Efficacy of new retinoids in the prevention of mammary cancers and correlations with short-term biomarkers

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A number of retinoid X receptor (RXR) agonists have proven to be highly effective in preventing methyl nitrosourea (MNU) induced mammary cancers. However, these agonists have side effects; particularly causing an increase in serum triglyceride levels. A series of ligands for RXR were designed based on computer modeling to the ligand binding domain (LBD) of the RXR receptors and on structure-activity relationships. The chemopreventive effects of these retinoids were evaluated in the relatively long-term MNU model. As a short-term assay to predict their efficacy, the ability of the retinoids to modulate cell proliferation and apoptosis was also determined in mammary cancers after only 7 days of treatment. The five UAB retinoids evaluated included two Class I UAB retinoids (UAB20, UAB112) and three Class II UAB retinoids (UAB30, 4-methyl-UAB30 and the benzosuberone-analog of UAB30). The previously evaluated RXR agonist targretin and the pan-agonist 9-cis-retinoic acid (9-cis-RA), which interacts with both RAR and RXR receptors, were included as positive agonists known to prevent cancer in the MNU model. In the prevention studies, in which the agents were administered beginning 5 days after MNU until the end of the study, targretin (150 mg/kg diet) and 4-methyl-UAB30 (200 mg/kg diet) were highly effective in decreasing cancer numbers by 75–85%. UAB30 (200 mg/kg diet) and 9-cis-RA (60 mg/kg diet) gave intermediate inhibitions of 60 and 45%, respectively. Targretin (15 mg/kg diet), UAB20 (200 mg/kg diet) and the benzosuberone analog of UAB30 (200 mg/kg diet) showed limited activity by decreasing cancer multiplicity 25–30%, while UAB112 had no effect on mammary cancer multiplicity. A direct correlation was observed between the long-term chemopreventive efficacy of these agents and their ability to decrease cell proliferation in mammary cancers after short-term treatment. Furthermore, the highly effective agents (4-methyl-UAB30 and targretin at 150 mg/kg diet) increased apoptosis 3–5 times, while agents with moderate or limited preventive efficacy failed to significantly increase apoptosis. Although the more effective retinoid treatments increased serum triglycerides 2.5- to 4.0-fold, one moderately effective agent (UAB30) had no significant effect on lipid levels. In summary, a short-term in vivo method has been identified for screening newly synthesized retinoids both for chemopreventive efficacy and for their adverse effect on serum triglycerides.

Abbreviations: BRDU, bromodeoxyuridine; ER, estrogen receptor; LBD, ligand binding domain; MNU, methyl nitrosourea; RXR, retinoid X receptor.

Introduction

Anti-hormonal agents [e.g., selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs)] have proven to be highly effective in the prevention and treatment of estrogen receptor (ER) positive mammary cancers in women and in experimental models (1–3). Although a wide variety of non-hormonal agents have been examined for their ability to prevent methyl nitrosourea (MNU)-induced mammary cancers, some of the most effective and least toxic agents have been the retinoids that target the retinoid X receptor (RXR) nuclear receptors. Targretin (4) and LGD100268 (5), for example, have proven to be highly active in both prevention and therapy of ER positive mammary cancers. Targretin has also been shown to be highly effective in preventing mammary tumors in transgenic mice expressing Neu or T antigen; both of which produce ER negative tumors (6,7).

