23

Monensin Induced Oxidative Stress Reduces Prostate Cancer Cell Migration and Cancer Stem Cell Population

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1. Introduction

Prostate cancer is the most common malignancy and second leading cause of cancer related death in males in developed countries (Jemal et al. 2011). Patients with localized and metastatic prostate cancer are treated with anti-androgens. Although prostate cancer cell proliferation is initially blocked or slowed down with anti-androgen therapy, eventually castration-resistant disease develops (Sharifi, Gulley & Dahut 2010). Therapeutic options for castration-resistant prostate cancer are limited and treatment responses to currently existing therapies are often unsatisfactory (Bracarda et al. 2011). For example, the cytotoxic therapy often causes severe toxicity and eventually leads also to the development of chemo-resistance (Tannock et al. 2004, Berthold et al. 2008, Bracarda et al. 2011). Thus, there is an urgent need for novel agents to block the proliferation and to inhibit the progression of the primary prostate cancer cells to the advanced stage as well as to target advanced and metastatic prostate cancer. Therefore, understanding of disease progression and drug resistance mechanisms may provide valuable insights into the development of novel treatment options to improve the survival of prostate cancer patients.

We have recently performed a high-throughput cell-based screening of 4,910 known drugs and drug-like molecules in four prostate cancer cell models and two non-tumorigenic prostate epithelial cell lines to identify prostate cancer cell growth selective inhibitors (Iljin et al. 2009). Only four compounds, antibiotic ionophore monensin, aldehyde dehydrogenase (ALDH) inhibitor disulfiram, histone deacetylase inhibitor trichostatin A and fungicide thiram inhibited selectively cancer cell growth at nanomolar concentrations. The mechanistic studies indicated that monensin and disulfiram inhibited prostate cancer cell growth by inducing oxidative stress (Iljin et al. 2009, Ketola et al. 2010). In contrast to disulfiram, monensin induced apoptosis, reduced androgen receptor signalling and showed a synergistic anti-proliferative effect with anti-androgens in prostate cancer cells. Moreover,

monensin increased the amount of reactive oxygen species (ROS) and induced an oxidative stress signature in prostate cancer cells (VCaP and LNCaP), but not in the non-malignant prostate epithelial cells (RWPE-1, EP156T) (Ketola et al. 2010). Furthermore, antioxidant vitamin C partially rescued the monensin induced growth inhibition, indicating that oxidative stress plays a key role in the antineoplastic effect of monensin in cultured prostate cancer cells (Ketola et al. 2010).

Oxidative stress occurs in the cell when redox regulation is imbalanced. Redox balance depends on the level of intracellular free radicals and reactive oxygen species as well as on the antioxidative capacity of the cell. Figure 1 illustrates the connection between the malignant progression and increase in intracellular ROS (Fig. 1).

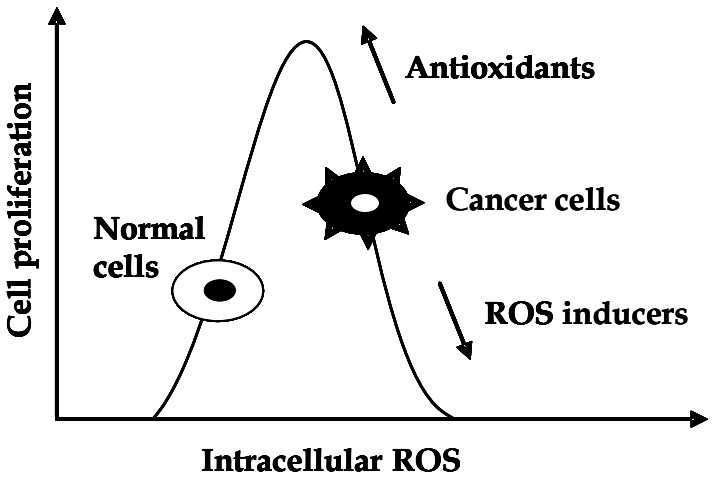

Fig. 1. The balance between cell proliferation and intracellular reactive oxygen species (ROS). The malignant progression and the effects of ROS inducers and antioxidants depend on the intracellular levels of ROS. In cancer cells, intracellular ROS levels are higher than in normal cells making them more vulnerable to agents inducing ROS. Figure idea adapted from Gupte & Mumper 2009.

Cancer cells benefit from the increased mutation rate induced via oxidative stress (Shibutani, Takeshita & Grollman 1991). Therefore, cancer cells need also an active antioxidative mechanism to be able to survive under high oxidative stress. The oxidative stress level is elevated in prostate cancer cells compared to non-malignant prostate epithelial cells (Khandrika et al. 2009, Yossepowitch et al. 2007, Kumar et al. 2008). Many oncogenes are known to protect cells from oxidative stress e.g. androgen receptor (AR), ERG and MYC are known to have antioxidative properties in cancer cells (Pinthus et al. 2007, Tam et al. 2003, Benassi et al. 2006, Swanson et al. 2011, DeNicola et al. 2011). Monensin may sensitize prostate cancer cells to oxidative stress via reducing the expression of these genes (Ketola et al. 2010). In addition, many other anti-neoplastic agents such as vinblastine, cisplatin, mitomycin C, doxorubicin, camptothecin, inostamycin, neocarzinostatin, etoposide, arsenic trioxide and nonsteroidal anti-inflammatory drugs are known to mediate their apoptotic

effect by inducing oxidative stress (Fang, Nakamura & Iyer 2007, Rigas, Sun 2008, Sun et al. 2011). The increased sensitivity to oxidative stress combined with dependency on anti-oxidative system may provide a way to selectivity inhibit cancer cell proliferation (Iljin et al. 2009, Ketola et al. 2010).

