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CHEMICAL BIOLOGY SCREEN FOR PROSTATE CANCER THERAPEUTICS

by

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"*Don't even try to understand all complex ways of tumorigenesis – just cure it*"

James D. Watson
Hallmarks and Horizons of Cancer
September 2011
Lausanne, Switzerland

Kirsi Ketola
Chemical Biology Screen for Prostate Cancer Therapeutics

From the Department of Biochemistry and Food Chemistry, University of Turku; Turku Centre for Biotechnology; Medical Biotechnology, VTT Technical Research Centre of Finland; and Drug Discovery Graduate School (DDGS), Turku, Finland
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ABSTRACT

Prostate cancer initially responds to hormone-based therapeutics such as anti-androgen treatment or chemotherapeutics but eventually becomes resistant. Novel treatment options are therefore urgently needed. This thesis study applied a high-throughput screen of 4910 known drugs and drug-like small molecules to identify compounds that selectively inhibit growth of prostate cancer cells. In addition, the mechanisms underlying the cellular sensitivity to potent cancer selective compounds were addressed.

Surprisingly, many of the compounds currently used in the clinics or studied in clinical trials were not cancer-selective. Only four drugs, aldehyde dehydrogenase inhibitor disulfiram (Antabus), antibiotic ionophore monensin, histone deacetylase inhibitor tricostatin A and fungicide thiram inhibited prostate cancer cell growth at nanomolar concentrations without major effects on non-malignant prostate epithelial cells. Disulfiram, monensin and a structurally similar compound to monensin, salinomycin, induced oxidative stress and inhibited aldehyde dehydrogenase activity. Moreover, monensin and salinomycin reduced androgen receptor signalling and steroidogenesis, enforced cell differentiation and reduced the overall levels of cancer stem cells.

Taken together, novel and potentially prostate cancer-selective therapeutic agents were identified in this study, including the description of a multitude of intoxicating mechanisms such as those relating to oxidative stress. The results provide novel insights into prostate cancer biology and exemplify useful means of considering novel approaches to cancer treatment.

Keywords: prostate cancer, oxidative stress, androgen receptor, disulfiram, monensin, salinomycin, aldehyde dehydrogenase, cancer stem cells

Kirsi Ketola
Lääkeaineiden tehoseulonta eturauhassyöpäsolujen kasvun estoon

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TIIVISTELMÄ

Eturauhassyöpä on miesten yleisin syöpä länsimaissa. Vaikka nykyiset hoidot kuten antiandrogeenihoito sekä kemoterapia tehoavat aluksi, kasvaimet tulevat lopulta resistenteiksi, jonka vuoksi eturauhassyövän hoitamiseksi tarvitaan uusia vaihtoehtoja. Väitöskirjatutkimuksessa selvitettiin 4910 tunnetun lääkkeen ja pienmolekyylin vaikutuksia eturauhassyöpäsolujen kasvuun sekä tutkittiin lääkkeiden vaikutusmekanismeja eturauhassyöpäsoluissa. Jatkotutkimuksiin valittiin lääkkeet, jotka estävät eturauhassyöpäsolujen kasvua, mutta eivät tehoa normaaleihin eturauhassoluihin. Tutkimuksen tarkoituksena oli myös saada lisätietoa eturauhassyövän molekyylimekanismeista.

Tulokset osoittivat, että monet tutkimuksessa mukana olevista lääkkeistä, joiden on aikaisemmin havaittu estävän eturauhassyöpäsolujen kasvua, olivat yhtä tehokkaita sekä syöpä- että normaalisoluissa. Väitöskirjatutkimuksessa osoitettiin neljän lääkkeen, aldehydidehydrogenaasi-inhibiittorin disulfiraamin (Antabus), monensiini-antibiootin, sienimyrkky thiramin ja histonideasetylaasi-inhibiittorin tricostatin A:n vaikuttavan eturauhassyöpäsolujen kasvua ehkäisevästi ilman vaikutusta normaalien eturauhassolujen kasvuun. Disulfiraamin, monensiinin ja monensiinin kanssa rakenteellisesti samankaltaisen yhdisteen salinomysiinin osoitettiin lisäävän mm. oksidatiivista stressiä sekä vähentävän aldehydidehydrogenaasientsyymin aktiivisuutta eturauhassyöpäsoluissa. Lisäksi havaittiin, että monensiini ja salinomysiini vähensivät androgeenireseptorin toimintaa ja steroidituotantoa, aiheuttivat solujen erilaistumista sekä vähensivät ns. syöpäkantasolujen määrää.

Tutkimuksessa löydettiin jo käytössä oleville lääkeaineille uusia käyttökohteita. Keskeisenä yhteisenä eturauhassyöpäsolujen kasvua estävänä mekanismina kuvattiin solunsisäisen oksidatiivisen stressin lisääntyminen. Uusien mahdollisten lääkeaineiden lisäksi tutkimustyöstä saatiin uutta tietoa eturauhassyövän taustalla olevista molekyylimekanismeista.