RXR receptors are promiscuous and form heterodimers with the widest range of nuclear receptors, including peroxisome proliferator-activated receptors (PPARs), retinoic acid receptors (RARs), liver X receptors (LXRs), the constitutive androstenedione receptor (CAR), the vitamin D receptor (VDR) and thyroxine receptor (TR) (8–9). The resulting heterodimers become transcriptional activators of a wide variety of genes. In certain heterodimers, the RXR receptor is an active partner and RXR agonists can enhance the activity of a cognate receptor (RARs) bound with an agonist or the RXR agonist can independently stimulate the transcriptional activation of genes without the presence of the ligands for the cognate nuclear receptors (PPARs, LXRs, CAR, etc.). For other heterodimers (VDR and TR), the RXR ligand does not appear to affect the transcriptional activation of the RXR heterodimers. There has been substantial interest in these rexinoid agonists and various analogs have been synthesized. Targretin has been approved for clinical use in refractory cutaneous T-cell lymphoma, and 9-cis-retinoic acid (9-cis-RA) (Panretin) has been approved for topical treatment of Kaposi’s sarcoma (10). However, the elevation of triglycerides levels by 9-cis-RA, targretin, and other retinoids has been known for many years and is a major concern in the use of these agents in a cancer prevention setting (10–13). A goal of our laboratories has been to modify the retinoid structure to produce RXR agonists that are effective agents with low adverse effect on serum triglycerides.
toxicity (14,15). At this time, it is possible using computer modeling to design agonists or antagonists that will bind to the RXR receptor. However, such efforts will not inform one regarding pharmacodynamics considerations or biological effects in specific tissues (e.g. triglycerides alterations). The biological effects of these agonists are likely to be mediated by the presence or absence of certain nuclear receptors that form heterodimers with the RXR receptors, as well as the presence or absence of a wide range of co-activators and co-repressors that interact with the resulting heterodimers. Thus, in short-term studies are unlikely to reflect the potentially great level of complexity reflected by heterodimer-ligand complex and the interaction with its co-repressors and co-activators.

The use of short-term biomarkers assays will allow the rapid evaluation of a large number of retinoids for their chemopreventive capabilities and reduce the necessity of synthesizing the large quantities needed for long-term chemoprevention studies. As potential surrogates for identifying agents for breast cancer prevention, the short-term effects of various RXR agonists on proliferation and apoptosis were examined in small mammary cancers, and the effects of the agents on serum triglycerides levels were measured. Proliferation and apoptosis were measured since these processes are closely associated with carcinogenesis (16). Mammary cancers were used rather than normal mammary tissue because proliferation and apoptosis occur too slowly in normal mammary epithelial cells to allow detection of significant changes by chemopreventive agents over a short time period. We have previously determined that effective anti-hormonal agents (e.g. AIs and SERMs) readily alter these parameters at doses necessary to achieve cancer prevention (17,18). More recently, we found that cancer preventive doses of targretin similarly altered cell proliferation and apoptosis (19). In the present studies, rats bearing small MNU-induced mammary cancers were treated with five UAB retinoids with different potency as agonists to the RXR receptors, as well as 9-cis-RA and targretin. The effects of these analogs on proliferation and apoptosis were compared with their long-term efficacy as preventive agents and their effects on serum triglycerides levels.

Materials and methods

Supplies

Chemicals and other materials were obtained as follows: trioctanoin and corn oil, Sigma Chemical, St Louis, MO; MNU, NCI Chemical Repository, Bethesda, MD and Teklad mash (4%) diet and Sprague-Dawley rats, Harlan Sprague-Dawley, Indianapolis, IN. Targretin and 9-cis-RA were made by custom synthesis. The three Class II UAB retinoids were synthesized by methods similar to the ones reported for the generation of UAB30 (15). Instead of using α-tetralone as a starting ketone, benzosuberone or 4-methyl-α-tetralone (Aldrich) were used for the generation of the benzosuberone-UAB30 and 4-methyl-UAB30, respectively. The two Class I UAB retinoids were prepared similar to the preparation of 9-cis-UAB8 (20). The structures of the retinoids are displayed in Figure 1, and the yields and detailed chemical methods will be reported elsewhere.

