Anti-Cancer Drugs 1996, **7**, pp. 312–320

Liarozole amplifies retinoid-induced apoptosis in human prostate cancer cells

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Beta-carotene, canthaxanthin and retinoic acid (RA) inhibited growth of human DU145 prostate cancer cells by 45, 56 and 18%, respectively. Lycopene was also found to inhibit cell growth. Other carotenoids including xanthophyll (lutein), cryptoxanthin and zeaxanthin were less effective. Liarozole (a novel imidazole-derived inhibitor of intracellular RA catabolism) had a modest effect upon cell growth, this drug significantly amplified the pro-apoptotic actions of β-carotene and RA. RA-induced expression of thymosin β-10, an apoptotic accelerant, was associated with increased nuclear DNA nicking as measured using TUNEL. Liarozole enhanced the pro-apoptotic actions of RA upon DNA fragmentation in a dose-dependent manner. These actions were accompanied by inhibition of the cell survival factor *bcl*-2. Liarozole may thus prove useful as a novel chemotherapeutic/chemopreventative agent by boosting retinoid-induced apoptosis in the prostate.

Key words: Apoptosis, liarozole, prostate cells, thymosin.

Introduction

Prostate cancer is the leading cause of death in men over the age of 60, with the incidence rate quoted as 25% in those individuals past the age of 70.[1] Indeed, prostate cancer is the second most common male malignancy in the US following lung cancer.[2] A combination of factors is implicated in the occurrence of this malignancy, these include race, sexual activity, socio-economic status, venereal disease as well as marital status. Black US males appear to be predisposed. Although recent epidemiological investigations suggest a link between dietary intake of carotenoid-rich vegetables and a reduced risk of certain cancers including that of the prostate,[3] little information is currently available regarding the molecular basis of these beneficial effects of β-carotene or the other carotenoids. It is known that the β-carotene metabolite, i.e. retinoic acid (RA), inhibits prostatic 5-α-reductase, an enzyme which plays a critical role in the development of benign prostatic hypertrophy (BPH).[4] More recent studies using *N*-(4-hydroxyphenyl)retinamide (4-HPR) indicate that these lipids can prevent the onset of cancer in the rat prostate.[5] It is for these reasons that increased attention has become focused upon elucidation of the molecular mechanisms by which chemopreventative carotenoids and their retinoid derivatives act to inhibit malignant transformation of the prostate.

It is a well established fact that the β-carotene metabolite RA induces and represses numerous genes which modulate cell growth and differentiation (reviewed[6] in Sporn and Roberts[6]). RA interacts with specific response elements via activation of ligand-specific nuclear receptors (RAR and RXR).[7] Ongoing research is now beginning to indicate that perhaps retinoids exert their anticancer actions by permitting cells to acquire the capacity to undergo appropriate programmed cell death (PCD). RA has now been shown to increase apoptosis in a number of cell and tissue types, including neuroblastoma cells.[8] Apoptosis is a vigorous form of cell suicide[9] and this PCD is characterized by a variety of easily identified morphological and biochemical criteria including, but not limited to, contraction, cell surface blebbing/ruffling, formation of 'apoptotic bodies', cell detachment and degradation of the genome.[10] PCD is modulated by an ever increasing list of proteins including *bcl*-2,[11] p53,[12] *myc*,[13] *bax*[14] and *bcl*-x.[15]

Recently, thymosin β-10, a 43 residue, 5 kDa (MW) protein was found to be highly expressed in many tumors including that of the prostate.[16] Additional work indicated that expression of this protein could be modulated by RA.[17] Increased thymosin β-10 is correlated with decreased cell proliferation.[17,18] These observations prompted the notion that perhaps this protein played a role in the cell cycle[19] or even in cell migration.[20] The β-thymosins were subsequently shown to be G-actin-binding proteins.[21–23] In turn, G-actin binds to and inhibits DNase I—an endonuclease believed to play a cardinal role in choreographing destruction of the genome during apoptosis.[24]

This work was supported by a grant from the National Institutes of Health (NIDDK) DK47588-04.

In this series of experiments the ability of RA to curtail division of human prostate cells is attributed in part to its ability to prepare cells to undergo apoptosis. The anti-cancer drug, liarozole, amplifies the pro-apoptotic propensity of RA—this is associated with inhibition of *bcl-2* and up-regulation of thymosin β-10, a protein which severs actin filaments.

Materials and methods

Drugs and chemicals

Liarozole fumarate (R 85246) was a generous gift from Dr Roland de Coster (Janssen Research, Beerse Belgium). All-*trans* RA (Ro 1-5488), lycopene (Ro 1-9251), lutein (Ro 5-6506), zeaxanthin (Ro 1-9371), cryptoxanthin (Ro 4- 0763), β-carotene (Ro 1-8300) and canthaxanthin (Ro 1-9915) were gifts from Hoffmann-La Roche (Nutley, NJ). Lithium chloride and phenol were obtained from Sigma. DMEM, antibiotics and fetal calf serum were from Gibco.

Cell line

The human prostate cancer cell line used in the present study (DU145) was purchased from the American Type Tissue Collection (Bethesda, MD), and maintained in DME containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml) under an atmosphere of 5% carbon dioxide in humidified air. These cells generally grew with a polygonal, non-motile, epithelial-like morphology.