Avainsanat: eturauhassyöpä, oksidatiivinen stressi, androgeenireseptori, disulfiraami, monensiini, salinomysiini, aldehydidehydrogenaasi, syöpäkantasolu

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ABBREVIATIONS

12,13-EODE	Leukotoxin B
AhR	Aryl hydrocarbon receptor
ALDH	Aldehyde dehydrogenase
ATF3	Activating transcription factor 3
AR	Androgen receptor
CD24	Cluster of Differentiation 24
CD44	Cluster of Differentiation 44
CSC	Cancer stem cell
DDIT3	DNA-damage-inducible transcript 3
DDIT4	DNA-damage-inducible transcript 4
DSF	Disulfiram, aldehyde dehydrogenase inhibitor
EMT	Epithelial-to-mesenchymal transition
ERG	Ets Related Gene
FDA	Food and Drug Administration
HDAC	Histone Deacetylase
HPV	Human papilloma virus
HTS	High-throughput screening
KLF6	Kruppel-like factor 6
MCM	Minichromosome maintenance complex
MT	Metallothionein
NF-kB	Nuclear factor kappa-B
PIN	Prostate intraepithelial neoplasia
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
siRNA	Small interfering ribonucleic acid
TMPRSS2	Transmembrane protease, serine 2
TSA	Tricostatin-A, Histone deacetylase inhibitor
TXNIP	Thioredoxin interacting protein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by Roman numerals I-IV. The original communications have been reproduced with the permission of the copyright holders. Unpublished data is also included.

I Iljin, K., Ketola, K., Vainio, P., Halonen, P., Kohonen, P., Fey, V., Grafström, R.C., Perälä, M., Kallioniemi, O., High-throughput cell-based screening of 4910 known drugs and drug-like small molecules identifies disulfiram as an inhibitor of prostate cancer cell growth. Clin Cancer Res., 2009, 15(19); 6070–6078.

II Ketola, K., Vainio, P., Fey, V., Kallioniemi, O. Iljin, I., Monensin Is a Potent Inducer of Oxidative Stress and Inhibitor of Androgen Signaling Leading to Apoptosis in Prostate Cancer Cells. Mol Cancer Ther., 2010, 9(12); 3175–85.

III Ketola, K., Vuoristo, A., Orešič, M., Kallioniemi, O. Iljin, I., Monensin induced oxidative stress reduces prostate cancer cell migration and cancer stem cell population. *In Press*, Oxidative stress 2, 2011.

IV Ketola, K., Hilvo, M., Hyötyläinen, T., Vuoristo, A., Ruskeepää, A-L., Orešič, M., Kallioniemi, O., Iljin, I., Salinomycin inhibits prostate cancer growth and migration via induction of oxidative stress. *In Press*, Br J Cancer, 2011.

INTRODUCTION

Prostate cancer is one of the most common malignancies and third leading cause of cancer deaths in developed countries (Jemal et al. 2011). The main treatment for prostate cancer is radical prostatectomy in which the tumour is surgically removed. In addition, anti-androgens are used in patients with localized and metastatic prostate cancer. Anti-androgen therapy initially reduces prostate cancer cell proliferation, but castration-resistant prostate cancer will develop eventually (Sharifi, Gulley & Dahut 2010). The therapeutic options for castration-resistant prostate cancer are limited and treatment responses to currently existing therapies are often unsatisfactory; cytotoxic therapy causes severe toxicity and eventually chemoresistance occurs (Tannock et al. 2004, Berthold et al. 2008). The median survival time for castration-resistant prostate cancer is approximately two years (Tannock et al. 2004). Therefore, novel agents to block the cell proliferation and to inhibit the primary prostate cancer cell progression to advanced stage are required. In addition, there is an urgent need for novel approaches to target advanced and metastatic prostate cancer cells. Moreover, better understanding of the disease development may provide new insights in the development of efficient therapeutic strategies to improve the survival of prostate cancer patients also in advanced stage.

The current pipeline of getting novel drugs to market is long. Drug target has to be validated and several in vitro assays and pharmacological profiling need to be performed before proceeding potential compound to preclinical and clinical trials. The drug discovery pipeline is also expensive: two billion euros are needed to gain one drug on the market in the EU. Moreover, the currently used chemotherapeutics are not cancer-selective and unwanted side effects and toxicity occur. Today, utilizing high-throughput cell-based screening assays is not an option in the industrial field alone; academic screening centres have also gained the facility to produce high quality high-throughput screening assays. The advantage of academic high-throughput screenings is that the desired outcome is not necessary a two billion drug; the screening may provide a novel indication for a less expensive alternative, “non-profit drug”, if the drug is already used and known for other diseases. In that case, proceeding to clinical trials may need support from the government if there are only small commercializing opportunities. However, these kinds of approaches may offer cost-effective solutions to gain novel drugs rapidly into clinical trials. In addition, a high-throughput screening can also provide important novelty about the biology and molecular mechanisms of the diseases. Therefore, a chemical biology screening approach can be used as a tool to provide novel information to target cancer cells when the molecular mechanisms of the cancer-selective drugs are known.

In this thesis, a chemical biology high-throughput screening approach was utilized to identify the most efficient prostate cancer cell growth inhibiting drugs among 4910 known drugs and small molecular compounds with already known pharmacological

and toxicological profiles. The aim was to find cancer-selective compounds which target prostate cancer cells with minimal effects on non-malignant prostate epithelial cells. The screening involved most of the drugs on the market today. As a result, these drugs are ready to be taken rapidly to pre-clinical and clinical studies. The focus was to identify novel inhibitors for prostate cancer cells containing TMPRSS2-ERG fusion, since ~40-70% of prostate cancer cases have been reported to contain this rearrangement (Tomlins et al. 2005, Iljin et al. 2006, Perner et al. 2006, Mosquera et al. 2007, Attard et al. 2009, Magi-Galluzzi et al. 2011). In addition to identifying novel indications for known drugs, the aim was to increase understanding of cancer biology by studying the cancer-selective drug responses in prostate cancer cells.

