Monensin Is a Potent Inducer of Oxidative Stress and Inhibitor of Androgen Signaling Leading to Apoptosis in Prostate Cancer Cells

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Abstract

Current treatment options for advanced and hormone refractory prostate cancer are limited and responses to commonly used androgen pathway inhibitors are often unsatisfactory. Our recent results indicated that sodium ionophore monensin is one of the most potent and cancer-specific inhibitors in a systematic sensitivity testing of most known drugs and drug-like molecules in a panel of prostate cancer cell models. Because monensin has been extensively used in veterinary applications to build muscle mass in cattle, the link to prostate cancer and androgen signaling was particularly interesting. Here, we showed that monensin effects at nanomolar concentrations are linked to induction of apoptosis and potent reduction of androgen receptor mRNA and protein in prostate cancer cells. Monensin also elevated intracellular oxidative stress in prostate cancer cells as evidenced by increased generation of intracellular reactive oxygen species and by induction of a transcriptional profile characteristic of an oxidative stress response. Importantly, the antiproliferative effects of monensin were potentiated by combinatorial treatment with the antiandrogens and antagonized by antioxidant vitamin C. Taken together, our results suggest monensin as a potential well-tolerated, in vivo compatible drug with strong proapoptotic effects in prostate cancer cells, and synergistic effects with antiandrogens. Moreover, our data suggest a general strategy by which the effects of antiandrogens could be enhanced by combinatorial administration with agents that increase oxidative stress in prostate cancer cells. Mol Cancer Ther; 9(12); 3175–85. ©2010 AACR.

Introduction

Prostate cancer is the most frequent cancer and the second leading cause of cancer deaths in the male population of the Western world. There is no efficient cure for advanced hormone refractory prostate cancer and novel ways to inhibit prostate cancer cell growth are needed. Because the majority of prostate tumors from patients with an androgen-independent disease overexpress androgen receptor (AR), AR and its coregulators are potential drug targets for the disease. Recently, a frequent gene fusion between the androgen-regulated prostate-specific protease TMPRSS2 and the ERG transcription factor has been discovered (1). In addition, other driver genes and oncogenic ETS factors (e.g., ETV1, ETV4, and ETV5) have been identified as gene fusions in prostate tumors (1–3).

To identify novel therapeutic opportunities for patients with prostate cancer, we have recently applied high-throughput screening to systematically explore most currently marketed drugs and drug-like molecules for their efficacy against a panel of prostate cells. The results indicated that most of the effective compounds, including antineoplastic agents, were nonspecific and inhibited both cancer and nonmalignant prostate epithelial cells in equal amounts. Only histone deacetylase inhibitor trichostatin A, thiram, disulfiram, and monensin were identified as potent and selective anti-neoplastic agents (4). The molecular mechanisms of trichostatin A–induced and disulfiram-induced growth inhibition in prostate cancer cells have been previously studied (4, 5). However, the antiproliferative effect of monensin in prostate cancer cells remains to be explored.

Monensin is a monocarboxylic acid ionophore veterinary drug produced by Streptomyces cinnamonensis with antibiotic and antimalarial activity (6–8). In addition to medicinal use, monensin has been shown to promote muscle growth in cattle (9), suggesting potential androgenic effects, which is interesting in the light of the anti–prostate cancer effects. Because monensin is one of the most potent prostate cancer–specific drugs and

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Drug-like molecules, and it has an excellent safety record in veterinary medicine, this provides an exciting opportunity for novel cancer treatment. In this study, we explored the mechanisms of monensin-induced growth inhibition in cultured prostate cancer cells.

Materials and Methods

Cells
The TMPRSS2-ERG gene fusion and AR-positive prostate carcinoma cell line VCaP was received from Drs. Adrie van Bokhoven (University of Colorado Health Sciences Center, Denver, CO) and Kenneth Pienta (University of Michigan, Ann Arbor, MI) and were grown in Dulbecco’s Modified Eagle’s Medium (10). LNCaP cells have a mutated AR (T877A) enabling progestagens, estradiol, and antiandrogens to activate androgen signaling (11) and were received from Dr. Marco Cecchini (University of Bern, Bern, Switzerland) and grown in T-Medium (Invitrogen Molecular Probes). The nonmalignant RWPE-1 prostate epithelial cells (12) were purchased from American Type Culture Collection and grown according to provider’s instructions. The nonmalignant EP156T prostate epithelial cells were received from Dr. Varda Rotter (Weizmann Institute of Science, Rehovot, Israel) and grown in the media recommended by the distributor (13). The identity of the cells was confirmed by array comparative genomic hybridization (Agilent Technologies), and the cells were passaged no longer than 6 months after receipt or resuscitation of frozen aliquots.

