



Research Article

Inhibition of Cytochrome P450 and Multidrug Resistance Proteins Potentiates the Efficacy of All-Trans Retinoic Acid in Pancreatic Cancer *In Vitro* and *In Vivo*

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Abstract

Objectives: All-trans retinoic acid (atRA) potentially induces differentiation and apoptosis in pancreatic cancer. However, the clinical use of retinoids is limited by retinoid resistance or development of toxicity at high doses. We tested the hypothesis that blocking atRA degradation and elimination from the cell would potentiate its effectiveness in pancreatic cancer therapy.

Methods: *In vitro*, AsPc-1 and HPAF cells were co-treated with atRA and inhibitors of either multidrug resistance (MDR: verapamil, LY335979, and quinidine) or cytochrome P450 (CYP450s: troleandomycin, clotrimazole and liarozole). In addition, cells were co treated with atRA and antisense oligonucleotides against MRP, Pgp, CYP26 and CYP3A4. Proliferation and apoptosis were investigated. *In vivo*, AsPc-1 xenografts were treated with atRA, verapamil, and troleandomycin alone or in combination.

Results: The anti-proliferative effect of atRA on AsPc-1 and HPAF cells was markedly potentiated by the inhibition of MDR and CYP450 or by antisense oligonucleotides to reduce their production. The combination also enhanced atRA-induced apoptosis. Co-administration of inhibitors of MDR and CYP450 also potentiated the inhibitory effect of atRA on growth of xenografts.

Conclusions: Co-treatment of pancreatic cancer with low non-toxic doses of atRA combined with MDR blockade and inhibition of CYP450 is effective suppressing tumor growth, suggesting a novel clinical application.

Keywords

Retinoic acid; Verapamil; Troleandomycin; Proliferation; Apoptosis; Pancreatic cancer

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in men and women in the United States [1]. The present treatment modalities for this devastating cancer; surgery and chemo-radiotherapy, fail to control the aggressive nature of the disease causing a significant

patient morbidity and mortality [2]. The overall 5-year survival for pancreatic cancer is approximately 5% [1]. The dismal prognosis of the disease has prompted the intensive investigation for therapeutic strategies that counteracts the biology of this tumor. All-trans retinoic acid (atRA), a vitamin A (retinol) derivative, regulates several essential cellular process including proliferation, differentiation and apoptosis in the normal epithelium. In addition, atRA suppresses tumorigenesis in a variety of epithelial tissues through multiple mechanisms including the induction of differentiation, inhibition of proliferation, inhibition of anti-apoptotic pathways, cell cycle arrest and down regulation of telomerase activity [3]. *In vitro* studies show that atRA reduces pancreatic cancer growth via similar mechanisms [4-7]. In preclinical studies retinoids suppressed the growth of carcinogenesis model of pancreatic adenocarcinoma [8,9]. In clinical trials single-agent retinoid treatments were not effective in pancreatic tumors, although combinations of retinoids with other cancer chemotherapy agents had limited efficacy combined with signs of toxicity [10,11]. A major challenge in retinoids cancer chemopreventive trials is the development of retinoic acid (RA) resistance and the occurrence of toxicity due to the prolonged use of high doses [12]. A potential mechanism implicated in the development of resistance is the induction of pathways involved in atRA metabolism [13], although there is also reduced expression of retinoic acid receptors in pancreatic cancer [14]. Furthermore, expression of cellular retinoic acid binding protein 2 (CRABP2) is associated with marked growth inhibition by atRA, while, in contrast, expression of fatty acid binding protein 5 (FABP5) is associated with minimal cytotoxicity in response to atRA [15].

Cytochrome P450 (CYP450) mediates the oxidation and thus the detoxification of atRA to its readily soluble products 4-oxo-RA, 4-hydroxy-RA and 5,6-epoxy-RA [15,16]. Two mammalian CYP450 subfamilies are known to be RA-inducible, namely CYP26A1 and B1 [17,18]. Pancreatic cancer tissues contain high levels of several drug metabolizing CYP450 enzymes known to target atRA catabolism and thus reduce its biological activity [19]. A second mechanism claimed to be involved in cancer chemoprevention resistance is the development of multidrug resistance (MDR) that involves the overexpression of ATP-dependent efflux transporters such as P-glycoprotein (Pgp) and multidrug resistance-associated protein (MRP) [20,21]. MRP and P-gp proteins pump cytotoxic chemotherapeutic drugs out the tumor cells [22] and have been associated with the atRA resistance [17,23]. Although pancreatic cancers express only traces of P-gp, the promoter of its gene is retinoid inducible. A high level of MRP expression is seen in pancreatic cancer [24,25]. Thus, retinoids with higher pharmacological indices are needed to improve retinoid-based chemotherapy in pancreatic cancer.

