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Modulation of endothelial cell proliferation by retinoid x receptor agonists

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Abstract

One feature that contraindicates the wide therapeutic use of natural retinoids is their adverse effects during systemic use and the lack of receptor selectivity. In contrast, synthetic retinoids are distinguishable from each other on the basis of their partial or exclusive preference in binding and activation of selective retinoid receptors. We examined the inhibitory activities of natural and synthetic retinoids for their ability to reverse basic fibroblast growth factor-induced endothelial cell proliferation. Both the naturally occurring retinoids at nanomolar concentrations reversed basic fibroblast growth factor-induced endothelial cell proliferation. Among the synthetic retinoids tested, retinoic acid receptor/retinoid x receptor pan-agonist AGN 191659 [(*E*)-5-[2-(5,6,7,8-tetrahydro-3, 5,5,8,8-pentamethyl-2-naphtyl) propen-1-yl]-2-thiophenecarboxylic acid] and retinoid x receptor pan-agonist AGN 191701 [(*E*)-2-[2-(5,6,7,8-tetrahydro-3, 5,5,8,8-pentamethyl-2-naphthyl) propen-1-yl]-4-thiophenecarboxylic acid] at nanomolar concentrations reversed the basic fibroblast growth factor-induced endothelial cell proliferation. Since none of the retinoic acid receptor agonists tested had any effect, the inhibitory effect of AGN 191659 could be attributed to its retinoid x receptor receptor activity. These results suggest that retinoid x receptor agonists may be more selective anti-angiogenic agents due to their ability to inhibit endothelial cell proliferation. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Angiogenesis; Retinoic acid receptor; Retinoid x receptor; Endothelial cell

1. Introduction

Retinol (vitamin A) and its derivatives-retinoids are involved in the regulation of a variety of physiological functions (Boehm et al., 1995). They are also key regulators of cellular proliferation and differentiation and, as such, play an important role in normal growth and development (Love and Gudas, 1994; Boehm et al., 1995). Angiogenesis is the formation of new capillary blood vessels by a processes of sprouting from existing microvascular vessels. Angiogenesis is important during fetal development, as well as, in the physiology of normal reproduction, formation of collateral vessels and wound healing. Angiogenesis has important roles under pathological conditions where it may contribute to the pathogenesis of a number of diseases such as diabetic retinopathy, tumor growth and rheumatoid arthritis (Liotta et al., 1991; Colville-Nash and Scott, 1992; Folkman and Shing, 1992; Montesano, 1992; Risau, 1997). In each system the formation of new capillaries involves a series of discrete, but overlapping events, including: (1) localized degradation of the endothelial cell basal lamina, (2) endothelial cell migration and extra-cellular matrix invasion, (3) endothelial cell proliferation and (4) formation of capillary lumina and reconstitution of the basal lamina (Folkman, 1986; Pepper, 1997). Interestingly, retinoids have been shown to alter the endothelial cell extracellular matrix (Paige et al., 1991; Braunhut and Moses, 1994), modulating their phenotype towards reduced growth rate and inhibition of endothelial cell proliferation (Braunhut and Palomares 1991). Majewski et al. (1989, 1994a, 1995) and Oikawa et al. (1989, 1993) have shown that retinoids and their synthetic analogues have anti-angiogenic effect on tumor-induced angiogenesis, suggesting the use of retinoids as anti-angiogeneic factors. In practice, systemic use of natural retinoids is associated with significant adverse effects, due to lack of receptor selectivity (Wilhite, 1990; Kochhar and Satre 1993). Targeting the diseased tissue and improving the selectivity of retinoids to activate only those pathways