REVIEW OF THE LITERATURE

1. Cancer

Cancer is a group of diseases in which the normal cell behaviour is disrupted by mutations or other abnormalities causing tumours. The six hallmarks of cancer - sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis - have recently been updated with two other hallmarks: reprogramming of energy metabolism and evading immune destruction (Hanahan, Weinberg 2000, Hanahan, Weinberg 2011). Since several cellular mechanisms have to be targeted to reduce cancer cell growth, the development of new treatments requires careful design of agents and/or synergistic approaches to target as many of these hallmarks as possible.

1.1 Prostate cancer

Prostate cancer is one of the most common cancers in the Western male population and it is the most common cancer among men in Finland. The first line treatment option for prostate cancer is surgical removal of the tumour. If the tumour recurs locally or metastasises, the circulating androgen levels that drive prostate cancer cell proliferation, are commonly diminished through castration or chemical castration using anti-androgens. The sentence "castration-resistant prostate cancer" used in this thesis describes advanced, androgen independent prostate cancer occurred locally or in metastases after chemical or surgical castration. Although the prostate cancer cells first respond to anti-androgen therapy, often castration-resistant prostate cancer occurs (Sharifi, Gulley & Dahut 2010). The survival time for castration-resistant prostate cancer is approximately two years (Tannock et al. 2004).

1.2. Molecular basis of prostate cancer

1.2.1. Androgen receptor -linked aberrations

The very first prostate cancer initiating mechanisms are not comprehensively known, due to the heterogeneity of the tumours. Many studies have though defined several molecular transformations in prostate cancer cells compared to prostate epithelial cells that contribute to the prostate cancer development and progression. The main signalling network contributing prostate cancer cell growth is androgen receptor (AR) signalling. In normal prostate epithelial cells, androgens stimulate AR that activates a signalling network regulating cell growth and production of seminal fluid. In prostate cancer, the malignant cells have gained the ability to harness the pro-proliferative effect of androgens via e.g. expressing high levels of AR. AR is a nuclear transcription

factor which has several target genes whose products control cell proliferation, survival and differentiation as well as lipid metabolism in the cells. The androgen ligand binding to AR induces conformation change, receptor dimerization and translocation of the androgen-AR complex to nucleus in which the complex binds to androgen response elements (ARE) and leads to transcription of AR target genes (Setlur, Rubin 2005, Berry, Maitland & Collins 2008). The AR activity can be modulated in different ways: for example, several coacitivators and corepressors have been described to control AR binding (Shang, Myers & Brown 2002, Cano et al. 2007). Thus, blocking of AR signalling is one of the main treatment options to reduce prostate cancer cell growth.

The AR signalling can first be blocked using antiandrogen treatment. Eventually, prostate cancer cells deregulate their AR signalling, gain androgen independence and become castration-resistant. Enhanced expression and sensitivity of androgen receptor (AR) as well as intratumoural androgen production via steroidogenesis have been proposed as mechanisms of prostate cancer androgen independence and development of castration-resistant prostate cancer (Yuan, Balk 2009, Feldman, Feldman 2001, Karlou, Tzelepi & Efstathiou 2010, Locke et al. 2008, Leon et al. 2009). Moreover, AR can be mutated which allows other steroids and antiandrogens to activate AR signalling (Harris et al. 1990, Culig et al. 1993). Prostate specific antigen (PSA) is commonly used as an indicator of active AR signalling. The levels of PSA are detected to identify prostate cancer and to indicate whether antiandrogen treatment has successfully reduced prostate cancer growth.

1.2.2. Disorder of other receptors

Several receptors are also known to be activated in prostate cancer. Those include an epidermal growth factor (EGFR), Hedgehog, Wnt/β-catenin, hyaluronan (HA)/CD44, transforming growth factor-β (TGF-β)/TGF-βR and stromal cell-derived factor-1 (SDF-1)/CXC chemokine receptor 4 (CXCR4) well reviewed by Mimeault and Batra (Mimeault, Batra 2011). Activation of these receptors in prostate cancer induces cell growth and survival as well as migration, invasion, metastasis and chemotherapy resistance.

1.2.3. Chromosomal abnormalities, tumour suppressors and proto-oncogenes

Androgen regulated TMPRSS2-ERG fusion has been found in 40 to 70% of the prostate cancer cases. In addition, other oncogenic gene fusions, e.g. with ETS factors ETV1, ETV4, ETV5, have been discovered in prostate tumours (Tomlins et al. 2005, Iljin et al. 2006, Perner et al. 2006, Mosquera et al. 2007, Attard et al. 2009, Magi-Galluzzi et al. 2011, Tomlins et al. 2006, Tomlins et al. 2007). It has been suggested though, that ERG fusion can be bypassed at later stages of cancer progression (Hermans et al. 2006). Moreover, transcription factor and oncogene MYC has a role in the early progression in prostate cancer (Gurel et al. 2008, Koh et al. 2010). Furthermore, it has been shown that MYC and ERG are closely cooperating in prostate cancer (Sun et al. 2008).

In addition, several tumour suppressors such as p53 and PTEN are down-regulated in prostate cancer whereas overexpression of oncogenes such as MYC and TMPRSS2-ERG fusion are linked to prostate cancer initiation, progression, invasion and metastasis (Tomlins et al. 2005, Kim et al. 2011a, Mellon et al. 1992, Feilotter et al. 1998, Fleming et al. 1986, Buttyan et al. 1987, Gurel et al. 2008, Hawksworth et al. 2010). Moreover, transcription factor Nuclear factor kappa B (NF-κB) that regulates cell survival by controlling several cellular response signals such as inflammatory and antiapoptotic responses, is highly active in several cancers including prostate cancer (Pikarsky et al. 2004, Karin, Greten 2005, Meng et al. 2010, Sarkar et al. 2008, Gluschnaider et al. 2010). Furthermore, NF-κB is activated by TMPRSS2-ERG fusion isoforms in prostate cancer indicating that NF-κB has a significant role in prostate cancer tumourigenesis (Wang et al. 2010). Taken together, several molecular alterations compared to normal prostate epithelial cells describe the malignant progression of prostate cancer.