In the present study, we hypothesized that retinoid resistance in pancreatic cancer is due to rapid catabolism by RA-induced CYP450 as well as an enhanced efflux by RA-induced multidrug resistance proteins, resulting in enhanced pumping and rapid clearance of cytosolic retinoids. Using *in vitro* and *in vivo* models, we investigated whether abrogating these two modalities has a synergistic effect on atRA efficacy in pancreatic cancer therapy.

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Materials and Methods

Chemicals

All-trans retinoic acid from Sigma Chemicals Co. (St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO) as a 100 mM stock, stored in dark brown tubes at -80 °C. Diluting stocks into working range of 1 nM - 10 μM reduced the concentrations of DMSO to ≤ 0.01%. Retinoid manipulations were carried out in subdued yellow light using previously optimized conditions [26]. Propidium iodide, verapamil, troleandomycin, quinidine and clotrimazole were from Sigma Chemicals Co. ³H-thymidine (25 Ci/mmol) was from Amersham Life Science (Arlington Heights, IL). Liarozole was a gift from the Janssen Research Foundation (Spring House, PN). LY335979 was a gift from Eli Lilly (Indianapolis, IN).

Cell culture and cotreatment schedule

Pleomorphic human pancreatic cancer cell lines AsPc-1, HPAF, and Capan-2 were purchased from American Type Culture Collection (ATCC) (Manassas, VA) were grown in a mixture of 1:1 ratio of Dulbecco's modified Eagle's medium (DMEM) and Nutrient Ham's F12 medium containing 10% fetal bovine serum (FBS), 2 mM glutamine and 100 μ/ml of each of penicillin and streptomycin. All retinoid experiments were conducted in the same mixture of serum-free and phenol red-free medium supplemented with 10 mM HEPES, 2.5 g/L glucose and 1 mM pyruvate. Cells were incubated at 37°C in an atmosphere of 5% CO₂. Cells were seeded in 24-well tissue culture plates in FBS-containing medium for 24 hours and then in serum-free condition for another 24 hours, after which retinoid treatment was started for various time periods. Inhibitors of CYP450s (troleandomycin, 100 μM, liarozole, 10 μM; or clotrimazole, 0.5 μM) and antagonists of MRPs (verapamil, 10 μM, LY335979, 0.1 μM; or quinidine, 30 μM) in DMSO were added to cells 2 hours before retinoid treatment and remained throughout the treatment period. Retinoids were added only once for time periods up to three days, but were otherwise renewed every other day.

Oligonucleotide transfection

The morpholino-antisense oligonucleotides or their scrambled controls were transfected into cells using a modified scrape-loading technique. In brief, cells at 75% confluence in 75 cm² tissue culture flasks were washed and detached with a cell scraper in the presence of 50 μM oligonucleotide in medium containing 0.1% FBS. Cells were dis-aggregated into a single cell suspension by forced pressure through a 21-gauge needle twice, counted and seeded in 48-well tissue culture plates for 12 hours in presence of 10% FBS to enhance recovery. Cells were then cultured in serum-free medium for 12 hours prior to three days of treatment with a single addition of retinoid. Proliferation was measured by ³H-thymidine incorporation. The sequences of the oligonucleotides used were: 5'-GAGCCCCAT GGCACGCTTCAG-3' antisense and 5'-GACGCCAGTGCACCGTTACG-3' scrambled control for CYP26; 5'-GGCTATGTGCATGGAGCTTTC-3' antisense and 5'-GGTCATGTCGATGGAGCTTCTC-3' scrambled control for CYP 3A4; 5'-CGCGTCCAGCCCTACCTAG-3' antisense and 5'-CGCGT CCCAGCCTCACTCAG-3' scrambled control for P-gp; and 5'-CCGGAGCGCCATGCCGGTGG-3' antisense and 5'-CGCGACGGCCATGCGCGTGG-3' scrambled control for MRP. These antisense oligonucleotides target the AUG and upstream sequences in the mRNAs and their scrambled controls

have four mismatches. Oligonucleotides were produced by AVI Biopharma (Corvallis, OR).