Drug-induced growth inhibition

Human DU145 cells (innoculated at 10 000 cells/well) were plated out in multiwell culture plates (Microtest III, 96-well; Falcon 3072, Becton-Dickinson) in 200 μl of DME. Beta-carotene and other lipids were added to the culture medium so as to give a final concentration of 10^{-10} and 10^{-6} M and cells cultured for up to 96 h. The number of viable cells was then determined using a non-radioactive cell proliferation kit (Cellititer-96; Promega), based upon the metabolic conversion of a tetrazolium salt into a blue formazan chromogen product, the abundance of which was ascertained at 570 nm in an automated ELISA plate reader.[27] Parallel experiments using a hemocytometer confirmed that MTT assay data was a true representation of the number of viable cells and not due to drug interference with the cellular metabolism of the prechromagen tetrazolium compound.

Morphometric analysis of cells

Cell cultures were examined under the light microscope for overt signs of cell death—which included morphological changes such as rounding up and detachment (indicated by increased refraction). Cell shape and density of staining with various antisera was analyzed by use of a scanning image analysis facility (ImageMaster; Pharmacia).

Fluorescence staining of chromatin

Cell monolayers were rinsed with 0.1 M phosphate buffered saline (pH 7.4) (PBS) and stained with Acridine Orange (10 μg/ml) in the dark for 20 min at room temperature. Excess dye was removed by extensive washing with PBS. Slides were mounted in glycerine mountant containing antifade and were visualized under fluorescent light. Heavy fluorescent staining of the nucleus was indicative of apoptotic cells.[25]

Immunohistochemistry

Cells were plated onto sterile poly-L-lysine coated cover slips in DMEM containing FCS (10%) and antibiotics. Post-treatment, cells were fixed *in situ* with 3.7% formaldehyde in PBS (pH 7.3), washed extensively in PBS and incubated in Block (3% normal Sheep serum, 0.2% Triton X-100, 0.5% sodium azide in PBS) overnight at 4°C. Following a wash in 1 × PBS, cell layers were incubated with primary antibody rabbit anti-thymosin β-10 at a dilution of 1 : 1000 for 2 days at 4°C. Cell layers were then washed in 1 × PBS (without sodium azide) and incubated with the secondary antibody [peroxidase-labeled affinity purified antirabbit IgG (H + L) (Vector)] overnight at 4°C. Slides were washed twice in 1 × PBS and developed in a solution of 500 μg/ml di-amino-benzidine (DAB) in 50 mM Tris–HCl, pH 7.6, containing hydrogen peroxide. Stained slides were analyzed using a laser-based digital imaging system (ImageMaster; Pharmacia). Thymosin β-10 protein levels were deduced and expressed as percent maximal response (optical density).

Cellular DNA fragmentation assay

Apoptosis was quantified using a commercially available DNA fragmentation ELISA. Exponentially proliferating DU145 human prostate cancer cells (approximately 4000 per 200 μl) were incubated with 5-bromo-2′-deoxy-uridine (BrdU) (10 μM) overnight at 37°C in a humidified atmosphere containing 5% CO_2. RA (10^{-6} to 10^{-10} M) and RA (10^{-7} M) plus liarozole fumarate (10^{-5} M) were added and culture continued for a further 24 h. Cell layers were lysed and aliquots of the medium transferred to 96-well microtiter plates pre-coated with anti-DNA antibody according to the manufacturer's specifications (Boehringer Mannheim) and incubated at 4°C for 16 h. Plates were washed three times with 250 μl of washing buffer [2 mM EDTA, Tween 20 (0.2%)] and anti-BrdU–peroxidase conjugate added for 90 min at 37°C. Following an additional wash, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate for 30 min at room temperature, the reaction was stopped by acidification with sulfuric acid and the absorbance (450 nm) read on an automated ELISA reader. Data are presented as percentage maximal response.

DNA nicking

Prostate cell apoptosis was also measured using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method.[43] Slide-bound prostate cells were fixed as above and endogenous peroxide suppressed with 0.3% hydrogen peroxidase in methanol for 30 min. Cell layers were then rinsed in PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Monolayers were washed again with PBS and 50 μl of TUNEL buffer added (containing TdT, TdT buffer and dNTPs) added to the surface. Monolayers were incubated at 37°C for 60 min, washed with PBS and incubated with antifluorescein antiserum for a further 30 min. Monolayers were washed again with PBS and developed using DAB for 10 min at room temperature. Cell nuclei exhibiting dark brown coloration were deemed TUNEL + apoptotic. The optical density of TUNEL+ nuclei was determined by image analysis and the degree of DNA nicking expressed as the percent maximal response.

Extraction of RNA

Control and treated cell cultures were washed several times in 3 ml of ice-cold 1 × PBS and homogenized in a solution containing 4 M guanidinium thiocyanate, 35 mM sodium citrate, pH 7.0, 100 mM 2-mercaptoethanol and 0.5% sarcosyl as described elsewhere.[20] Ethanol precipitated RNA pellets were resuspended in diethylpyrocarbonate-treated sterile water. RNA obtained by this method usually exhibited an $A_{260/280}$ of greater than 1.8.

Radiolabeling and specificity of thymosin β-10 cDNA

Molecular cloning and sequencing experiments have unambiguously demonstrated that the 3′ untranslated regions of thymosin β-10 and thymosin β-4 mRNAs exhibit less than 25% identity.[17] Hence, use was made of the 3′ untranslated region of the human thymosin β-10 cDNA to monitor thymosin β-10 levels in response to RA, β-carotene and liarozole. The random priming technique was used to label clone 6[16] to a specific activity of 4×10^9 c.p.m./μg DNA with [^{32}P]dCTP (Amersham). This cDNA was highly specific for thymosin β-10 transcripts as previously demonstrated.[18]

An 18S ribosomal RNA cDNA was used as a control (housekeeping gene). The *bcl-2* cDNA was purchased from Cambridge Bioscience (Cambridge, UK).

