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Flexible heteroarotinoids (Flex-Hets) exhibit improved therapeutic ratios as anti-cancer agents over retinoic acid receptor agonists

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Summary

The anti-cancer activities and toxicities of retinoic acid (RA) and synthetic retinoids are mediated through nuclear RA receptors (RARs) and retinoid X receptors (RXRs) that act as transcription factors. Heteroarotinoids (Hets), which contain a heteroatom in the cyclic ring of an arotinoid structure, exhibit similar anti-cancer activities, but reduced toxicity *in vivo*, in comparison to parent retinoids and RA. A new class of Flexible Hets (Flex-Hets), which contain 3-atom urea or thiourea linkers, regulate growth and differentiation similar to RA, but do not activate RARs or RXRs. In addition, Flex-Hets induce potent apoptosis in ovarian cancer and in head and neck cancer cell lines through the intrinsic mitochondrial pathway. In this study, 4 cervical cancer cell lines were growth inhibited by micromolar concentrations of Flex-Hets to greater extents than RAR/RXR active retinoids. The most potent Flex-Het (SHetA2) inhibited each cell line of the National Cancer Institute's human tumor cell line panel at micromolar concentrations. Oral administration of Flex-Hets (SHetA2 and SHetA4) inhibited growth of OVCAR-3 ovarian cancer xenografts to similar extents as administration of a RAR/RXR-panagonist (SHet50) and Fenretinide (4-HPR) *in vivo*. None of these compounds induced evidence of skin, bone or liver toxicity, or increased levels of serum alanine aminotransferase (ALT) in the treated mice. Topical application of Flex-Hets did not induce skin irritation *in vivo*, whereas a RAR/RXR-panagonist (NHet17) and a RARγ-selective agonist (SHet65) induced similar irritancy as RA. In conclusion, Flex-Hets exhibit improved therapeutic ratios for multiple cancer types over RAR and/or RXR agonists.

Introduction

The majority of cancer-related deaths occur after primary therapy has been completed, mostly due to recurrence of the cancer or development of second cancers. Major efforts are underway to develop pharmaceuticals that can prolong the disease-free interval after primary therapy by preventing recurrence of the cancer or the development of new cancers. Only agents that lack significant toxicity are acceptable in this setting. One of the most promising classes of cancer chemoprevention agents designated by the Chemoprevention Working Group to the American Association for Cancer Research (AACR) is retinoids [1]. These compounds, which are modeled after the active vitamin A metabolite, retinoic acid (RA), offer promise as cancer chemoprevention agents because of their abilities to regulate growth, differentiation, apoptosis, angiogenesis, metastasis and immune function. Despite limited success of various isomers of RA (All-*trans*-RA, 13-*cis*-RA and 9-*cis*-RA) and a synthetic retinoid in chemoprevention trials (Fenretinide, 4-HPR), structural alterations of the compounds are needed to improve the therapeutic ratio (efficacy/toxicity) before clinical application of a retinoid strategy for chemoprevention [2–5].

The toxicities associated with chronic retinoid treatment affect the skin, mucus membranes, hair, eyes, gastrointestinal system, liver, neuromuscular system, endocrine system, kidneys and bone, and are collectively termed hypervitaminosis A [6]. These individual toxicities and teratogenicities have been shown to be induced

through activation of nuclear retinoic acid receptors (RARα, RARβ, RARγ) and retinoid X receptors (RXRα, RXRβ, RXRγ) that act as transcription factors [7, 8]. Early efforts to improve the therapeutic ratio involved constraining the RA double-bonds, by inclusion in an aromatic ring of chemical structures called arotinoids. The first arotinoid evaluated, TTNPB, was 10-fold more potent than RA in biological assays of efficacy, but considerably more toxic [9–12].

Our strategy to reduce the toxicity of arotinoids was to retard metabolic oxidation of the compounds by incorporation of oxygen or sulfur heteroatoms to replace one of the *gem*-dimethyl groups in the tetrahydronaphthalene ring of TTNPB (Figure 1). The resulting compounds, called Heteroarotinoids (Hets), exhibited the similar biological activities to RA [9, 13], but significantly reduced toxicities [12]. Thus, inclusion of the heteroatom in the arotinoid structure was shown to greatly improve the therapeutic ratio (efficacy/toxicity) in animal models [9, 12]. The clinical application of a Het called Tazarotene (produced by Allergan) for treatment of psoriasis, has confirmed the improved therapeutic ratio predicted for compounds with heteroatoms [14].

Individual structural alterations of Hets greatly affected their selectivities for individual RAR and RXRs (Figure 1) [12, 15–17]. A Het that activated RXRs only (OHet72) was found to be sufficient to inhibit establishment of head and neck xenograft tumors, while a retinoid that activated both RARs and RXRs (SHet50) exerted greater growth inhibitory activity [17]. The importance of RARγ activation in skin cancer was demonstrated by comparisons of Hets, which differed by single structural