Compounds
Monensin (monensin sodium salt) and flutamide were purchased from Sigma-Aldrich and diluted in ethanol. Bicalutamide was purchased from Sequoia Research Products and diluted in ethanol. Disulfiram was purchased from Fluka and diluted in dimethyl sulfoxide.

Cell viability and apoptosis assays
Cell viability and apoptosis assays were done on 384-well plates (Falcon). A total of 2,000 cells per well were plated in 35 μL of their respective growth media and left to attach overnight. Compound dilutions were added to the cells and incubated for 48 hours. Cell viability was determined with CellTiter-Blue (CTB) or viability and apoptosis assays were then done according to the manufacturer’s instructions. The flurometric signal from CTB (excitation FITC 485 nm, emission FITC 535 nm) and luminescence signals (700 nm) from CTG and apoptosis assays were quantified using Envision Multilabel Plate Reader (Perkin-Elmer).

RNA extraction and quantitative reverse transcriptase PCR
Total RNA was extracted from cultured cells using RNeasy (Qiagen) according to the manufacturer’s protocol. Reverse transcription using 500 ng of total RNA was done with Applied Biosystems’ cDNA synthesis kit. TaqMan gene expression probes and primers from the Universal Probe Library (Roche Diagnostics) were used to study AR, ERG, MYC, Kruppel-like factor 6 (KLF6), activating transcription factor 3 (ATF3), metallothioneins MT1G and MT1F, thioredoxin binding protein (TXNIP), DNA damage-inducible transcripts 3 and 4 (DDIT3 and DDIT4), and β-actin mRNA expression. Primer sequences are listed in Supplementary Table S1. Real-time quantitative PCR was done using ABI Prism 7900 (Applied Biosystems). Quantitation was carried out using the ΔΔCT method with RQ manager 1.2 software (Applied Biosystems). β-Actin was used as an endogenous control. Average expression of the untreated control samples was considered for the calculation of the fold changes. Two to four replicate samples were studied for quantitation of mRNA expression.

Gene expression analysis using bead arrays
VCaP cells were grown into approximately 70% confluence and treated with 1 μmol/L monensin for 3, 6, and 24 hours before harvesting. Total RNA was extracted and RNA integrity was monitored prior to amplification and hybridization using an Experion electrophoresis station (Bio-Rad Laboratories) according to manufacturer’s instructions. The RNA quality indicator value for all samples was 10, indicating high-quality, intact RNA. Purified RNA (300 ng) was used for amplification with the Illumina RNA TotalPrep Amplification kit (Ambion, Austin, TX) and the biotin-labeled cRNA was hybridized to Sentrix HumanRef-8 vs.3 Expression BeadChips (Illumina). The arrays were scanned with the BeadArray Reader (Illumina). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-TABM-1049.

Statistical analysis of gene expression data
The raw gene expression data were quantile-normalized and analyzed with the R/Bioconductor software (14). Statistical analysis of differential gene expression after the compound treatments was done using the empirical Bayes statistics implemented in the eBayes function of the limma package (15). Gene expression profiles of the compound treated samples were compared with the negative control samples. The threshold for differential expression was q < 0.05 after the Benjamini–Hochberg multiple testing correction. The functional gene ontology and pathway annotation were analyzed for the sets of differentially expressed genes using DAVID (16, 17). To identify drugs with similar or opposite effects on gene expression, Connectivity Map 02 was used (18).
**Western blot analysis and subcellular proteome extraction**