1.3. Redox regulation in prostate cancer

Induction of oxidative stress during aging is considered one of the main causes of prostate cancer. Almost all men are will eventually develop microscopic prostate cancer (Minelli et al. 2009). Oxidative stress occurs when redox regulation is in imbalance: intracellular free radicals and reactive oxygen species (ROS) form due to incomplete antioxidative defence capacity. ROS are important regulators of signalling pathways in normal and cancer cells and ROS are involved in tumourigenesis (Weinberg, Chandel 2009). Thus, it has been suggested that supplementation of antioxidants might reduce the risk of prostate cancer and delay carcinogenic process. Indeed, compared to non-malignant prostate epithelial cells, the level of oxidative stress has been found to be elevated in prostate cancer cells (Davydov, Dobaeva & Bozhkov 2004, Tam et al. 2003, Khandrika et al. 2009, Yossepowitch et al. 2007, Kumar et al. 2008, Dakhova et al. 2009).

However, since cancer cells operate under higher levels of ROS, they need an effective antioxidative capacity. Thus, redox regulation and active antioxidant mechanisms are important in maintaining the cell's ability to get more mutations and proliferate without exposing the cell to too high level of oxidative stress. Interestingly, many of the key prostate cancer oncogenes have been described to have antioxidative properties and to take part in the antioxidant defence system. AR signalling is known to increase the levels of ROS and reduce the antioxidative capacity in prostate cancer cells (Pinthus et al. 2007, Tam et al. 2003, Schultz, Abdel-Mageed & Mondal 2010). MYC and ERG expressions are also involved in the protection of cells against oxidative stress (Benassi et al. 2006, Swanson et al. 2011, DeNicola et al. 2011). In addition, activation of NF-κB is one of the key cellular defence mechanisms for reactive oxygen species. NF-κB activity promotes cell viability, tumourigenesis and metastasis as well as enhances the resistance to oxidative stress (Meng et al. 2010, Gloire, Legrand-Poels & Piette 2006, Sarkar et al. 2008, Gluschnaider et al. 2010). Thus, the studies suggest that targeting oxidative defence mechanisms could be a potential means to target prostate

cancer cells. This exposes the cancer cells to unmanageable levels of oxidative stress. Impairing redox control can selectively target cancer cells for example via ROS induction of reduction of antioxidative enzymes (Pelicano, Carney & Huang 2004, Gupte, Mumper 2009). Interestingly, many anti-neoplastic agents such as vinblastine, cisplatin, mitomycin C, doxorubicin, camptothecin, inostamycin, neocarzinostatin, etoposide, arsenic trioxide and nonsteroidal anti-inflammatory drugs induce oxidative stress in prostate cancer cells (Fang, Nakamura & Iyer 2007, Rigas, Sun 2008, Sun et al. 2011). Moreover, the reduction of AR signalling through oxidative stress induction has been suggested as an emerging theme for novel prostate cancer therapeutics (Fajardo et al. 2011). Figure 1 summarizes the malignant progression and levels of ROS.

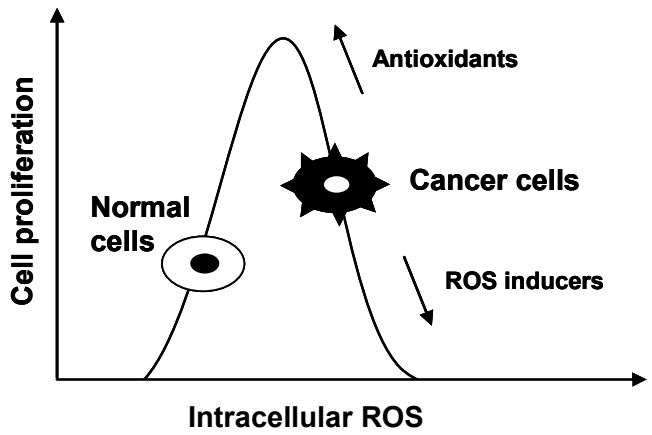

Figure 1. The malignant progression and the effects of reactive oxygen species (ROS) inducers and antioxidants depend on the basal intracellular levels of ROS in normal and cancer cells. Figure idea adapted from Gupte, Mumper 2009 (III).

1.4. Prostate cancer stem cells, epithelial-to-mesenchymal transition and metastasis

Metastasised prostate cancer is the main cause of prostate cancer mortality. Cancer metastasis occurs when tumour cells get characteristics to leave the primary tumour, adapt to the microenvironment of other tissue, colonize and form a new tumour (Hanahan, Weinberg 2000, Hanahan, Weinberg 2011, Chaffer, Weinberg 2011). The two hypotheses - cancer stem cells (CSC) and epithelial-to-mesenchymal transition (EMT) - have recently arisen as the main hypotheses describing the metastatic behaviour of the tumour cells (Chaffer, Weinberg 2011, Floor et al. 2011). Cancer stem cell hypothesis, first described by John Dick and Bonnett in leukemia, reveals that only a small subset of cancer cells have the ability to initiate tumours (Bonnet, Dick 1997). Several solid tumours have been shown to include cancer stem cells (Reya et al. 2001, Al-Hajj et al. 2003, Singh et al. 2004, O'Brien et al. 2007, Li et al. 2007a). In the EMT

theory, every single tumour cell has a capacity to transform, gain metastatic potential to migrate and invade. Epithelial cells are polarized and are normally attached to basement membrane in an organ whereas mesenchymal cells are more invasive and resistant to apoptosis. Localized cancer cells that undergo EMT, gain properties to migrate and leave the original tumour tissue and eventually form metastasis. Some studies have suggested that cells gaining EMT characteristics also become cancer stem cells. In other words, cancer stem cells have been formed from cancer cells through EMT (Chaffer, Weinberg 2011, Floor et al. 2011). Nevertheless, cells that have been modified through EMT, are more migratorial and have characteristics of cells that can move from one place to another. Figure 2 describes the CSC and EMT hypotheses and metastatic potential of cancer cells (Chaffer, Weinberg 2011).