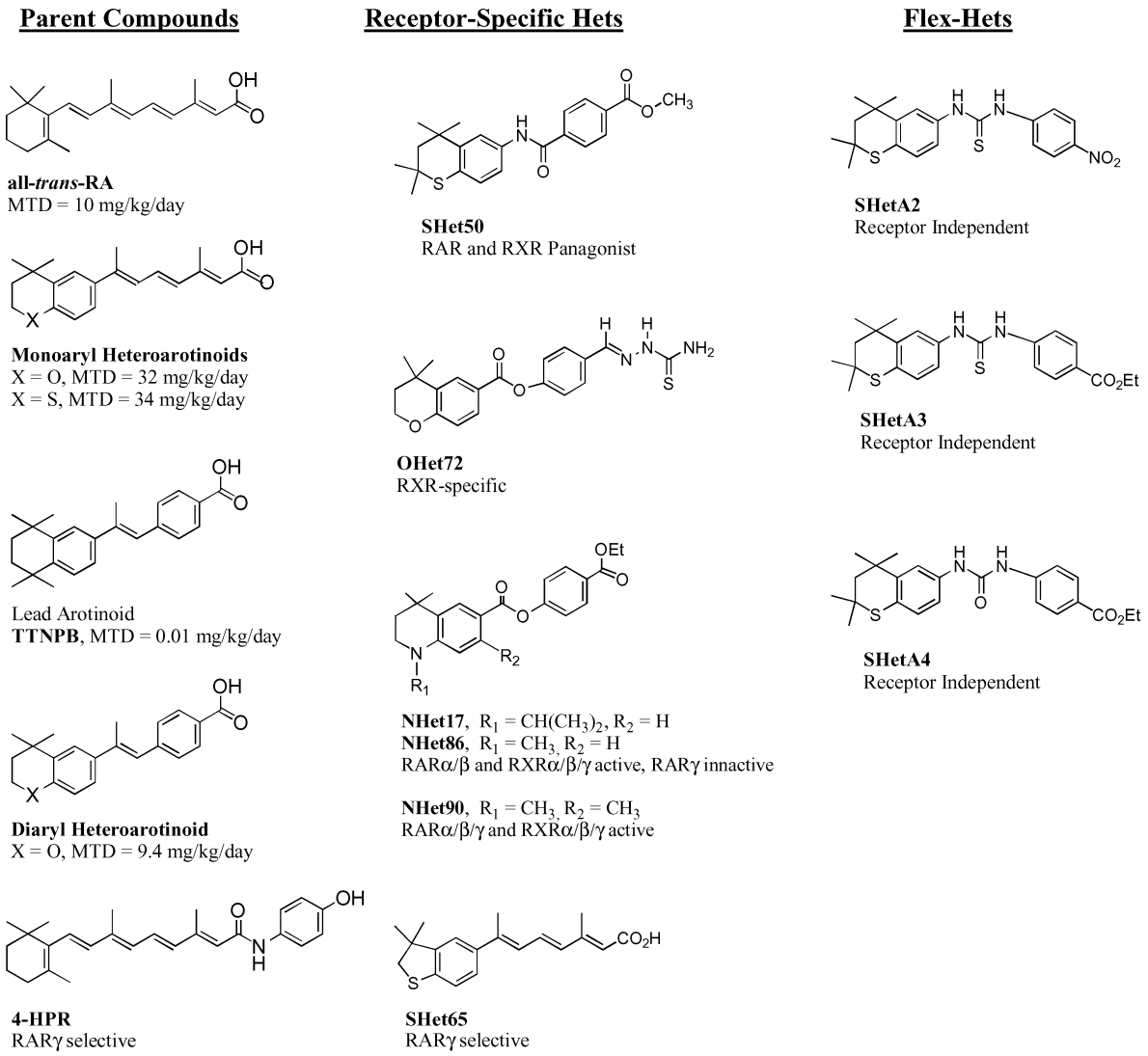

Figure 1. Chemical structures of retinoids and Hets. (MTD = Maximum Tolerated Dose.)

alterations that regulated their abilities to activate the RARγ receptor. A Het that activates all six nuclear receptors (NHet90) induced significantly greater growth inhibition of vulvar carcinoma cell lines in comparison to structurally related compounds, that activate all retinoid receptors except RARγ (NHet17 and NHet86) [16]. Interestingly, Hets containing three-atom urea or thiourea linkers, which increased the flexibility of their conformations, regulated growth and differentiation similar to RA, but did not activate the RARs and RXRs [18]. These flexible Hets (Flex-Hets) exhibited significantly greater growth inhibition activity against epithelial ovarian cancer and borderline-cancer cells than benign epithelial ovarian cells and normal endometrial cells [19]. The most potent Flex-Het, SHetA2, was characterized for the mechanism of this strong growth inhibition in head and neck cancer cell lines, and was found to induce apoptosis through G2 cell cycle arrest, alterations in mitochondrial membrane permeability, release of cytochrome *c* from the mitochondria, generation of reactive oxygen species (ROS), and activation of caspase 3 [19, 20]. Generation of ROS was also demonstrated in SHetA2-treated ovarian cancer cell lines [19].

While natural RA isomers and classical retinoids are weak apoptosis inducers, some retinoids, 4-HPR, CD437/AHPN and MS3350-1, which are selective for RARγ activation, exhibit potent apoptosis-inducing activity similar to Flex-Hets [21]. 4-HPR also weakly activates RARβ, and activation of multiple retinoid receptors by 4-HPR is involved in the mechanism of growth inhibition in leukemia cells [22, 23]. The additional non-retinoid activities possessed by these compounds have led to their classification as retinoid-related molecules (RRMs). While the ability of these compounds to induce apoptosis is only partially independent of the retinoid receptors, Flex-Hets are unique in that they induce apoptosis completely independent of RAR and RXR activation [20, 24, 25]. Several clinical trials of 4-HPR demonstrated limited cancer chemoprevention activity at low doses, and tolerable toxicity at higher doses sufficient to induce apoptosis [3, 5, 26]

The objective of this study was to assess the potential of Flex-Hets as anti-cancer pharmaceutical agents. Positive controls included RA isomers (all-*trans*-RA, 9-*cis*-RA), pan-agonist Hets that activate all RARs and RXRs (SHet50, NHet17), an RRM that activates retinoid receptors (4-HPR) and/or an agonist Het selective for RARγ (SHet65) [15] (see Figure 1 for structures). The ranges of growth inhibitory activities were evaluated in 59 cell lines representing 10 cancer types. *In vivo* activity and toxicity was evaluated in an ovarian cancer xenograft animal model and in an animal model of skin irritation.

Materials and methods

Drugs

The Hets were synthesized and their receptor specificity determined as previously described (SHet65 is compound 15 in reference [15], NHet17 is compound 3 in reference [16], SHet50 is compound 16 in reference [17], while SHetA2, SHetA3 and SHetA4 are described in reference [19]). 9-*cis*-RA was purchased from Biomol and 4-HPR was provided by Johnson and Johnson. Drugs were dissolved differently for each *in vitro* and *in vivo* assay as described below.