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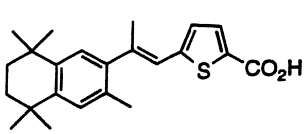
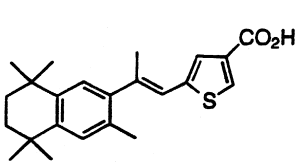
that may be involved in angiogenesis may improve the efficacy of retinoids and may minimize their adverse effects. Since the discovery and characterization of retinoid receptors beginning in 1987, (Giguere et al., 1987; Petkovich et al., 1987) knowledge of the retinoid mechanism of action has advanced, allowing the opportunity to design drugs with selective receptor function. There are two structurally and pharmacologically distinct families of retinoid receptors, the retinoic acid receptor family and the retinoid x receptor family (Fig. 1). Each family is represented by three distinct sub-types α, β and γ, encoded by three different genes. The sequences of retinoic acid receptor α, β and γ are more homologous to each other than to the retinoid x receptor genes (Table 1) (Mangelsdorf et al., 1990, 1994; Ruberte et al., 1991; Pfahl, 1993). From cell-free systems and transfected cells, two distinct pathways for transcriptional activation of responsive genes by retinoid receptors have been identified. In the first pathway, one of the retinoic acid receptor and one of the retinoid x receptor bind as heterodimers to specific half sites in the promoters of the target genes in such a way that retinoic acid receptor component is located downstream of the binding site. All-*trans*-retinoic acid requires such heterodimers (retinoid x receptor/retinoic acid receptor) to activate transcription. The other pathway utilizes a different type of binding site that retinoid x receptors use to bind as homodimers (retinoid x receptor/retinoid x receptor), and 9-*cis*-retinoic acid has been proposed to be the physiological ligand for retinoid x receptors (Heyman et al., 1992; Levin et al., 1992; Zhang et al., 1992; Kurokawa et al., 1994). The existence of several types of retinoid receptors means that the pharmacological effects of retinoids may vary with their receptor selectivity. Keeping this in mind, we employed an in vitro screening system to evaluate a series of retinoid analogues with specificity for different retinoid receptors. Among the retinoid analogs tested AGN 191659 [(*E*)-5-[2-(5,6,7,8-tetrahydro-3, 5,5,8, 8-pentamethyl-2-naphtyl) propen-1-yl]-2-thiophenecarboxylic acid] a retinoic acid receptor/retinoid x receptor



AGN 191659



AGN 191701

Fig. 1. Chemical structures and code names of effective retinoids.

Table 1
Receptor affinity information for natural and effective retinoids

Ligand	K_d (nM)					
	Retinoic acid receptor			Retinoid x receptor		
	α	β	γ	α	β	γ
Retinoic acid	15	13	18	[a]	[a]	[a]
13 *cis*-RA[b]	90	112	141	1413	1044	1751
9 *cis*-RA[b]	7	7	17	32	12	4
AGN 191659	[c]	2700	6900	120	139	214
AGN 191701	[c]	9000	[c]	308	387	301

[a] K_d for receptor of >1000 nM (Agarwal et al. 1996).
[b] RA: retinoic acid.
[c] K_d for receptor of $>10{,}000$ nM.

pan-agonist and AGN 191701 [(*E*)-2-[2-(5,6,7,8-tetrahydro-3, 5,5,8,8-pentamethyl-2-naphthyl) propen-1-yl]-4-thiophenecarboxylic acid] a selective retinoid x receptor pan-agonist prevented the basic fibroblast growth factor-induced proliferation of endothelial cells at nanomolar concentrations. These results suggest that retinoid x receptor agonists may be useful as selective inhibitors of endothelial cell proliferation and may be important in disease processes where prevention of angiogenesis may be important.

2. Materials and methods

2.1. Retinoids

All the retinoids used in this study were obtained from Allergen (Irvine, CA). All procedures involving manipulation of the retinoids were performed in the dark under dim yellow light to retard photodegradation. All the retinoids were dissolved in absolute ethanol and stored in the dark at 100 mM from which fresh dilutions were made before use. The final concentration of ethanol in the medium was below 0.1%, where ethanol did not have a significant effect on the [^{3}H]thymidine incorporation.

2.2. Isolation culture and characterization of canine aortic endothelial cells

Canine aortic endothelial cells were isolated by collagenase digestion using the method described by Pakala et al. (1994). Briefly, aorta were rinsed twice in Hank's balanced salt solution (HBSS, Sigma), all branches and one end of aorta were tied and filled with 0.01% w/v collagenase (Sigma) in Dulbecco's modified eagles medium (DMEM, Cellgro). After 10 min, the collagenase solution was collected and the aorta rinsed again and both solutions were pooled and centrifuged at $500 \times g$ for 10 min. The pellet was resuspended in 3 ml of DMEM containing 20% fetal bovine serum (FBS, Cellgro). After 24 h of incubation at 37°C, the medium was replaced with DMEM

containing 10% FBS and cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air (v/v). The cells were passaged by treatment with 3 ml of trypsin/EDTA in HBSS. The action of trypsin was stopped after 2–4 min by adding 9 ml of medium containing 10% of fetal bovine serum. The cells were harvested by centrifugation at 500 $\times g$ and suspended in fresh DMEM containing 10% of FBS. The endothelial cells were characterized by staining for the presence of Von Willebrand Factor and the absence of α-actin.