Analysis of retinoids

The purity and homogeneity of each of the various retinoids in the diet were analyzed by photospectroscopy and high-performance liquid chromatography (HPLC). Feed containing a retinoid (0.5 g) was homogenized in 2 ml of an aqueous solution containing 0.5 mg/ml each of EDTA and ascorbic acid. These preparations were extracted with 23 ml of methanol-butanol (1:1, v/v). HPLC analyses of the methanol-butanol extracts were performed using a Spherisorb ODS-1 5 μm column (4.6 × 250 mm, Phase Separations, Norwalk, CT). The eluent was 90% methanol:10% water (except for 9-cis-RA which was 80% acetonitrile:20% acidified water), flow rate was 1 ml/min, detection was by ultraviolet at the wavelength of maximum absorbance and quantification was by peak area integration and external standardization. The retention time for the compounds were as follows: 4-methyl-UAB30 (4.33 min at 330 nm), benzosuberone-UAB30 (4.5 min at 320 nm), UAB20 (3.7 min at 325 nm), UAB30 (5.05 min at 330 nm), UAB112 (13.72 min at 335 nm), 9-cis-RA (8.70 min at 340 nm) and targretin (6.12 min at 260 nm).

Binding Affinities to the LBD of RXRα

A plasmid was received from Dr Ellen Li at Washington University in St. Louis, Missouri. The plasmid was ligated into the pET17b vector and transformed into Escherichia coli cells for expression. The His-tagged LBD was purified on a nickel affinity column (Hi Trap Chelating HP column) using an AKTApurifier (Amersham Biosciences). Thrombin cleavage of the His tag produced the LBD of RXRα (Mr 27 236). The protein was further purified by size-exclusion chromatography (Hi Load 26/60 Superdex 75) to isolate low molecular mass homodimers. SDS-PAGE, MALDI-TOF and native PAGE confirmed the purity and monomeric state of the protein. To evaluate the binding of retinoids to this domain, fluorescence experiments were performed using a Cary Eclipse Spectrofluorimeter. One micromolar solutions of protein in Tris-HCl (pH 8) containing 50 mM NaCl, 0.5 mM EDTA and 2 mM DTT was titrated with microliter additions of the test retinoid in methanol to a stirred cell quenched at 25°C. Decrease in the protein fluorescence at 337 nm was used to construct a binding isotherm. After correction for non-specific binding and inner filter effects, the fluorescence quenching data were fit to an apparent K<sub>d</sub> value using a single site model as previously reported for other UAB retinoids and 9-cis-RA binding to retinoid binding proteins (20).

Chemoprevention studies

Diets were prepared by mixing the various retinoids with Teklad (4%) mash diet using a liquid-solid blender ( Patterson-Kelly, East Stroudsburg, PA). Targretin was mixed directly into the diet (i.e. no vehicle required). For the remaining retinoids, each compound was mixed with ethanol: trioctanoin (12 and 19 g/kg diet, respectively) prior to incorporation into the diet (13,14). Tenox-20 and α-tocopherol (0.05 ml of each per kg diet) were also added as antioxidants. Female Sprague-Dawley rats were obtained at 28 days of age and housed in polycarbonate cages (5 rats/cage). At 50 days of age, the rats received one injection of MNU (50 mg/kg body wt) via the jugular vein. Treatment with the retinoids was initiated 5 days after the administration of MNU (15 rats/group). The UAB compounds were administered at a dose of 200 mg/kg diet. Targretin was given at 150 and 15 mg/kg diet, and 9-cis-RA at a dose level of 60 mg/kg diet. These dose levels did not alter body weights of the rats or cause other clinical signs of toxicity. The studies were terminated 126 days after MNU treatment.
Rats were palpated for mammary tumors twice each week and weighed once every week. Mammary tumors were excised, weighed and processed for histological classification at termination of the studies. Statistical analyses of cancer incidence and latency were determined using Logrank analysis (21), and differences in cancer multiplicity were determined by the Armitage test (22).

Cancer cell proliferation and apoptosis
Animals bearing small palpable MNU-induced mammary cancers (~1.0 cm in diameter) were treated with each of the retinoids for a period of 7 days. Rats were injected with bromodeoxyuridine (BRDU), 100 mg/kg body wt in saline, i.p., 2 h prior to the time of killing. After the animals were killed by CO₂ asphyxiation, cancers were removed and fixed overnight in 10% formalin for assessment of histopathology, BRDU labeling and apoptosis.