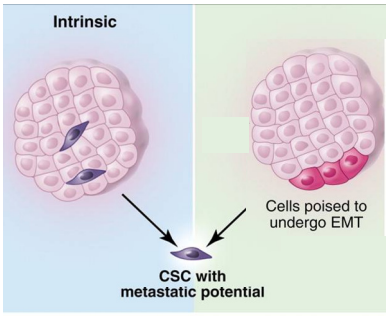

Figure 2. Two hypotheses of generation of cancer stem cells (CSC): induced through EMT or intrinsic derived from normal tissue stem cells. Figure adapted and modified from Chaffer, Weinberg 2011.

The concept of CSC and EMT has been most comprehensively studied in breast cancer. However, CSCs have been identified in cell lines models and in xenograft tumours also in prostate cancer (Collins et al. 2005, Patrawala et al. 2006, Patrawala et al. 2005, Patrawala et al. 2007, Pfeiffer, Schalken 2010, Gu et al. 2007, Li et al. 2008, Eaton et al. 2010). Thus, these studies indicate that prostate cancer tumours contain cancer stem cells. Prostate CSCs have been identified to have characteristics of normal stem cells such as high aldehyde dehydrogenase (ALDH) and CD44 expression (Burger et al. 2009, van den Hoogen et al. 2010, Klarmann et al. 2009, Patrawala et al. 2006). For example, high-ALDH cells are more potent to migrate and form metastases in prostate cancer *in vitro* and *in vivo* than low-ALDH cells (van den Hoogen et al. 2010). However, the origin of prostate CSCs remains to be clarified. Prostate CSCs are suggested to derive from basal stem cells (expressing low levels of AR) or luminal progenitor cells (expressing AR) through transformation or they may have evolved through reprogramming of already differentiated cells, most likely through EMT (Li et al. 2007b, Lawson, Witte 2007, Lawson et al. 2010, Wang et al. 2009, Werbowetski-Ogilvie, Bhatia 2008, Li, Tang 2011, Wang, Shen 2011).

The EMT induced prostate cancer cells also have characteristics of stem cells and the EMT phenotype associates with metastatic invasion and therapeutic resistance

phenotypes in prostate cancer (Kong et al. 2010, Xie et al. 2010). EMT induced cells are known to gain CSC characteristics also in breast cancer (Mani et al. 2008). Interestingly, androgen was recently identified to induce cell migration in prostate cancer cells (Castoria et al. 2011). Moreover, TMPRSS2-ERG is known to promote EMT (Gupta et al. 2010, Leshem et al. 2011). Thus, it has been suggested that EMT and CSC hypotheses are closely linked and contribute to prostate cancer metastatic potential (Floor et al. 2011, Li, Tang 2011).

However, despite the origin of prostate cancer stem cells, those cells have been suggested to be very different compared to the original tumour initiating cell and thus may not have any characteristics of the original cell of origin in the end (Fig. 3) (Wang, Shen 2011). In this study, the phrase "cancer stem cell" describes the fraction of cultured cells that have high ALDH or $CD44^{+}/CD24^{-}$ expression and thus potentially have cancer-initiating properties.

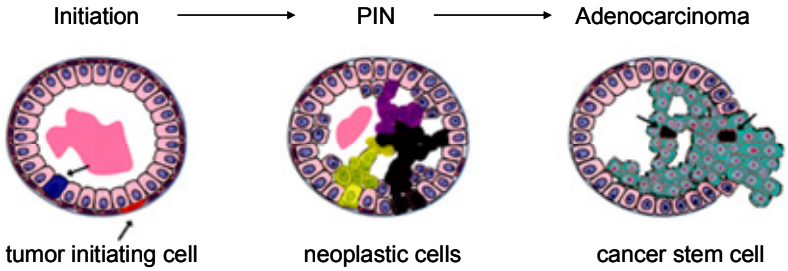

Figure 3. Cancer stem cell model for prostate cancer progression. Cancer initiation may occur in genetically mutated basal (red) or luminal (blue) cells. As neoplastic cells (yellow, purple and black) accumulate more mutations in prostatic intraepithelial neoplasia (PIN) lesions, some of their progeny may become selected to acquire CSC properties (brown). Such CSCs would give rise to the bulk of tumour cells with less proliferative potential (green). Note that the prostate CSCs may not have any features of the original cell of origin in this model. Figure and the figure legend adapted and modified from Wang, Shen 2011.

In conclusion, an emerging interest has arisen recently towards the research of CSCs and EMT in several cancers including prostate cancer. However, to understand the biology, cell competition and Darwinian evolution of CSCs and EMT induced cells in tumour microenvironment, and to find specific markers for these cells still requires further studies (Floor et al. 2011). Table 1 illustrates the similar characteristics that have currently been accepted for cancer stem cells and EMT cells, such as capability to start tumours and resistance to different therapies (Floor et al. 2011).