Recently, redox control including antioxidative defence mechanisms and ROS-scavenging systems has been identified as an important regulator of cancer stem cell potential, metastasis and chemoresistance (Kobayashi, Suda 2011b, Cairns, Harris & Mak 2011, Pani, Galeotti & Chiarugi 2010). Aldehyde dehydrogenase activity is widely used as a marker for cancer stem cells and its activity has been shown to correlate with poor outcome in several cancers such as in prostate cancer (Davydov, Dobaeva & Bozhkov 2004, Burger et al. 2009, Li et al. 2010, Yu et al. 2011, Zhang et al. 2009a). Aldehyde dehydrogenases are detoxifying enzymes that are responsible for the oxidation of intracellular aldehydes (Duester 2000, Magni et al. 1996, Sophos, Vasiliou 2003, Yoshida et al. 1998). Moreover, ALDH oxidize retinol to retinoic acid (vitamin A) known to reduce oxidative stress whereas retinoic acid depletion induces oxidative stress and mitochondrial dysfunction (Ahlemeyer, Krieglstein 1998, Ahlemeyer et al. 2001, Chiu, Fischman & Hammerling 2008, Duester 2000, Chute et al. 2006). Thus, ALDH increases the antioxidative capacity in cells and protects cells from oxidative stress induction. Moreover, the inhibition of ALDH activity has recently been linked to reduced chemotherapy and radiation resistance in cancer stem cells (Croker, Allan 2011). These results suggest that not only cancer cells, but also cancer stem cells could be targeted by oxidative stress induction and/or reduction of antioxidative capacity. Interestingly, the results from mechanistic studies of prostate cancer selective compounds indicated that both disulfiram and monensin reduced the ALDH activity in prostate cancer cells (Iljin et al. 2009, Ketola et al., 2010).

In this study, we explored further the molecular mechanism of monensin induced growth inhibition in cultured prostate cancer cells. Cancer pathway reporter assays and steroid profiling was performed to get insights into altered signalling and metabolite levels in monensin exposed prostate cancer cells. Since monensin reduces ALDH activity, the putative effect on cancer stem cells was evaluated. Furthermore, we studied the effect of monensin on prostate cancer cell differentiation and motility.

2. Materials and methods

2.1 Cells

VCaP prostate carcinoma cells (TMPRSS2-ERG positive, received from Drs. Adrie van Bokhoven, University of Colorado Health Sciences Center, Denver, Colorado and Kenneth Pienta, University of Michigan, Michigan) were grown in Dulbecco's Modified Eagle's Medium (Korenchuk et al. 2001b, Korenchuk et al. 2001a). LNCaP prostate carcinoma cells (received from Dr. Marco Cecchini, University of Bern, Bern, Switzerland) were grown in T-Medium (Invitrogen). PC-3 prostate carcinoma cells were purchased from American Type Culture Collection (LGC Promochem AB) and grown according to provider's instructions. All cells were cultured in appropriate growth media described above including 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

2.2 Compounds

Monensin was purchased from Sigma-Aldrich and diluted in ethanol.

2.3 Cancer 10-pathway reporter array

Cancer 10-pathway Reporter Luciferase Kit (Wnt (TCF/LEF), Notch (RBP-Jκ), p53/DNA damage, TGF-β (SMAD2/3/4), Cell cycle/pRb-E2F (E2F/DP1), NF-κB, Myc/Max, Hypoxia (HIF1A), MAPK/ERK (Elk-1/SRF) and MAPK/JNK (AP-1) was used to study the monensin modulated signalling (SABiosciences, Frederick, MD). The assay was performed according to manufacturer's instructions. In brief, inducible transcription factor responsive firefly luciferase reporters with constitutively expressing Renilla construct transcription factor reporters were plated onto 96-well plates with transfection reagent (siLentFect, Bio-Rad Laboratories), followed by addition of cells and incubation for 24 hours. A mixture of non-inducible firefly luciferase reporter and constitutively expressing Renilla construct was used as the negative control. After 24 hours of transfection, monensin (100 nM) and control treatment were added onto the cells and plates were incubated for 18 hours. The Dual-LuciferaseReporter (DLR™) Assay System (Promega) was utilized in quantitation of reporters and results according to the manufactorer's instructions. The change in the activity of each signalling pathway was determined by comparing the normalized luciferase activity of the reporters in monensin or solvent exposed cells.

2.4 Wound healing assay

The effect of monensin (10 nM, 100 nM and 1 μM) on prostate cancer cell migration was studied using a scratch wound assay. PC-3 cells were plated on 96-well plates (Essen ImageLock, Essen Instruments, UK) and a wound was scratched with wound scratcher (Essen Instruments). Compounds and appropriate controls were added immediately after wound scratching and wound confluence was monitored with Incucyte Live-Cell Imaging System and software (Essen Instruments). Wound closure was calculated for every hour for 24 hours by comparing the mean relative wound density of three biological replicates in each experiment.

