Suppression of human pancreatic cancer cell proliferation by AGN194204, an RXR-selective retinoid

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Retinoids may be useful agents for the treatment of pancreatic cancer. However, retinoic acid receptor (RAR)-selective retinoids produce unwanted side effects. In contrast, retinoid X receptor (RXR)-selective retinoids produce fewer side effects; however, it was not known whether RXR-selective retinoids could reduce pancreatic tumor cell proliferation. In the present study, the novel RXR-selective retinoid, AGN194204, was compared with that of other retinoids for the ability to suppress pancreatic cancer cell proliferation. We treated various pancreatic cancer cell lines with receptor-selective ligands and cytotoxic agents and monitored the effects on cell proliferation, markers of apoptosis and cell cycle. Our results indicate that AGN194204, at concentrations 4-fold lower than those of RAR-selective retinoids, could reduce pancreatic tumor cell proliferation. In contrast, retinoid X receptor (RXR)-selective retinoids, while producing less toxicity, did not reduce pancreatic tumor cell proliferation. AGN194204 targeted RXR receptors, resulting in an additive but not synergistic reduction in MIA PaCa-2 cell proliferation. In addition, AGN194204 significantly increased the expression of the cyclin-dependent kinase inhibitor p27 and decreased the expression of the cyclins E and D1.

Materials and methods

Reagents
atRA, 9-cRA, 13-cis-retinoic acid (13-cRA) and cisplatin were purchased from Sigma (St Louis, MO). AGN194204, AGN193109, AGN195393, SR11217 and TTNPB (Table I) were synthesized in the Department of Chemistry, Allergan. Retinoids were prepared as 1000-fold stocks in dimethylsulfoxide and stored in aliquots at −70°C. Recombinant human interferon-gamma (IFNγ), prepared in 10 mM acetic acid containing 0.1% human serum albumin.

Abbreviations: atRA, All-trans-retinoic acid; cdk, cyclin-dependent kinase; 9-cRA, 9-cis-retinoic acid; 13-cRA, 13-cis-retinoic acid; 5-FU, 5-fluorouracil; RAR, retinoic acid receptors; RXR, retinoid X receptors.

Introduction

Pancreatic cancer is the fifth leading cause of cancer-related death in western countries (1). Despite improvements in diagnosis, staging and treatment, the prognosis of advanced pancreatic cancer has not substantially improved during the past three decades. More than 80% of the patients have advanced regional disease or metastasis at the time of diagnosis, and the 5-year survival is <2% with a median survival time of ~5 months (1,2). Therefore, new therapeutic strategies focusing on inhibition of pancreatic cancer cell function are urgently needed.

Retinoids are natural or synthetic derivatives of vitamin A. They mediate their biological effects by binding to nuclear, ligand-activated receptors (3,4). Upon ligand binding, these receptors bind to specific DNA elements to regulate transcription (5–7). These receptors are divided into the retinoic acid receptor (RAR) and retinoid X receptor (RXR) families. Each family includes α, β and γ receptor subtypes. RARs display a high affinity for all-trans-retinoic acid (atRA) and related ligands, while RXRs bind 9-cis-retinoic acid (9-cRA) and other RXR ligands (6,8,9). An important anticancer strategy is the design of retinoids that have high efficacy and low toxicity for the treatment of disease. In particular, investigators have designed ligands that target various subclasses of the retinoid receptors (10–13).

Various retinoids have been shown to be effective in pancreatic cancer cell models. These agents act to inhibit cell proliferation, induce differentiation and promote apoptosis (14–17). However, treatment of human cancers with retinoids has been limited by significant toxicities including mucocutaneous irritation, hyperglycemia and teratogenicity (18,19). Therefore, identification of new retinoids having better therapeutic index with less toxicity is an important goal. It is also appreciated that it would be desirable to have retinoids that work efficiently in conjunction with existing therapeutic agents. RXR-selective retinoids are useful candidate therapeutic agents, as they may display less toxicity than RAR-selective retinoids.

