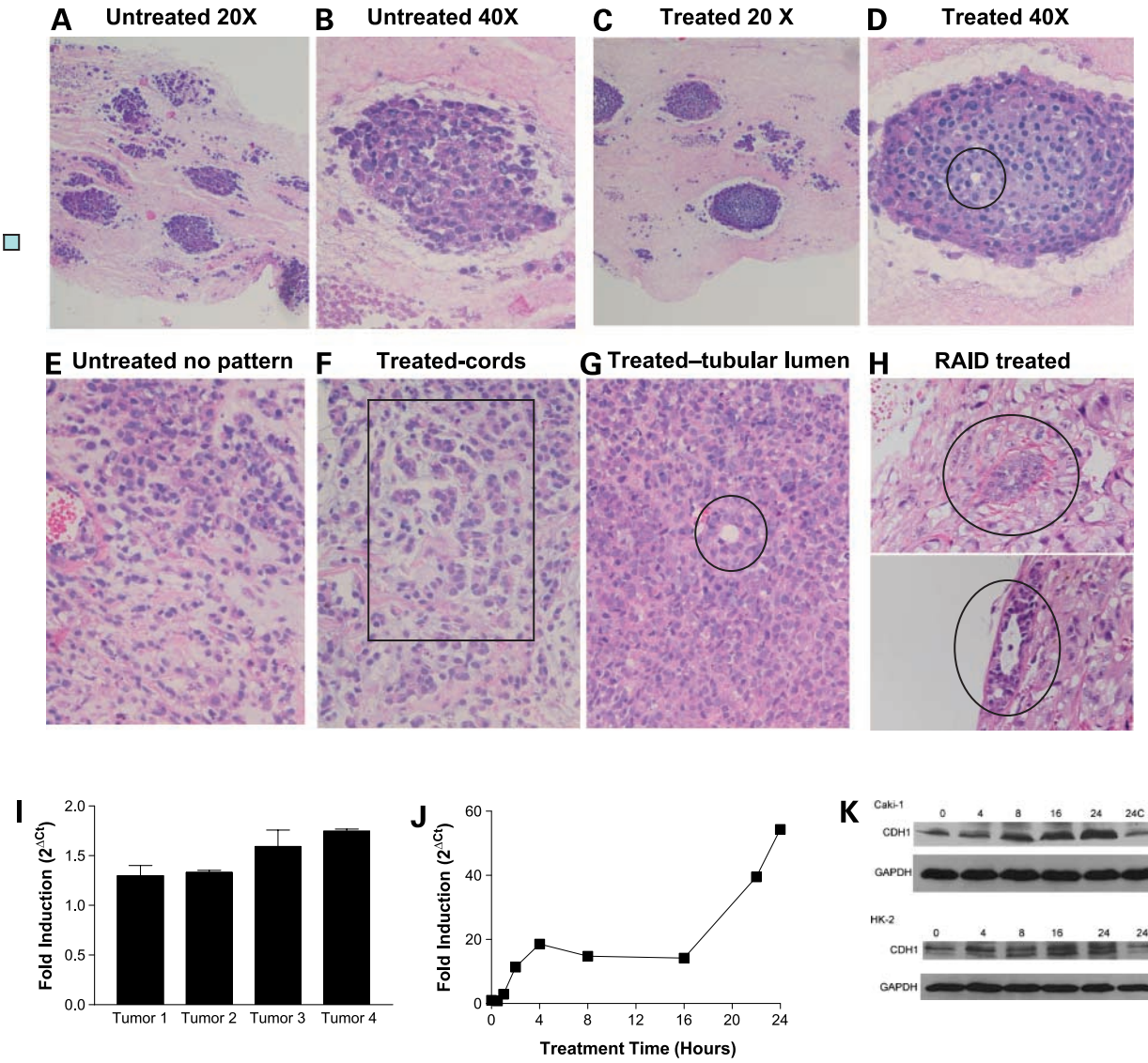


Figure 4. Induction of tubule differentiation. *Top,* H&E-stained sections of Caki-1 organotypic cultures grown in the absence (**A** and **B**) or presence of 1 μmol/L SHetA2 (**C** and **D**) for 2 wk before histochemical analysis. *Middle,* H&E-stained sections of xenograft tumors from untreated control mice (**E**) showing that most of the malignant cells are arranged singly and separate one from the other, compared with the more orderly arrangement of cells in tumors from mice treated with oral SHetA2 (60 mg/kg/d) 5 d per week for 30 d at the University of Oklahoma (**F** and **G**) or every day for 30 d at the RAID program (**H**). In some areas of the treated tumors, collections of tumor cells formed cords that were similar to tubules but no lumen was present (identified by a rectangle in **F**). In other areas of the treated tumors, a few tubules with lumens were present (circled in **G** and **H**). Neither cords nor lumens were present in the control tumors. **I,** rt-PCR analysis of E-Cadherin expression in tumors from the RAID xenografts in (*bottom*; **H**). To calculate fold induction, the expression levels in the individual treated tumors were compared with the average expression observed in three separate untreated tumors. **J,** real-time rtPCR analysis of E-Cadherin in Caki-1 cells treated with SHetA2 for the indicated times. Results are representative of two independent experiments. **K,** Western blot analysis of E-Cadherin expression in cell cultures treated with SHetA2. *24C,* control cultures treated with solvent only for 24 h.

control group, 6 in the group treated at 60 mg/kg/day, 6 in the group treated with 40 mg/kg/day, and 3 in the group treated with 20 mg/kg/day. None of the treatment concentrations altered the body weight of the animals compared with the control group (Fig. 6D). Another xenograft model using the A498 cancer cell line did not exhibit significant sensitivity to SHetA2 at 20, 40, or 60 mg/kg/day or significant animal body weight loss (data not shown).

Structure-Activity Relationships of Flexible Heteroarotionoids