Table 1. Similar properties of CSCs and EMT cells. Table adapted from Floor et al. 2011.

Characteristics	Classical CSCs	EMT cells
Xenografts	+	+
Participating to tumorospheres	+	+
Capable of generating whole cancers	+	+
Differentiation	0	0
Resistance to chemotherapy	+	+
Resistance to RX	+	+
Resistance to UV	+	+
Resistance to apoptosis	+	+
Proliferation	0 or ++	0
Found in cancer cell lines	+?	+
EMT gene expression	+?	+
CSC gene expression	+	+?
$CD44^{+}/CD24^{+/-}$ (breast)	+	+?
Reversible state	0?? (Permanent?)	+ (Reversible)

1.5. Oxidative stress, prostate cancer stem cells and metastasis

Recently, CSC targeting has been proposed as a prominent way to target cancer drug resistance and prevent metastases (Mimeault, Batra 2011, Clayton, Mousa 2011). Redox control, antioxidative defence mechanisms and ROS-scavenging systems have also been identified as important regulators of CSC potential, metastasis and chemoresistance (Kobayashi, Suda 2011, Cairns, Harris & Mak 2011, Pani, Galeotti & Chiarugi 2010). In addition, prostate CSC marker CD44 has been identified as an antioxidative gene which regulates redox status in cancer cells (Ishimoto et al. 2011). Moreover, CSC marker ALDH correlates with the poor outcome in prostate and other cancers, detoxifies intracellular aldehydes and contributes to induced chemotherapy and radiation resistance in CSCs (Davydov, Dobaeva & Bozhkov 2004, Burger et al. 2009, Li et al. 2010, Yu et al. 2011, Zhang et al. 2009a, Duester 2000, Magni et al. 1996, Sophos, Vasiliou 2003, Yoshida et al. 1998, Croker, Allan 2011). Furthermore, ERG oncogene is known to have an important role in stem cell biology (Loughran et al. 2008). ERG has also been suggested to have a role in regulating the self-renewal processes in prostate cancer and thus promote the progression of prostate tumours (Blum et al. 2009). In addition, MYC increases the fraction of tumour-initiating cells and causes stem cell-like transcriptional program in differentiated adult cells (Eilers, Eisenman 2008). It is also known that NF-κB regulates cell migration and its inhibition causes apoptosis in prostate CSCs (Zhang et al. 2009b, Rajasekhar et al. 2011, Birnie et al. 2008, Jin et al. 2008). Interestingly, induced steroidogenic enzymes and stem cell markers have also been identified in castration-resistant prostate cancer and several studies have shown that steroidogenesis is inhibited by ROS (Blum et al. 2009, Pfeiffer et al. 2011, Tsai et al. 2003, Kodaman, Aten & Behrman 1994, Lee et al. 2009a, Abidi et al. 2008).

As a summary, the recent studies suggest that redox control can be impaired by affecting key prostate cancer oncogenes and regulators. Moreover the studies suggest that deregulated redox control offers a potential way to target prostate cancer and prostate cancer stem cells.

1.6. Current therapy for prostate cancer

Prostate cancer is currently diagnosed measuring the levels of prostate specific antigen (PSA) in the blood. The tumour size is first monitored, since the progression of severe prostate cancer normally takes many years. Since the treatment of prostate cancer can cause unwanted side-effects, patient age, general health and other risk factors are considered before treatment. The main treatment for prostate cancer is surgical removal of tumour in prostatectomy, radiation therapy and in the case of recurrence chemical castration via androgen deprivation. Flutamide and bicalutamide are the most common antiandrogens used for the treatment of prostate cancer. In addition, novel antiandrogens are studied *in vitro* and in clinical trials (Chen, Clegg & Scher 2009). However, prostate cancer becomes eventually resistant to antiandrogens and there is currently no efficient treatment for castration-resistant prostate cancer.

Docetaxel chemotherapy is a treatment option for metastatic castration-resistant prostate cancer and it may prolong survival and quality of life for a few months, but cannot cure the disease. In addition, it has toxicity problems which limit the treatment dose and duration (Tannock et al. 2004). The survival time for castration-resistant prostate cancer remains around two years (Tannock et al. 2004). Interestingly, several novel chemotherapeutics from novel androgen receptor blockers to angiogenesis inhibitors such as Bevacizumab, Aflibercept, Lenalidomide, ZD4054, Astrasentan, Dasatinib, Denosumab, Sipuleucel-T (Provenge), Abiraterone, Sunitinib, Cabazitaxel, Abiraterone and MDV3100 are studied in Phase III clinical trials (Table 2) (Bracarda et al. 2011). In addition to Sipuleucel-T, another prostate cancer vaccine Ipilimumab is in Phase III clinical trials (identifier NCT00861614). Castration-resistant prostate cancer has been thought to be very independent from androgen signalling. However, new AR blockers have recently been proposed as novel options for castration-resistant prostate cancer (Ryan, Tindall 2011, Yap et al. 2011). Although several novel therapeutics have reached clinical trials, some of the trials have failed and survival times have not been extended significantly. Thus, novel therapeutic options to target castration-resistant and metastatic prostate cancers are still needed. In addition, neither prostate cancer stem cell inhibitors nor reducers of EMT in prostate cancer have been identified.

Table 2. Selected Phase III clinical trials for first*- and second-line metastatic castration-resistant prostate cancer. Table and table legend adapted from Bracarda et al. 2011.