2.5 Cell viability assay

Cell viability was determined with CellTiter-Glo (CTG) cell viability assay (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 2,000 cells per well were plated in 35 μl of their respective growth media and left to attach overnight. Monensin (100 nM) was added to the cells and incubated for 12 or 24 hours. CTG reagent was added and the signals were quantified using Envision Multilabel Plate Reader (Perkin-Elmer, Massachusetts, MA).

2.6 RNA extraction and quantitative real-time PCR

VCaP cells were exposed to monensin for 6 hours, total RNA was extracted and quantitative real-time PCR was done as previously described (Ketola et al. 2010). TaqMan gene expression probes and primers from the Universal Probe Library (Roche Diagnostics, Espoo, Finland) were used to study E-cadherin (5´-cccgggacaacgtttattac-3´ and 5´-gctggctcaagtcaaagtcc-3´) and

β-actin (5´-ccaaccgcgagaagatga -3´ and 5´-ccagaggcgtacagggatag -3´) mRNA expression. Three replicate samples were studied.

2.7 Fluorescence-Activated Cell Sorting analysis (FACS)

VCaP and LNCaP cells were exposed to monensin (1 μM) for 6 hours, samples were fixed with 2% paraformaldehyde, and stained with CD44 (FITC-conjugated mouse monoclonal anti-human, BD Pharmingen™ 555478) and CD24 (PE-conjugated rat monoclonal anti-human, Abcam ab25281) antibodies for 45 minutes at 4°C in the dark. Cells were washed and the fluorescence intensity was measured using Accuri C6 Flow Cytometer.

2.8 Immuofluorescence staining

For immunofluorescence stainings, VCaP cells were grown on cover slip slides and exposed to monensin for 6 hours. Cells were fixed with 4 % paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS for 15 min, and blocked with 2 % BSA/PBS for 30 min. Cells were stained with E-cadherin antibody (1:100 dilution, polyclonal rabbit anti-human, Cell Signaling Technology, MA, USA) and Alexa-conjugated polyclonal donkey anti-rabbit antibody was used for secondary staining (1:300 dilution, Invitrogen, Molecular Probes, Carlsbad, CA). Cell nuclei were stained with DAPI present in Vectashield mounting medium (Vector Labs) 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. 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., CO, USA).

2.9 Statistical analyses

Stars in the figures indicate the significance of the experiments calculated using Student's t-test (*P<0.05, **P<0.01, *** P<0.001).

2.10 Steroid quantification

VCaP cells ($1x10^7$ cells) were exposed to 1 μM monensin for 6 hours, harvested and counted. An internal standard (labeled C16:0) and chloroform:methanol (2:5) mixture were added, the samples were homogenized with Retsch system (5 min, 20 Hz), centrifuged and the supernatants were collected and evaporated. MOX (25 μl, TS-45950, Thermo Scientific, Helsinki, Finland) was added and the mixture was incubated at 45°C for 60 minutes. Next, 100 μl of MSTFA with 1% trimethylchlorosilane (Fluka, St. Louis, MO) was added and the mixture was incubated at 70°C for 60 minutes. Injection standard was added to the mixture before gas chromatography-mass spectrometry analysis (GC-MS, Agilent 6890 gas chromatograph (GC) combined with Agilent 5973 mass selective detector (MSD)). The injector (injection volume 1 μl with pulsed splitless injection) and MSD temperatures were 230°C (MS Source) and 150°C (MS Quad). The analyses were performed on Supelco 38499-02C capillary column. Selective ion monitoring using specific masses for each target analyte was used in the detection. The following steroids were quantified: 7-ketocholesterol, aldosterone, progesterone, pregnenolone, estrone, 17B-estradiol, 4B-hydroxycholesterol,

25-hydroxycholesterol, 5a,6a-epoxycholesterol (Mono-TMS), dihydrotestosterone and testosterone (the standards were from Steraloids, Newport, RI)).

3. Results

3.1 Monensin reduces NF-κB pathway activity in prostate cancer cells

Here, we studied the effect of monensin exposure on the activities of ten cancer signalling pathways using Cancer pathway Reporter Array in prostate cancer cells. Inducible transcription factor responsive firefly luciferase reporters were transfected to VCaP and LNCaP cells with constitutively active Renilla reporters and incubated for 24 hours. Monensin (100 nM) or solvent control was added onto the transfected cells for 18 hours followed by measure of luciferase activities. The results are presented in Fig. 2. Comparison of the basal pathway activities in VCaP prostate cancer cells indicated that NF-κB was