Cell proliferation. Proliferating cells in mammary cancers were labeled in vivo with BRDU as previously described (17,18). The nuclei labeled with BRDU were identified employing an anti-BRDU monoclonal antibody (Beckton Dickenson, Palo Alto, CA) and ABC kit. More than 1000 cells were randomly scored from each cancer and the percent of BRDU-labeled cells (BRDU-LI) was determined.

Cell apoptosis. Apoptotic cells were identified by the TUNEL method as previously described (18) using methods recommended by the ApoTag in situ hybridization detection kit (Oncor, Gaithersburg, MD). The top sections of each slide, which were incubated without digoxigenin-dUTP, were used as a negative control. Rat mammary glands taken 6 days after ovariectomy (when the number of apoptotic cells was high) were used as positive controls. Tissue sections were counterstained by methyl green for visualization of tumor morphology. From each tumor, >1500 cells were evaluated for the presence of apoptotic cells. Statistical evaluations of cell proliferation apoptosis were performed employing Wilcoxon rank analysis.

Serum triglycerides
Blood was collected at the time of killing the rats (7 days after initial retinoid treatment) and placed on ice until centrifuged. Serum was frozen at −85°C until analyzed for triglycerides (23). The Infinity™ triglycerides assay kit was purchased from Thermo DMA (Louisville, MO)

Results
Structure and binding affinity of retinoids to the LBD of RXRɑ
The X-ray crystal structure of the complex between RXRɑ LBD and 9-cis-RA is displayed in Figure 2. In this crystal structure the flexible 6–7 bond is twisted 70° from a planar geometry (Figure 1), and 9-cis-RA adopts a non-planar L-shaped geometry in the LBDs of RXRs (24). In contrast, 9-cis-RA adopts a different angle about the 6–7 bond when bound to the LBDs of RARs; the shape of the ligand binding pocket is long and planar in these receptors, and this binding site is able to accommodate the all-trans isomer of retinoic acid (25). The binding affinity of the pan-agonist 9-cis-RA to RXRɑ LBD was evaluated at 25°C using fluorescence quenching methods as was done for the binding of 9-cis-RA to RXRɑ-AB-His (26). The $K_d$ value for 9-cis-RA was 14 nM, which is similar to that reported by Schimerlik et al. (26). UAB30 uses a six-member ring to constrain the 6–7 bond and contains a twisted 8–9 bond arising from steric interactions (between the C-9 methyl and ring methylene groups). This retinoid has been demonstrated to be highly selective for binding and activating RXR subtypes (20). Using molecular modeling, UAB30 was inserted into the 9-cis-RA binding site and after energy minimization a model of its binding geometry was found. As displayed in Figure 2, UAB30 adopts a non-linear and L-shaped geometry that is very similar to that of 9-cis-RA. The binding affinity for UAB30 was determined, and a $K_d$ of 53 nM was found. This is consistent with previous data that evaluated the inhibition constants at 50% ($IC_{50}$) of each of these retinoids to the entire RXRɑ receptor using a radiolabel assay (27).

