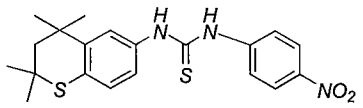
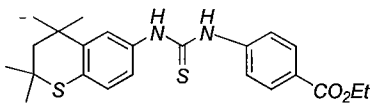
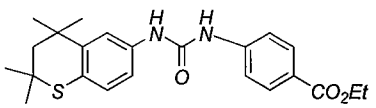
Design of 3-atom linker Receptor-Independent Flex-Hets. Through several structure-function studies we demonstrated that single substitutions of the Het structure could dramatically alter the retinoic acid receptor selectivity of the compounds in correlation with specific biologic activities (1-3). Further studies demonstrated that the type of linker between the aromatic rings of heteroarotinoids has a strong effect on their biological activity profiles. Hets with 3-atom thiourea or urea linkers, such as SHetA2, SHetA3, and SHetA4 (4), do not activate the retinoic acid receptors, despite their abilities to induce several retinoid biologic activities. The Hets with 3-atom linkers have more flexibility in their structure and are thus called flexible Hets (Flex-Hets).



SHetA2



SHetA3



SHetA4

The abilities of Het compounds to activate the RAR and RXR retinoid receptors were evaluated in two different assays. One assay evaluated the ability of the compounds to activate endogenous receptors in the retinoid-sensitive SW962 squamous cancer cell line and the other assay evaluated the ability of the compounds to activate retinoid receptors expressed from cDNA clones in the CV-1 cells. Although 9-*cis*-RA exhibited a dose-responsive induction of the retinoid receptors in both assays, the Hets and 4-HPR did not exhibit any activity in either assay (data not shown). Although retinoic acid and other receptor-active Hets induce weak growth inhibition activities against ovarian cancer cell lines, the 3-atom linker Hets exerted significantly higher levels of growth inhibition indicative of apoptosis (5). In our studies and those of collaborators summarized below, SHetA2 induced the highest levels of apoptosis and differentiation of all retinoids tested.

Differential Effects of SHetA2 on Cancer versus Normal Cells. Given the potential for clinical application of these compounds for wide-spread use in cancer chemoprevention, it is important to determine if they harm normal cells. To evaluate effects on normal cells, primary cultures derived from a benign (non-cancerous) ovarian cyst and from normal endometrial cells were

Also, the potential for use of these compounds as chemoprevention agents was evaluated by testing their effects on a primary culture derived from a borderline ovarian tumor. A borderline ovarian tumor is not considered to be cancer, but instead represents a pre-malignant state with increased potential to become cancerous. The ideal clinical agent should exhibit a large differential between the growth effects on the borderline and cancerous cultures in comparison to the benign and normal cultures. The growth inhibition curves of SHetA2 in Figure 2, illustrate the greater efficacies and potencies of SHetA2 on pre-cancer and cancer cells in comparison to normal and benign cells.

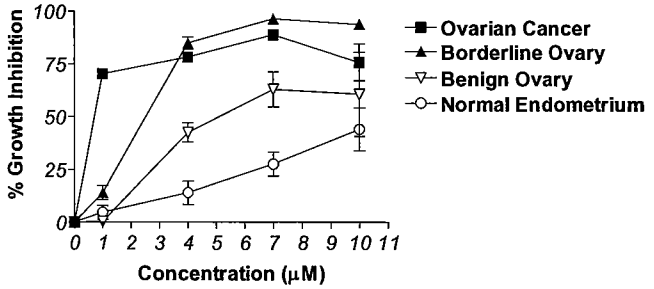


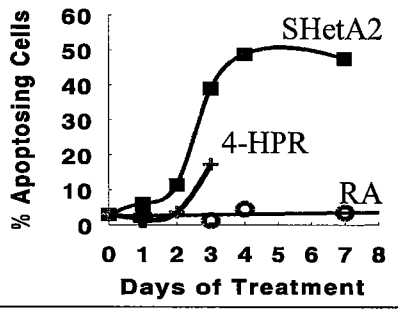
Figure 2. Differential Effects of SHetA2. Cultures were treated with a range of SHetA2 concentrations or the same solvent as a control. The percentage of growth inhibition after 3 days of treatment was determined using the MTS assay. The results represent the average and standard deviation of 3 experiments performed in triplicate.