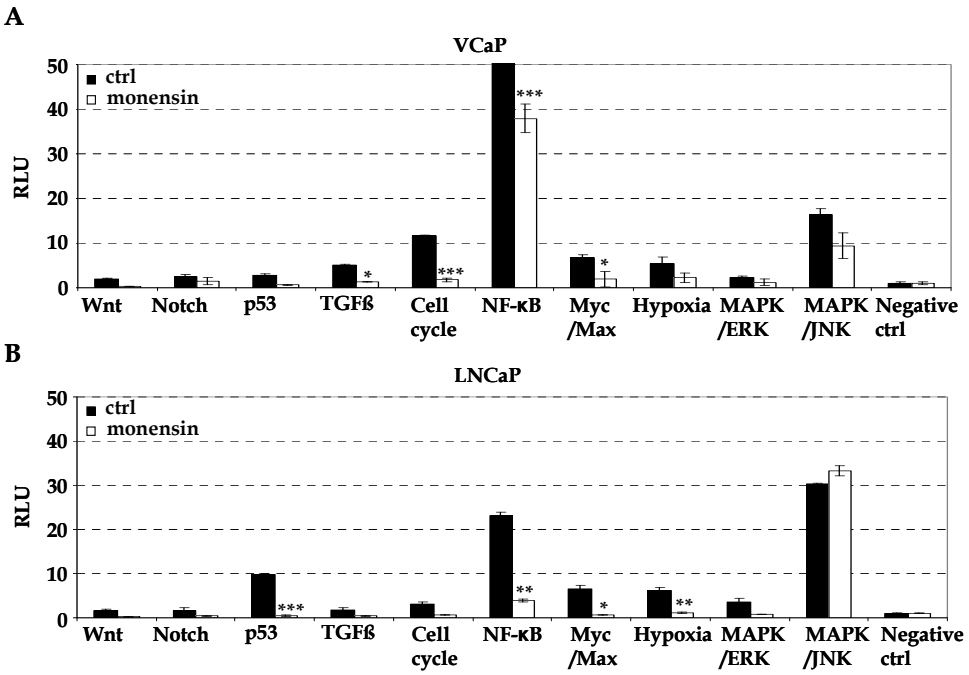

Fig. 2. Cancer pathway activities in VCaP and LNCaP prostate cancer cells. A) VCaP and B) LNCaP cells were exposed to ethanol or 100 nM monensin for 24 hours and pathway activities were measured as described in the text. Results with negative control measuring the background firefly luciferase activity (non-inducible firefly luciferase reporter and constitutively expressing Renilla construct) are indicated and used to determine the basal pathway activities in VCaP and LNCaP cells. The y-axis was set to 50 to allow direct comparison of relative luciferase units (RLU) in VCaP and LNCaP cells, although the basal NK-κB activity in VCaP cells extended RLU 324. Statistical significance of monensin induced changes are shown for the active pathways *P<0.05, **P<0.01, ***P<0.001.

clearly the most active pathway (fold change FC > 300, compared to the negative control). In addition, TGF-ß (FC = 5), cell cycle (FC = 12), Myc/Max (FC = 7) and hypoxia (FC = 5) were active in VCaP cells. In LNCaP cells, MAPK/JNK was the most active pathway (FC = 30 compared to the negative control), followed by NF-κB (FC = 23), p53 (FC = 10), Myc/Max (FC = 7) and hypoxia (FC = 6). Interestingly, NF-κB pathway was 14 times more active in TMPRSS2-ERG fusion positive VCaP cells than in ERG negative LNCaP cells. These results are in agreement with previous data indicating that ERG induces NF-κB activity in prostate cells *in vitro* and *in vivo* (Wang et al. 2010). The results with p53 pathway are also in accordance with the previous literature since VCaP cells are known to have an inactivating mutation in p53 (Trp-248) whereas LNCaP cells express the active form, supporting further the overall functionality of the assay (van Bokhoven et al. 2003).

Monensin exposure inhibited NF-κB activity in both VCaP (by 88%) and LNCaP (by 83%) cells. NF-κB is a transcription factor that regulates various cellular processes such as cellular antioxidant defence capacity (Gloire, Legrand-Poels & Piette 2006). These results suggest that monensin induced oxidative stress may result from reduced NF-κB signalling. In addition, monensin reduced TGF-ß (by 75%), cell cycle (by 85%) and Myc/Max (by 71%) activities in VCaP cells and p53 (by 94%), hypoxia (by 82%) and Myc/Max (by 90%) activities in LNCaP cells. The reduced Myc/Max signalling is supported by reduced MYC mRNA expression seen in monensin exposed VCaP and LNCaP cells (Ketola et al. 2010). Taken together, monensin reduced the activities of multiple signalling pathways such as NF-κB, TGF-ß, hypoxia and Myc/Max, all associated with cancer cell survival, oxidative stress, stem cell potential and metastasis (Jones, Pu & Kyprianou 2009, Blum et al. 2009, Mimeault, M. & Batra, S.K. 2011, Benassi et al. 2006, Koh et al. 2010).

3.2 Monensin reduces the cancer stem cell population in prostate cancer cell cultures

Monensin exposure reduced the activities of multiple pathways maintaining antioxidative capacity and promoting the growth and survival of cancer stem cells such as NF-κB, HIF1A, MYC and ALDH, in cultured prostate cancer cells. Therefore, the effect of monensin on prostate cancer stem cell population was studied. Prostate cancer stem cells can be identified by high expression of CD44 and low expression of CD24 antigens (Klarmann et al. 2009). Interestingly, CD44 cell surface glycoprotein has recently been shown to increase antioxidative capacity in cancer cells, indicating that cancer initiating cells could be targeted by impairing oxidative stress defence mechanisms (Ishimoto et al. 2011). Thus, we analyzed the effect of monensin exposure to the fraction of $CD44^+/CD24^-$ cells in cultured prostate cancer cells. VCaP and LNCaP cells exposed to monensin (1 μM) for 6 hours were stained with CD44 and CD24 recognizing antibodies and the samples were analyzed using FACS. The results showed that monensin reduced the fraction of $CD44^+/CD24^-$ cells in both VCaP (from 3 to 1.3%) and LNCaP (3.1 to 2.6%) cells (Fig. 3). In agreement with these results, genome-wide gene expression analysis indicated that monensin decreased CD44 (by 20%) and induced CD24 (by 30%) mRNAs compared to the levels in ethanol control at 6-hour time point (Ketola et al. 2010). Taken together, these results indicate that monensin reduces the fraction of cancer stem cells in prostate cancer cell cultures.