In vitro cytotoxicity assays in cervical carcinoma cell lines

The SHetA2, SHetA3, SHetA4, SHet50 and 9-cis-RA compounds were evaluated in 4 cervical carcinoma cell lines. The SiHa, CC-1 and C33a human cervical cell lines were maintained in Minimal Essential Media (MEM) containing Earle's salts and *L*-glutamine supplemented with nonessential amino acids, 1% sodium pyruvate, 10% fetal bovine serum (FBS) and antibiotic/antimycotic. The HT-3 cervical carcinoma cell line was cultured in McCoy's 5a medium supplemented with 10% FBS and antibiotic/antimycotic. Cells were inoculated into 96 well microtiter plates at densities of 1000 cells/well and incubated for 24 h prior to addition of drugs. Drugs were dissolved in dimethyl sulfoxide (DMSO) and diluted in complete medium, prior to addition to triplicate culture wells to achieve final concentrations of 1, 4, 7 and 10 μM. Following drug addition, the plates were incubated for 72 h. The assay was terminated by the addition of cold TCA and the cells were stained with 0.4% sulforhodamine B (SRB) in 1% acetic acid. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 560 nm. Growth inhibition was determined by dividing the average Optical Density (OD) of the triplicated treated cultures by the average OD of the triplicate control cultures treated with DMSO solvent only. This ratio was converted into a percentage by multiplying by 100. The potency (concentration required to induce half of the maximal activity - GI_{50}) were derived from dose-response graphs generated with results from 3 to 5 individual experiments using GraphPad Software.

SHetA2 cytotoxicity in NCI human tumor cell line panel

The SHetA2 compound was evaluated in the National Cancer Institute (NCI) human tumor cell line panel by the Developmental Therapeutics Program (DTP). The human

tumor cell lines were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM *L*-glutamine. Cells were inoculated into 96 well microtiter plates at densities ranging from 5,000 to 40,000 cells/well depending on the doubling time of individual cell lines and incubated for 24 h prior to addition of SHetA2. After 24 h, two plates of each cell line were fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Drugs were dissolved in dimethyl sulfoxide (DMSO) and diluted in complete medium containing 50 μg/ml gentamicin, prior to addition to the culture wells. Final drug concentrations ranged from 10^{-4} to 10^{-8} M in a series of 10-fold dilutions. After 24 h, two plates of each cell line were fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Following drug addition, the plates were incubated for an additional 48 h and then the assay was terminated by the addition of cold TCA and stained as described in the preceding section. The absorbance was read on an automated plate reader at a wavelength of 515 nm. Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the GI_{50} was calculated from $[(Ti - Tz)/(C - Tz)] \times 100 = 50$. The NCI results are presented as the average and standard deviation of two independent experiments.

RAR and RXR receptor expression

The cervical cancer cell lines were plated at a density of 10^6 cells on 100 mm plates. After allowing the cells to adhere overnight, the cultures were treated with 0 (DMSO solvent only), 1 and 10 μM SHetA2. After 4 and 24 hours of incubation, RNA was isolated from the cultures using the RNeasy Mini RNA isolation Kit (Qiagen) in combination with the QIAShredder Spin Column (Qiagen). The previously published PCR primers and conditions for reverse transcriptase polymerase chain reaction (RT-PCR) were utilized with the exception that First Strand cDNA Synthesis Kit (AMV) (Roche) was used for reverse transcriptase and the Platininum® Taq DNA polymerase (Invitrogen) was used for amplification [27].

Xenograft tumor animal model

The OVCAR-3 cultures were maintained in RPMI media supplemented with 10% fetal bovine serum. All animal experimentation described in this manuscript was conducted in accord with accepted standards of humane animal care. Twenty-five female NU/NU CD-1 female mice (Charles Rivers Laboratories) were housed in a laminar flow room under sterile conditions at 83–85° F. The mice were quarantined for one week prior to the beginning of the study and were allowed access to autoclaved food (Purina 5001 mouse/rat sterilizable diet, St. Louis, MO) and water *ad libitum*. OVCAR-3 cells in log phase growth were harvested by trypsinization, resuspended in RPMI culture medium, and centrifuged at 3,000 rpm for 10 min. The pellets were resuspended in RPMI culture medium at a concentration of 7×10^6 cells/ml before implantation into mice. Animals were injected with 3.5×10^6 cells into the right scapular region with a 24-gauge needle/1cc tuberculin syringe. Twenty-four hours after tumor implantation, animals were randomized into 5 groups of 5 animals each.

The Hets, SHet50 and SHetA2 were synthesized and their receptor specificity determined as previously described (SHet50 is compound 6 in reference [17] and SHetA2 is compound 15c in reference [19]. The 4-HPR compound was a gift from R.W. Johnson Pharmaceutical Research Institute, Raritan, NJ. All retinoids were dissolved in super refined sesame oil (Croda, Inc., Parsippany, NJ) and stored at −80°C. Since the retinoids are light sensitive, all manipulations involving retinoids were performed under subdued lighting. Drugs were administered daily by gavage beginning 35 days after tumor implantation with a 20-gauge intragastric feeding tube (Popper & Sons, New Hyde Park, NY), 5 days/week, at 10 mg/kg/day in 0.1 ml of oil for each treatment group. A control group received 0.1 ml of oil without retinoid. Tumors were measured with calipers weekly, and tumor volumes were calculated using the formula: volume = length × width × height. Tumor growth was determined by the dividing the volume of the tumor at each weekly measurement by the volume on the first day of treatment. Animal weights and clinical signs of overall health status and cutaneous toxicities were recorded weekly.