The 4-methyl-UAB30 adopts a very similar geometry in the 9-cis-RA binding site as found for UAB30. The extra methyl group interacted with key residues on Helix 11, which was important for the correct positioning of Helix 12 in its agonist conformation. The hydrophobic methyl group was expected to increase the binding affinity of this UAB30 analog over UAB30. To confirm this, the binding affinity of 4-methyl-UAB30 to RXRɑ LBD was examined. The apparent $K_d$ value for this analog of Class II UAB retinoids was ~8 nM, and both the binding affinity and transcriptional activity were about 5-fold better than UAB30. This affinity potency was similar to targeatin ($K_d = 19$ nM). To offer a more flexible ring system than the six-member ring used in UAB30, a seven-member ring analog using benzosuberone was designed. The benzosuberone-UAB30 fit well into the binding site, and it had an apparent binding affinity to RXRɑ LBD of 10 nM. Transient transcriptional studies demonstrated that the benzosuberone-UAB30 was a full
agonist with potency at least as great as that of UAB30. Two UAB retinoids that belong to a series of Class I UAB retinoids were also examined. The general structure is provided in Figure 1. Like Class II UAB retinoids, a six-member ring was used to constrain the 6–7 bond and to maintain a non-linear geometry of the retinoid. To provide non-covalent interactions with the residues on the protein in Helix 7 and Helix 11, different groups were placed at R1 and R2. Previously, we demonstrated that the 9-cis-isomer of UAB8, which contains an ethyl group at R1 and an isopropyl group at R2, was a very potent agonist for the RXR receptors with a $K_d$ value of 16 nM (28). The agonist activity decreased with increasing bulk of the group at R1. Using a phenyl group at this position decreased the binding affinity to RXRα LBD ($K_d = 42$ nM) and reduced the ability of this retinoid to act as an agonist (Donald D. Muccio, unpublished data). From modeling studies, this owed to the inability of UAB20 to fit properly in the ligand binding pocket of the RXRα LBD. UAB76 had an iso-pentyl group at R2 with an ethyl group at R1, and was a more potent agonist than UAB8 ($K_d = 12$ nM). Like UAB20, the phenyl substitution on UAB112 abolished the agonist qualities of UAB76 and reduced its binding affinity ($K_d = 160$ nM), and therefore was a poor agonist of RXR transcription.

Chemopreventive efficacy of retinoids
The retinoids were administered in the diet beginning 5 days after MNU treatment. Dose levels for targretin and 9-cis-RA were selected (based on prior studies in our laboratories) that did not cause a significant decrease in body weight gain or cause other overt signs of toxicity. The maximum tolerated dose for 9-cis-RA was found in our laboratories to be <150 mg/kg diet, and doses from 60 to 120 mg/kg diet showed similar chemopreventive efficacy. For the UAB retinoids (4-methyl-UAB30; benzosuberone-UAB30, UAB20, UAB30 and UAB112), a dose level of 200 mg/kg diet was used. On average, the final body weights of the rats receiving the retinoids were 10–18 g (4–7%) higher than the controls (which averaged 255 g). Mammary cancer multiplicities were decreased 74, 30, 15 and 1% by 4-methyl-UAB30, benzosuberone-UAB30, UAB20, UAB30 and UAB112, respectively (Table I and Figure 3A–D). The weights of the mammary cancers in rats receiving 4-methyl-UAB30 were also greatly decreased (72%). The 60 mg/kg diet dose level of 9-cis-RA caused a 51% decrease in mammary cancer multiplicity and a 77% decrease in the weight of the cancers. We previously reported (19) that targretin at a dose level of 150 mg/kg diet significantly decreased mammary cancer multiplicity (74%), while a lower dose (15 mg/kg diet) of targretin decreased tumor multiplicity by 38%. We have also reported (14) that UAB30 at a dose level of 200 mg/kg diet decreased cancer numbers by 63%. The published data on targretin and UAB30 were included in Table I for comparison with other retinoids and because the other parameters measured for these two retinoids had not been previously reported.

**Effects of retinoids in the prevention of mammary cancers and on short-term biomarkers in female Sprague-Dawley rats**

**Chemopreventive efficacy of retinoids**
- The retinoids were administered in the diet beginning 5 days after MNU treatment. Dose levels for targretin and 9-cis-RA were selected (based on prior studies in our laboratories) that did not cause a significant decrease in body weight gain or cause other overt signs of toxicity.
- The maximum tolerated dose for 9-cis-RA was found in our laboratories to be <150 mg/kg diet, and doses from 60 to 120 mg/kg diet showed similar chemopreventive efficacy.
- For the UAB retinoids (4-methyl-UAB30; benzosuberone-UAB30, UAB20, UAB30 and UAB112), a dose level of 200 mg/kg diet was used.
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**Effects on proliferation and apoptosis**
- Rats bearing small mammary cancers were given retinoids for 7 days at the same dose levels as those used in the chemoprevention studies. The effects of the compounds on cancer cell proliferation and apoptosis are summarized in Table I. In all cases, the retinoids decreased cell proliferation index and increased the apoptotic index; but to different degrees. It should be noted that a dose–response in these parameters was observed with targretin. However, for UAB30, increasing this dose by 4-fold (from 200 to 800 mg/kg diet) did not greatly change the effect on cell proliferation, although the higher dose did increase apoptosis. To illustrate the variability of the retinoids’ response in cancer bearing animals within a group, the actual values for targretin, UAB30, 4-methyl-UAB30 and UAB112 are shown in Figure 4A and B. It can readily be observed that retinoids that were highly active in preventing mammary cancers (e.g. 4-methyl-UAB30 and high dose targretin) yielded a highly consistent decrease in cell proliferation and an increase in apoptosis. We further illustrated these results by calculating a PI:AI ratio, which simultaneously demonstrates the effects on proliferation and apoptosis (Table I).