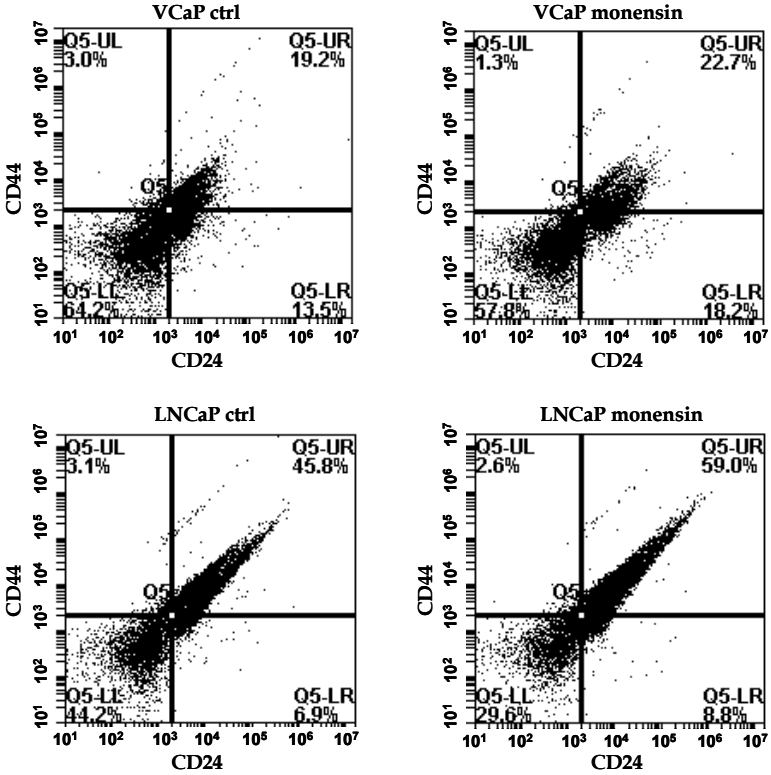

Fig. 3. FACS analysis of CD44 and CD24 immunostained VCaP and LNCaP prostate cancer cells. Monensin reduces the fraction of CD44+/CD24- cells in cultured prostate cancer cells. VCaP and LNCaP cells were stained with CD44 and CD24 antibodies in response to six hour monensin or ethanol exposure. Representative images from one out of four replicates are shown.

3.3 Monensin induces cell differentiation in prostate cancer cells

Cancer stem cells have been hypothesized to arise intrinsically through oncogenic transformation of normal tissue stem or progenitor cells in the early stage of the tumorigenesis or through induction of epithelial-to-mesenchymal transition (EMT) at later stages (Chaffer, Weinberg 2011). The expression of E-cadherin is considered as a marker of epithelial differentiation and it is lost during EMT. Interestingly, ERG knock-down in VCaP cells has been shown to induce E-cadherin expression (Gupta et al. 2010). Moreover, our previous results indicated that monensin reduced ERG expression in VCaP cells (Ketola et al. 2010). Thus, to study whether monensin affects prostate cancer cell differentiation, E-cadherin expression was analysed in monensin exposed VCaP cells. The results from immunochemical staining and quantitative RT-PCR showed that monensin induced E-cadherin expression in VCaP cells (Fig. 4A and B), indicating that monensin promotes cell differentiation in cultured prostate cancer cells.

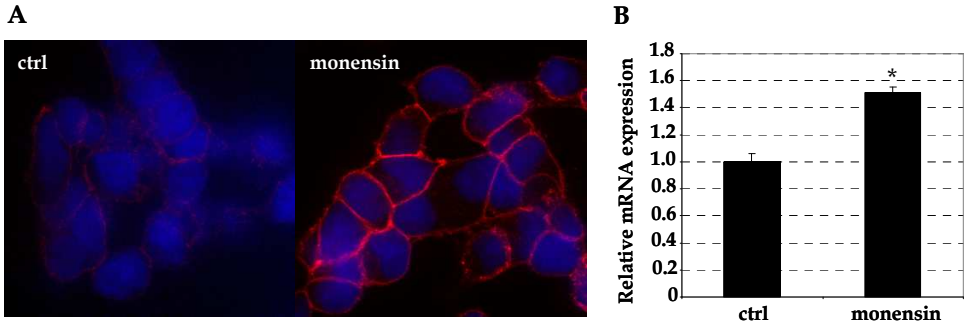

Fig. 4. Monensin induces E-cadherin expression in VCaP prostate cancer cells. A) Immunofluorescence staining of E-cadherin (red) in response to 1 μM monensin or ethanol (ctrl) for 6-hour in VCaP cells. Nuclei are stained with DAPI (blue). B) Relative mRNA expression of E-cadherin in response to 1 μM monensin or ethanol (ctrl) exposure for 6-hour in VCaP cells.