Proliferation assays

Treated AsPc-1, HPAF and Capan-2 cells were washed with phosphate buffered saline (PBS) and incubated with ³H-thymidine 0.5μ Ci/well, 25 Ci/mmol (Amersham Life Science, Arlington Heights, IL) in serum free medium for 2 hours. Cells were processed to measure new DNA synthesis as previously described [27].

Apoptosis assays

Apoptosis was assessed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Treated adherent AsPc-1 and HPAF (1×10⁶ cells/ml) and floating cells in medium were pooled following trypsinization. The mean green fluorescence of the incorporated fluorescein-12-dUTP analysis of TUNEL stained cells (Promega, Madison, WI) against red fluorescence of propidium iodide was quantified by flow cytometry as previously described [27].

Animal studies, drug dosage and treatment protocol

Athymic nude mice (female BALB/c, nu/nu, 6 to 8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Mice were randomized into 6 treatment groups (n=8-10 per group receiving treatment with either (a) atRA: 2 mg/kg/day; (b) atRA: 6 mg/kg/day; (c) verapamil: 42 mg/kg/day and troleandomycin: 200 mg/kg/day; (d) atRA 2 mg/kg/day; with verapamil and troleandomycin; and (e) atRA 6 mg/kg/day with verapamil and troleandomycin, or (f) the DMSO vehicle- by oral gavage. In brief, 5×10⁶ AsPc-1 cells in (50 μL medium) were injected sub-cutaneously into mice flanks (two injection sites per mouse). One day following xenograft induction, mice in different groups received their treatment for five days/ week for four weeks when the experiment was ended because of the tumor burden in the control groups. Tumor sizes were measured by caliper twice a week, and the tumor size was calculated as (L x W X (L + W/2) x 0.526 where L=length (mm) and W=width (mm) [28]. Body weights of mice were monitored once a week and excised sc. tumors weighed at euthanasia. All animal procedures were performed in accordance with American Association of Laboratory Animal Care guidelines and approved by the Institutional Animal Care and Use Committee.

Data Analysis

Data was analysed by analysis of variance (ANOVA) using Dunnett's or Bonferroni's corrections for multiple data points as appropriate.

Results

Decreased proliferation of AsPc-1, HPAF and Capan-2 cells by combined treatment

The effect of atRA alone or combined with pharmacological antagonists of MDR proteins was tested in AsPc-1, HPAF and Capan-2 cells. Compared with vehicle-treated cells, treatment with atRA alone caused a modest decrease in proliferation (Figure 1). Combination of atRA with quinidine, LY335979, or verapamil for three days was much more effective than with atRA alone (all P<0.001, Figure 1A, 1C and 1E). The effect of co-treatment with atRA and CYP450 inhibitors was also tested in AsPc-1 and HPAF cells. The proliferation inhibitory