**Effect on serum triglycerides levels**
- Serum triglycerides levels were measured in the rats after 7 days of treatment with the retinoids (Table I).

### Table I. Effects of retinoids in the prevention of mammary cancers and on short-term biomarkers in female Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mammary cancers/rat$^a$</th>
<th>Proliferation index (PI)$^b$</th>
<th>Apoptotic index (AI)$^b$</th>
<th>PI/AI index</th>
<th>Serum triglycerides level (mg/dl)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>4.6–5.5$^c$</td>
<td>12.8</td>
<td>0.6</td>
<td>20</td>
<td>62</td>
</tr>
<tr>
<td>2</td>
<td>UAB112, 200 mg/kg diet</td>
<td>1 (1)$^d$</td>
<td>41 (1)</td>
<td>100 (1)</td>
<td>6.3</td>
<td>76 (1)</td>
</tr>
<tr>
<td>3</td>
<td>UAB20, 200 mg/kg diet</td>
<td>15 (1)</td>
<td>44 (1)</td>
<td>100 (1)</td>
<td>6.4</td>
<td>6 (1)</td>
</tr>
<tr>
<td>4</td>
<td>Benzosuberone-UAB30, 200 mg/kg diet</td>
<td>30 (1)</td>
<td>50 (1)$^e$</td>
<td>33 (1)</td>
<td>8.0</td>
<td>144 (1)$^e$</td>
</tr>
<tr>
<td>5</td>
<td>UAB30, 800 mg/kg diet</td>
<td>$-$</td>
<td>73 (1)$^e$</td>
<td>116 (1)$^e$</td>
<td>2.0</td>
<td>77 (1)</td>
</tr>
<tr>
<td>6</td>
<td>UAB30, 200 mg/kg diet</td>
<td>65 (1)$^e$</td>
<td>65 (1)$^e$</td>
<td>33 (1)</td>
<td>5.0</td>
<td>65 (1)</td>
</tr>
<tr>
<td>7</td>
<td>9-cis-RA, 60 mg/kg diet</td>
<td>51 (1)</td>
<td>56 (1)$^e$</td>
<td>67 (1)</td>
<td>4.8</td>
<td>326 (1)$^e$</td>
</tr>
<tr>
<td>8</td>
<td>Targretin, 15 mg/kg diet</td>
<td>40 (1)</td>
<td>40 (1)</td>
<td>83 (1)</td>
<td>7.6</td>
<td>81 (1)</td>
</tr>
<tr>
<td>9</td>
<td>Targretin, 150 mg/kg diet</td>
<td>70 (1)$^e$</td>
<td>89 (1)$^e$</td>
<td>483 (1)$^e$</td>
<td>0.4</td>
<td>283 (1)$^e$</td>
</tr>
<tr>
<td>10</td>
<td>4-methyl-UAB30, 200 mg/kg diet</td>
<td>74 (1)</td>
<td>92 (1)$^e$</td>
<td>317 (1)$^e$</td>
<td>0.4</td>
<td>566 (1)$^e$</td>
</tr>
</tbody>
</table>

$^a$MNU was administered to female Sprague-Dawley rats at 50 days of age ($N = 15$ rats/group). Beginning at 55 days of age, the retinoids were given in the diet. Number of mammary cancers at end of study (126 days after MNU).