Given the inconsistent *in vivo* efficacy of SHetA2, structural alterations theorized to improve the pharmacologic properties in kidney cancer by increasing hydrophilicity were evaluated. A series of Flex-Hets that differ by single structural alterations were compared for their ability to inhibit growth of two renal cancer cell lines (Caki-1 and 786-O),

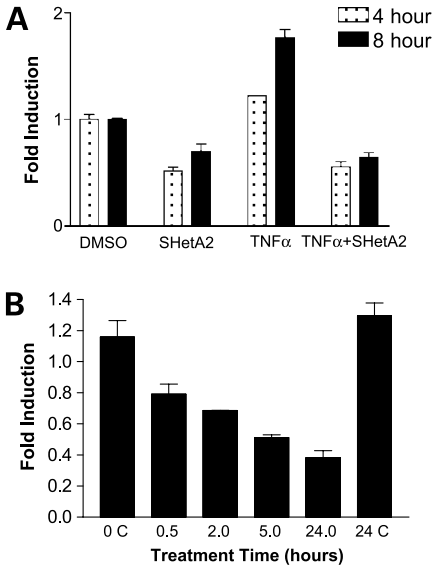


Figure 5. Repression of NF-κB activity. Caki-1 renal cancer cells were cotransfected with pNF-κB-Luc reporter and pRLTK transfection control plasmids and treated with DMSO or 10 μmol/L SHetA2 with or without 20 ng/mL tumor necrosis factor α (*TNFα*) for 4 or 8 h (**A**) or 10 μmol/L SHetA2 only (**B**) for various amounts of time before lysing the cells and evaluating for luciferase activity. *c,* control treated with solvent only. Tumor necrosis factor α was added to last 30 min of treatment. The firefly luciferase (NF-κB reporter) was normalized to the renilla luciferase (transfection control) activity.

an immortalized normal kidney cell line (HK-2), and primary cultures of normal kidney cells (RTC91696; Table 1). The efficacies (maximal growth inhibition), but not the potencies (concentration required to induce half maximal growth), were greater in the cancer cell lines compared with the normal cell cultures. Compounds with NO_2 substitutions (SHetC2 and SHetA2) on the single aromatic ring exhibited the greatest activity. In all cases, compounds with urea linkers exhibited slightly greater activity compared with the matching thiourea linkers (compare SHetC2 with SHetA2, SHetA4 with SHetA3, and SHetD3 with SHetD4). Although the increased hydrophilicity of the ethyl and methyl esters slightly decreased the efficacy, it did not eliminate the differential effect on cancer over normal cells. Relocation of the methyl ester on the cyclic ring did not significantly alter the activity of SHetD5 compared with SHetD4. All *trans*-retinoic acid did not inhibit the growth of the cancer cell lines and had minimal activity in the normal cells. Previous studies showed that SHetA2, SHetA3, SHetA4, and SHetC2 work through similar mechanisms of action in ovarian cells (7, 16).