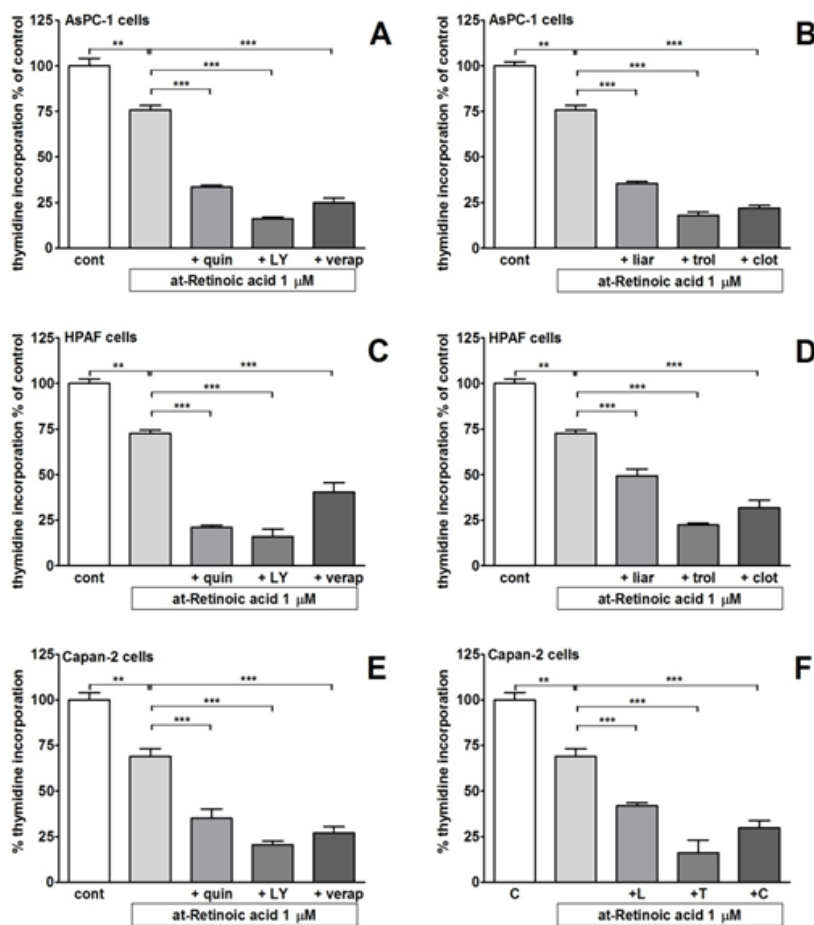


Figure 1: Effects of co-treatment with all-trans retinoic acid and multidrug resistance protein antagonists (A, C and E) (quinidine (quin), LY335979 (LY), or verapamil (verap) or inhibitors of cytochrome-P450 enzymes (B, D and F) (liarozole (liar), troleandomycin (trol), or clotrimazole (clot)) for 3 days on proliferation of AsPC-1, HPAF and Capan-2 cells respectively, measured as thymidine incorporation. Data shown are mean \pm SEM of the % of untreated control, from four separate experiments performed in triplicate, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

effect of single-agent atRA was markedly potentiated when combined with liarozole, troleandomycin, or clotrimazole (all $P < 0.001$) (Figure 1B, 1D and 1F). When used alone quinidine, LY335979, verapamil, liarozole, troleandomycin, and clotrimazole had no significant effects on proliferation (Figure 2). Because pharmacologic inhibitors have inherent nonspecific effects, specific morpholino-antisense oligonucleotides against MRP, Pgp, CYP450 3A4, and CYP26 were tested to see if these would also potentiate the antiproliferative effect of atRA. AsPC-1 and HPAF cells were treated for 3 days with atRA alone, atRA/scrambled antisense, atRA/MRP antisense, or vehicle treated and proliferation was measured by ^3H -thymidine incorporation. Antisense oligonucleotides directed to MRP caused a marked increase in the efficacy of atRA ($P < 0.001$), whilst Pgp antisense had a small but significant ($P < 0.01$) enhancing effect when compared with atRA alone or in combination with the respective scrambled oligonucleotide controls (Figure 3A and 3C). Similarly, antisense oligonucleotides directed towards CYP3A4 or CYP26 also markedly increased the efficacy of atRA in inhibiting proliferation of the AsPC-1 cells compared with atRA alone or the scrambled controls (both $P < 0.001$) (Figure 3B and 3D).

Potentiated induction of apoptosis in AsPC-1 and HPAF cells by combined treatment

Co-treatment of atRA with MDR protein antagonists and CYP450 inhibitors was then tested to see if the apoptotic effect of RA was also potentiated. Apoptosis was assessed by the TUNEL assay. AsPC-1 and HPAF cells cultured with atRA alone, or with verapamil, LY335979 or inhibitors of CYP450 (liarozole or troleandomycin). Compared with vehicle control, atRA caused a significant induction of apoptosis in both AsPC-1 cells ($P < 0.001$) and HPAF cells ($P < 0.01$) (Figure 4). The induction of apoptosis by atRA was markedly enhanced when the atRA was combined with verapamil or LY335979 (all $P < 0.001$ vs. atRA alone) (Figure 4A and 4C) or when combined with liarozole or troleandomycin (all $P < 0.001$ except liarozole in HPAF $P < 0.01$, vs atRA alone), (Figure 4B and 4D).