For protein extraction and Western blot analysis, VCaP and LNCaP cells were plated at 70% confluency and left to attach overnight before treatments with indicated compounds. Subcellular protein extraction was done with the Compartment Protein Extraction Kit (Chemicon). The cytoplasmic and nuclear protein fractions were analyzed from VCaP cells. Protein concentrations were determined using a DC Bradford assay kit (Bio-Rad Laboratories). Ten μg of total protein was denatured at 95°C for 5 minutes in Laemmli buffer, separated on a 7% SDS-polyacrylamide gel, and transferred to a Protran nitrocellulose transfer membrane (Schleicher & Schuell). Western blot analysis was done using specific antibodies against AR (1:1,000 dilution, mouse monoclonal; Labvision), histone H3 (1:1,000, rabbit polyclonal; DakoCytomation), histone H3 (1:1,000, rabbit polyclonal; Abcam), and β-actin (1:4,000 dilution, mouse-monoclonal; Becton Dickinson). Signal was detected with 1:4,000 dilutions of appropriate HRP-conjugated secondary antibodies (all from Invitrogen Molecular Probes) followed by visualization with the enhanced chemiluminescence reagent (Amersham Biosciences).

**Subcellular localization of androgen receptor**

For immunofluorescence stainings, VCaP cells were androgen deprived by culturing cells in starvation medium with 10% charcoal-stripped serum for 18 hours in absence or presence of 10 μmol/L flutamide prior to subsequent addition of 35 nmol/L monensin for 24 hours and stimulation with R1881 for the last 18 hours. For controls, samples without monensin and/or R1881 were prepared. Cells were fixed with 3.7% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS for 15 minutes, and blocked with 2% bovine serum albumin or PBS for 30 minutes. Cells were stained with AR antibody (1:33 dilution, mouse monoclonal; Labvision) and Alexa-conjugated secondary anti-mouse antibody was used for secondary staining (1:300 dilution; Invitrogen Molecular Probes). Cell nucleus was stained with VectaShield mounting medium (Vector Laboratories, Ltd.) containing 4’,6-diamidino-2-phenylindole and images were taken with Zeiss Axiovert 200M Microscope with the spinning disc confocal unit Yokogawa CSU22 and a Zeiss Plan-Neofluar 63 oil 1.4 NA objective (Carl Zeiss MicroImaging). Z-stacks with 1 airy unit optical slices were acquired with a step size of 0.5 μm between slices, and the maximum intensity projections were created with SlideBook 4.2.0.7 software (Intelligent Imaging Innovations Inc.).

**Endocytosis of transferrin receptor**

To study whether monensin affects transferrin internalization to the cell, cells were grown on cover slips in 6-well plates. Monensin (10 μmol/L) or ethanol control was added to the cells prior to incubation with 546-labeled transferrin (5 pg/mL, Molecular Probes Europe BV) for 0, 15, or 30 minutes. Total incubation time for monensin in every sample was 1.5 hours. The amount of transferrin in the cells was detected with the confocal microscope described above.

**Determination of combinatorial drug effects**

The nature of interaction and the degree of synergy between monensin and antiandrogen flutamide or bicalutamide were analyzed using the combination index (CI) method (19). The concentration dependence of antiproliferative effects was determined for both compounds, either alone or in combination. Synergy was then analyzed according to the fixed concentration method, that is, fixed concentration of flutamide or bicalutamide together with increasing concentrations of monensin (18, 35, 70, and 140 nmol/L), thus providing a measure of the enhancement of monensin activity at various levels of diminished AR activity. The data were analyzed with CalcuSyn software (Biosoft), and the combination index (CI) was calculated from the median effect plots according to the following equation: $CI = D1/DX1 + D2/DX2$, where DX1 and DX2 are the concentrations of compounds 1 and 2 needed to produce the same level of antiproliferative effect when used alone, whereas D1 and D2 are their concentrations that produce the same effect when used in combination. A CI of 0.9 to 1.1 indicates additive interaction, values below 0.9 indicate synergism, and values more than 1.1 indicate antagonism.

**Reactive oxygen species detection**

Reactive oxygen species (ROS) assay was done on 384-well plates (Falcon). Two thousand cells per well were plated in 35 μL of medium and left to attach overnight. Monensin or hydrogen peroxide (H₂O₂) as positive control was added to the cells and incubated for 48 hours. Medium was removed and cells were washed with PBS. Carboxy-H₂DCFDA (100 μmol/L) was added to the cells in 10 μL of medium and incubated in the dark at 37°C for 30 minutes. Reagents were removed, cells washed with PBS, and oxidation of the probe was measured in PBS by monitoring the increase in fluorescence with Envision Multilabel Plate Reader (Perkin-Elmer).