Lack of DNA Mutagenicity

Because benzisothiazoles compounds possessing an aromatic nitro group have been found to be mutagenic (17), the mutagenicity of NO_2-subsituted SHetA2 was compared with its ethyl ester substituted SHetA3 in five different bacterial strains. In this assay, five bacterial strains that each contained a different type of mutation that prohibits their growth in histidine deficient media were incubated with the positive controls, SHetA2, SHetA3, or DMSO solvent. The positive controls caused revertant mutations that allowed the bacteria to grow in histidine-deficient media, as indicated by a significantly increase in the number of revertant colonies (Table 2). In contrast, neither SHetA2 nor SHetA3 induced substantial increases in the revertant colony counts in any of the five bacterial strains. Preincubation with S9 microsomal fractions was done to determine if SHetA2 or SHetA3 metabolites were mutagenic. Again, there was no effect on the number of revertant colonies in any of the five bacterial

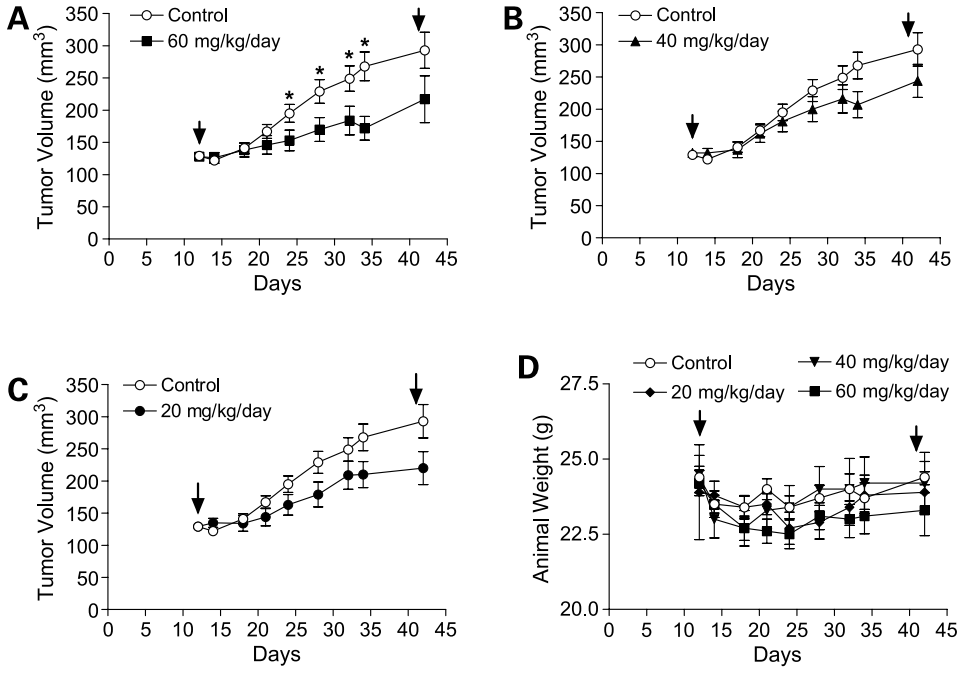
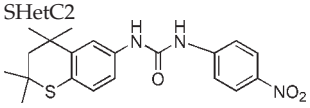
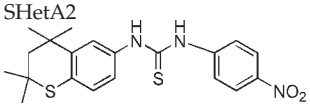
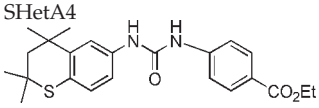
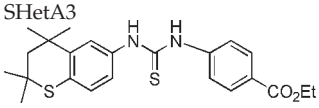
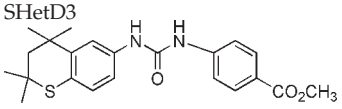
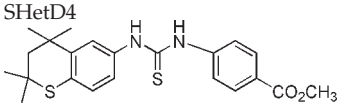
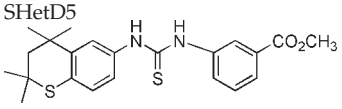
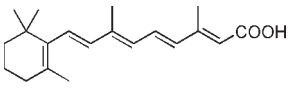


Figure 6. Effects of oral SHetA2 on Caki-1 tumor size. The mean and SE of the tumor volumes of the control group gavaged with PEG400 vehicle is shown compared with SHetA2 gavaged at 60, 40, and 20 mg/kg/d for 30 d. *Arrows,* the initiation and termination of treatment. *, statistically significant differences between the treated and vehicle control tumor sizes determined by *t* tests (day 24, $P = 0.035$; day 28, $P = 0.021$; day 32, $P = 0.028$; day 34, $P = 0.006$).