Reduced growth of AsPC-1 xenograft in athymic mice by combined treatment

The effects of combining atRA with troleandomycin or verapamil were tested *in vivo* using the mouse xenograft model with AsPC-1 cells. From our experience, HPAF cells do not form reliable xenografts

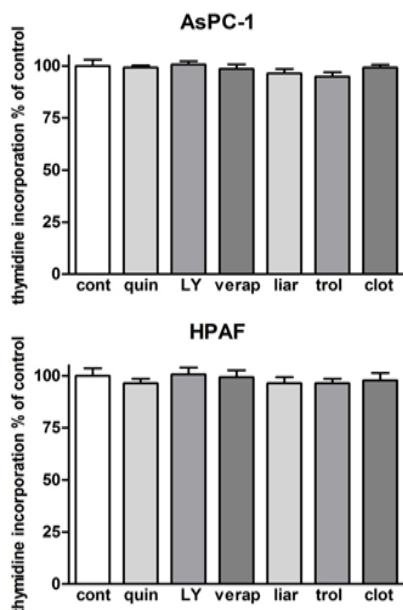


Figure 2: Effects of quinidine (quin), LY335979 (LY) verapamil (verap), liarozole (liar), troleandomycin (trol), or clotrimazole (clot) for 3 days on proliferation of AsPC-1 (top panel) and HPAF (bottom panel) cells measured as thymidine incorporation. Data shown are mean \pm SEM of the % of untreated control, from four separate experiments performed in triplicate. None of these individual treatments showed any significant effect.

and so this cell line was not used for the *in vivo* study. Mice that received either verapamil (42 mg/kg/day) and troleandomycin; atRA at 2 or 6 mg/kg/day, or atRA at 2 or 6 mg/kg/day in combination with verapamil and troleandomycin, all demonstrated a significant reduction of tumor growth as early as 9 days compared with vehicle-treated mice (Figure 4A). However, the greatest reduction in tumor size and weight was seen with the two combinations of atRA with verapamil and troleandomycin (both $P < 0.0001$, Figure 5A). Tumor size was affected by time ($F = 8.26$, $P < 0.001$) and by treatment ($F = 23.3$, $P < 0.0001$). Treatment with the two doses of atRA alone or a combination of verapamil with troleandomycin caused a significant reduction in tumor weight (all $P < 0.001$, Figure 5B). The combinations of atRA with verapamil and troleandomycin caused significantly greater reduction in tumor weight than the respective doses of atRA alone ($P < 0.05$ at 2 mg/kg/day, $P < 0.01$ at 6 mg/kg/day, Figure 4B). There were no significant differences in animal weights between the treatment groups at the end of the experiment. Similar results were obtained from four separate experiments using combinations of atRA with verapamil and troleandomycin at various doses. The reduced tumor growth in mice received the combined treatment is consistent with the *in vitro* potentiated suppression of proliferation and induction of apoptosis following the combined treatment of AsPc-1 *in vitro*. Therefore, combined administration of low nontoxic doses of atRA, verapamil, and troleandomycin has a significant benefit for pancreatic cancer treatment.