Histochemical and immunohistochemical evaluation of xenograft tumors

On the last day of treatment, the animals were sacrificed and then the tumors were removed, fixed in neutral buffered formalin and embedded in paraffin. Five micron sections of the paraffin-embedded tumors were stained with hematoxylin and eosin (H&E) for histologic evaluation, and with the TUNEL (terminal deoxynucleodidyl transferase-mediated biotin-deoxyuridine triphosphate nick) FragEL kit (Oncogene Research Products, Boston, MA) for evaluation of apoptosis according to manufacturers instructions. MUC-1 expression was detected in tumor sections using a mouse monoclonal antibody to human MUC-1 (Pharmingen, San Diego, CA) and the Histostain-Plus AEC Kit (Zymed Laboratories, San Francisco, CA) according to manufacturers

instructions. Briefly, endogenous peroxidase activity in deparaffinized sections was quenched with 3% H_2O_2 in methanol and then sections were incubated in serum blocking solution, and subsequently in primary antibody, diluted 1:50 in phosphate buffered saline (PBS), overnight at 4°C in a humid chamber. Sections were then washed with PBS, treated with biotinylated secondary antibody for 15 minutes, followed by enzyme conjugate for 10 minutes. AEC (3-amino-9-ethyl carbazole) chromagen was applied for 15 minutes followed by counterstaining with Harris' hematoxylin. Sections of a mucinous gastric carcinoma were used as a positive control (New Comer Supply, Middleton, WI). Omission of the primary antibody was used as a negative control. Both the MUC-1 and the TUNEL stains were repeated three times on separate sections cut at three different levels of the tumors. The slides were coded so that laboratory personnel and the pathologist were blinded from the treatment groups. Tumor sections were scored for number of glands (quantified in one H&E stained sections and 3 MUC-1-stained sections) and MUC-1 and TUNEL staining (based on staining intensity and percent positive cells). Scores from three separate sections taken from different locations within the tumors were averaged. A students t-test was used to compare the scores between the different treatment groups. P values of less than 0.05 were considered statistically significant.

Alanine aminotransferase (ALT) activity

At the end of the treatment period, blood was drawn from each animal. Plasma was separated by centrifugation and stored at −70°C. The activity of ALT was determined in aliquots of plasma by standard spectrophotometric enzymatic techniques which are based on the reduction of pyruvate by lactate dehydrogenase [28]. The method measures NADH disappearance with time of incubation at 37°C. The activities are expressed as μm/L/min. Two different amounts of plasma were used in each assay to assure measurement of the maximum rate.

Topical irritancy

Topical irritancy was evaluated according to published procedures [29]. Briefly, 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 compound.

Results

In vitro cytotoxicity of multiple cell lines

Three structurally-related Flex-Hets, SHetA2, SHetA3 and SHetA4 were evaluated for their ability to inhibit the growth of cervical cancer cell lines over a range of concentrations. Since these compounds do not activate the RARs and RXRs they were compared to the most potent RAR/RXR pan-agonist Het (SHet50) and RAR/RXR pan-agonist RA isomer (9-*cis*-RA). Each compound inhibited growth of all cell lines in the micromolar range. The efficacies of the compounds were compared by measuring the maximal growth inhibition, defined by the percentage growth inhibition induced by 10 μM drug in comparison to the untreated control (Table 1). The Flex-Hets exhibited statistically significant greater efficacies than the receptor-active compounds across all cell lines as determined by a two-tailed paired t-test ($p < 0.05$). The efficacies of the receptor active Het (SHet50) on the other hand, were not significantly different than 9-*cis*-RA across all cell lines (two tailed paired t-test: $p > 0.05$). The potencies were compared by deriving the GI_{50} values (concentrations that induced half of the maximal growth inhibition activity) from graphs of growth inhibition versus drug concentration (summarized in Table 1). SHetA2 consistently exhibited the greatest efficacies and potencies in comparison to all other compounds tested against the cervical cancer cell lines, as was observed in previous studies for ovarian cancer cell lines [19] and head and neck cancer cell lines [20]. The cervical cancer cell lines however, were more resistant to Flex-Hets, than ovarian and head and neck cancer cell lines, which exhibited greater than 90% growth inhibition when treated with 10 μM Flex-Hets in previous studies [19, 20].

To evaluate the spectrum of cancers sensitive to Flex-Hets, the most potent compound, SHetA2, was submitted to the National Cancer Institutes (NCI's) human tumor cell line screen for evaluation in 55 cell lines representing 9 different cancer types. All cell lines representing leukemia, non-small cell lung cancer, colon cancer, central nervous system cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer were growth inhibited with GI_{50} values in the micromolar range (Figure 2).

RAR and RXR receptor expression

Although Flex-Hets do not activate the RARs or RXRs, their effects on RAR/RXR expression levels have not been evaluated. To determine if SHetA2 regulates RAR and RXR expression, rt-PCR was performed on RNA isolated from cervical cancer cell lines treated with 0, 1 and 10 μM SHetA2 for 4 and 24 hours. No effects on receptor