$^b$When a rat developed a mammary cancer 150–250 mm$^3$, the rat was assigned to a group ($N = 10$) to receive a retinoid. After 7 days of treatment, the animal was killed.

$^c$Since the retinoids were evaluated in different chemoprevention studies, the range of cancer multiplicity in the various studies is shown.

$^d$Values are percent difference from the respective control group (Group 1); either increase (↑) or decrease (↓).

$^e$Significantly different from controls ($P <0.05$).

$^f$UAB30 at this dose was not evaluated for preventive efficacy owing to lack of compound.

$^g$Data for UAB30 and Targretin previously published (14,19).
4-Methyl-UAB30, 9-cis-Retinoic acid and targretin (high dose) greatly increased (2- to 5-fold) serum triglycerides, while UAB20, UAB112, UAB30 and Targretin (low dose) caused non-significant increases. Although many of the retinoids were similar in structure (Figure 1), their effect on triglycerides levels differed greatly. It was of interest that a dose–response was observed with targretin, but not with UAB30. Triglycerides in UAB30 treated rats did not increase significantly despite a 4-fold increase in the dose administered.

Discussion

The improvements in X-ray crystallography and computer-based methods have made it easier to design agonists for the RXR receptors that will bind with high affinity to the receptor. Developing specific agonists for the individual RXR receptors (α, β, γ), however, may be difficult because the ligand binding pockets for all three receptors are very similar. Furthermore, although it may be relatively easy to predict the primary biological effects of an antagonist for a specific class of enzymes (e.g. COX-2 inhibitors), dealing with a promiscuous nuclear receptor that interacts with many other nuclear receptors may be more difficult. Thus, tamoxifen which is an ER alpha antagonist in breast cancer is an ER alpha agonist in endometrium and bone. In contrast, more recent SERMs such as raloxifene have antagonist activity in both breast and endometrium, but still have agonist activity in bone. Presumably, such dissimilar activities can be obtained because slight structural variations in the agent may significantly alter the interaction of the receptor complex with co-activators and co-repressors that then alter tissue specific expression. These variations may potentially be even greater when dealing with RXR agonists that themselves may interact with the widest range of receptors (PPAR, CAR, VDR, LXR, etc.) (8,9). Furthermore, the resulting heterodimers may have differences in interactions with different co-activators and co-repressors based on minor structural changes in specific ligands for either the RXR receptor or the other nuclear receptors.

Because of this tissue specific complexity, it will be extremely difficult to model by computer or in vitro studies. Therefore, we determined the ability of these analogs to modulate specific biological responses following short-term exposure in vivo. The endpoints examined were proliferation and apoptosis in small palpable cancers following exposure to the various agents for only 7 days. The appeal of these particular parameters is that proliferation and apoptosis are directly related to the carcinogenic process and that these endpoints have been employed clinically (16). Our laboratories have previously shown that anti-hormonal agents that are highly effective in this model will modulate both proliferation and apoptosis (17,18).
shown that targretin caused a dose dependent decrease in proliferation and an increase in apoptosis in this model (19).

Screening for new agents in a short time period is desirable for two reasons. First, the agents can be screened in a 1–2 week time frame (including data analysis) in contrast to a complete mammary cancer prevention study that takes 3–4 months; this model is a relatively short assay compared with many prevention studies that take 9–18 months. Second, the 1 week assay requires <5% of the amount of the retinoid needed for a prevention study. Employing limited quantities of a compound is particularly important since scale-up synthesis methods are not needed. Thus, a short-term in vivo procedure allows one to predict agents with preventive efficacy in the target tissue as well as providing data regarding the pharmacokinetics of the agent.