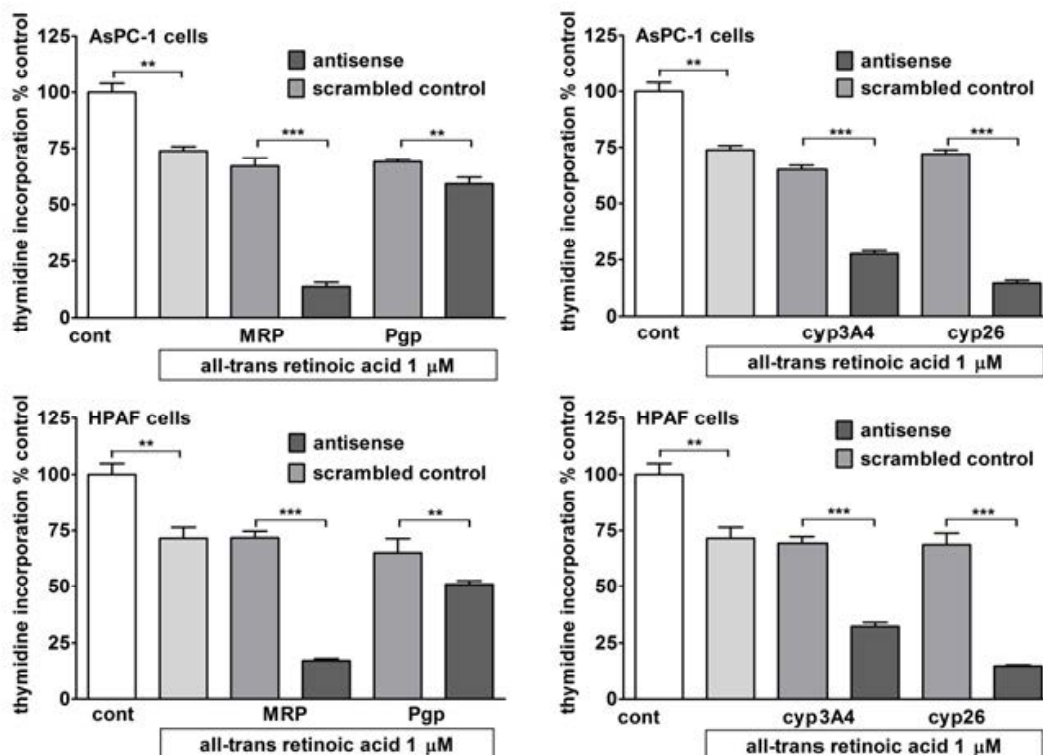


Figure 3: Effect of co-treatment with all-trans retinoic acid and antisense oligonucleotides against multidrug resistance proteins (MRP or Pgp) (A and C) or against CYP450 enzymes (3A4 or CYP26) (B and D) for three days on proliferation of AsPC-1 and HPAF cells respectively, measured as thymidine incorporation. Data shown are mean \pm SEM of the % of untreated control, from four separate experiments performed in triplicate. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