**Determination of DNA double-strand breaks**

The amount of DNA double-strand breaks in VCaP and LNCaP cells was measured by monitoring the induction of serine 139 phosphorylation of histone variant H2AX (γH2AX). Cells were grown on cover slip slides and incubated for 3, 6, or 24 hours with 1 μmol/L monensin or vehicle control. Cells were stained with the rabbit polyclonal phosphorylated histone variant H2AX (Ser 139) antibody (Abcam). Secondary staining was done with anti-rabbit Alexa555 antibody (Abcam) and the results were obtained with confocal microscope.

**Determination of aldehyde dehydrogenase activity**

The activity of aldehyde dehydrogenase (ALDH) was determined with Aldefluor reagent (Stemcell Tech-
Monensin inhibits prostate cancer cell growth and induces apoptosis

Monensin inhibits prostate cancer cell viability at nanomolar concentrations whereas nonmalignant prostate epithelial cells are more resistant (>20-fold) to the compound (Fig. 1A; ref. 4). The effect of monensin on cell viability was clearly seen in VCaP (EC50 = 35 nmol/L) and LNCaP (EC50 = 90 nmol/L) prostate cancer cells, whereas nontumorigenic RWPE-1 (EC50 > 10 μmol/L) and EP156T (EC50 > 1 μmol/L) prostate epithelial cells were not affected at nanomolar concentrations (Fig. 1A). To determine whether the observed decrease in cell growth in response to monensin exposure was because of induction of apoptosis, caspase-3 and caspase-7 activities were determined by a quantitative fluorometric assay. Caspase activity was significantly induced in response to monensin exposure in VCaP cells (from 10 nmol/L) and in LNCaP cells (from 1 μmol/L). No statistically significant induction of apoptosis was seen in the nontumorigenic RWPE-1 and EP156T prostate cells, although weak increase in caspase activity was observed with 10 μmol/L monensin in EP156T cells (Fig. 1B).

Monensin reduces the expression of androgen receptor

To get further insights into the growth inhibitory mechanism of monensin, VCaP and LNCaP cells were exposed to 1 μmol/L of monensin for 3, 6, or 24 hours and AR mRNA expression was measured using quantitative PCR. Monensin reduced AR mRNA levels by 22% (P < 0.02) and 48% (P < 0.03) in VCaP cells at 6- and 24-hour time points (Fig. 2A). In LNCaP cells, AR mRNA level was decreased by 45% (P < 0.02) in response to 24-hour monensin exposure. Accordingly, a significant decrease was observed in AR protein levels (Fig. 2B). Besides AR, the mRNA expression of androgen-regulated ERG oncogene was reduced by 30% (P < 0.001) in VCaP cells, and MYC oncogene was reduced by 60% (P < 0.01) in VCaP cells and 54% (P < 0.03) in LNCaP cells in response to 24-hour monensin exposure (Fig. 2D).

Monensin does not affect the subcellular localization of androgen receptor

Monensin has been described to alter protein transport and transferrin endocytosis (9, 20). Our results on endocytosis indicated that monensin reduces transferrin internalization in VCaP cells (Supplementary Fig. S1). Because monensin reduced AR expression in the prostate cancer cells, we studied whether monensin affects nuclear transport of AR in VCaP cells. The results from immunofluorescence staining showed slightly reduced AR levels both in the cytoplasm and in the nucleus in response to 35-nmol/L monensin exposure (Fig. 3A). To further confirm the result, cytoplasmic and nuclear protein lysates were prepared and separated and run on SDS-PAGE followed by Western blot analysis (Supplementary Fig. S2). Flutamide and monensin exposures alone resulted in significant reduction in AR protein expression in the nuclear fractions in comparison to the control. A combinatorial treatment with monensin and flutamide reduced AR expression even more than either of the compounds alone, both in the cytoplasmic and nuclear fractions. In response to flutamide and
monensin cotreatment, basal PSA protein level was reduced in both cytoplasmic and nuclear fractions of the cells in comparison to flutamide-only–treated cells (Supplementary Fig. S2). A similar combinatorial effect on AR expression was seen in LNCaP cells (Supplementary Fig. S2). Taken together, these results indicate that monensin reduces intracellular AR levels especially in combination with flutamide.