In the present study, we examine the ability of a novel, high affinity, highly selective, RXR-retinoid, AGN194204, to inhibit the function of pancreatic cancer cells. Our results suggest that RXR-selective retinoids are more efficient inhibitors of pancreatic cell function than RAR-selective retinoids, but that not all pancreatic cancer cell lines are retinoid sensitive.
Table I. Receptor binding affinity of retinoid ligands

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The relative binding affinity of each retinoid was determined for the RAR and RXR subtypes using baculovirus-expressed RAR and RXRα, β and γ as described previously.

was purchased from R&D Systems. Interferon-alpha (IFNs), prepared in phosphate-buffered saline, was obtained from Biochemical. Mouse monoclonal antibodies specific for p27 (sc-1641), p21 (sc-6246), rabbit polyclonal antibodies specific for cyclin E (sc-481), cyclin-dependent kinase (cdk)2 (sc-163), cdk4 (sc-601), cdk8 (sc-7181) caspase-9 (sc-8355) and goat polyclonal anti-caspase-3 (sc-6136) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-caspase-3 was purchased from Biosource (AHZ052). Mouse monoclonal anti-β-actin was obtained from Sigma (A5441) and anti-cyclin D1 (554180) was from BD PharMingen.

Cell lines and culture conditions

Human pancreatic cancer cell lines MIA PaCa-2, BxPC-3 and AsPC-1 were obtained from American Type Culture Collection (ATCC, Rockville, MD). MIA PaCa-2 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 4 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 10% fetal bovine serum (FBS) and 2.5% horse serum. BxPC-3 cells were grown in RPMI supplemented with 4 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES, 10% FBS and 4.5 g/l glucose. AsPC-1 cells were maintained in RPMI supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 1 mM sodium pyruvate, 20 mM HEPES, 20% FBS and 4.5 g/l glucose.

Proliferation assays

Pancreatic cancer cells were seeded into 9.6 cm² dishes at 1000 cells/cm² for MIA PaCa-2, and 3000 cells/cm² for BxPC-3 and AsPC-1 in complete medium and allowed to attach for 24 h. The cells were treated by the addition of fresh medium, containing increasing concentrations of AGN194204 (10⁻⁷ to 10⁻⁴ M) for 6 days. After 6 days, the cells were harvested with 0.025% trypsin, 1 mM EDTA and counted using a coulter counter. The cell counts are expressed as the mean ± standard error of the mean for a minimum of three repeats.

Co-treatment with cytotoxic agents

MIA PaCa-2 cells were plated at 1000 cells/cm² in 9.6 cm² dishes and allowed to attach overnight. The cells were given fresh DMEM medium containing 0–1 μg cisplatin/ml, 0–4000 IU IFNa/ml, or 0–200 IU IFNγ/ml, 0–12.5 mM gencitabine, or 0–20 μM 5-fluorouracil delivered in combination with 0–10 μM AGN194204. The final concentration of the DMSO vehicle did not exceed 0.2% in the culture medium, and all control dishes received the appropriate concentration of vehicle. After 6 days, with fresh treatment on alternate days, the cells were harvested with trypsin–EDTA and counted using a coulter counter. The cell counts are expressed as the mean ± standard error of the mean for a minimum of three repeats.

Western blot analysis

For immunoblot, an equivalent amount of protein was electrophoresed on denaturing 8–10% polyacrylamide gels, transferred to nitrocellulose membrane, and the membrane was incubated in 10 mM Tris-HCl pH 7.2 containing 100 mM NaCl, 0.1% Tween-20 and 5% non-fat dry milk to block non-specific binding. After incubation with primary antibody, the blot was washed and exposed to the appropriate horseradish peroxidase-conjugated secondary antibody. Specific antibody binding was visualized using chemiluminescence detection reagents (Amersham Biosciences).

Cell cycle analysis

MIA PaCa-2 cells were grown in DMEM with supplements as above. When 30% confluent, the cells were treated with DMSO or 1 or 10 μM AGN194204 for 24 or 48 h. The cultures were trypsinized, washed with phosphate-buffered saline, and collected by centrifugation. The cells were re-suspended in phosphate-buffered saline and fixed in cold methanol. After an additional wash in phosphate-buffered saline, the cells were incubated with RNase (10 μg/ml) at 37°C for 10 min, and stained with 50 μg/ml propidium iodide for 60 min. The cells were then analyzed by flow cytometry. Cell cycle analysis was performed three separate times with similar results in each case.