Northern blot analysis

Total RNA (10 μg) was mixed with RNA loading buffer containing formaldehyde and glycerol, heat-denatured for 15 min at 70°C, cooled to 4°C and fractionated in a 1.2% agarose gel containing 2.2 M formaldehyde overnight.[29] Gel-bound RNA was transferred to Nylon membranes (Nytran) overnight by the capillary blotting technique.[29] Filters were baked at 80°C for 1 h in a vacuum oven.

Hybridization and dehybridization

For the thymosin β-10 cDNA filters were prehybridized at 42°C for 3 h in a solution containing 0.75 mM sodium chloride, 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 10 × Denhardt's solution, 0.2% SDS, 2% glycine and 50% formamide. Hybridization was for 16 h at 42°C in a solution containing 0.75 mM sodium chloride, 50 mM sodium phosphate (pH 7.0), 50 mM EDTA, 0.2% SDS, 2% glycine and 2 × Denhardt's 50% formamide. Final filter washings were carried out at 65°C in 0.2 × SSC containing 0.1% SDS.

Analysis of data

Data analysis was performed using the Student's *t*-test.

Results

Inhibitory actions of RA and carotenoids upon prostate cell division

Addition of β-carotene (10^{-8} to 10^{-10} M for 5 days) to cultures of prostate cancer cells resulted in a significant ($p < 0.02$) inhibition of cell proliferation. The antiproliferative action of β-carotene was most evident at 10^{-8} M ($p < 0.01$): any increase above this concentration had little further inhibitory effect. Data shown in Figure 1 indicate differing degrees of inhibition of cell proliferation with equivalent concentrations of an assortment of carotenoids (as well as RA) (10^{-8} M for 5 days). Remarkably, canthaxanthin and β-carotene proved to be the most effective inhibitors of cell proliferation. Surprisingly, lycopene was apparently a more efficacious inhibitor of cell proliferation than RA (Figure 1). Xanthophyll, cryptoxanthin and zeaxanthin were all less potent antiproliferative agents than RA, β-carotene or canthaxanthin.

Effects of liarozole on cell growth inhibition

Figure 2 shows that liarozole (fumarate salt) alone has little or no effect on cell growth—although a mild stimulatory action was consistently observed with very high concentrations of the drug (10^{-5} M). Liarozole potentiated the antiproliferative actions of both RA (10^{-7} M) and β-carotene (10^{-8} M) in a concentration-dependent fashion. In fact, coincubation with liarozole (10^{-5} M) effectively doubled the degree of inhibition achieved by RA or β-carotene alone (Figure 2). Time-course experiments revealed that the amplifying actions of liarozole (10^{-5} M) upon the antiproliferative actions of RA (10^{-7} M) became detectable as early as 24 h, evident after 5 days and extremely pronounced 10 days after addition of the drugs to cell cultures (data not shown).

Effects of RA, β-carotene and liarozole upon expression of the thymosin β-10 gene

It was previously shown that the RAR-α gene is expressed in the DU145 prostate cancer cell line,[32] hence this cell line possesses a retinoid receptor signal transduction pathway. In view of the fact that previous experiments had indicated that the thymosin β-10 gene is responsive to anticancer retinoids such as RA,[17] it was deemed prudent to examine expression of this gene in DU145 cells. DU145 cells expressed a single 600 nucleotide species of thymosin β-10 mRNA (Figure 3). RA (10^{-7} M), β-carotene (10^{-8} M) and canthaxanthin (10^{-8} to 10^{-10} M) all *stimulated* the steady-state levels of thymosin β-10 mRNA (Figure 3). Actions of RA were significantly amplified when liarozole was included in the culture medium (Figure 4). Liarozole itself had no discernable effect upon thymosin β-10 mRNA at 'low' concentrations—but affected β-10 gene expression at higher doses. When liarozole (10^{-5} M) was added in combination with RA (10^{-7} M), stimulation of thymosin β-10 mRNA was much more pronounced (16-fold increase above control) than that achieved with RA alone (6-fold).

The cytoplasmic compartment of the vehicle-treated human DU145 prostate cancer cells exhibited relatively low but detectable levels of the thymosin β-10 polypeptide (Figure 5). Nuclei were essentially devoid of immunostaining for thymosin β-10 (data not shown). Inclusion of RA (2×10^{-7} M for 3 days) resulted in an increased expression of the protein (2- to 5-fold increase above control values) (Figure 5). This enhanced level of β-10 expression was preferentially localized inside cells which displayed a change in their morphology (i.e. rounding up, ruffled surfaces, condensation of nuclear chromatin) (data not shown). Inclusion of β-carotene (10^{-8} M for 3 days) also stimulated the level of thymosin β-10 detected by this immunostaining procedure (data not shown). The combined effect of liarozole and RA was to increase the proportion of dead/apoptotic prostate cells which appeared to be more refractory under the light microscope.