**Antiandrogens potentiate monensin induced growth inhibition in VCaP and LNCaP cells**

Because monensin exposure reduced AR expression and a combinatorial treatment with monensin and flutamide reduced AR protein expression even more than either compound alone, the potential combinatorial effect of androgen deprivation and monensin on cell viability was studied in VCaP and LNCaP cells. Cells were treated with 10 μmol/L flutamide or bicalutamide for 24 hours prior to subsequent exposure to increasing concentrations of monensin for 48 hours. A synergistic effect (CI < 0.9) of flutamide and monensin was observed with 18–140 nmol/L of monensin in both VCaP and LNCaP cells (Fig. 3B and C). Synergy was seen also between bicalutamide and monensin (35–140 nmol/L) in VCaP and LNCaP cells (Supplementary Fig. S3).

**Monensin induces genes involved in cholesterol synthesis and oxidative stress defense and shows agonistic effects to terfenadine and antagonistic effects to progesterone**

Genome-wide expression analysis was done in VCaP cells at early time points (3, 6, and 24 hours) to identify potential early primary effects of monensin as opposed to secondary alterations. The most significantly altered biological processes in gene ontology analysis (Supplementary Table S2) indicated that cholesterol and lipid metabolic activity was enriched at 3- and 6-hour time points. The 6-hour treatment also induced the expression of metal binding genes including metallothioneins whereas 24-hour exposure affected genes involved in protein transport, cell differentiation, cell cycle, and DNA repair.

Differentially expressed genes at the 6-hour time point were compared with the gene expression profiles representing drug responses to more than 1,309 compounds using the Connectivity Map approach (Supplementary Table S3). Progesterone and pregnenolone, as well as progesterone agonists etynoldiol and dehydrogesterone, were among the drugs altering gene expression in an opposite direction than monensin. Progesterone and pregnenolone are precursors of androgens and their levels are upregulated in castrate prostate cancer.

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**Figure 2. Monensin reduces the expression levels of key oncogenes androgen receptor (AR), ERG and MYC in prostate cancer cells.**

A, AR mRNA expression in response to 3-, 6-, and 24-hour monensin exposure in VCaP and LNCaP cells; B, AR protein expression in response to 6- and 24-hour monensin exposure in VCaP and LNCaP cells; C, ERG mRNA expression in response to 3-, 6-, and 24-hour monensin exposure in VCaP cells; and D, MYC mRNA expression in response to 3-, 6-, and 24-hour monensin exposure in VCaP and LNCaP cells. Asterisks indicate statistical significance. *, P < 0.005; **, P < 0.01; ***, P < 0.001.
resistant prostate cancers (21, 22). Terfenadine, a histamine H1 receptor antagonist, was the most enriched compound altering gene expression in the same direction as monensin. Terfenadine induces apoptosis in melanoma cells (23) and causes massive production of H2O2 in cultured cerebellar neurons (24). Taken together, these results suggest that monensin has opposite effects to androgen precursors and may induce oxidative stress in prostate cancer cells.

Monensin induces a gene expression signature characteristic of oxidative stress response

Analysis of gene expression profiles also indicated that the expression of several previously identified oxidative stress-associated markers (25) was altered (<3 fold change >3) in monensin exposed VCaP cells. The gene expression changes were validated in VCaP, LNCaP, RWPE-1, and EP156T cells using quantitative PCR (Fig. 4A; Supplementary Figs. S4 and S5). At 3-hour time point, thioredoxin-interacting protein (TXNIP) was the most downregulated mRNA in VCaP and LNCaP cells (Fig. 4A; Supplementary Fig. S4). TXNIP is an oxidative stress responsive gene regulating intracellular ROS by inhibiting thioredoxin activity or limiting its bioavailability (26–29). Metallothioneins were the most rapidly induced genes in response to monensin exposure in VCaP and LNCaP cells (Fig. 4A; Supplementary Fig. S4). Metallothioneins are known to protect cells against oxidative stress by binding to free metals and H2O2 (30). Interestingly, our previous results indicate that VCaP prostate cancer cells are sensitive to
oxidative stress and metallothioneins were also induced in response to disulfiram exposure (4). MT1F and MT1G mRNA expression levels were highly induced in response to 3- and 6-hour monensin exposure in VCaP cells (10- to 30-fold). In LNCaP cells the induction of metallothioneins was seen (2- to 20-fold), at 6- and 24-hour time points. In addition, 2 apoptosis-associated transcription factors, tumor suppressor KLF6 and ATF3 were induced by monensin in a time-dependent manner (Fig. 4A; Supplementary Fig. S5) in VCaP and LNCaP cells. ATF3 is a proapoptotic protein that is upregulated under stress conditions such as oxidative stress and DNA damage (31). KLF6 has been shown to regulate ATF3 expression in prostate cancer and they are both needed for cancer cell apoptosis in stress conditions (32). Moreover, DNA damage-inducible transcripts 3 (DDIT3, GADD153, CHOP) and 4 (DDIT4, REDD1) were induced in monensin exposed prostate cancer cells (Fig. 4B; Supplementary Fig. S5). DDIT3 and DDIT4 are expressed in endoplasmic reticulum stress–induced apoptosis and DNA damage (33, 34). γH2AX immunofluorescence staining was also positive in monensin-treated VCaP and LNCaP cells, reflecting DNA damage and onset of apoptosis (Fig. 4B).