Validation of Results in Other Labs and Organs. A series of Hets, were sent to 4 laboratories around the world under material transfer agreements. Each laboratory identified SHetA2 as the most efficacious and

potent compound. The investigators were Dr. Reuben Lotan at the MD Anderson Cancer Center in Houston, Texas, USA, who evaluated head and neck squamous cancer cell (HNSCC) lines (17a, 17b, 22b, 38, 886, 1186,1483, sQCC/y1, SCC38); Dr. Michael Birrer at the National Cancer Institute at Frederick, Maryland, USA, who evaluated ovarian cancer cell lines (222, UCI 101, CP70, AD10, A224, SK-OV-3 and UCI 107), Denver Hendricks at the University of Cape Town, Cape Town, South Africa who evaluated oesophageal cell lines (WHCO6, WHCO1), and Dr. Xie Qian at the Medical Center of Fudan University in Shanghai, China who evaluated liver cancer cell lines (SMMC7721, SF7721, HepG2, Hep3B, MHCC, 7402, 7402D).

Induction of Apoptosis. Two different assays (Annexin-V-Flous and TUNEL) performed in several cell lines demonstrated that cell loss in SHetA2-treated cultures was due to induction of apoptosis. The Annexin-V-Flous assay, demonstrated that SHetA2 induced apoptosis cancer cell lines in the same concentration range as 4-HPR (6) (Figure 3).

Figure 3 Apoptosis. SW962 vulvar carcinoma cultures were treated with 10 μM of the indicated retinoid or the same volume of solvent. At the specified times, the cultures were labeled with Annexin-V-Fluos and propidium iodide (PI), and the number of apoptotic cells was quantified using dual-laser flow cytometric analysis.



Effects of Clinically Relevant Concentrations. Although micromolar concentrations of retinoids have been achieved in phase I clinical trials, lower effective doses are desirable. A demonstration that less than micromolar concentrations of a retinoid could exhibit chemopreventative activity was demonstrated for 4-HPR, which was evaluated in a randomized clinical trial conducted at the Istituto Nazionale Tumori in Italy. In this trial, women who had surgery for breast cancer were randomly assigned to receive 200 mg of 4-HPR per day or a placebo for five years to determine whether 4-HPR reduced the incidence of second primary tumors (7, 8). A secondary finding of this trial was that statistically significantly fewer ovarian cancers developed in women who took 4-HPR. 4-HPR is similar to classical retinoids in that it both can induce differentiation in a receptor-dependent manner at 1 μM (9). At 3 to 12.5 μM however, 4-HPR differs from classical retinoids in that it induces apoptosis in a variety of cell lines through a receptor-independent mechanism (9-15). In the Italian trial, the plasma concentration of 4-HPR achieved in treated women ranged from 340 to 868 nM (16). Therefore, the concentrations in the ovarian tissue of treated women were not likely to have reached the levels required for apoptosis. We hypothesized that the 4-HPR chemoprevention observed was due to induction of differentiation or apoptosis in ovarian cancer tissue by chronic exposure to less than micromolar 4-HPR concentrations.

To test our hypotheses, we evaluated the effects of clinically achievable concentrations of 4-HPR and our Hets on growth, differentiation and apoptosis in ovarian tissue. A series of compounds with a variety of receptor activation profiles were evaluated. In order to evaluate therapeutic effects, we developed an organotypic culture model of ovarian tumors by growing established cell lines and primary cultures inside collagen gels (5, 17). Monolayer cultures of cells are not accurate representations of *in vivo* tissue because they lack the types of interactions that cells have with other cells and with the extracellular matrix *in vivo*. These interactions are known to influence gene expression and thus the constitution and behavior of cells. In our organotypic model, the growth of tumor cells inside the collagen allows a representation of tumors that have invaded stromal tissue. In the presence of fibroblasts, established cell lines and primary cultures of