2.3. [^{3}H]thymidine incorporation

Endothelial cells from passages 2 and 3 were seeded into 35 mm diameter plates at a density of 65,000–75,000 cells/plate in DMEM containing 10% FBS and allowed to proliferate for approximately 72 h. After 72 h, the medium was replaced with 2 ml DMEM containing 0.1% FBS and incubated for approximately 72 h for arresting cell growth and synchronization. After growth arrest, the medium was replaced with 2 ml DMEM containing 500 μg/ml bovine serum albumin, 10 μg/ml human transferrin, 10 μg/ml bovine insulin, 25 ng/ml selenium (Sigma), basic fibroblast growth factor with or without retinoids. After 20 h of incubation, 1 μCi of [^{3}H]thymidine (NEN) was added to each plate and incubated for an additional 4 h, then medium was removed and the plates washed three times with ice cold phosphate-buffered saline followed by addition of 6% trichloroacetic acid to the cells and the acid-insoluble thymidine collected on a glass fiber filter. The filters were washed with 100% ethanol, air-dried and [^{3}H]thymidine was quantified using a liquid scintillation counter. Equal volume absolute ethanol controls were added to make sure that the inhibitory effects were not due to ethanol. The cells were counted (Coulter counter) on the day of seeding and before changing to 0.1% serum medium to ensure they were growing, and finally on day 6 to determine that the cells were growth arrested. All experiments were performed in triplicate and each experiment repeated three times with different batches of cells. To examine the effect of basic fibroblast growth factor, serum-free medium containing different concentrations of basic fibroblast growth factor was added to cells and [^{3}H]thymidine incorporation determined as described above. To examine the cytotoxic effect of retinoids, cells were incubated with indicated concentrations of retinoids. After incubating for 24 h, retinoid containing medium was aspirated and 10% FBS containing medium was added and the amount of [^{3}H]thymidine incorporation determined as described above. To examine the antiproliferative effect of retinoids, they were added along with 10 ng/ml basic fibroblast growth factor.

2.4. Cell number

Serum-free medium containing 10 ng/ml basic fibroblast growth factor without (control) or with indicated concentrations of retinoids was added to growth arrested and synchronized cells. Two days after addition of compounds, 0.3 ml 2% crude pancreatic trypsin (Sigma) in phosphate-buffered saline was added to each dish. The contents of each dish were diluted to 10 ml with isoton 11 and cell numbers determined with a cell counter (Coulter model ZM). Triplicate counts were taken for each plate and triplicate plates were used in each experiment. Each experiment was repeated three times with different batches of cells. Results are expressed as increase in cell number over 0.1% ethanol-treated plates.

3. Results

3.1. Effects of basic fibroblast growth factor on canine aortic endothelial cell proliferation

The effect of increasing concentrations of basic fibroblast growth factor (0 to 20 ng/ml) on [^{3}H]thymidine incorporation into the DNA is shown in Fig. 2. Addition of basic fibroblast growth factor in serum-free medium to growth arrested endothelial cells resulted in a dose dependent increase in the amount of [^{3}H]thymidine incorporated into the DNA. At ≈8 ng/ml of basic fibroblast growth factor there was a 3.5-fold increase in the amount of [^{3}H]thymidine incorporated as compared to the unstimulated control. Even with further increases in the basic fibroblast growth factor concentration (up to 20 ng/ml), the amount of [^{3}H]thymidine incorporated remained the same, indicating that higher concentrations of basic fibroblast growth factor did not induce additional growth and also were not cytotoxic or growth inhibitory to cells.

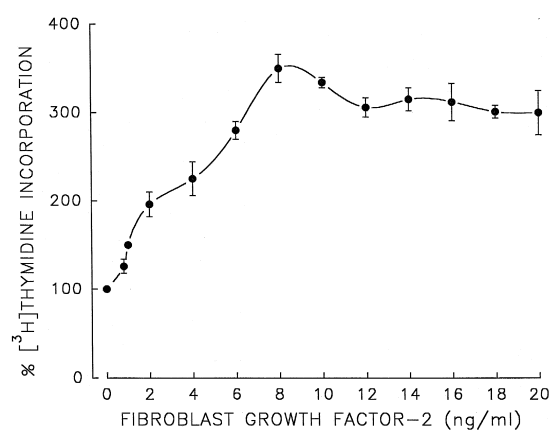


Fig. 2. Effect of basic fibroblast growth factor on endothelial cell proliferation. Growth arrested endothelial cells were stimulated with different concentrations of basic fibroblast growth factor in serum-free medium for 24 h and the amount of [^{3}H]thymidine incorporated into the DNA measured as described in Materials and methods. Results are expressed as percentage increase from the baseline. 100% = baseline value of [^{3}H]thymidine incorporation into DNA of control cells. 100% = 6750 ± 380 CPM/10^6 cells. Experiments were performed with three different batches of cells and each experiment done in triplicate.