Table 1. Structures and Activities of Flex-Hets and *t*-RA in cancer and normal renal cells

	Caki-1 cancer	786-O cancer	HK-2 normal	RTC91696 normal
SHetC2 	85.71% ± 4.53% 4.91 ± 0.29 μmol/L	78.53% ± 6.11% 7.48 ± 0.34 μmol/L	52.67% ± 1.84% 4.47 ± 0.49 μmol/L	52.31% ± 7.38% 7.67 ± 0.49 μmol/L
SHetA2 	84.40% ± 3.86% 4.93 ± 0.22 μmol/L	72.00% ± 5.06% 7.55 ± 0.38 μmol/L	51.02% ± 7.07% 4.50 ± 0.28 μmol/L	36.84% ± 6.80% 4.57 ± 0.49 μmol/L
SHetA4 	68.92% ± 6.94% 5.07 ± 0.15 μmol/L	45.04% ± 4.80% 2.56 ± 0.34 μmol/L	26.35% ± 0.80% 5.19 ± 0.25 μmol/L	39.67% ± 2.52% 7.55 ± 0.51 μmol/L
SHetA3 	56.48% ± 14.58% 7.28 ± 0.27 μmol/L	54.10% ± 13.66% 7.2 ± 0.72 μmol/L	35.34% ± 2.09% 5.14 ± 0.26 μmol/L	28.23% ± 3.07% 5.92 ± 1.91 μmol/L
SHetD3 	56.99% ± 12.12% 7.14 ± 0.22 μmol/L	61.52% ± 1.44% 7.15 ± 0.81 μmol/L	26.37% ± 0.22% 6.91 ± 0.66 μmol/L	46.27% ± 0.95% 9.42 ± 0.43 μmol/L
SHetD4 	38.67% ± 1.50% 7.56 ± 0.15 μmol/L	44.55% ± 17.56% 5.08 ± 0.71 μmol/L	43.67% ± 0.44% 7.42 ± 0.20 μmol/L	37.74% ± 6.61% 5.36 ± 0.60 μmol/L
SHetD5 	34.10% ± 1.20% 7.22 ± 0.25 μmol/L	41.80% ± 16.91% 5.14 ± 0.70 μmol/L	47.97% ± 1.00% 4.96 ± 0.25 μmol/L	ND
t-RA 	6.46% ± 9.40% 4.64 ± 1.14 μmol/L	0.00% ± 1.43% 23.60 ± 0.0 μmol/L	15.81% ± 0.47% 6.28 ± 2.19 μmol/L	ND

NOTE: The top value represents the efficacy (maximum growth inhibition observed) and the bottom value represents the potency (EC_{50}, concentration needed to induce half maximal efficacy). The hydrophilicity of the compounds is increased by the end group with NO_2 being the least hydrophobic, CO_2Et intermediate, and CO_2CH_3 being the most hydrophilic.

Abbreviations: ND, not determined; *t*-RA, *trans*-retinoic acid.

strains. Partial or complete absence of the background lawn of nonrevertant bacteria and reduction in revertant colony counts were obtained with three of five bacterial strains after exposure to SHetA2 at higher dose levels (Table 2). SHetA3 induced toxicity at the highest does in only one of the five bacterial strains (Table 2). Because there was no indication of any mutagenicity at surviving doses or in the presence of S9, this toxicity is not considered to have had any material effect on the validity of the result or the conclusion obtained.