Agent	Target	Randomization	Identifier
Bevacizumab*	VEGF	Docetaxel plus prednisone ± bevacizumab	NCT00110214
Aflibercept*	VEGF	Docetaxel plus prednisone ± aflibercept	NCT00519285
Lenalidomide*		Docetaxel plus prednisone ± lenalidomide	NCT00988208
ZD4054*	Endothelin receptor	Docetaxel ± ZD4054	NCT00617669
Astrasentan*	Endothelin receptor	Docetaxel plus prednisone ± astrasentan	NCT00134056
Dasatinib*	Src family kinases	Docetaxel plus prednisone ± dasatinib	NCT00744497
Denosumab*	RANKL	Denosumab vs zoledronic acid	NCT00321620
Sipuleucel-T*	Immune therapy	Sipuleucel-T vs placebo	NCT00065442
Abiraterone*	CYP17	Abiraterone + prednisone/prednisolone vs placebo + prednisone/prednisolone	NCT00887198
Sunitinib	VEGF receptor	Sunitinib + prednisone vs placebo prednisone	NCT00676650
Cabazitaxel	Tubulin	Cabazitaxel + prednisone vs mitoxantrone + prednisone	NCT00417079
Abiraterone	CYP17	Abiraterone + prednisone/prednisolone vs placebo + prednisone/prednisolone	NCT00638690
MDV3100	Androgen receptor	MDV3100 vs placebo	NCT00974311

AIMS OF THE STUDY

The treatment options for prostate cancer are limited and novel approaches to target prostate cancer cells are needed. In addition, further knowledge of the molecular biology of prostate cancer is needed to help design novel therapeutic strategies. In this study, a chemical biology high-throughput screening approach was utilized to identify inhibitors of prostate cancer cell growth among all marketed drugs and small molecular compounds.

The specific aims of this doctoral thesis were:

I) Identification of novel compounds inhibiting prostate cancer cell growth

II) Validation of the cancer-selective growth inhibitory compounds

III) Characterization of the molecular mechanisms underlying cancer-selectiveness of the compounds

MATERIALS AND METHODS

High-throughput compound screening protocol

High-throughput compound screening was utilized to identify selective antineoplastic compounds among 4910 small molecule compounds including marketed drugs. The growth rates of the cell lines were titrated to linear range and the cell viability was detected after incubation of the compounds for 3 days. The principle of the high-throughput screening (HTS) protocol is described in more detail in Figure 4 and publication I.

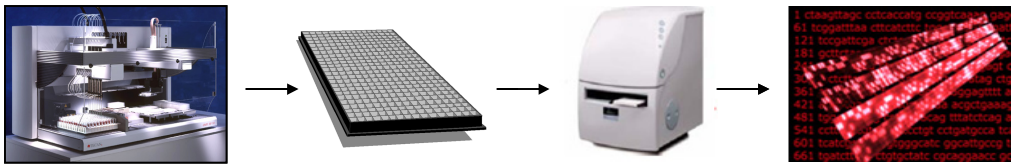

Figure 4. Protocol for the HTS approach. Compound libraries of Biomol (80 known kinase and phosphatase inhibitors), LOPAC (1 280 existing Food and Drug Administration (FDA) - approved drugs and other compounds with pharmacologically relevant structures), IBIS (1 473 compounds derived from natural sources), Microsource Spectrum (2 000 compounds including most of the known drugs and other bioactive compounds and natural products) and an inhouse library (77 experimental compounds) were plated in different concentrations on four prostate cancer (VCaP, LNCaP, DU 145, and PC-3) and two non-malignant prostate epithelial cell lines (RWPE-1 and EP156T). The cell viabilities were measured using CellTiter-Blue cell viability assay (Promega, Inc.) and The Envision Multilabel Plate Reader (Perkin-Elmer). The more detailed protocol and data analysis is described in publication I.

Cell lines

Cell line name	Species	AR/ERG expression	Tissue of origin	Used in
VCaP	human	$^{+}/^{+}$	prostate adenocarcinoma, vertebral metastasis	I-IV
DuCap	human	$^{+}/^{+}$	prostate adenocarcinoma, vertebral metastasis	I
LNCaP	human	$^{+}/^{-}$	prostate adenocarcinoma, lymph node metastasis	I-IV

Cell line name	Species	AR/ERG expression	Tissue of origin	Used in
LNCaP C4-2	human	$^{+}/^{-}$	prostate adenocarcinoma, lymph node metastasis	I
PC-3	human	$^{-}/^{-}$	prostate adenocarcinoma, bone metastasis	I
DU-145	human	$^{-}/^{-}$	prostate adenocarcinoma, brain metastasis	I, IV
PrEC	human	$^{-}/^{-}$	prostate epithelium	I, IV
EP156T	human	$^{+}/^{-}$	prostate epithelium, hTERT immortalized	I, II, IV
RWPE-1	human	$^{+}/^{-}$	prostate epithelium, HPV-18 immortalized	I, II, IV

Reagents and chemicals

Compound	Supplier	Dissolved in	Used in
Compound library	Biomol	DMSO	I
Compound library	LOPAC	DMSO	I
Compound library	Microsource Spectrum	DMSO	I
Compound library	IBIS	DMSO	I
Disulfiram (DSF)	Fluka	DMSO (or olive oil in xenograft study)	I, II
Monensin	Sigma	ethanol	II, III
Salinomycin	Sigma	ethanol	IV
Vitamin C	Sigma	DMSO	III, IV
Flutamide	Sigma	ethanol	II
Bicalutamide	Sequoia Research Products	ethanol	II
carboxy-H2DCFDA	Invitrogen		II, IV
Hydrogen peroxide	Sigma-Aldrich		II, III
Transferrin	Molecular Probes		II
CellTiter-Blue (CTB) cell viability assay	Promega, Inc.		I
CellTiter-Glo (CTG) cell viability assay	Promega, Inc.		I, II, IV
Apo-ONE	Promega, Inc.		I, II, IV
ECL reagent	Amersham Biosciences		I-IV
Aldefluor	Stemcell Technologies		III, IV
Diethylaminobenzaldehyde	Stemcell Technologies		III, IV