ovarian cancer formed colonies inside the collagen, while normal or benign ovarian epithelial cells remained as single cells inside the collagen. Human ovarian cancer cell lines (OVCAR-3, Caov-3, SK-OV-3, A2780, UCI 101) and primary human cultures (borderline ovarian tumor (O1), serous papillary tumor (O2), benign ovarian cyst (O3)) were cultured inside and on top of a collagen gel seeded with 3T3 fibroblasts for one week. After further incubation in the presence or absence of 1 μM retinoid (media concentration) for 3, 7 or 14 days, cultures were fixed and sectioned for immunohistochemical analysis. Eleven different retinoids were evaluated and SHetA2 was found to be the most potent regulator of growth, differentiation and apoptosis as published (5) and summarized below.

Induction of Differentiation. All retinoid treatments of the ovarian cancer organotypic cultures reversed characteristics associated with the cancerous phenotype. In general, this was observed as: increased cytoplasm, decreased nuclear to cytoplasmic ratio, decreased nuclear pleomorphism, decreased size and number of nucleoli, and a more orderly organization of the cells. In retinoid treated OVCAR-3, Caov-3 and A2780 cultures, well-formed glands were consistently observed as cells arranged around a central space with the individual cells having the cytoplasm pointed toward the space, and their nuclei located at the outer periphery of the cells (Figure 4).

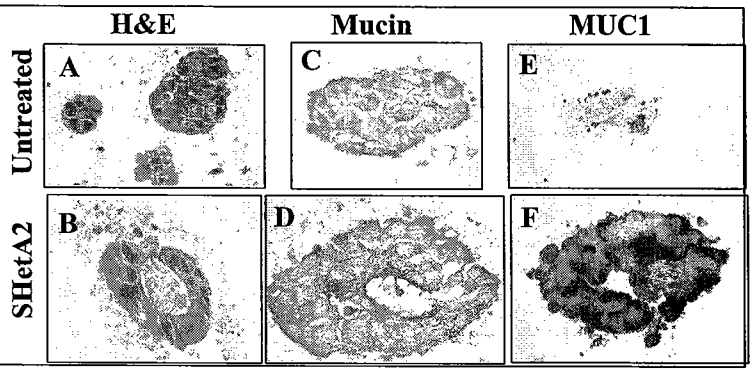
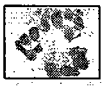


Figure 4. Differentiation. Photomicrographs (40X) of Caov-3 organotypic cultures sections (A and B) stained with hematoxylin and eosin (H&E) and OVCAR-3 organotypic cultures (C, D, E and F) stained with Periodic Acid Schiff (PAS – purple color is positive) to detect mucin proteins (C and D) and immunohistochemically stained for MUC1 expression (Reddish brown color is positive) (E and F). Cultures were growth in the absence (top row) and presence of 1 μM SHetA2 for two weeks.

To determine whether mucin was being produced in these glands, sections of the organotypic cultures were stained with PAS. Positive PAS staining was observed in some cells and inside the glandular lumens (Figure 4D). The intensity and percent of cells staining PAS positive were estimated semi-quantitatively by pathology review and used to generate a score. In the absence of retinoids, OVCAR-3 expressed a PAS score of 6 out of 12. Of all the retinoids evaluated, SHetA2 induced the highest PAS score of 12 out of 12. Expression of the mucin 1 (MUC1) protein was found to be greatly elevated in SHetA2 OVCAR-3 organotypic cultures (Figure 4) and correlated with induction of differentiation and apoptosis (5). Although increased MUC1 expression is implicated in metastases, this type of aberrant expression in cancer cells is due to increased expression on all cell surfaces and aberrant glycosylation of the MUC-1 protein (18-22). In contrast, induction of MUC1 expression by retinoids is limited to the correct cellular location at the luminal surface of glands (Figure 4D&F).