Discussion

Evaluation of molecular events induced during SHetA2 regulation of apoptosis, growth, and differentiation in kidney cancer cells showed repression of NF-κB activity and regulation of multiple downstream events. These results are consistent with a systems biology analysis that implicated multiple genes driven by NF-κB and its inducer, tumor necrosis factor α, in the mechanism of SHetA2 chemoprevention of dimethylbenz(a)anthracene–induced endometrial carcinogenesis (18). The cellular redox state plays an important role in regulation of NF-κB activity, with activation or repression dependent on the individual cellular context (19, 20). SHetA2 modulates the cellular redox state via generation of reactive oxygen species, which may be produced as a consequence of the known binding to glutathione and mitochondrial swelling (6, 12, 21). Treatment with a range of antioxidants under conditions validated to repress SHetA2-induced generation of both cellular and mitochondrial reactive oxygen species did not prevent mitochondrial swelling or apoptosis, indicating that alterations in cellular redox are a consequence, and not a cause, of the mitochondrial and cellular events induced by SHetA2 (7). In this study, the inability of exogenous glutathione or NAC to prevent apoptosis further supports the conclusion that redox regulation is not required for SHetA2 action. Also, the inconsistent repression of SHetA2-induced G_1 arrest in Caki-1, but not normal HK-2 cells, suggests that glutathione binding can contribute, but is not required, for the mechanism of G_1

arrest. Lack of repression of SHetA2-induced G_1 arrest in ovarian cancer cell lines by glutathione and NAC also support this conclusion.

Although glutathione binding is a potential source of toxicity, the widespread safe use of the analgesic paracetamol (acetaminophen), which also binds glutathione, suggests that this toxicity can be managed. The main toxicity of acetaminophen is hepatotoxicity, which can be effectively treated with NAC (22). Although livers from SHetA2-treated animals were not evaluated in this study, evaluation of

Table 2. Bacterial mutation test

Strain	Treatment	μg/plate	SHetA2				SHetA3			
			−S9		+S9		−S9		+S9	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD
TA1535	DMSO (μmol/L)	0	21	3	21	7	21	3	21	7
	0.125	50	19	9	15	10	27	3	23	3
	0.393	158	26	9	20	6	21	3	19	3
	1.245	500	18	7	22	2	19	7	19	3
	3.937	1581	21	7	19	1	18	2	16	5
	12.452	5000	16	5	22	4	20	6	17	2
	PC: NaAZ	0.5	304	54	N/A	N/A	304	54	N/A	N/A
	PC: 2AA	5	N/A	N/A	324	81	N/A	N/A	324	81
TA1537	DMSO (μmol/L)	0	15	4	21	11	15	4	21	11
	0.004	1.58	9	6	23	3	ND	ND	ND	ND
	0.012	5.0	T	T	21	2	13	1	15	3
	0.039	15.8	T	T	21	4	11	2	17	7
	0.125	50	T	T	T	T	13	5	22	3
	0.393	158	T	T	T	T	11	3	15	2
	1.245	500	ND	ND	ND	ND	11	2	16	8
	3.937	1581	ND	ND	ND	ND	6	2	6	3
	PC: 9AZ	10	673	108	N/A	N/A	673	108	N/A	N/A
	PC: BaP	5	N/A	N/A	119	5	N/A	N/A	119	5
TA98	DMSO (μmol/L)	0	30	5	45	13	30	5	45	13
	0.004	1.58	16	6	48	2	ND	ND	ND	ND
	0.012	5.0	T	T	45	8	ND	ND	ND	ND
	0.039	15.8	T	T	52	10	ND	ND	ND	ND
	0.125	50	T	T	49	8	31	6	55	8
	0.393	158	T	T	12	6	24	4	61	2
	1.245	500	ND	ND	ND	ND	27	6	55	6
	3.937	1581	ND	ND	ND	ND	22	3	62	7
	12.452	5000	ND	ND	ND	ND	18	7	57	6
	PC: 2NF	1	108	7	N/A	N/A	108	7	N/A	N/A
	PC: BaP	5	N/A	N/A	375	13	N/A	N/A	375	13
TA100	DMSO (μmol/L)	0	124	4	160	12	124	4	160	12
	0.004	1.58	136	17	136	9	ND	ND	ND	ND
	0.012	5.0	T	T	124	14	151	16	ND	ND
	0.039	15.8	T	T	171	24	138	12	ND	ND
	0.125	50	T	T	T	T	132	5	168	19
	0.393	158	ND	ND	T	T	117	14	150	12
	1.245	500	ND	ND	T	T	123	6	213	27
	3.937	1581	ND	ND	ND	ND	T	T	217	4
	PC: NaAZ	0.5	511	35	N/A	N/A	511	35	N/A	N/A
	PC: NQO	0.5	N/A	N/A	965	70	N/A	N/A	965	70
WP2 *uvrA*	DMSO (μmol/L)	0	19	5	33	7	19	5	33	7
	0.125	50	17	3	27	4	18	1	37	15
	0.393	158	14	3	18	8	19	3	36	4
	1.245	500	18	1	22	2	15	2	36	6
	3.937	1581	10	2	17	3	15	3	29	9
	12.452	5000	12	3	13	3	15	3	20	4
	PC: 2AA	0.5	594	45	N/A	N/A	594	45	N/A	N/A
	PC: 2AA	15	N/A	N/A	145	10	N/A	N/A	145	10