Bcl-2 expression

Bcl-2 promotes cell survival by counteracting PCD via an as yet unidentified mechanism.[11] As a rule, *bcl*-2 is not expressed in the normal human prostate gland, whilst prostate cancer is associated with increased expression of this gene. *Bcl*-2 mRNA and

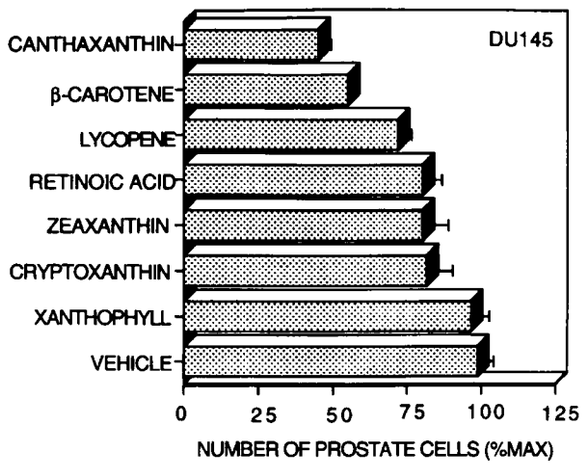

Figure 1. Comparative antiproliferative actions of RA and various carotenoids (all at 10^{-8} M for 3 days) in human DU145 cells. Each point on the graph represents the mean + SD for five independent determinations using the MTT dye assay.

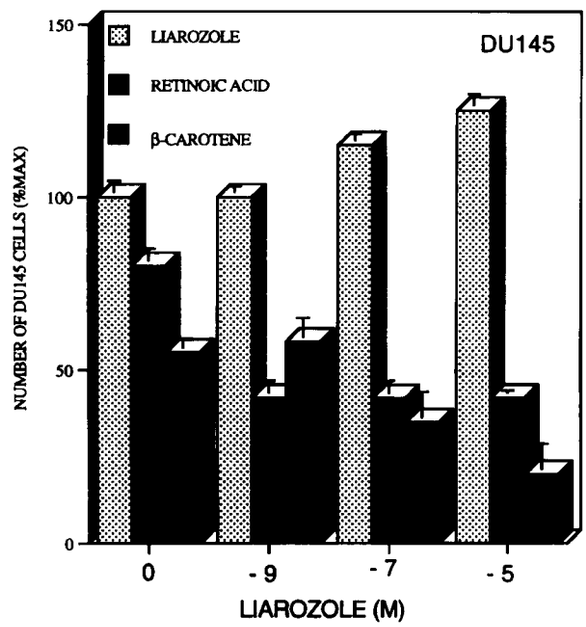

Figure 2. Effects of liarozole fumarate (R85246) upon the relative antiproliferative efficacy of RA (10^{-7} M) and β-carotene (10^{-7} M) in DU145 cells. Cells were exposed to increasing amounts of liarozole in the presence or absence of RA or β-carotene for 5 days. Each point represents the mean + SD.

protein were both found to be present in the DU145 cell line. Generally, RA inhibited prostate cell levels of *bcl*-2 message and protein. RA together with liarozole were more effective in suppressing the *bcl*-2 gene than either agent alone (Figure 6). Thus, *bcl*-2

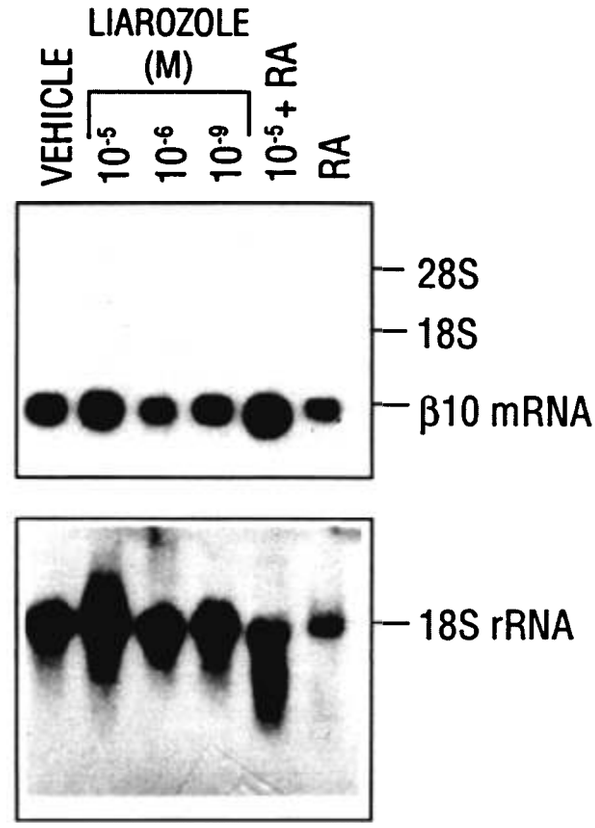

Figure 3. Northern blot analysis of thymosin β-10 mRNA in DU145 cells. Cells were treated with RA and/or with liarozole. Each lane of the gel contained 10 μg of total RNA.

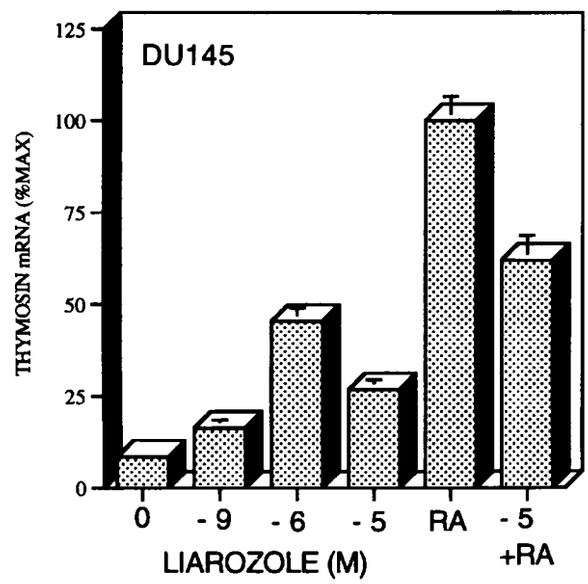

Figure 4. Relative abundance of thymosin β-10 mRNA in DU145 prostate cells following 3 days exposure to liarozole alone or together with RA (10^{-7} M). Each point is the mean + SD of the percentage maximal densitometer response.