3.4 Monensin reduces migration in cultured prostate cancer cells

Epithelial-to-mesenchymal transition is a perquisite for cancer cell migration (Baum, Settleman & Quinlan 2008). Moreover, in addition to the role in promoting cancer stem cell growth and survival, NF-κB and ALDH activities as well as high CD44 and low CD24 expressions are known to enhance prostate cancer cell migration (van den Hoogen et al. 2010, Klarmann et al. 2009). Since monensin induced cell differentiation, the effect of monensin exposure on prostate cancer cell migration was studied. VCaP and LNCaP cells do not migrate and therefore PC-3 prostate cancer cells were used as a model in migration assay. The results are presented in Fig. 5. Interestingly, already at 10 nM concentration, monensin significantly reduced cell migration. The anti-migratorial effect was stronger at higher concentrations (100 nM and 1 μM) (Fig. 5A). Thus, monensin was able to reduce cell migration at nanomolar concentrations in PC-3 cells although the same concentrations did not significantly decrease cell viability in these cells even at 24 hour time point (Fig. 5B). Pictures showing the decrease in wound closure in monensin exposed PC-3 cells in comparison to control are shown at 12 and 24 hour time points in Fig. 5C.

3.5 Monensin increases oxidative stress inducing steroids as well as reduces the level of androgen precursor and antioxidative steroid

Our previous results indicated that monensin induced oxidative stress and altered the expression of genes involved in cholesterol and steroid biosynthesis (Ketola et al. 2010). Moreover, monensin reduced androgen receptor (AR) signalling and showed synergistic growth inhibitory effects with anti-androgens in prostate cancer cells. To validate the monensin induced changes in cellular steroid levels, steroid profiling was performed in VCaP prostate cancer cells. Cells were exposed to ethanol or monensin (1 μM) for six hours and steroid profiles were studied using gas chromatocraphy - mass spectrometry (GC-MS). The results presented as a heat-map in Fig. 6 show that the most prominent changes in response to monensin exposure were the induction of 7-ketocholesterol and aldosterone

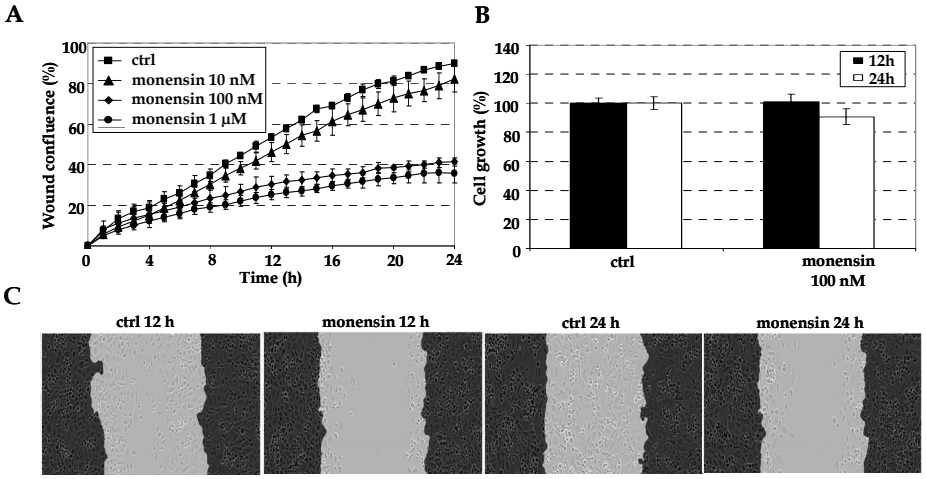

Fig. 5. The effect of monensin exposure on PC-3 prostate cancer cell migration. A) Relative wound confluency in response to monensin (10 nM, 100 nM and 1 µM) or control (ethanol) was monitored for 24 hours. B) Results from cell viability assay in response to monensin exposure (100 nM) for 12 and 24 hours in PC-3 cells. C) Pictures of scratch-wounded wells in response to 100 nM monensin or ethanol exposure at 12- and 24-hour time points. The wound margin in the beginning of the experiment is coloured in dark grey.

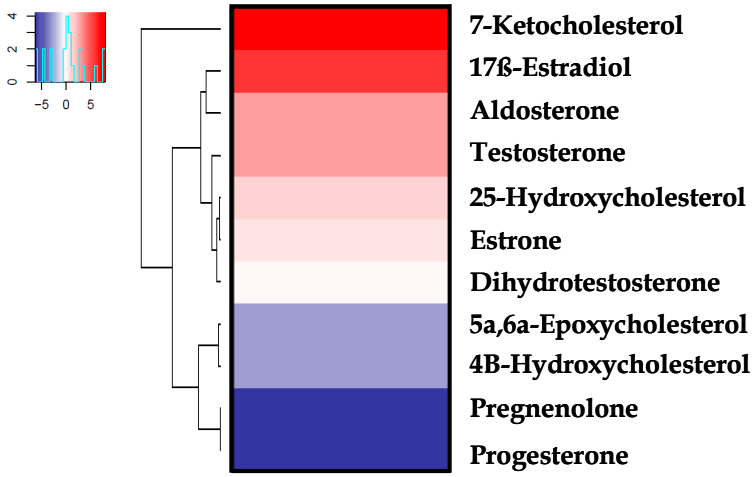

Fig. 6. Steroid profiling heat-map of monensin exposed VCaP prostate cancer cells. The cells were exposed to monensin (1 µM) for 6 hours and the steroid profile was measured with gas chromatography-mass spectrometry (GC-MS). The levels of individual steroids in monensin exposed cells were compared to ethanol exposed samples (presented as fold change, red: induction, blue: reduction).