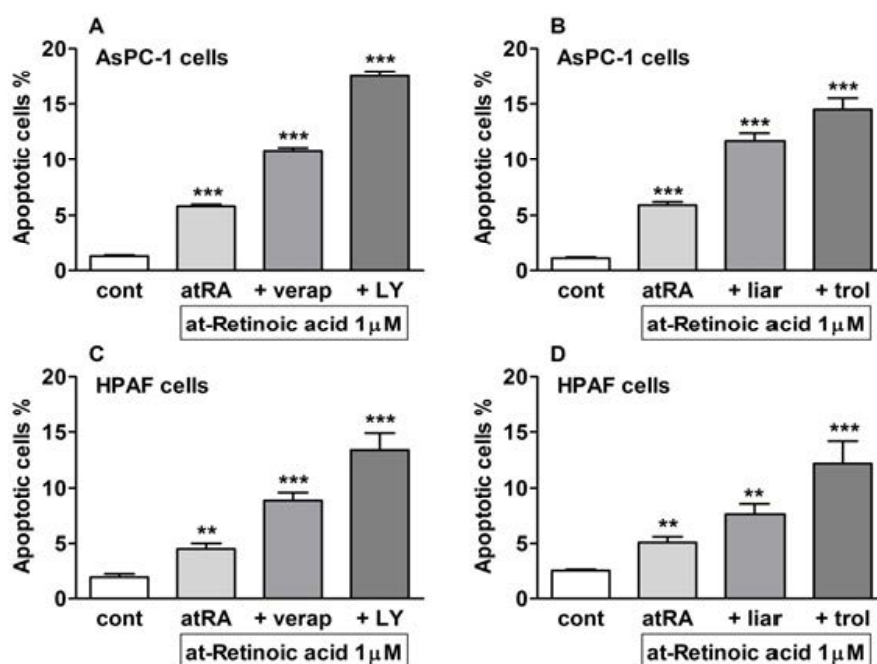


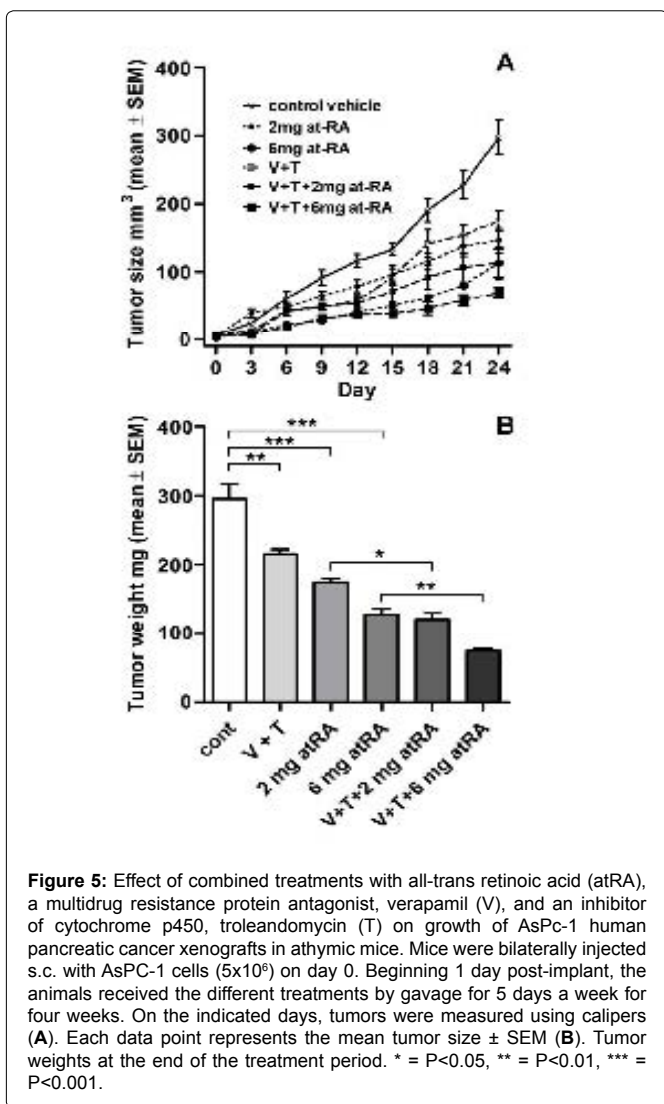
Figure 4: Effect of co-treatment of AsPC-1 cells with all-trans retinoic acid (at-retinoic acid) and multidrug resistance protein antagonists (A and C) or inhibitors of cytochrome-P450 enzymes (B and D) on apoptosis of AsPC-1 and HPAF cells respectively, measured by the TUNEL assay. Cells were treated for three days with atRA alone or in combination with verapamil (verap), LY335979 (LY), liarozole (liar), troleandomycin (trol). Floating cells were pooled to harvested adherent cells, fixed, TUNEL assay stained, counterstained with propidium iodide and analyzed by flow cytometry. Data shown are mean \pm SEM of the % of apoptotic cells from four separate experiments in triplicate. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Discussion

In the belief that redifferentiation therapy is a rational approach to anticancer therapy, the present study was designed to provide further information on the therapeutic use of retinoids. At high concentrations with limited side-effects, at RA has promising *in vitro* anticancer activities on human pancreatic adenocarcinoma cell lines. However, atRA efficiently induces its own catabolism by cytochrome P450-dependent mechanisms that limit its intracellular bioavailability. Another limiting mechanism is through exclusion of retinoids from cells through multidrug resistance proteins. Over-expression of multidrug resistance proteins and CYP enzymes in tumor tissues, including pancreas, is associated with resistance to drug treatment. Multidrug resistance proteins, such as MRP induces resistance by pumping cytotoxic drugs out of tumor cells [22], while CYP enzymes work by inactivation of anti-cancer drugs [29].

In the present study, we tested the ability of pharmacological inhibitors of each pathway to potentiate the anti-cancer effects of atRA. The studies tested the effects of verapamil and troleandomycin in combination with atRA. The model used was an aggressive human pancreatic adenocarcinoma (AsPc-1) cell line previously reported to be retinoid resistant *in vitro* and in athymic nude mice as a xenograft. The *in vitro* studies used verapamil, LY335979, or quinidine as structurally unrelated pharmacological antagonists of MRP [30], and troleandomycin, liarozole, or clotrimazole as structurally unrelated pharmacological inhibitors of CYP450s [31,32]. As single agents or in combination, these drugs did not show significant antiproliferative or apoptosis-inducing effects on AsPc-1 or HPAF cells *in vitro*. On the other hand, atRA alone at a low concentration significantly reduced