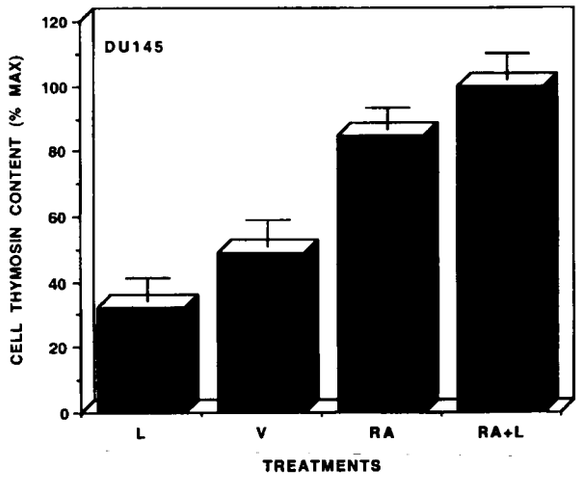

Figure 5. Effects of liarozole and RA upon prostate cell thymosin β-10 protein content. Each bar represents the mean + SD of the relative concentration of thymosin as deduced by digital analysis of cells stained with a specific antiserum. Data are expressed as the percent maximal response. L, liarozole; V, vehicle; RA, retinoic acid.

and thymosin β-10 gene expression appeared to be mutually exclusive in these experiments.

Effects of RA and liarozole upon DNA fragmentation

To examine the possibility that RA could be controlling prostate cell PCD, the effect of this lipid upon cellular DNA cleavage were ascertained.

RA (10^{-9} to 10^{-6} M for 24 h) provoked a dose-dependent enhancement of the amount of BrdU-labeled DNA fragments released into the prostate cell cytoplasm and detected by the ELISA (Figure 7). DNA degradation was increased slightly in the presence of 10^{-8} M RA ($p > 0.05$), but was significantly stimulated by the higher concentrations of RA (10^{-6} M) ($p < 0.05$) (Figure 7). Addition of liarozole (10^{-5} M) itself for 24 h induced a very small increment in endonuclease activity, but this was not significantly different to the effect obtained with ethanol vehicle (below 0.001% v/v) ($p > 0.05$). Simultaneous addition of liarozole (10^{-5} M) together with RA (10^{-6} M) resulted in a highly significant stimulation of prostate cell DNA fragmentation ($p < 0.01$). These effects were obtained within 24 h—other data indicated that retinoid-induced DNA degradation could be achieved in less than 4 h using the same assay (data not shown). Hence, even within 24 h, liarozole was seen to enhance the apoptotic efficacy of this natural retinoid. These

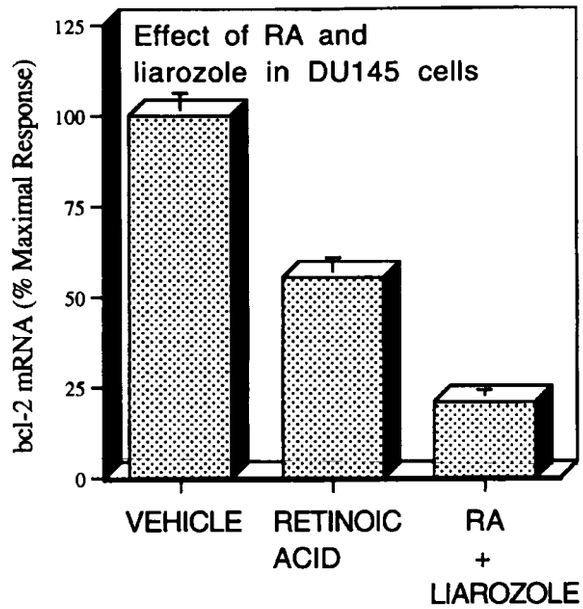

Figure 6. Effects of liarozole and RA upon *bcl*-2 mRNA in human DU145 prostate cells. Cells were exposed to liarozole (10^{-5} M) or a combination of RA (10^{-7} M) plus liarozole (10^{-5} M) for 3 days. Each point on the graph represents the mean + SD of the percentage maximum densitometer response derived from scanning several *bcl*-2 probed slot-blots.

results are the first to demonstrate that the antitumor drug liarozole increases retinoid-induced apoptosis in human cancer cells.

Effects of liarozole and RA upon DNA nicking

As an additional check, the effects of RA and liarozole upon prostate cell PCD were also monitored visually using TUNEL to detect intranuclear DNA damage *in situ*.[43] Figure 8 indicates that apoptosis measured by TUNEL was very similar to that observed in the DNA fragmentation assay. Again, a combination of liarozole and RA resulted in a synergistic stimulation of nuclear DNA nicking.