Results

Retinoids

Table I lists the binding affinity of each of the retinoids used in this study. These ligands were selected to include a potent RAR-selective retinoid (TTNPB) (20) that has a low affinity for RXR receptors, a moderate affinity RXR-selective retinoid (SR11217), and a high affinity RXR-selective retinoid (AGN194204) (11). The table also lists the binding affinities of an RAR-selective antagonist (AGN193109) (21–23) and an RXR-selective antagonist (AGN195393).

AGN194204 regulation of pancreatic cancer cell proliferation

To test the effect of AGN194204 on pancreatic cancer cell proliferation, cells were treated with increasing concentrations of AGN194204, TTNPB or SR11217 (0–10 μM) for 6 days. As shown in Figure 1, AsPC-1 cell number was modestly reduced (20%) by all three retinoids. BxPC-3 cell number was reduced 40% by treatment with 1 μM AGN194204, 20% by treatment with 1 μM SR11217, and 10% by treatment with TTNPB. MIA PaCa-2 cell number was reduced 60% by treatment with 1 μM AGN194204, and 20% by treatment with 1 μM SR11217 or TTNPB. These results indicate that AGN194204-dependent suppression of cell number varies for different pancreatic cell lines, and that the RXR-selective AGN194204 is, overall, a more effective inhibitor of pancreatic cancer cell proliferation than the RAR-selective, TTNPB. The partially RAR-selective ligand, SR11217, has an intermediate efficiency.

AGN194204-dependent suppression of cell number is mediated by RXR

To confirm that the AGN194204-dependent suppression is mediated by RXR, we monitored the ability of RAR and RAR-selective antagonists to inhibit the AGN194204 response. MIA PaCa-2 cells were incubated with either 100 nM TTNBP (RAR-selective agonist) or 100 nM AGN194204 (RXR-selective agonist) in the presence of increasing concentrations of the RAR-selective antagonist, AGN193109. After 6 days of treatment, the cells were harvested and counted. As shown in Figure 2A, addition of TTNBP results in a 20% reduction in cell number and AGN193109 produces a concentration-dependent reversal of this inhibition. In contrast, AGN193109 does not reverse the 40% reduction in cell number produced by AGN194204. The experiment shown in Figure 2B indicates that addition of AGN195393, an RXR-selective antagonist, reverses the AGN194204-dependent inhibition of cell number, but that this agent does not reverse the TTNBP-dependent reduction. These findings suggest that the TTNBP-dependent inhibition is mediated exclusively by RAR subtypes, and that the AGN194204-dependent inhibition of cell number is mediated exclusively by RXR subtypes.

AGN194204 modifies the level of some cell cycle regulators

We next determined whether AGN194204 influences cell cycle regulatory protein expression in MIA PaCa-2 cells. This analysis shows that cyclin E and cdk6 levels are suppressed, to 40 and 10% of control level, respectively, after 48 h of treatment with 1 or 10 μM AGN194204 (Figure 3A). In contrast, the levels of cyclin D1, cdk2 and cdk4 are not modified. In addition, the levels of the cdk inhibitor, p27, is...
increased 2-fold following treatment with 10 μM AGN194204. p21 was not detected. Thus, AGN194204 changes the expression level of only selected cell cycle-associated regulators.

**RXR antagonist reversal of AGN194204-dependent changes in cyclin E, cdk6 and p27 level**

As noted above, the RXR antagonist, AGN195393, can reverse the AGN194204-dependent reduction in cell number. To determine whether the AGN194204-dependent modification of cell cycle regulatory protein level can be reversed by this ligand, MIA PaCa-2 cells were treated with 10 μM AGN194204 in the presence of RAR-selective antagonist AGN193109, or RXR-selective antagonist AGN195393. As shown in Figure 3B, AGN193109 and AGN195393 do not alter cyclin E, cdk6 or p27 level. In addition, the AGN194204-dependent suppression of cyclin E and cdk6 expression and the increase in p27 expression is not reversed by treatment with RAR antagonist. However, the RXR-selective antagonist, AGN195393, reverses these AGN194204-dependent changes.