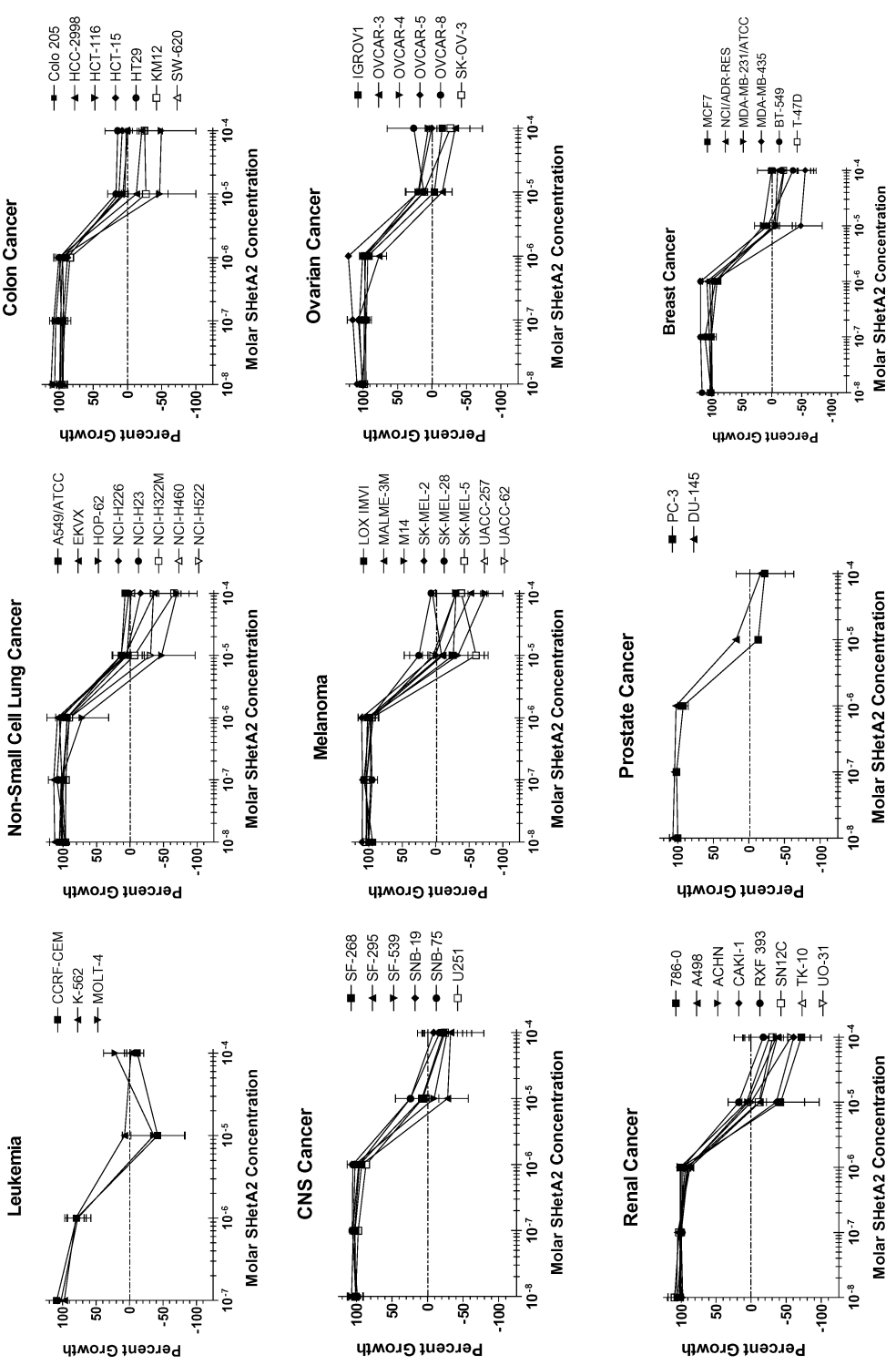

Figure 2. *In vitro* growth inhibition of the National Cancer Institute's Human Tumor Cell Line Panel. Each line represents the dose-response growth inhibition curve of the individual cell lines as indicated in the graph legend. (CNS = central nervous system).

Table 1. Efficacy (top line), potency (middle line) and statistical significance (bottom line) of Flex-Hets (SHetA2, SHetA3 and SHetA4) and a receptor active Het (SHet50) versus 9-cis-RA on cervical cancer cell lines

Cell Line	SHetA2	SHetA3	SHetA4	SHet50	9-*cis*-RA
SiHa	68%	58%	67%	35%	24%
	$3.8 \pm 2.9\,\mu$M	$7.5 \pm 0.2\,\mu$M	$5.9 \pm 2.3\,\mu$M	$11.9 \pm 1.2\,\mu$M	$5.5 \pm 0.7\,\mu$M
	$p = 0.038$	$p = 0.047$	$p = 0.013$	$p = 0.173$	
CC-1	58%	42%	59%	15%	28%
	$2.3 \pm 0.1\,\mu$M	$7.1 \pm 0.3\,\mu$M	$6.5 \pm 0.1\,\mu$M	ND	ND
	$p = 0.002$	$p = 0.047$	$p = .013$	$P = 0.143$	
C33a	87%	84%	85%	67%	45%
	$3.3 \pm 0.6\,\mu$M	$4.8 \pm 1.3\,\mu$M	$4.3 \pm 0.8\,\mu$M	$3.9 \pm 0.1\,\mu$M	$5.6 \pm 0.6\,\mu$M
	$p = 0.023$	$p = 0.005$	$p = 0.025$	$p = 0.189$	
HT-3	92%	65%	76%	47%	47%
	$3.9 \pm 0.1\,\mu$M	$7.2 \pm 1.7\,\mu$M	$5.9 \pm 0.9\,\mu$M	$5.5 \pm 0.1\,\mu M$	$8.6 \pm 1.1\,\mu$M
	$p = 0.049$	$p = 0.030$	$p = 0.042$	$p = 1$	

The efficacy is the percent growth inhibition relative to the untreated control induced by $10\,\mu$M compound. The potency is the concentration required to induce half maximal activity (GI_{50}) ND = not determined. The p values are from a two-tailed paired t-test comparing the efficacy of the Flex-Hets or SHet50 versus 9-*cis*-RA.

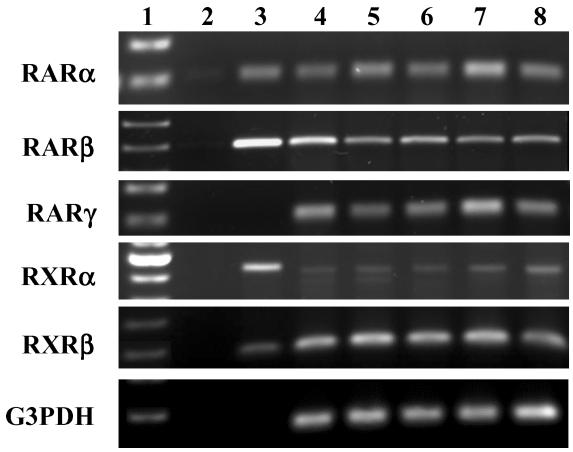

Figure 3. Lack of SHetA2-regulation of RAR or RXR expression in cervical cancer cell lines. RNA isolated from C33a cultures was reverse transcribed and evaluated for RAR and RXR receptor expression by rt-PCR. Reaction products from the linear phase of amplification were electrophoresed into a 1% agarose gel and visualized with ethidium bromide staining. Lane 1: DNA molecular weight markers; Lane 2: no RNA negative control; Lane 3: positive control containing cDNA of the specific receptor, note that no positive control was used for the RARγ and G3PDH primers; Lane 4: untreated control treated with DMSO solvent only; Lane 5: treated with $1\,\mu$M SHetA2 for 4 hours Lane 6: treated with $10\,\mu$M SHetA2 for 24 hours; Lane 7: treated with $10\,\mu$M SHetA2 for 4 hours; Lane 8: treated with $10\,\mu$M SHetA2 for 24 hours.

expression were observed for any of the cell lines evaluated in Table 1. Figure 3 presents the results for the C33a cell line.