Monensin induces oxidative stress, and the effect of monensin on cell viability is antagonized by antioxidant vitamin C

To validate the oxidative stress induction in response to monensin exposure, the levels of ROS were detected using carboxy-H2DCFDA, a cell-permeant indicator for ROS. VCaP, LNCaP, RWPE-1, and EP156T cells were exposed to monensin or solvent control for 48 hours. As a positive ROS control, cells were exposed to 400 μmol/L H2O2 for 6 hours. Results indicated that monensin significantly increases ROS levels in VCaP cells (Fig. 5A). ROS levels were also increased in LNCaP cells, although not as strongly as in the VCaP cells. In contrast, monensin did not induce ROS in nontumorigenic RWPE-1 and EP156T cells.

Vitamin C (l-ascorbic acid) is an antioxidant that acts as a scavenger for a wide range of ROS. Recently, the cytotoxic effects of antineoplastic drugs that produce mitochondrial dysfunction, such as loss of mitochondrial membrane potential and an increase in ROS levels, were shown to be inhibited by vitamin C in leukemic cells (35). To find out whether monensin-induced cell death can be antagonized by vitamin C treatment, the effect of 10 μmol/L vitamin C and 100 nmol/L monensin on VCaP and LNCaP cell viability was analyzed after 48 hours of
incubation. Monensin alone reduced cell proliferation by 55% (P < 0.01), whereas vitamin C antagonized the effect (Fig. 5B). Taken together, these results indicate that monensin-induced antiproliferative effect can be partially reversed by vitamin C.

**Monensin inhibits aldehyde dehydrogenase activity in VCaP and LNCaP cells**

Because vitamin C antagonized the monensin-induced antiproliferative effect, we hypothesized that monensin would inhibit the induction of antioxidative enzymes. Our previous results showed that the ALDH inhibitor disulfiram caused oxidative stress and reduced prostate cancer cell viability (4). Because ALDH protects cells from aldehyde-induced oxidative stress, we explored whether monensin also induced oxidative stress by reducing ALDH activity. The ALDH activity was studied in response to 48-hour monensin or disulfiram exposure in VCaP, LNCaP, RWPE-1, and EP156T cells using Aldefluor reagent (Fig. 6). The results indicated that monensin reduces ALDH activity by 42% in VCaP cells (P < 0.01) and 32% in LNCaP cells (P < 0.001), and that disulfiram reduces ALDH activity by 46% in VCaP cells (P < 0.001) and 52% in LNCaP cells (P < 0.0001). No changes were detected in RWPE-1 and EP156T cells. Aldehydes accumulate in the cell in oxidative stress and aldehydes can be metabolized by ALDH (36). The results suggest that the reduced ALDH activity after monensin exposure allows aldehydes to accumulate and induce oxidative stress in prostate cancer cells.

**Discussion**

Sodium ionophore monensin inhibits prostate cancer cell viability at nanomolar concentrations in vitro (4) and is among the most potent and cancer-specific inhibitors of prostate cell growth among all the tested known drugs and drug-like molecules. Because monensin has a favorable safety profile in animal systems, it was of interest to determine its molecular mechanisms of action. Here, we showed that monensin inhibits cell viability and AR signaling, and induces apoptosis and oxidative stress in prostate cancer cells.