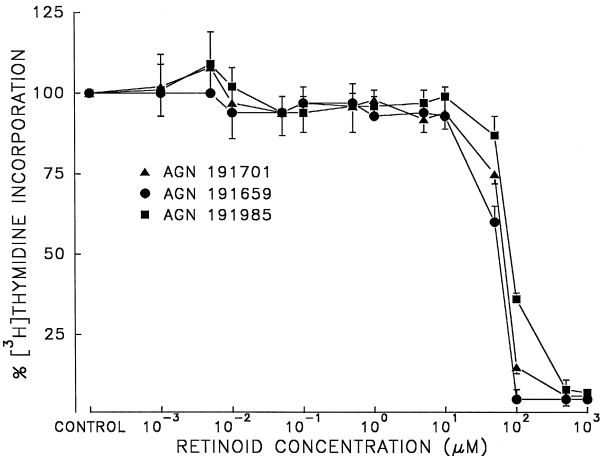


Fig. 3. Effect of retinoids on endothelial cell growth. Growth arrested endothelial cells were incubated with different concentrations of retinoids in serum-free medium. Twenty-four hours after addition of retinoids, the medium containing retinoids was aspirated and 10% fetal bovine serum containing medium was added and [^{3}H]thymidine incorporation determined as described in Materials and methods. Control cells were incubated with 0.1% ethanol. Results are expressed as percentage of control. Control = 100% = 30150 ± 2650 CPM/10^6 cells. Experiments were performed with three different batches of cells and each experiment done in triplicate.

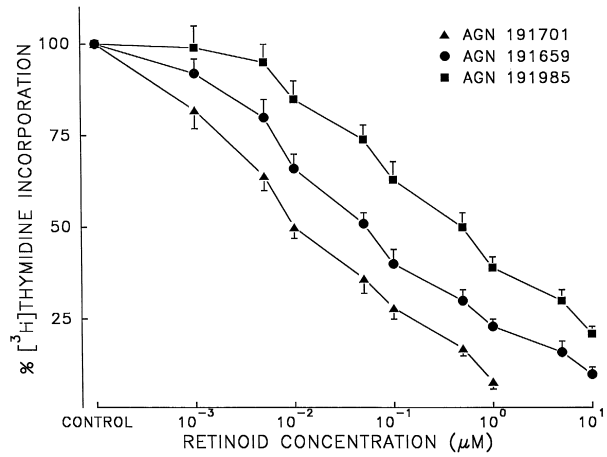


Fig. 4. Effect of AGN 191653, AGN 191701 and AGN191985 on basic fibroblast growth factor-induced endothelial cell proliferation. Growth arrested endothelial cells were stimulated with 10 ng/ml basic fibroblast growth factor with or without the given concentrations of the retinoids in serum-free medium for 24 h and the amount of [^{3}H]-thymidine incorporated into the DNA measured as described in Materials and methods. Results are expressed as percentage decrease from baseline. 100% = baseline value of [^{3}H]thymidine incorporation into DNA of 10 ng/ml basic fibroblast growth factor stimulated cells. 100% = 23,450 ± 1150 CPM/10^6 cells. Experiments were performed with three different batches of cells and each experiment done in triplicate.

3.2. Effect of retinoids on basic fibroblast growth factor-induced endothelial cell proliferation

A total number of 16 retinoids including all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 9-*cis* retinoic acid were tested for their ability to prevent basic fibroblast growth factor-induced endothelial cell proliferation. To ensure that the inhibitory effect of retinoids on endothelial cells were not due to their cytotoxicity, all the retinoids were tested for their cytotoxic effect, by observing the morphology, cell detachment (data not presented) and ability to resume growth upon returning to normal growth conditions, (Fig. 3). Only non-cytotoxic concentrations of retinoids were used for evaluating their growth inhibitory effects. Table 2 records the micromolar concentrations at which these retinoids produced half maximal (IC_{50}) inhibition of the

Table 2
Comparison of molar concentrations of retinoids need for half maximal (IC_{50}). Inhibition of basic fibroblast growth factor-induced endothelial cell proliferation