Induction of apoptosis. The TUNEL assay was used to evaluate apoptosis in fixed, paraffin-embedded, cut sections of the treated and control cultures (Figure 5). Apoptosis was induced by 4-HPR and all of the 3-atom linker receptor-independent Hets, SHetA2, SHetA3 and SHetA4, and by two potent receptor pan agonist retinoids, 9-*cis*-RA and SHet50. The 2-atom ester linker Hets exhibiting a variety of receptor specificities did not induce apoptosis. SHetA2 induced the highest level of apoptosis. Extensive review of multiple sections and repetitions of this experiment did not reveal any cellular clumps in the organotypic

cultures that were undergoing simultaneous differentiation and apoptosis. These two cellular outcomes appeared to be mutually exclusive.

Figure 5. TUNEL assay demonstrates apoptosis in a clump in a SHetA2-treated culture. 40X 

Inhibition of Proliferation. To determine if both classes of retinoids were capable of decreasing the growth fraction, the sections were immunohistochemically stained for Ki-67, which is considered a marker of proliferating cells. All of the retinoids, including SHetA2 significantly decreased the percentage of Ki-67 positive cells (5).

In Vivo Inhibition of Xenograft Tumor Growth. The ability of retinoids to inhibit the growth of ovarian carcinomas *in vivo* was evaluated in a nude mouse xenograft model using the human OVCAR-3 ovarian carcinoma cell line. The xenograft tumors were extremely heterogeneous in size ranging from 36 to 2200 mm^3, which is reflective of the heterogeneous population of cells characteristic of the OVCAR-3 cell line. All of the retinoids, 4-HPR, SHet50 and SHetA2, significantly inhibited the growth of the xenograft tumors (Figure 6). After 15 days of treatment, the relative volumes of the tumors in the retinoid-treated groups were significantly smaller than the control group treated with sesame oil alone (t-test on day 29: $p = 0.028$ for 4-HPR; $p = 0.010$ for SHet50; $p = 0.046$ for SHetA2). There were no significant differences between the degrees of growth inhibition exerted by the different retinoids.

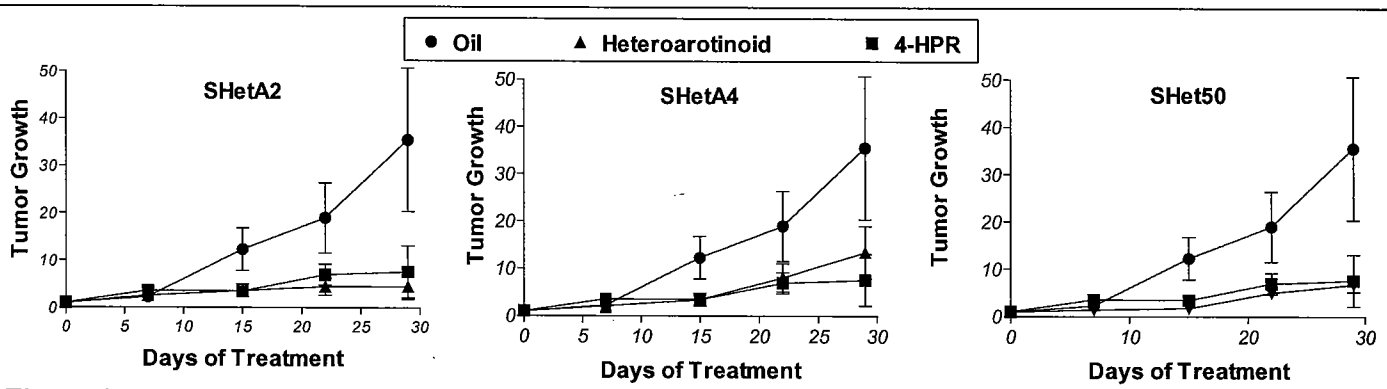


Figure 6. Xenograft Tumor Growth. Thirty nu/nu mice were injected subcutaneously with one million OVCAR-3 cells and arbitrarily divided into five groups of six mice each. After three weeks of tumor growth, the animals were gavaged with sesame oil, 10 mg/kg/day 4-HPR, or the indicated Het five times per week for four weeks and the tumors were measured once per week. The tumor sizes were averaged and divided by the average size for each group on the day that treatment was initiated (day 0).