Monensin exposure in cultured cells has been shown to increase intracellular calcium levels due to influx of Na⁺ and efflux of H⁺ and K⁺ (37), leading to swelling of mitochondria, decrease of ATP production, lipid peroxidation, and loss of cell membrane integrity (9). In addition, monensin has been shown to inhibit the growth of various cancer cells by inducing cell cycle arrest and apoptosis in vitro (38–41). Because monensin is commonly used in veterinary medicine and has anabolic effects on muscle, it was of special interest to study the effects on androgen signaling and the molecular mechanisms in prostate cancer. Monensin reduced AR expression and signaling in prostate cancer cells and its effects on cell growth were potentiated by antiandrogens. Our results with monensin, therefore, add up to a growing body of evidence suggesting that antiandrogen response and induction of oxidative stress are closely linked, and their combined induction could provide a powerful cancer-specific therapeutic strategy. Androgen deprivation is
known to decrease antioxidative capacity, elevate ROS anabolism, and sensitize AR-positive prostate cancer to oxidative stress–induced death \textit{in vitro} and \textit{in vivo} (42, 43). Because the basal level of oxidative stress is higher in prostate cancer cells than in nonmalignant prostate cells, a strategy that takes advantage of prostate cancer sensitivity to acute exposure of oxidative stress has been proposed (44–47). The results from this study indicate that monensin induced a gene expression signature characteristic of oxidative stress response and elevated intracellular levels of ROS in prostate cancer cells, but not in normal prostate epithelial cells studied. We hypothesize that monensin leads to rapid induction of oxidative stress followed by downregulation of AR signaling, which then further increases sensitivity of prostate cancer cells to oxidative stress. Moreover, the effect of monensin on cell viability was antagonized by antioxidant vitamin C, indicating that the monensin-induced antiproliferative effect was dependent on the elevated level of ROS.

Monensin changed the gene expression profile of prostate cancer cells in an opposite direction than progesterone, a precursor of androgens with documented antioxidant properties (48). Interestingly, progesterone levels are known to be elevated in hormone refractory prostate cancers (21, 22). Monensin also reduced the expression of oncogenes ERG and MYC ERG expression is regulated by androgens in \textit{TMPRSS2-ERG} positive prostate cancers (1, 49) and MYC is a known downstream target of ERG. MYC plays a role in cell cycle progression, apoptosis, and cellular transformation and is frequently amplified and overexpressed in prostate cancer (50). MYC is also known to protect cells against oxidative stress (51) and in leukemia cells, the oxidative stress–induced MYC mRNA downregulation has been identified as a mechanism of 2-methoxyestradiol–induced apoptosis (52). Thus, these results also support the idea that monensin induces growth inhibition by sensitizing prostate cancer cells to oxidative stress.

Furthermore, monensin reduced the ALDH activity in prostate cancer cells. ALDH is constituted of antioxidant and detoxifying enzymes that metabolize aldehydes accumulated in the cell under oxidative stress conditions (36). We have previously shown that prostate cancer cells are sensitive to ALDH inhibitor disulfiram (4). Overexpression of ALDH 1A1 has been shown to reduce oxidation–induced toxicity in neuroblastoma cells (53). Recently, ALDH 1A1 has also been identified as a marker for malignant prostate stem cells and predictor of outcome for prostate cancer patients (54, 55). Interestingly, cancer stem cells overexpress ROS detoxifying genes and thus have a stronger antioxidant defense system than their nontumorigenic counterparts (56). Moreover, 2 structurally highly similar antibiotic ionophores to monensin—salinomycin and nigericin—have recently been identified as inhibitors of breast cancer stem cells by the high-throughput screening (57). Inhibition of ALDH activity and the structural similarity to known cancer stem cell inhibitors suggest that monensin may have additional effects on cancer stem cells and their sensitization to oxidative stress–induced cell death.

We propose monensin as a potential well-tolerated, \textit{in vivo} compatible drug with strong proapoptotic effects that are specific to prostate cancer cells, and synergistic effects with antiandrogens. Overdoses of monensin have been linked with toxic effects in some animal species (58) indicating that possible studies with monensin in humans should be designed carefully. Moreover, our data suggest a general strategy by which the effects of antiandrogens could be enhanced by combinatorial administration with agents that increase oxidative stress in prostate cancer cells.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest have been disclosed.

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