NOTE: Mean and standard deviations of the number of revertant colonies from triplicate results are presented.
Abbreviations: PC, positive control; N/A, not applicable; T, toxic as indicated by incomplete/no background lawn.

livers of SHetA2-treated mice from a previous xenograft model showed no liver damage as indicated by necrosis, fatty changes, or inflammation in portal tracts (5). Differences in the mechanisms of cell death induced by acetaminophen and SHetA2 suggest that the glutathione binding by SHetA2 will not cause the same toxicities as acetaminophen. The major pathway of cell death induced by acetaminophen is initiated by disturbances in cellular calcium homeostasis, activation of the proapoptotic Bax and Bid proteins, and release of proteins from the mitochondria that cause a caspase-independent DNA laddering and necrotic cell death (23). In contrast, results presented in this report on kidney cancer and in previous publications on ovarian, head and neck, and lung cancer show that SHetA2 does not induce necrosis, but instead causes cell death through induction of apoptosis via mechanisms involving decreased levels of the antiapoptotic Bcl-2 proteins with no effect on Bax, leading to release of cytochrome *c* from the mitochondria and activation of caspases (7, 21, 24, 25).[6] Furthermore, NAC can prevent acetaminophen induced cell death but not SHetA2-induced cell death (23). Although acetaminophen also inhibits NF-κB activity, the mechanism occurs through repression of reactive oxygen species similar to NAC, whereas SHetA2, inhibition of NF-κB activity is repressed by NAC.

If glutathione binding proves to be a significant hindrance to progression of SHetA2 to clinical trials, SHetC2, which contains a urea linker in place of the thiourea linker, is a viable alternative. Theoretically, the urea linker cannot be oxidized to a product that binds glutathione. In addition, the oxygen atom of the urea linker in SHetC2 has increased H-bonding capability and may therefore bind to potential protein targets with greater affinity. On the other hand, the increased H-bonding capability might increase binding to plasma proteins and decrease the bioavailability. Because SHetA2 was shown to be bound by plasma proteins at 99.3% to 99.5% (27), increased binding activity could be detrimental to the pharmaceutical qualities of Flex-Het. Previous studies showed that SHetC2 is also capable of inducing apoptosis (16). The equal potencies and efficacies of SHetC2 with SHetA2 for inhibiting the growth of renal cancer cell lines and similar reduced effects in normal kidney cells indicates that SHetC2 is a reasonable alternative lead compound for SHetA2 that would avoid the potential toxicity of glutathione binding. The switch of SHetC2 for SHetA2 as a lead compound is not an easy decision, however, because millions of dollars have already been invested by the NCI RAID and RAPID programs in the preclinical development of SHetA2 (NSC 726189) and no significant toxicities have been noted to date.

The differential effect of SHetA2 on apoptosis in cancer versus normal cells further supports the potential safety of this compound. The resistance of primary cultures of kidney epithelial tubule cells and the HK-1 normal kidney epithelial cell line to SHetA2 apoptosis is consistent with the resistance of normal primary cultures and cell lines previously reported for ovarian, endometrial, and endothelial cells (6, 7, 28). The mechanism of this differential effect involves resistance of normal cells to perturbations in the balance of Bcl-2 proteins and mitochondrial swelling induced by SHetA2 and related Flex-Hets (7).