In the present studies, we employed a short-term biological assay that provided information about: (i) the biologic effects that may predict for the long-term mammary chemoprevention assay and (ii) the effect of the RXR agonists on serum triglycerides levels (Table I). The long-term biological assay used was the prevention of MNU-induced mammary cancers in rats (2,14). Previous studies had shown that the RXR agonist targretin was highly effective in preventing these ER+ tumors (4). This agent was also effective against ER- mammary cancers as well (6,7). The results in Figure 3A–D and in Table I show that poor RXR agonists like UAB20 and UAB112 have low activity in the chemoprevention assay and a high PI/AI ratio, and that potent RXR agonists like 4-methyl-UAB30, UAB30 and targretin have high activity with a low PI/AI ratio.
The results with benzosuberone-UAB30 was surprising since this retinoid agonist exhibited high activity in nuclear receptor binding and activation (equal to or better than UAB30) and was expected to prevent mammary cancers in the MNU model. However, it displayed poor activity in the chemoprevention assay (comparable with UAB20) and had a high PI/AI ratio consistent with its low activity. The origins of this lack of activity probably owes to rapid metabolism of the retinoid (or poor absorption) and hence poor distribution to mammary tissue and cancers. This result highlights the importance of the rapid screen for predicting chemopreventive activity since in vitro studies and receptor modeling do not predict for such biological behavior.

Of interest, the dose–response relationship for RXR analogs (e.g. targetretin) was relatively flat. While a dose of 150 mg/kg diet was highly effective, a dose of 15 mg/kg diet still decreased tumorigenesis 35–40%. Thus, unlike many preventative agents that may have a relatively steep dose–response curve, retinoids even at suboptimal doses may yield substantial activity.

As shown in Table I, all the agents decreased tumor proliferation following limited treatment. However, the highly effective agents (4-methyl-UAB30 and high dose targetretin) decreased proliferation by roughly 90%, while the moderately effective agents (e.g. 9-cis-RA and UAB30) had more limited (56–65% decreases) but still significant effects. Also, 4-methyl-UAB30 and high dose targetretin significantly affected apoptosis, while agents with minimal efficacy did not significantly alter this parameter. We tested the use of a compound index (AI:PI) and found that it identified those agents with high preventive efficacy as contrasted with agents with moderate or minimal activity. Our demonstration that the high dose of targetretin has strong therapeutic activity (19) would also predict activity for 4-methyl-UAB30 based on the present results. A preliminary study with UAB30 on a limited number of established mammary tumors has demonstrated therapeutic activity for this compound.

Triglycerides levels were also measured in serum of the rats after 7 days of treatment. Most of the agents increased triglycerides levels and the two highly effective agents, targetretin at 150 mg/kg diet and 4-methyl-UAB30, increased serum levels by 283 and 566%, respectively (Table I). The only effective preventative agent that had non-significant effects on triglycerides was UAB30. In a separate 28 days toxicity study, the administration of 800 mg UAB30/kg diet minimally altered triglycerides levels. Since increased triglycerides levels are a major impediment to development of RXR agonists for long-term use (e.g. cancer prevention), it is important to overcome this limitation. This could be done by either decreasing the dose of a highly effective agent or by increasing the dose of a moderately effective agent. For targetretin, decreasing the dose greatly decreased its preventive efficacy and still resulted in an increase in serum triglycerides levels. As our example of the second method, increasing the dose of UAB30 from 200 mg/kg diet to 800 mg/kg diet decreased proliferation by 73% and increased apoptosis by 116%, while only slightly increasing serum triglycerides. Studies have been designed to demonstrate the efficacy of high doses of UAB30 against both mammary and lung cancers. An additional approach is to combine new UAB retinoids with SERMs or AIs as has been demonstrated previously with retinoids (14,29).

In summary, a short-term series of biomarker assays have been identified that permit the evaluation of new retinoids. The short-term study allowed us to readily identify a highly active RXR agent (4-methyl-UAB30), as well as an effective agonist that did not increase triglycerides levels (UAB30). This same approach should be relevant to other classes of agents as well, although potential toxic markers will obviously be distinct for each class.

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References

defined retinoic acid analogues. 5. large-scale synthesis and mammary cancer chemopreventive activity for (2E, 4E, 6Z, 8E)-8-(3'04-Dihydro-1'(2'H)-naphthalen-1'-ylidene)-3,7-dimethyl-2,4,6-octatrienoic acid (9kUAB30). J. Med. Chem., 46, 3766–3769.


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