Ligand	Receptor specificity	IC_{50} μM
Retinoic Acid	RAR[a], Pan	0.1
9 *cis*-RA[b]	RAR[a] Pan, RXR[c] Pan	0.05
13 *cis*-RA[b]	RAR[a] Pan, Weak RXR[c] Pan	1.0
AGN 191659	RXR[c] Pan, Weak RAR[a] β,γ	0.05
AGN 191183	Pan RAR[a], β, γ Specific	> 10
AGN 190521	Pan RAR[a], β, γ Specific	> 10
AGN 190121	Pan RAR[a], β Specific	4.25
AGN 191379	Pan RAR[a], β Specific	> 10
AGN 190205	Pan RAR[a], γ Specific	> 10
AGN 192326	RAR[a] β	> 10
AGN 192327	RAR[a] β	> 10
AGN 192509	RAR[a] β	> 10
AGN 192870	RAR[a] β	4.5
AGN 191701	RXR[c]	0.01
AGN 191985	Weak RXR[c]	0.50
AGN 192620	Weak RXR[c]	> 10

[a]RAR: Retinoic acid receptor.
[b]RA: Retinoic acid.
[c]RXR: Retinoid x receptor.

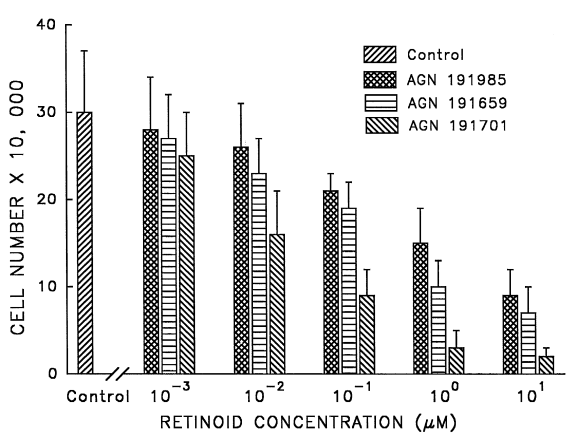


Fig. 5. Effect of AGN 191653, AGN 191701 and AGN 191985 on basic fibroblast growth factor-induced endothelial cell number. Growth arrested endothelial cells were incubated with basic fibroblasts growth factor alone (control) or with indicated concentrations of retinoids in serum-free medium for 48 h and the cell numbers determined as described in Materials and methods. Triplicate counts were taken for each plate and triplicate plates were used in each experiment repeated a minimum of three times with different batches of cells.

basic fibroblast growth factor-induced [^{3}H]thymidine incorporation by endothelial cells. Among the naturally occurring retinoids tested, retinoid x receptor ligand specific 9-*cis*-retinoic acid (IC_{50}, 50 nM) is a more potent inhibitor of basic fibroblast growth factor-induced endothelial cell proliferation as compared to retinoic acid receptor ligand specific all-*trans*-retinoic acid (IC_{50}, 100 nM). Among the synthetic retinoids tested retinoic acid receptor/retinoid x receptor pan-agonist AGN 191659 (IC_{50}, 50 nM), retinoid x receptor pan-agonist AGN 191701 (IC_{50}, 10 nM) and weak retinoid x receptor pan-agonist AGN 191985 (IC_{50}, 500 nM) inhibited basic fibroblast growth factor-induced [^{3}H]thymidine incorporation into the DNA (Table 2, Fig. 4) and cell number (Fig. 5) of endothelial cells at nanomolar concentrations. The IC_{50} values for the other retinoids were very high, and at such concentrations these retinoids lose their receptor specificity.

4. Discussion

Retinoids have been shown to inhibit angiogenesis induced by tumor cells. Systemic treatment of mice with retinoids retards vessel in-growth induced by tumor cells implanted into the cornea (Arensman and Stolar 1979) or injected intradermally in the skin (Majewski et al., 1994a,b, 1996; Rudnicka et al., 1991). These effects of retinoids are thought to be due in large part to the direct action of retinoids on the tumor cells themselves, because angiogenesis is usually inhibited if the tumor cells are treated with retinoids before their implantation into an untreated mouse. However, it is possible that in addition to its primary effects on tumor cells, retinoids can also contribute to the inhibition of tumor neovascularization by blocking the activation of the endothelial cells of the host animal. Retinoids, and retinoic acid in particular, can inhibit embryonic neovascularization in the absence of either tumor cells or macrophage infiltration, for when pellets containing retinoic acid are implanted in the chorioallantoic membrane of the developing chick, embryonic neovascularization is inhibited (Ingber and Folkman 1988; Oikawa et al., 1989). When cultured endothelial cells are exposed to retinoids, their capacity to invade collagen gels and form tubules (Pepper et al., 1994) and to migrate are hindered (Pepper et al., 1994).