In vivo inhibition of xenograft tumor growth

The *in vivo* efficacies of the two strongest Flex-Hets, SHetA2 and SHetA4, and the strongest RAR/RXR pan-agonist Het, SHet50, were evaluated in an animal xenograft model using the OVCAR-3 ovarian cancer cell line. The well-characterized RRM, 4-HPR, was administered for comparison, and sesame oil only served as a negative control. Drug treatment was initiated after the tumors were established and growing, which was 35 days after injection of the tumor cells into the animals. Since the ultimate route of administration in humans will be oral, the drugs were administered by gavage 5 days per week for 4 weeks. At the end of the treatment, 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. Each of the compounds significantly inhibited the growth of the xenograft tumors (Figure 4). After 15 days of treatment, the growth of the tumors in each treatment group were significantly less than the growth of the control group treated with sesame oil alone (t-tests on day 29: $p = 0.028$ for 4-HPR; $p = 0.010$ for SHet50; $p = 0.046$ for SHetA2; $P = 0.033$ for SHetA4). There were no significant differences between the degrees of growth inhibition exerted by the different compounds.

Differentiation and apoptosis in xenograft tumors

At the end of the experiment, the xenograft tumors were evaluated for histology, differentiation and apoptosis. Differentiation in ovarian cancer is routinely defined by gland formation and mucin expression. Pathologic review revealed that the tumors were arranged into large irregular nests and islands of cells separated by thin strands of fibrous connective tissue (Figure 5). The majority of epithelial cell nests exhibited a differentiated phenotype, however smaller areas of dedifferentiated cells also were observed within the tumors. In the differentiated areas,

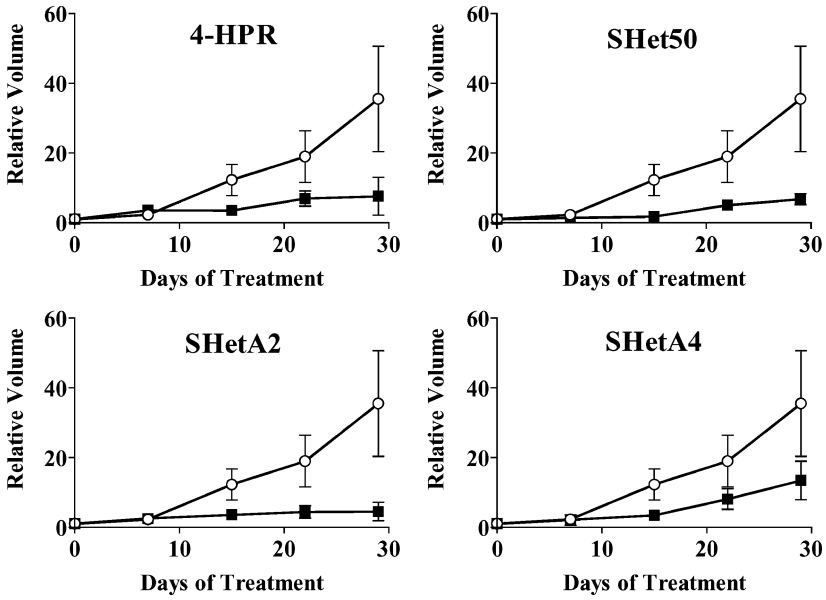

Figure 4. Inhibition of xenograft tumor growth by Hets and 4-HPR. Groups of 5 mice bearing OVCAR-3 xenografted tumors were gavaged daily with the indicated drugs in sesame oil (■ closed square) or with sesame oil alone (○ open circle). Tumor volumes were measured in 3 dimensions with calipers weekly. Each data point represents the average and standard error of the tumor volumes on the indicated treatment day relative to the tumor volume on day zero.

there were small glands and gland-like structures, with some of the cells lined up in a duct-like pattern. Occasionally papillations were present. The majority of cells in these differentiated areas were quite large with abundant cytoplasm and pleomorphic nuclei containing clumped chromatin and prominent nucleoli. In the dedifferentiated areas, the sizes of cells were markedly reduced, the cytoplasm was not apparent, and the nuclei tended to be more spindle in shape and were very hyperchromatic.

The tumors from the treated animals exhibited more differentiated characteristics in comparison to the untreated control tumors. As seen in Figure 5, cells in the treated tumors are arranged in a flatter more organized fashion instead of being piled up one cell on top of the other as in the untreated culture. Even more clearly the "punched out" holes in the treated cultures are glands that have formed. The numbers of glands in 3 sections taken from different areas of each tumor were quantified and compared between the different treatment groups. Tumors from each of the three treatment groups exhibited significantly greater numbers of glands than the untreated control group (Figure 5). The receptor-independent Het, SHetA2, exhibited the greatest degree of gland induction.

To evaluate the induction of differentiation at the molecular level, 3 sections taken from different areas of each tumor were stained immunohistochemically for expression of the mucin-1 (MUC-1) protein. MUC-1 expression was noted in the differentiated areas of the tumors with specific expression in the apical surface of glandular lumens, but not in the areas of dedifferentiated cells (Figure 5). An experienced Pathologist (S.L.) evaluated each section for the percentage of positively stained cells and the intensity of staining and provided a score that incorporated both parameters. The average scores of the sections for each treatment group were compared. All drugs significantly increased the level of MUC-1 expression in the tumors (Figure 5). Apoptosis was measured in tumor sections by the TUNEL assay (Figure 5). Although the levels of apoptosis appeared higher in the treated tumors, the increase was not statistically significant.

Evaluation of oral toxicity

During the course of the xenograft mouse model experiment, the animals were monitored daily for visually-observable signs of retinoid toxicity to the skin or bone. None of the retinoids induced evidence of the skin or bone toxicities, which were observed in previous experiments when animals were treated with 10 mg/kg/day of the all-*trans* RA isomer [17]. To determine if 4-HPR and the 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 2, t-test: $p > 0.05$).