Conclusion

Whilst evidence exists to support the notion that retinoids and to some extent their carotene precursor β-carotene can inhibit growth of many cell-types including prostate cells,[6] relatively little is known about the basic mechanisms by which these lipids exert their anticancer actions. Retinoids and β-car-

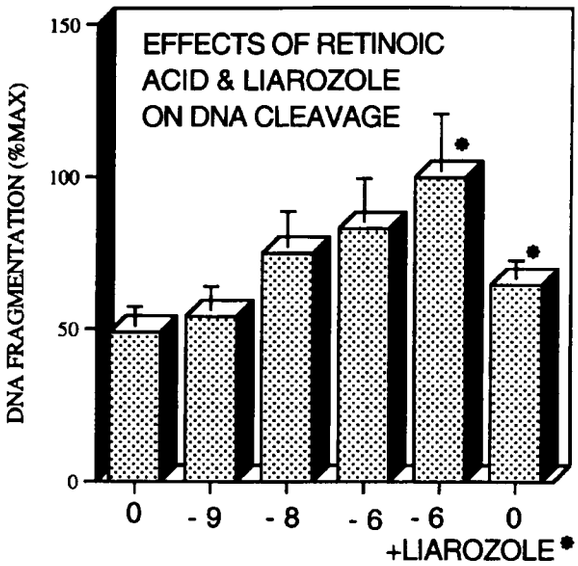

Figure 7. Effects of RA and liarozole upon cellular DNA fragmentation. DU145 cells were prelabeled with BrdU for 24 h and then exposed to RA (10^{-6} to 10^{-9} M) of RA (10^{-6} M) plus liarozole (10^{-5} M) for 24 h*. Each point represents the mean + SD of the percentage maximal DNA fragmentation response.

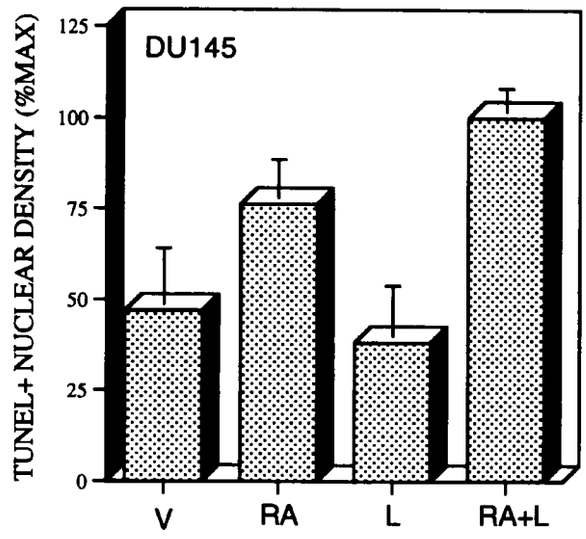

Figure 8. TUNEL analysis of the pro-apoptotic actions of RA and liarozole in human prostate cancer cells. Each bar value corresponds to the mean + SD of the degree of TUNEL+ nuclear staining as deduced by image analysis (optical density) of cell nuclei.

otene can act inside the target cell as antioxidants by scavenging free oxygen radicals.[33] Other experiments indicate that retinoids, via specific nuclear receptors, modulate expression of genes which act at critical phases as cell cycle check-points.[6] The present series of experiments has attempted to analyze the effects of RA, related carotenoids and a novel drug—liarozole (which inhibits intracellular RA degradation)—upon prostate cell proliferation and apoptosis. RA, β-carotene and canthaxanthin induce significant antiproliferative actions in the DU145 cell line. Surprisingly, β-carotene appeared to be a more potent inhibitor of cell growth than its biologically active metabolite, RA. As intracellular degradation of RA could have been a factor here, the effects of liarozole were studied. Liarozole (fumarate salt), a novel imidazole-derived antitumor inhibitor of cytochrome P-450-dependent RA 4-hydroxylase,[34] known to increase intratumor retinoid accumulations,[35] dramatically enhanced the antiproliferative actions of RA as well as that of β-carotene (Figures 2 and 3). This amplifying effect of liarozole was dose- and time-dependent, and was consistent with its actions upon other tumor cell lines (MCF-7).[31]

As very little is known regarding the molecular mechanism by which liarozole acts to deter tumor growth and metastasis, it was deemed logical to identify possible targets for this novel drug. Previous observations had already demonstrated that RA could stimulate expression of thymosin β-10 in a number of tumor cell lines.[18] It became evident that the stimulatory action of RA upon prostate cell thymosin β-10 mRNA (6-fold increase above control levels, $p < 0.05$) was significantly amplified in the presence of liarozole (10^{-5} M) (16-fold increase over control values). Thymosin β-10 is a major intracellular scavenger of monomeric G-actin[21,22] and G-actin inhibits DNase 1.[24] More recently thymosin β-10 was shown to accelerate PCD,[42] possibly by removing G-actin inhibition of this endonuclease.[36] Certainly then, in the present study, *increased* thymosin β-10 expression is associated with *decreased* cell growth and increased apoptosis. These results are consistent with those recently reported in human neuroblastoma cells (LA-N-5),[8] where PCD induced by a synthetic retinoid (4-HPR) is clearly associated with cell detachment.[8]

Apoptosis constitutes a natural and fundamental physiological suicide process which is controlled by a wide spectrum of both genetic and epigenetic pathways, and which plays a cardinal role in maintenance of tissue phenotype.[10] The list of genes and agents that control apoptosis continues to expand.[36] Significantly, the 24–26 kDa (MW) *bcl*-2 protein blocks cell death provoked by many stimuli by interfering with the cell's lipid peroxidation pathway.[11,37] *Bcl*-2 (and its other related family

members) are of paramount importance in cancer biology because these survival proteins promote cellular immortality/oncogenic transformation.[11] Expression of *bcl*-2 is associated with unfavorable histology (and N-*myc* amplification) in human neuroblastoma.[38] However, *bcl*-2 inhibits chemotherapy-induced apoptosis in neuroblastoma.[39] In view of these observations, the effects of RA and liarozole were examined upon expression of this tumor promoting protein in the prostate cells. The ability of RA to repress *bcl*-2 mRNA was strengthened by coincubation with liarozole: this effect was mirrored by inhibition of the *bcl*-2 protein itself. Hence RA induces apoptotic competency in this prostate cancer cell line in part by inhibiting expression of *bcl*-2, presumably via up-regulating p53. Liarozole enhanced this effect by inhibition of intracellular RA breakdown.[34] These findings support the hypothesis that β-carotene and its active primary metabolite RA are capable of inhibiting prostate cancer cell proliferation partly by increasing cell loss via PCD.