In the present study, a total of 16 natural and synthetic retinoids were examined to assess their ability to inhibit basic fibroblast growth factor (a known angiogenic factor)-induced endothelial cell proliferation. Our results indicate that both the naturally occurring retinoids (all-*trans* retinoic acid, 9-*cis*-retinoic acid) and three synthetic retinoids (AGN 191659, AGN 191701, AGN 191985) at nanomolar concentrations inhibited basic fibroblast growth factor-induced endothelial cell proliferation. Among the synthetic retinoids, as expected AGN 191701, which is a retinoid x receptor pan agonist (like 9-*cis*-retinoic acid), inhibited basic fibroblast growth factor-induced endothelial cell proliferation at nanomolar concentration (IC_{50}, 10 nM). Another synthetic retinoid AGN 191659 which is a pan retinoid x receptor and weak retinoic acid receptor β agonist (IC_{50}, 50 nM) also inhibited basic fibroblast growth factor-induced endothelial cell proliferation. Since none of the retinoic acid receptor α, β-agonists (AGN 190121, AGN 190205, AGN 191379) or retinoic acid receptor β-agonists (AGN 192326, AGN 192327) tested had any effect on basic fibroblast growth factor-induced endothelial cell proliferation, the inhibitory effect of AGN 191659 can be attributed to retinoid x receptor specificity. In agreement with this, AGN 191985, which is a weak retinoid x receptor agonist, inhibited basic fibroblast growth factor-induced endothelial cell proliferation at higher concentration (IC_{50}, 500 nM). The inhibitory effect of all-*trans*-retinoic acid, which is a retinoic acid receptor agonist, can be explained by the fact that in in vivo conditions all-*trans*-retinoic acid has been shown to isomerize to 9-*cis*-retonic acid and 9-*cis* retinoic acid to all-*trans*-retinoic acid (Kojima et al., 1994). Thus, these results indicate that retinoid x receptor agonists may be more selective inhibitors of endothelial cell proliferation. Another interesting feature is that even though AGN 191701, a retinoid x receptor agonist, has higher K_d than 9-*cis* retinoic acid, physiological ligand for retinoids x receptors, AGN 191701 is a better inhibitor of endothelial cell proliferation which may probably be because of receptor selectivity of AGN 191701 over 9-*cis* retinoic acid.

In conclusion, preliminary results reported here support that retinoid x receptor agonists are better inhibitors of endothelial cell proliferation than retinoic acid receptor agonists. Mechanistic explanations invoking molecular interactions may be premature at this stage because most of the information is derived from in vitro cell systems. However, it is established that retinoic acid receptor agonists predominantly act through retinoic acid receptor/retinoid x receptor heterodimers (Mangelsdorf et al., 1990; Stunneberg, 1993; Chambon, 1994). The role of retinoid x receptor in transactivation is only to facilitate binding of retinoic acid receptor to the responsive promoters and that it is the retinoic acid receptor which is directly responsible for transcriptional activity (Stunneberg, 1993; Valcarcel et al., 1994). Although it is not yet known if retinoid x receptor/retinoid x receptor homodimers exist in nature, their formation has been shown to be preferentially induced in transfected cells by treatment with retinoid x receptor-specific ligands (Heyman et al., 1992; Yang et al., 1993; Kurokawa et al., 1994). If retinoid x receptor homodimer formation were also to occur in vivo, our results suggest the possibility that response pathways mediated by such homodimers are likely to be anti-angiogenic. It is important to note, however, that retinoid x receptors are absolutely essential in normal development because retinoid x receptors null mutant embryos die in utero and show heart and eye malformations, presumably due to abroga-

tion of the retinoic acid receptor/retinoid x receptor function (Kastner et al., 1994; Sucov et al., 1994). Suggesting that retinoic acid receptor and retinoid x receptor ligands, presently bumped together as retinoids have distinct activities in vivo. Moreover, retinoid x receptor agonists which appear to lack some classical retinoid toxicicities (Jiang et al., 1995; Standeven et al., 1996) may be retinoid of choice for anti-angiogenic therapy.

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