Although normal cells are resistant to SHetA2-induced apoptosis, they are still growth inhibited. The results presented here showed that this growth inhibition is caused by arrest of the cell cycle at the G_1-S border in association with loss of Cyclin D1. The decreased levels of Cyclin D1 seem to be the result of alterations in both protein synthesis and degradation because inhibition of these activities with cyclohexamide and MG132, respectively, could attenuate the Cyclin D1 decrease. The repression of Cyclin D1 transcription is consistent with the theory that SHetA2 repression of NF-κB initiates a series of events responsible for the cellular outcomes, because Cyclin D1 transcription can be induced by NF-κB through multiple NF-κB sites in the Cyclin D1 promoter (29).

In addition to the growth inhibition and apoptosis activities of SHetA2, the ability to reverse abnormal cellular differentiation in ovarian organotypic cultures and xenografts offers promise for utility of this compound in chemoprevention (5, 10). The results presented here confirm that SHetA2 also can induce differentiation in kidney cancer organotypic cultures and xenografts. SHetA2 up-regulation of E-Cadherin expression is a likely candidate for the molecular mechanism of differentiation induction and is consistent with repression of NF-κB, because this transcription factor has been shown to decrease E-Cadherin expression (30). In addition to a role in differentiation, loss of E-Cadherin expression in kidney cancer is associated with increased proliferation through down-regulation of p27Kip1 (p27), a cyclin-dependent kinase inhibitor, and induction of activating phosphorylation of the epidermal growth factor receptor (31). E-Cadherin seems to play an important role specifically in kidney carcinogenesis due to its regulation by the von Hippel-Lindau protein, which is defective or lost in von Hippel-Lindau hereditary cancer syndrome and most sporadic clear cell renal cell cancers in association with decreased E-Cadherin expression (32). The von Hippel-Lindau protein indirectly represses E-Cadherin expression through binding to the ubiquitin E3 ligase complex, resulting in degradation of E-Cadherin (32, 33). Decreased or nuclear E-cadherin expression is associated with poor prognosis in kidney cancer (34, 35). Loss of E-cadherin occurs during epithelial to mesenchymal transition that confers the ability to migrate and invade, making restoration of E-Cadherin a therapeutic goal of anticancer strategies (13). Caution is needed in up-regulation of E-Cadherin as an anticancer strategy because this up-regulation could promote mesenchymal-epithelial reverting transitions and result in the ability of cancer cells to form metastic lesions after they have disseminated from the primary tumor site (36).

The reason for effective growth inhibition of Caki-1 xenografts, but not A498 xenografts, by SHetA2 is unclear,

[6] Chengedza S, Benbrook DM. NF-KB repression by SHetA2 overcomes TNFα-resistance in ovarian cancer cells. 2009: In preparation.

because both cell lines are equally sensitive to this compound *in vitro* (5). A large study comparing the *in vitro* growth inhibition activity of compounds with their ability to inhibit xenograft tumor growth found that only 18% to 44% of the compounds that were active *in vitro* exhibited activity against the corresponding xenograft *in vivo* (37). Thus, the pharmacologic properties of SHetA2 may need to be improved. In this and previous studies, compounds with nitro substitutions on the phenyl ring (SHetC2 and SHetA2) consistently exerted greater growth inhibitory and apoptosis activity in comparisons to related compounds with methyl or ethyl ester substitutions (SHetA3, SHetA4, SHetD3, and SHetD4; refs. 6, 16). Although aromatic nitro groups in the context of bz-nitro–substituted amino 2,1-benzisothiazoles have been shown to be mutagenic, the structures of these compounds are very different from Flex-Hets (17) and neither SHetA2 nor SHetA3 were mutagenic as direct agents or after metabolism by S9 liver microsomal fraction. This is consistent with the lack of teratogenicity of SHetA2 in mouse embryos (8). Higher doses of SHetA2 were toxic to three of the five bacterial strains, whereas SHetA3 exhibited limited tocixity in only one of the five bacterial strains, which is consistent with the greater cytotoxicity of SHetA2 in eukaryotic cells. Previous studies found no differences in the cytotoxicities of SHetA2, SHetA3, and SHetA4 against Mycobacterium bovis Bacillus Calmette-Guerin (38).

In conclusion, SHetA2 regulates growth, differentiation, and apoptosis in kidney cancer cell lines through multiple molecular events downstream of NF-κB repression and independent of cellular redox regulation. Flex-Hets with greater hydrophilicity than SHetA2 retain the differential effect on cancer cells over normal cells, thus offering alternatives for improvement of therapeutic value.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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