the growth of the cells following 3 days of treatment in our previously optimized phenol red- and serum-free medium. The combined treatment each of these drugs significantly potentiated the anti-proliferative and apoptosis-inducing effects of atRA in AsPc-1 and HPAF cells. In addition, specific antisense oligonucleotides against the cytochrome P450 (CYP450 3A4, and CYP26) isozymes, which are implicated in retinoid catabolism, and antisense against MRP and Pgp, implicated in retinoid efflux, potentiated the *in vitro* antiproliferative effect of atRA on AsPc-1 cells. In contrast, their scrambled control antisense oligonucleotides were not effective. The *in vitro* effects of the combined therapy on proliferation were also reproduced in Capan-2 pancreatic cancer cells. Overall, our *in vitro* results show that co-administration of Pgp and CYP450 inhibitors improves the responsiveness to atRA, presumably through improved intracellular accumulation of active atRA. The *in vivo* effects of low doses of oral atRA combined with verapamil and troleandomycin on the growth of AsPc-1 cells xenografts in athymic mice were then investigated. When administered alone, atRA therapy caused significant reduction of tumor growth at 2 and 6 mg/kg/day. However, treatment with atRA in combination with verapamil and troleandomycin was substantially more effective. *In vivo* the combination of verapamil and troleandomycin also caused significant reduction in tumor growth, although no effect was seen with these drugs *in vitro*. It is possible that the combination of these pharmacological agents enhanced the growth inhibitory effect of endogenous retinoids. The results support our *in vitro* findings and affirm our proposal that interfering with mechanisms that reduce intracellular levels of atRA can enhance the inhibitory effects of atRA on pancreatic cancer growth, suggesting that this regimen might be useful in chemoprevention or treatment of



pancreatic neoplasms. The inhibitory effects of different retinoids on pancreatic cancer have been extensively investigated [4-11]. However, the potentiation of the anti-cancer effects of non-toxic very low doses of atRA both *in vitro* and *in vivo* by interfering with mechanisms that antagonize the buildup of an effective intracellular atRA levels are novel findings. We hypothesized that the effect could be due to increased exposure of the tumor cells to atRA. The direct biological effects of atRA and its analogues on pancreatic cancer are complex. We have shown that retinoids induce antiproliferation, redifferentiation, trans differentiation, and apoptosis of pancreatic cancer cells *in vitro* [5,26,27]. The anticancer effects of retinoids on pancreatic cancer are attributed at least in part to the involvement of growth inhibitory and apoptosis-inducing mechanisms including a decrease in the Bcl-2/Bax ratio [4] as well as the induction of signaling through the TGF-β2 pathway [7]. A major obstacle to cancer treatment is the development of acquired drug resistance. Because retinoids are critical regulators of numerous physiological functions, retinoid target cells, such as pancreatic cancer cells, are equipped with several mechanisms that act in concert to buffer retinoid excess [33,34]. MRP is the major resistance factor in several pancreatic cancer cell lines [33-37]. Verapamil, a widely-used non-specific antagonist for such ATP-dependent membrane pumps, had no antiproliferative effect at the

concentration used; however, combined with atRA it synergistically inhibited proliferation and induced apoptosis. CYP450 enzymes, on the other hand, provide the major catabolic pathway for retinoids. Induction of CYP450 activity correlates with the development of retinoid resistance and lower plasma retinoid levels *in vitro* and *in vivo* [12,34,38-40]. The normal pancreas expresses a pattern of CYP450 subfamilies. These enzymes are over expressed in pancreatic cancer cells and expression is further induced by retinoids [19,41]. This study is the first to show the enhanced therapeutic index of atRA in pancreatic cancer cells resulting from the combined administration of CYP450 inhibitors and antisense oligonucleotides. Interestingly, Osanai et al. [42] suggested an indirect oncogenic role of CYP26A found to be upregulated in a number of cancers. This is a consequence of the enhanced atRA catabolism that renders cells less susceptible to apoptosis. However, several other possible mechanisms that have to be investigated could underline the positive activity of this combined regimen. For example, a CYP450 inhibitor of the same family as clotrimazole, namely ketoconazole not only inhibits CYP450 activity but also antagonizes MRPs [43]. Likewise, ketoconazole potentiates the anticancer efficacy of systemic atRA in bladder cancer patients [44]. The ability of nonspecific inhibitors, antagonists, and specific antisense oligonucleotides against MRPs and CYP450 to potentiate the effectiveness of retinoids substantiates the hypothesis that failure of delivery and rapid clearance of retinoids reduces their cellular sensitivity. Although approaches using atRA have been reported for the chemoprevention of different types of tumors including pancreatic cancer, there have been no reports investigating an adjuvant approach in combination with inhibitors targeting MRPs and CYP450 enzymes for pancreatic cancer, especially for *in vivo* models. Our findings reveal the promising potential for using natural retinoids in the treatment of pancreatic cancer, particularly when used in combination regimen with clinically safe drugs that maintain effective intracellular concentrations with avoidance of toxicity. The preclinical efficacy of the studied combination of therapy is warranted. There are several other possible mechanisms of action that have to be investigated and could underlie the positive activity of this regimen.

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