**Simultaneous treatment with AGN194204 and other growth inhibitors**

Simultaneous treatment with retinoids and other growth inhibitory agents may be useful for the treatment of pancreatic cancer. To assess whether co-treatment results in enhanced inhibition of cell proliferation, we examined the effect of treatment with selected growth inhibitors in conjunction with AGN194204 treatment. In Figure 4A MIA PaCa-2 cells were treated with increasing concentrations of IFNα in the absence (closed circles) or presence (open circles) of 100 nM AGN194204. IFNα produced a concentration-dependent reduction in cell number with a 50% suppression at an IFNα concentration of 4000 IU/ml. We next tested the response of IFNα in the presence of 100 nM AGN194204. As shown in Figure 4A, the inclusion of AGN194204 shifted the IFNα dose-response downward by 30%, but did not change the slope. Figure 4B shows a similar response for the inverse experiment in which cells were treated with increasing concentrations of AGN194204 in the absence or presence of 500 IU/ml IFNα. The addition of IFNα shifts the AGN194204 response curve downward, but does not change the slope. We also examined the effects of co-treatment of MIA PaCa-2 cells with AGN194204 and IFNg. Figure 5 shows that, similar to IFNα, IFNg produces an additive inhibition when co-administered with AGN194204. This holds whether the cells are treated at a fixed concentration of AGN194204 with increasing IFNg (Figure 5A), or treated at fixed IFNg with increasing AGN194204 (Figure 5B).

Cisplatin is a cytotoxic agent that is widely used as a chemotherapeutic agent. Figure 6A shows that cisplatin treatment produces a concentration-dependent inhibition of MIA PaCa-2 cell proliferation. When combined with 100 nM AGN194204, the cisplatin suppression curve is moved lower, but the slope of the curve does not change—thus the agents produce additive inhibition. The inverse experiment, using a fixed cisplatin concentration, with increasing AGN194204, reveals a similar additive inhibition (Figure 6B).

Gemcitabine and 5-Fluorouracil (5-FU) are also used presently to treat pancreatic cancer. Gemcitabine is at present the most effective chemotherapeutic drug for the treatment of this disease (24). As shown in Figure 7A, 5-FU treatment reduces MIA PaCa-2 cell number, while co-treatment with
100 M AGN194204 shifts the 5-FU response curve but does not change the slope. Figure 7B indicates a similar relationship for the inverse experiment where a fixed concentration of 5-FU (2.5 μM) is used with increasing concentrations of AGN194204. Figure 8A shows a concentration-dependent inhibition of MIA PaCa-2 cell proliferation by gemcitabine. Treatment with 7.5 nM gemcitabine results in a 52% reduction in cell number. Addition of 100 nM AGN194204 shifts the gemcitabine dose-response downward, but did not change the slope. Treatment with 5 nM gemcitabine and increasing concentration of AGN194204 results in a similar additive inhibition (Figure 8B).

Effect of AGN194204 compared with natural retinoids
Figure 9 compares the ability of AGN194204 and the other natural retinoids to suppress MIA PaCa-2 cell number. AGN194204 (10 μM) reduces cell number by 72%. atRA, 9-cRA and 13-cRA, at 10 μM, inhibit by 67, 66 and 55%, respectively.

AGN194204 does not enhance MIA PaCa-2 cell apoptosis
The above findings suggest that AGN194204 suppresses MIA PaCa-2 cell proliferation, in conjunction with a reduction in cyclin E and cdk6 expression, and increase in p27 level. To determine whether apoptotic processes are also involved, we treated cells with the agents as indicated in Figure 10 and then assayed for the ability of each agent to reduce procaspase-8, -9 and -3 levels. As shown in Figure 10, none of the agents tested promote enhanced cleavage of procaspase-8. With respect to procaspase-9 and -3, only 5-FU promotes a reduction in level.