levels as well as decrease in progesterone and pregnenolone levels. Aldosterone and 7-ketocholesterol are known oxidative stress inducers whereas progesterone has antioxidant properties (Leonarduzzi et al., 2006, Gramajo et al., 2010, Lee et al., 2009, Calo et al., 2010, Queisser et al., 2011, Ozacmak et al., 2009). Moreover, progesterone and pregnenolone are androgen precursors which have been suggested to play a major role in prostate cancer cell survival (Locke et al. 2008). Interestingly, progesterone has also been shown to promote mammary stem cell expansion (Joshi et al. 2010). The steroid profiling validates also the previous Connectivity Map results which indicated that monensin has opposite effects to progesterone and pregnenolone (Ketola et al. 2010). Taken together, monensin induced alterations in cellular steroid profile indicated that monensin induced oxidative stress results from increased aldosterone and 7-ketocholesterol levels as well as decreased progesterone and pregnenolone levels.

4. Conclusion

In this study, we explored the molecular consequences of monensin exposure in cultured prostate cancer cells. Our previous study indicated that monensin inhibited selectively prostate cancer cell viability at nanomolar concentrations by inducing oxidative stress. Cancer cells are constantly under pro-oxidative state (Szatrowski, Nathan 1991, Toyokuni et al. 1995). Long-term oxidative stress stimulates cell growth and proliferation, contributes to metastatic process and promotes cancer cell invasiveness and migration (Mori, Shibanuma & Nose 2004, Sung et al. 2006). Therefore, cancer cells need strong antioxidant mechanisms to survive and profit from these oxidative stress induced changes. Interfering redox balance has been suggested as a potential mean to selectively target cancer cells for example by increasing the cellular ROS level or reducing the expression of antioxidative enzymes (Pelicano, Carney & Huang 2004). Our results with monensin support this hypothesis.

Our previous Connectivity Map results indicated that monensin has agonistic effects to NF-κB inactivator and oxidative stress inducer niclosamide supporting monensin as a potent NF-κB inhibitor. Here, we showed that although monensin reduced the activities of several pathways known to play a role in tumourigenesis, the strongest reduction was seen in NF κB signalling. NF-κB activity promotes cell viability, tumorigenesis and metastasis as well as correlates with poor prognosis in prostate cancer patients (Blum et al. 2009, Sarkar et al. 2008). Importantly, NF-κB regulates the expression of genes responsible for antioxidant defence capacity and its inhibition induces oxidative stress as well as reduces tumourigenesis, metastasis and cancer stem cell potential (Gloire, Legrand-Poels & Piette 2006, Sarkar et al. 2008, Gluschnaider et al. 2010). NF-κB inhibitors are known to decrease AR signalling *in vitro* and reduce the growth of androgen deprivation-resistant prostate cancer xenografts *in vivo* (Jin et al. 2008, Zhang et al. 2009b). However, at present no specific NF-κB inhibitors have reached the stage of clinical trials for prostate cancer treatment (Mahon et al. 2011). Our results support NF-κB as the main mediator of monensin induced oxidative stress, which may also contribute to the reduced androgen signalling and induction of apoptosis in monensin exposed prostate cancer cells.

Recently, cancer stem cell targeting has raised a lot of interest as a prominent way to target cancer drug resistance and metastasic growth (Mimeault, Batra 2011, Clayton, Mousa 2011). In comparison to cancer cells, ROS levels in cancer stem cells are lower due to controlled

redox balance system such as high ALDH and CD44 expression protecting cancer stem cells from oxidative stress (Kobayashi, Suda 2011a, Croker, Allan 2011, Ishimoto et al. 2011). Interestingly, NF-κB inhibition induces apoptosis in prostate cancer stem cells and thus NF-κB is considered as an attractive chemotherapeutic target also against cancer stem cells (Jin et al. 2008, Birnie et al. 2008). Since monensin reduced ALDH and NF-κB activities, we studied the fraction of $CD44^+/CD24^-$ cells in prostate cancer cell cultures in response to monensin exposure. The results confirmed that monensin reduced the amount of prostate cancer stem cells. Moreover, monensin induced epithelial cell differentiation and reduced motility in cultured prostate cancer cells, suggesting that monensin inhibits prostate tumorigenesis by multiple ways. Cancer stem cell inhibitor and cell differentiation inducer salinomycin shares a similar structure as monensin, supporting the functional similarities between these two compounds (Gupta et al. 2009).