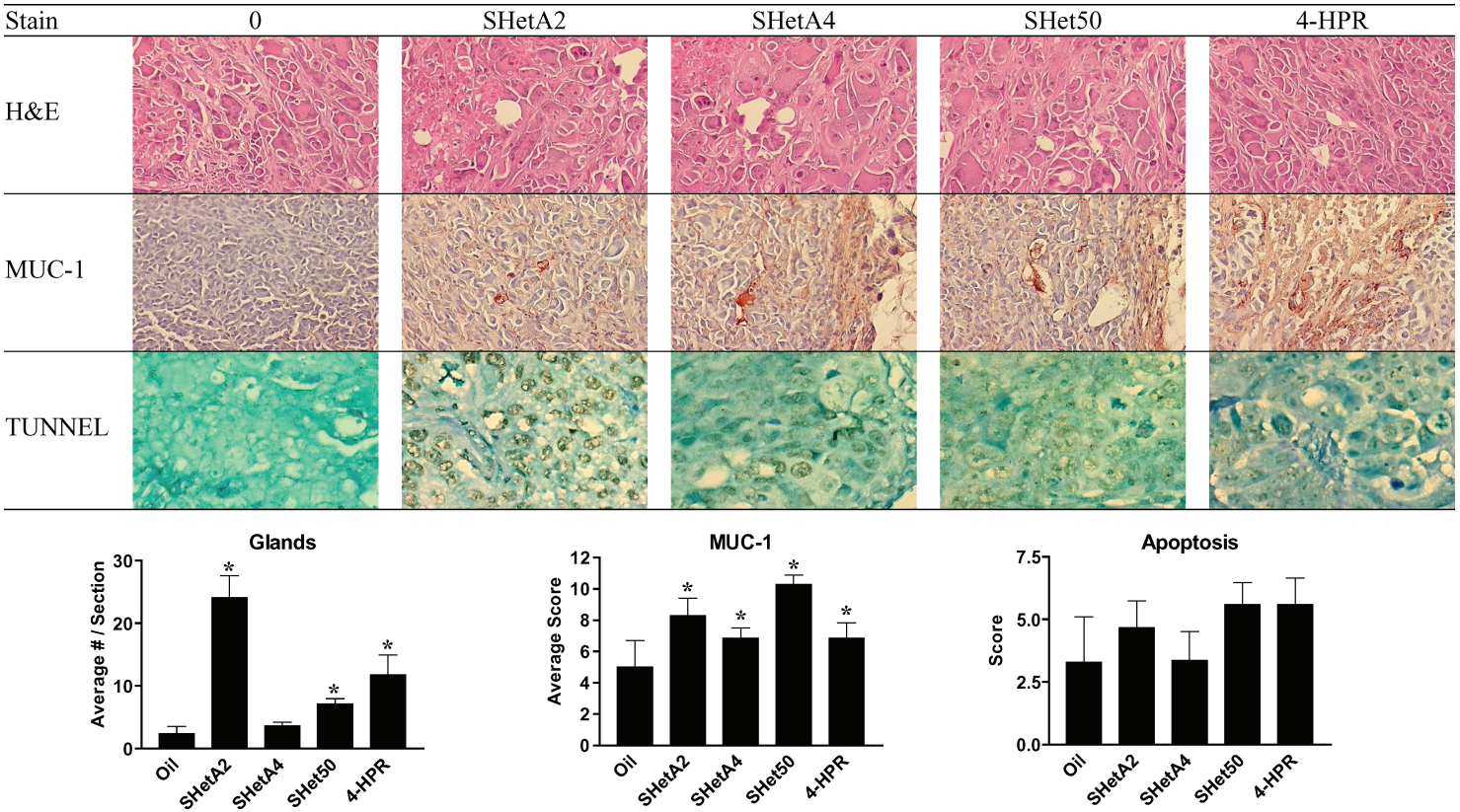

Figure 5. Effects of Hets and 4-HPR on gland formation, MUC-1 expression and apoptosis in tumors of treated animals. Statistically significant higher levels of glands and MUC-1 expression in the tumors of treated animals in comparison to controls treated with sesame oil are indicated by asterisks (*) and were determined by *t*-tests. For gland formation, $p < 0.001$ for SHetA2, $p = 0.097$ for SHetA4, $p = 0.001$ for SHet50, and $p = 0.007$ for 4-HPR. For MUC-1 scores, $p = 0.011$ for SHetA2, $p = 0.050$ for SHetA4, $p = 0.040$ for SHet50, and $p = 0.001$ for 4-HPR. The higher levels of TUNEL scores in the tumors of treated animals in comparison to control animals approached significance (*t*-test, $p = 0.27$ for SHetA2, $p = 0.48$ for SHetA4, $p = 0.20$ for SHet50, and $p = 0.12$ for 4-HPR).

Table 2. ALT activity in plasma of mice

Treatment	ALT Activity (μm/liter/min)
Sesame Oil Control	103.0 ± 60.3
4-HPR	149.7 ± 140.9
SHet50	65.5 ± 46.3
SHetA2	118.5 ± 153.4
SHetA3	64.5 ± 40.3

*Normal range 18–184 (μM/L/min).

Each of the ALT 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, Wilmington, MA). Necrosis, fatty changes or inflammation in portal tracts were not observed in histologic evaluation of liver sections.

Skin irritancy

The potential for utilizing Hets for treatment of cervical, vulvar, melanoma and other skin cancers as a topical formulation will depend upon the levels of irritancy induced by the compounds. Therefore, topical irritancy of receptor-agonist Hets and Flex-Hets were evaluated in an animal model in comparison to all-*trans*-RA. A pan-agonist Het that activates all RARs and RXRs (NHet17) and a Het selective for RARγ (SHet65) induced topical irritancy scores in a dose-responsive manner similar to all-*trans*-RA (Figure 6A). In contrast, the RAR/RXR-independent Flex-Hets, SHetA2, SHetA3 and SHetA4, did not induce topical irritancy in this model (Figure 6B).