Discussion
Retinoids as cancer therapeutics
Retinoids are important regulators of cell proliferation and differentiation. As such they are important candidates for the treatment of disease conditions, including cancer (12). The retinoid receptor family includes six distinct members—RARα, β and γ, and RXRα, β and γ (3,4,6,9,25). These
receptors cooperate to regulate cell function by forming homo- and heterodimers that act to regulate cell transcription in a cell type- and receptor subtype-specific manner. Because of the tissue-selective pattern of expression and the selective role of the RAR and RXR subtypes in generating specific biological responses, retinoid development has focused on identifying retinoid analogs that selectively interact with specific retinoid receptor subtypes (10,26). The ultimate goal is the design of a specific cancer-targeted therapy with minimal side effects. RXR-selective ligands are frequently less toxic than RAR-selective ligands (11,27). For example, Wu et al. (28), working with C3(1)-SV40 Tag mice, a mammary tumorigenesis model, showed that a RAR-selective ligand was modestly chemopreventive and highly toxic. In contrast, LGD1069, a RXR ligand, was an effective chemopreventive agent without toxicity. Based on this and other studies, it is hoped that RXR-selective ligands may be efficacious inhibitors of pancreatic tumor cell growth, without producing significant unwanted side effects (28,29). The major goal of the present study is to evaluate the ability of an RXR-selective retinoid, AGN194204, to inhibit the growth of several pancreatic cancer cell lines.

**Inhibition of pancreatic cancer cell proliferation by AGN194204**

The antitumor effects of retinoids have been noted in a variety of tissues and model systems, including pancreatic cancer cells (15,30–32). Previous reports indicate that a range of natural retinoids, including atRA, 9-cRA and 13-cRA, can inhibit pancreatic cancer cell proliferation (14–16,31,33,34).
Additional studies suggest that the response requires specific RAR receptor subtypes. Some investigators report that the retinoid-associated inhibition of pancreatic cancer cell proliferation requires the RARγ receptor (14,35), while others attribute the suppression to the RARα receptor (36). For example, treating DSL-6A/C1 pancreatic adenocarcinoma cells with an RARα-selective synthetic retinoid, Ro 40-6055, suppresses cell proliferation (36)—co-treatment with a synthetic RARα-antagonist, Ro 41-5253, inhibits this response. Another RARα-selective synthetic retinoid, TAC-101, inhibits proliferation of BxPC-3, MIA PaCa-2 and AsPC-1 cells (37). In addition, mofarotene (Ro 40-8757), a synthetic retinoid that does not bind to or activate any retinoid receptor, also suppresses pancreatic cancer cell proliferation (38). All of these studies focus on the potential utility of RAR-selective retinoids. Thus, a potential role for RXR-targeting ligands has not been investigated.

In the present study we investigate the anti-proliferative effects of two RXR receptor-selective retinoids, SR11217 and AGN194204 (39). SR11217 selectively binds, with moderate affinity, to RXR receptors, while AGN194204 binds to RXR receptors with high selectivity and affinity (39). Our studies indicate that MIA PaCa-2 cells are markedly growth suppressed by retinoid treatment. BxPC-3 cells, in contrast, are moderately retinoid-sensitive and AsPC-1 cells are the least sensitive. Among the various compounds tested, AGN194204 is the most effective—more effective than SR11217 or the high-affinity RXR-selective retinoid, TTNPB (20). These results suggest that RXR-selective ligands, acting via RXR receptors, can suppress pancreatic cancer cell proliferation. This idea that the suppression requires RXR receptor function is supported by the observation that the AGN194204-dependent suppression of proliferation is reversed by treatment with the RXR-selective antagonist, AGN195393, but is not reversed by treatment AGN193109, a RAR-selective antagonist (11). These findings indicate that high-affinity RXR-selective ligands can efficiently suppress pancreatic cancer cell proliferation. The relative difference in sensitivity of the cell lines is not likely to be due to differences in retinoid receptor subtype expression, as most pancreatic cell lines express RXRα and RXRB (32). The response difference could be due to differing expression of co-activators and co-repressors (7,40–42), however, this has not been examined.