Apoptosis is *the* major mechanism for cell elimination in tumors. Hence it would follow that proteins which contribute to or accelerate this process will be more abundant in tumor tissue—presumably, a low level of apoptosis in a particular tumor would be indicative of a poor prognosis. Thymosin β-10 (and β-4) are overexpressed in many tumor types, including prostate tumors.[16] To some extent the pro-apoptotic influence of RA could be due to its ability to increase tumor cell expression of thymosin β-10—which would tend to destabilize the cell actin cytoskeleton[22] and thence increase a particular cell's apoptotic proficiency.[42] The results of the present study indicate that liarozole acts at the molecular level by amplifying the ability of RA and β-carotene to inhibit the *bcl*-2 whilst up-regulating the G-actin binding protein thymosin β-10. The combined effect of these actions results in increased death of prostate cancer cells by apoptosis. These data together with other unpublished findings indicate that liarozole could also be beneficial in the treatment of other neoplasms including neuroblastoma, especially when given in combination with 4-HPR or other anti-cancer drugs.

Acknowledgments

I thank the Janssen Foundation for supplying the liarozole fumarate. I thank Dr PF Sorter (Hoffmann La-Roche, Nutley, NJ) for providing the synthetic carotenoids and retinoic acid. Professor S Christakos (UMDNJ, Newark, NJ) provided the 18S cDNA probe. I am grateful to Dr G Evangelatos (National Center for Scientific Research, Athens, Greece) for providing the antibody for thymosin β-10.

References

1. Mills PK, Beeson LW, Phillips RL, Fraser GF. Cohort study of diet and lifestyle and prostate cancer in adventist men. *Cancer* 1989; **64**: 598–604.
2. Byers TE, Graham S, Haughey BP, Marshall JR, Swanson MK. Diet and lung cancer: findings from the Western New York study. *Am J Epidemiol* 1987; **125**: 351–63.
3. Hsing AW, Comstock GW, Abbey H, Polk BF. Serologic precursor of cancer. Retinol and carotenoids and tocopherol and the risk of prostate cancer. *J Natl Cancer Inst* 1990; **82**: 941–6.
4. Halgunset J, Sunde A, Lundmo PI. Retinoic acid: an inhibitor of 5-alpha-reductase in human prostate cancer cells. *J Steroid Biochem* 1987; **28**: 731–6.
5. Pollard M, Luckert, PH, Sporn MB. Prevention of primary prostate cancer in Lobund-Wistar rats by *N*-(4-hydroxyphenyl)retinamide. *Cancer Res* 1991; **51**: 3610–1.
6. Sporn MB, Roberts AB. Role of retinoids in differentiation and carcinogenesis. *Cancer Res* 1983; **43**: 3034–40.
7. Mangelsdorf DJ, Borgmeyer U, Heyman RA, *et al.* Characterization of three RXR genes that mediate the action of 9-*cis* retinoic acid. *Genes Dev* 1992; **6**: 329–44.
8. Vinci AD, Geido E, Infusini E, Giaretti W. Neuroblastoma cell apoptosis induced by synthetic retinoid *N*-(4-hydroxyphenyl)retinamide. *Int J Cancer* 1994; **59**: 422–6.
9. Ellis RE, Yuan J, Horvitz HR. Mechanisms and functions of cell death. *Annu Rev Cell Biol* 1991; **7**: 663–98.
10. Schwartzman RA, Cidlowski JA. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocrine Rev* 1993; **14**: 133–51.
11. Hockenbery D, Nunez G, Milliman C, Schreiber R, Korsmeyer SJ. *Bcl*-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990; **348**: 334–6.
12. Isaacs WB, Carter BS, Ewing CM. Wild-type p53 suppresses growth of human prostate cancer cells containing p53 alleles. *Cancer Res* 1991; **51**: 4716–20.
13. Bissonette RP, Echeverri F, Mahboubi AD, Green DR. Apoptotic cell death induced by c-*myc* is inhibited by *bcl*-2. *Nature* 1992; **8**: 552–4.
14. Oltvai ZN, Milliman CL, Korsmeyer SJ. *Bcl*-2 heterodimerizes *in vivo* with a conserved homolog, *Bax*, that accelerates programmed cell death. *Cell* 1993; **74**: 609–19.
15. Boise LH, Gonzalez-Garcia M, Posta CE, *et al. Bcl*-x, a *bcl*-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 1993; **74**: 597–608.
16. Hall AK. Differential expression of thymosin genes in human tumors and in the developing human kidney. *Int J Cancer* 1991; **48**: 672–7.
17. Hall AK, Hempstead J, Morgan JI. Thymosin beta-10 levels in developing human brain and its regulation by retinoic acid in the HTB-10 neuroblastoma. *Mol Brain Res* 1990; **8**: 129–36.