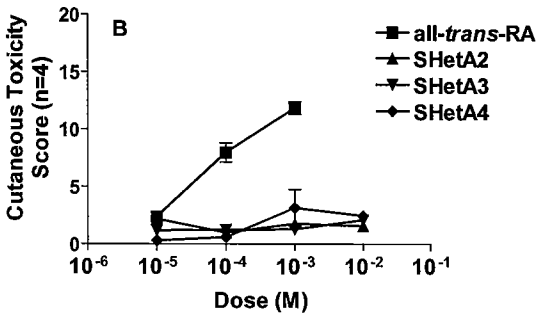
Evaluation of Toxicity. During the course of the xenograft nude-mouse model experiment, the animals were continuously monitored for visually observable signs of retinoid toxicity to the skin or bone. None of the retinoids induced any signs of skin or bone toxicity, which were observed in previous experiments when animals were treated with 10 mg/kg/day of all-*trans* retinoic acid. To determine if 4-HPR and the three Hets induced hepatotoxicity in this animal model, plasma alanine aminotransferase (ALT) activity levels were measured and histological liver sections were examined at the end of the treatment period. There were no statistically significant differences between the ALT activities in the different treatment groups or between the treatment groups and the control group (Table 1 on the next page, t-test:$p>0.05$). Each of these values was in the normal range of ALT values (28-184 μM/L/min) for this species of mouse as reported by the supplier (Charles Rivers Laboratories). Necrosis, fatty changes or inflammation in portal tracts were not observed in histological evaluation of liver sections.

Table 1. ALT Activity in Plasma of Mice.
*Normal range 18-184 (μ*M*/L/min).

Treatment	ALT Activity (μ*M*/L/min)*
Sesame Oil	103.0 ± 60.3
4-HPR	149.7 ± 140.9
SHet50	65.5 ± 46.3
SHetA2	118.5 ± 153.4
SHetA4	64.5 ± 40.3

Evaluation of Topical Irritation. The potential for utilizing Hets in the topical treatment of cervix, vulva and other skin cancers, topical irritancy was tested in an animal model in comparison to RA according to published procedures (23). In this model, retinoic acid (all-trans-RA) and Hets that activate the RAR retinoic acid receptors, RA, SHet65 and NHet17 caused topical irritancy while, topical application of the receptor-independent retinoids SHetA2, SHetA3 and SHetA4 did not induce topical irritancy in this model (Figure 8B).

Figure 8. Topical Irritancy. Female Skh hairless mice, 6-8 weeks old, were treated topically on the dorsal skin for 4 days over a 2-log concentration dose range with various compounds. Daily flaking and abrasion scores were combined to calculate a single cutaneous toxicity score for each mouse. Each treatment group consists of 4 mice, and group averages are used to plot cutaneous toxicity against dose of the retinoid.



Mechanism of SHetA2 Apoptosis. The first clues as to the molecular mechanism of SHetA2-induced apoptosis arose from a collaborative study of head and neck squamous cancer cell lines (HNSCCs) (24). These results have been eliminated from the revised version in order to allow sufficient space to include the new data on microarray analysis. A reprint of this study is provided in the appendix. In summary, these results confirmed that SHetA2 is the most potent of the heteroarotinoids, and that it induces apoptosis through a mechanism that is independent of the retinoic acid receptors, and that involves G2 cell cycle arrest, alterations in the mitochondrial membrane permeability transition, release of cytochrome c from the mitochondria, generation of reactive oxygen species, and activation of caspase 3.

Similar Molecular Mechanisms in Ovarian and Head and Neck Cancers. SHetA2 was also evaluated for generation of ROS and induction of caspase in two ovarian cancer cell lines, UCI 101 and 222, by Dr. Michael Birrer's laboratory (25). The growth of the UCI 101 cell line was inhibited by 80 and 95%, respectively, when treated with 5 and 10 μ*M* of SHetA2. The 222 cell line was inhibited 70 and 90%, respectively, when treated with 5 and 10 μ*M* of SHetA2. Both cell lines exhibited a dose-responsive induction of ROS by SHetA2 and a dose-responsive induction of caspase 3.

Current unpublished results demonstrate that SHetA2 works through two mechanisms of action that may be independent or integrated. SHetA2 directly targets the mitochondria and regulates gene expression.

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