**AGN194204 regulation of cell cycle protein expression**

Pancreatic cancer cells have defective G1 cell cycle control (43). To determine whether the AGN194204-dependent reduction in cell number is associated with specific changes in the level of cell cycle regulatory proteins, we measured the effect of AGN194204 on expression of these regulators. No change in cyclin D1, cdk2 or cdk4 level is observed following AGN194204 treatment. However, a reduction in cyclin E and cdk6 level is observed at 48 h of treatment. In addition, a moderate increase of p27 is observed. p21 is not detected. Treatment with AGN195393, the RXR-antagonist (44), reverses the AGN194204-dependent changes in cell cycle protein expression. Previous studies describe retinoid effects on cell cycle regulatory proteins in pancreatic cancer cells. In MIA PaCa-2 cells, TAC-101, a RARα-selective synthetic retinoid, inhibits cell proliferation leading to arrest in G1 phase (37). This arrest is associated with reduced pRB and cyclin A level, and increased p21 and p27 level (37). Another study reports that the synthetic retinoid, Ro 40-8757, inhibits pancreatic cancer cell proliferation by increasing p21 and p27 levels (16). Thus, although the protein targets are subtly different, both RAR- and RXR-selective ligands appear to target cell cycle regulatory proteins that control events in the G1 cell cycle phase. A consistent observation for both RAR- and RXR-selective ligands is that growth suppression correlates with increased cdk inhibitor expression (i.e. p21 and/or p27). These results anticipate arrest in the G1 phase of the cell cycle. Indeed, cell cycle analysis of untreated cells reveals 46% in G1, 24% in G2/M and 30% in S phase. In contrast, the distribution was 64% G1, 18% G2/M and 18% S phase in cells treated for 24 or 48 h with 1 or 10 μM AGN194204 (not shown).

In addition to regulation of cell cycle progression, retinoids have also been shown to influence cancer cell apoptosis
Procaspase cleavage is a commonly used marker of apoptosis. Thus, we measured the ability of the RAR- and RXR-selective retinoids and the various cytotoxic agents to promote cleavage of procaspase 3, 8 and 9. Our studies indicate that neither RAR- nor RXR-selective retinoid enhance apoptosis, as measured by enhanced procaspase cleavage. Thus, at least in MIA PaCa-2 cells, the main regulation appears to be regulation of cell cycle-associated events.

Regulation of MIA PaCa-2 cell proliferation by AGN194204 in combination with cytotoxic agents

Clinical experience indicates that pancreatic cancer is not controlled by chemotherapy or other conventional treatments (30). Because it is possible that co-therapy with cytotoxic agents and retinoids may improve the activity of chemotherapeutic anticancer agents, we evaluated the ability of AGN194204 to enhance the action of various cytotoxic
agents and growth suppressors. We selected INFα, INFγ, cisplatin, gemcitabine and 5-FU for testing, as these agents are used to treat pancreatic cancer patients (30). Treatment with 13-cRA and IFNα produces partial remission in some pancreatic cancer patients and prompts disease stabilization in others (48), and co-treatment with IFNβ and retinol palmitate has been reported to be effective in treating metastatic pancreatic carcinoma patients (18). However, an important complicating issue is that these treatments produce toxic side effects, some of which are due to the retinoid therapy (18,48). In this respect, RXR-selective ligands have an important advantage, as in vivo side effects are minimal compared with RAR-selective ligands (28,29).

Our results indicate that IFNα inhibits MIA PaCa-2 cell proliferation, but not as efficiently as IFNγ. Gemcitabine, cisplatin and 5-FU treatment also produced a concentration-dependent reduction in cell number. Moreover, the combination of AGN194204 with IFNα, INFγ, gemcitabine, cisplatin or 5-FU enhances inhibition in an additive, but not synergistic, manner. This is in contrast to a report that suggests that IFNα can act synergistically with retinoids (19). On balance, our present studies suggest that RXR-selective retinoids may provide a less-toxic alternative to RAR-selective ligands for the treatment of pancreatic cancer.

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References


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