Discussion

The results of this study demonstrate that Flex-Hets exhibit improved therapeutic ratios over other Hets, as well as natural and synthetic retinoids, which are RAR and/or RXR agonists. Despite the lack of RAR or RXR activation by Flex-Hets at the molecular level, these compounds exhibit retinoid-like activities such as growth inhibition and differentiation induction at the cellular level. On the efficacy side of the therapeutic ratio, the growth inhibition induced by Flex-Hets is greater than the RAR/RXR-active compounds in cervical cancer cell lines (Table 1). Previous studies in ovarian and in head and neck cancer cell lines demonstrated that this high level of growth inhibition is due to induction of apoptosis [18–20]. Apoptosis is likely to contribute to the mechanism of growth inhibition induced in leukemia, non-small cell lung, colon, central nervous system, melanoma, renal, prostate and breast cancer as indicated by the negative percent growth levels induced by 10 and 100 μM SHetA2 in the majority of cell lines of the NCI's human tumor cell line panel (Figure 2). Although 10 μM SHetA2 concentrations are required to induce apoptosis in a 48 hour treatment period, our previous study demonstrated that longer treatment times with 1 μM SHetA2, SHetA3, SHetA4 and 4-HPR could induce apoptosis in a more biologically relevant organotypic culture model of ovarian cancer [18]. Interestingly, all of the other potent apoptosis-inducing RRM's characterized to date, 4-HPR, CD437/AHPN and MS3350-1, are selective for RARγ [21], suggesting that the RRM's and possibly Flex-Hets induce apoptosis through an unidentified nuclear receptor with a similar ligand binding pocket to that of RARγ.

In contrast to the *in vitro* results from OVCAR-3 organotypic cultures, induction of apoptosis was not observed *in vivo* in the OVCAR-3 xenograft tumors in this study. The lack of apoptosing cells measured at the end of the treatment period however, does not preclude the possibility that apoptosis could have contributed to the growth inhibition observed during the treatment period. In the *in vivo* situation, cells induced into apoptosis by the treatments could have been eliminated through the innate immune system present in these animals [30]. In the *in vitro* assays

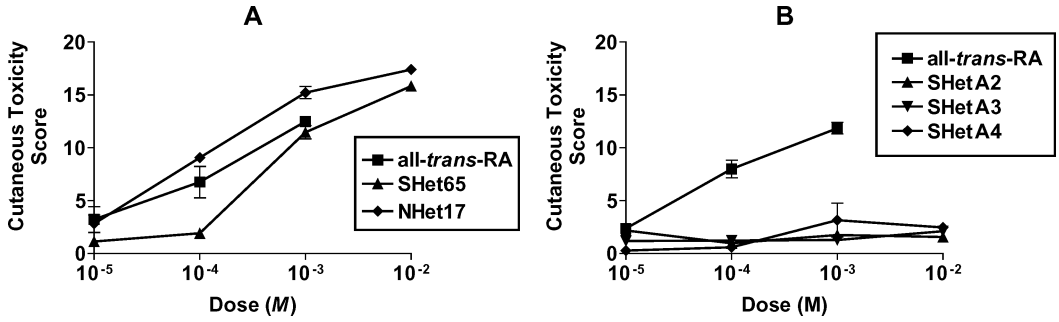

Figure 6. Association of topical irritancy with RAR/RXR activation. Female Skh hairless mice, 6–8 weeks old, were treated topically on the dorsal skin for 4 days over a 2-log concentration dose range with various compounds. Retinoic acid receptor-Hets were evaluated in A, while retinoic acid receptor-independent Flex-Hets were evaluated in B. Daily flaking and abrasion scores were combined to calculate a single cutaneous toxicity score for each mouse. Each treatment group consisted of 4 mice, and group averages were used to plot cutaneous toxicity against doses of the compounds.

however, there is no biological system to eliminate apoptotic cellular debris, and therefore sufficient numbers of cells could accumulate to allow detection of drug-induced apoptosis [18]. The trend toward higher percentages of apoptosis in the tumors from all treatment groups in this study however, indicates that apoptosis did contribute to the mechanism of growth inhibition by these drugs *in vivo*.

The lack of evidence for oral toxicity or topical irritancy induced by Flex-Hets in this study is consistent with their lack of RAR/RXR activation. The lack of teratogenicity induced by SHetA2 in a mouse model is also consistent with RAR/RXR independence [31]. An additional toxicity that needs to be avoided in improving retinoids is the potential harm that can be done to patients who continue to smoke during treatment. Clinical trials for prevention of second cancers have found that RA and β carotene, which is a nutrient that can be metabolized to RA, may be harmful to patients if they continue smoking during treatment [32–34]. Although the exact mechanism of these harmful effects is not clear, these findings demonstrate the importance of developing novel pharmaceuticals, such as Flex-Hets, that can induce the same anticancer activities as retinoids, but through different molecular mechanisms.

The lead Flex-Het, SHetA2, exhibited the greatest efficacy and potencies of all of the other Flex-Hets and retinoids tested. This greater activity was not associated with any detectable increase in oral toxicity or skin irritancy. It could be postulated that the most active Flex-Het would also be the most toxic, but greater levels of toxicity or irritancy were not observed in SHetA2-treated animals in comparison to animals in the other treatment groups. This supports the previous observation that the induction of apoptosis by Flex-Hets occurs through a mechanism that is selective for cancer cells over normal cells [19]. The efficacy, toxicity, pharmacokinetics and formulation of SHetA2 are currently being evaluated in the National Cancer Institute's (NCI's) Rapid Access to Intervention Development (RAID) program (Application 196, Compound NSC 726189). The RAID pharmacokinetic studies demonstrated that micromolar concentrations of SHetA2 can be achieved and maintained in mice [35] indicating that concentrations sufficient to differentially induce apoptosis in cancer cells over normal cells can be targeted in clinical trials.

In conclusion, Flex-Hets exhibit improved therapeutic ratios for multiple cancer types over RAR and/or RXR agonists, in that they exert similar effects on growth, differentiation and apoptosis without activating the RARs and RXRs and without inducing the oral toxicity, skin irritancy and teratogenicity associated with receptor activation.

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