18. Hall AK, Chen, S-C, Hempstead J, Morgan JI. Retinoic acid regulated thymosin beta-10 levels in rat neuroblastoma cells. *J Neurochem* 1991; **56**: 462–8.
19. Schobitz BG, Netzker R, Hannappel E, Brand K. Cell-cycle-regulated expression of thymosin beta-4 in thymocytes. *Eur J Biochem* 1991; **199**: 257–62.
20. Border BG, Lin S-C, Griffin, ST, Pardue S, Morrison-Bogorad M. Alterations in actin-binding beta-thymosin expression accompany neuronal differentiation and migration in rat cerebellum. *J Neurochem* 1993; **61**: 2104–14.
21. Safer D, Elzinga M, Nachmias VT. Thymosin beta-4 and Fx, an actin-sequestering peptide, are indistinguishable. *J Biol Chem* 1991; **266**: 4029–32.
22. Yu F-X, Lin S-C, Morrison-Bogorad M, Yin HL. Effects of thymosin beta-4 and thymosin beta-10 on actin structures in living cells. *Cell Motil Cytoskel* 1994; **27**: 13–25.
23. Sanders MC, Goldstein AL, Wang YL. Thymosin beta-4 (Fx peptide) is a peptide regulator of actin polymerization in living cells. *Proc Natl Acad Sci USA* 1992; **89**: 4678–82.
24. Lazarides E, Lindberg U. Actin is the naturally occurring inhibitor of deoxyribonuclease 1. *Proc Natl Acad Sci USA* 1974; **71**: 4742–6.
25. Waters CM, Moser W, Walkinshaw G, Mitchell IJ. Death of neurons in the neonatal rodent and primate globus pallidus occurs by a mechanism of apoptosis. *Neurscience* 1994; **63**: 384–94.
26. Lin SC, Morrison-Bogorad M. Cloning and characterization of testis- specific thymosin beta-10 cDNA: expression in post-meiotic male germ cells. *J Biol Chem* 1991; **266**: 23347–53.
27. Mosmann T. Rapid colorimetric assay for cellular growth and survival—application to proliferation and cytotoxicity assays. *J Immunol Methods* 1987; **65**: 55–63.
28. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987; **162**: 156–9.
29. Thomas P. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci USA* 1980; **77**: 5201–5.
30. De Coster R, Waters W, Van Ginckel R, *et al.* Experimental studies with liarozole (R 75 251): and antitumoral agent which inhibits retinoic acid breakdown. *J Steroid Biochem Mol Biol* 1992; **43**: 197–201.
31. Wouters W, v Dun J, Dillen A, Coene M-C, Cools W, De Coster R. Effects of liarozole, a new antitumoral compound, on retinoic acid-induced inhibition of cell growth and on retinoic acid metabolism in MCF-7 human breast cancer cells. *Cancer Res* 1992; **52**: 2841–6.
32. Hall AK. Unpublished observations 1991.
33. Tsuchiya M, Scita G, Thompson DFT, Kagen VE, Livrea MA. Retinoids and carotenoids are peroxyl radical scavengers. In: Livrea MA, Packer L, eds. *Retinoids: progress in research and clinical applications.* New York: Marcel Dekker: 525–36.
34. Van Wauwe J, Ceone M-C, Goossens J, Van Nijen G, Cools G, Lauwers W. Effects of cytochrome P-450 inhibition on the *in vivo* metabolism of *all-trans* retinoic acid in rats. *J Pharmac Exp Ther* 1990; **252**: 365–9.
35. End DW, Garrabrant TA. Effects of liarozole fumarate (R85246) on the levels of endogenous retinoic acid in murine solid tumors: comparison to exogenous retinoic acid. *Proc Am Ass Cancer Res* 1993; **34**: 108.
36. Hall AK. Molecular interactions between G-actin, DNase 1 and the beta-thymosins in apoptosis: a hypothesis. *Med Hypoth* 1994; **43**: 125–31.
37. Hockenbery DM, Oltvai ZN, Yin X-M, Milliman CL, Korsmeyer SJ. *Bcl-2* functions in an antioxidant pathway to prevent apoptosis. *Cell* 1993; **75**: 241–51.
38. Castle VP, Heidelberger KP, Bromberg J, Ou X, Dole M, Nunez G. Expression of the apoptosis-suppressing protein, *bcl-2*, in neuroblastoma is associated with unfavorable histology and N-*myc* amplification. *Am J Pathol* 1993; **143**: 1543–50.
39. Dole M, Nunez G, Merchant AK, *et al. Bcl-2* inhibits chemotherapy-induced apoptosis in neuroblastoma. *Cancer Res* 1994; **54**: 3253–9.
40. Bacher M, Rausch U, Goebel H-W, Polzar B, Mannherz HG, Aumuller G. Stromal and epithelial cells from rat ventreal prostate during androgen deprivation and estrogen treatment—regulation of transcription. *Exp Clin Endocrinol* 1993; **101**: 78–86.
41. Stearns ME, Wang M, Fudge K. Liarozole and 13-*cis*-retinoic acid anti-prostatic tumor activity. *Cancer Res* 1993; **53**: 3073–7.
42. Hall AK. Thymosin beta-10 accelerates apoptosis. *Cell Mol Biol Res* 1995; **41**: 167–80.
43. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death *in situ* via specific labeling of DNA fragmentation. *J Cell Biol* 1992; **119**: 493–502.

(Received 16 January 1996; accepted 13 February 1996)