Steroidogenic enzymes as well as stem cell markers are induced in castration-resistant prostate cancer both *in vitro* and *in vivo* (Blum et al. 2009, Pfeiffer et al. 2011). Several studies have shown that steroidogenesis is inhibited by ROS (Tsai et al. 2003, Stocco, Wells & Clark 1993, Kodaman, Aten & Behrman 1994, Lee et al. 2009, Abidi et al. 2008). Our previous results indicated that monensin reduced androgen receptor signalling. Here, we showed that monensin increases the levels of oxidative stress inducing steroids, 7-ketocholesterol and aldosterone, and reduces androgen precursor and antioxidative steroid progesterone in cultured prostate cancer cells. Interestingly, 7-ketocholesterol is a ligand for aryl hydrocarbon receptor (AhR) and acts as AhR antagonist (Savouret et al. 2001). AhR expression is elevated in malignant prostate cells and its signalling is activated in prostate cancer stem cells (Blum et al. 2009, Gluschnaider et al. 2010). AhR pathway increases the expression of ALDH proteins and protects cells against oxidative stress and foreign chemicals (Lindros et al. 1998, Vrzal, Ulrichova & Dvorak 2004, Nebert et al. 2000, Kohle, Bock 2007). Interestingly, AhR binds to NF-κB, induces MYC activation and reduces E-cadherin expression in breast cancer cells (Kim et al. 2000, Dietrich, Kaina 2010). Moreover, AhR can form a complex with androgen receptor and protect prostate cancer cells during androgen ablation (Ohtake, Fujii-Kuriyama & Kato 2009, Gluschnaider et al. 2010). Thus, our results indicate that monensin induced oxidative stress is potentially transmitted via reduced AhR signalling. This hypothesis is further supported by our previous gene expression results indicating that although AhR itself was not altered, the expression of AhR target gene mRNAs were decreased in response to monensin exposure (Ketola et al. 2010).

Taken together, we hypothesize that monensin induced anti-neoplastic effects result mainly due to increase in oxidative stress. The overview figure 7 illustrates the various changes occurring in prostate cancer cells in response to monensin exposure. The cancer selectiveness could be explained by increased intracellular ROS due to reduced antioxidative capacity sensitizing prostate cancer cells to oxidative stress. Since normal prostate epithelial cells are not under intensive oxidative stress and therefore, are less dependent on the function of antioxidative genes, they are not as sensitive to monensin exposure as cancer cells. In conclusion, our results support the idea that impairing the redox control, which has a crucial role in cancer cells enabling survival in high intracellular ROS, is a potent way to target prostate cancer cells.

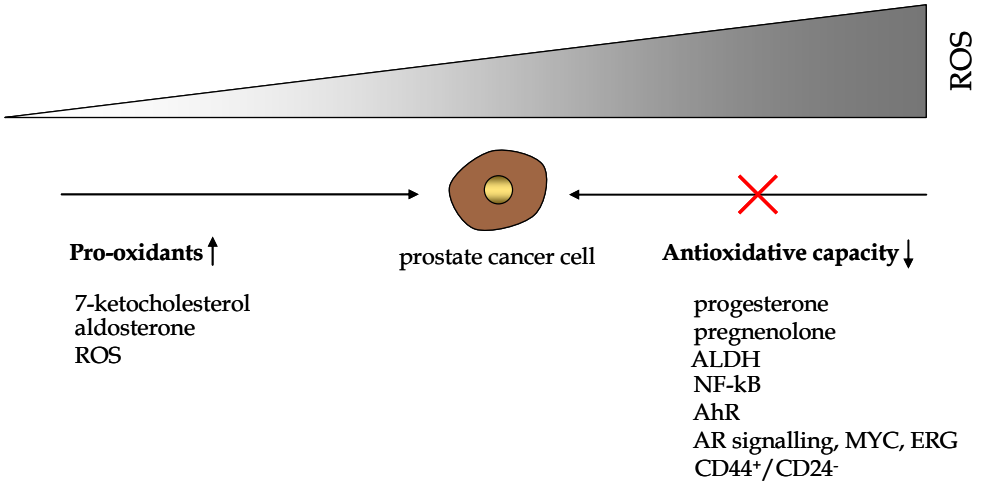

Fig. 7. Overview of monensin induced changes in prostate cancer cells. The figure idea adapted from Cairns, R.A., Harris, I.S. & Mak, T.W. 2011.

5. Acknowledgement

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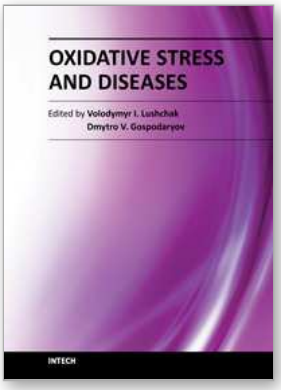

Oxidative Stress and Diseases

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The development of hypothesis of oxidative stress in the 1980s stimulated the interest of biological and biomedical sciences that extends to this day. The contributions in this book provide the reader with the knowledge accumulated to date on the involvement of reactive oxygen species in different pathologies in humans and animals. The chapters are organized into sections based on specific groups of pathologies such as cardiovascular diseases, diabetes, cancer, neuronal, hormonal, and systemic ones. A special section highlights potential of antioxidants to protect organisms against deleterious effects of reactive species. This book should appeal to many researchers, who should find its information useful for advancing their fields.

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