Compound	Supplier	Dissolved in	Used in
(DEAB)			
Carboxy-H2DCFDA	Invitrogen	DMSO	III, IV
siLentFect	Bio-Rad Laboratories		I, III, IV
Cancer 10-pathway Reporter Luciferase Kit	SABiosciences		III, IV
Dual-LuciferaseReporter (DLR™) Assay System	Promega, Inc.		III, IV
Vectashield mounting medium	Vector Labs		II, III, IV
$CuCl_2$	Sigma	DMSO	I
$ZnCl_2$	Sigma	DMSO	I
R1881	PerkinElmer	EtOH	II
MCM5 siRNA	Qiagen		I
MT1A siRNA	Qiagen		I
MT1B siRNA	Qiagen		I
MT1F siRNA	Qiagen		I
MT1G siRNA	Qiagen		I
MT1X siRNA	Qiagen		I
MT2A siRNA	Qiagen		I
AllStars negative control siRNA	Qiagen		I
Positive control (PLK1)	Qiagen		I
siLentFect	Bio-Rad Laboratories		I
Steroid standards	Steraloids		III, IV

Methodology

Method	Used in
Cell culture	I-IV
Cell proliferation assay	I-IV
Cell apoptosis assay	I,II,IV
Gene expression analysis	I,II,IV
Compound treatments	I-V
Compound EC_{50} value calculations	I, IV
Connectivity Map	I,II,IV
RNA extraction	I-IV
Reverse transfection	I-IV
Quantitative Real-time PCR	I-IV
RNA interference	I
Combinatorial analyses, Calcucyn software	III
Detection of reactive oxygen species	II, IV
Detection of aldehyde dehydrogenase activity	II, IV
Determination of DNA double-strand breaks	II
Confocal microscope	II, III, IV
Flow cytometry	III, IV

Method	Used in
Bioinformatics	I, II, IV
Statistical analyses	I
HTS	I
Immunofluorescence staining	II, III
Western blot analysis	I, II, IV
Subcellular proteome extraction	II
Transferrin receptor endocytosis assay	II
Wound healing assay	III, IV
Cancer luciferase reporter assay	III, IV
Steroid analysis	III, IV
Live-Cell Imaging	III, IV
Mouse xenograft study	I

Equipment and softwares

Type	Supplier	Used in
384-well standard plates	Greiner	I-IV
96-well plates	Essen ImageLock	III, IV
Automated liquid handling robot	Hamilton	I-IV
Automated liquid dispenser	ThermoFisher	I
EnVision Multilabel plate reader	PerkinElmer Inc., Wallac Oy	I, II, IV
BeadArray Reader	Illumina	I, II, IV
Bioanalyzer 2100	Agilent Technologies	I, II, IV
7900HT Fast Real-Time PCR System	Applied Biosystems	I, II, IV
Universal ProbeLibrary Assay Design Center	Roche Diagnostics	I-IV
Gas chromatography-mass spectrometer	Agilent	III, IV
Accuri C6 Flow cytometer	Accuri	III, IV
Zeiss spinning disc confocal microscope	Zeiss	II, III
Slidebook 4.0 software	Intelligent Imaging Innovations, Inc.	II, III
Bioconductor software		I
Incucyte Live-Cell Imaging System and software	Essen Instruments	III, IV
Wound Maker 96 Tool	Essen Instruments	III, IV
Ingenuity Pathway Analysis (IPA) Software	Ingenuity Systems Inc.,	IV
CalcuSyn software	Biosoft	II

Antibodies

Antigen	Species	Antibody	Used in
AR	mouse	Neomarkers	I
AR	rabbit	Neomarkers	II, III
β-actin	mouse	Becton Dickinson	I-IV
PSA	rabbit	DakoCytomation	I
E-cadherin	rabbit	Cell Signaling Technology	III, IV
CD24	mouse	Cell Signaling Technology	III, IV
CD44	rabbit	Cell Signaling Technology	III, IV
cPARP	rabbit	Abcam	I
HRP-conjugated secondary antibodies		Invitrogen Molecular Probes	
Alexa Fluor antibodies		Invitrogen Molecular Probes and Abcam	II, III, IV
Phosphorylated histone variant H2AX (Ser 139)	rabbit	Abcam	II

Primers

Gene	Forward primer	Reverse primer	Used in
ACTB	ccaaccgcgagaagatga	ccagaggcgtacagggatag	I-IV
AR	gccttgctctctagcctcaa	gtcgtccacgtgtaagttgc	I, II, IV,
ATF3	cgtgagtcctcggtgctc	gcctgggtgttgaagcat	II, IV,
DDIT3	cagagctggaacctgaggag	tggatcagtctggaaaagca	II, IV,
DDIT4	ctggagagctcggactgc	tccaggtaagccgtgtcttc	II, IV,
MYC	caccagcagcgactctga	gatccagactctgacctttgc	II, IV,
ERG	caggtgaatggctcaagga	agttcatcccaacggtgtct	I, II, IV,
TXNIP	acgcttcttctggaagacca	aagctcaaagccgaacttgt	II, IV,
MT1A	tgggatctccaacctcacc	atttgcaggagccagtgc	I
MT1B	gaactccaggcttgtcttgg	catttgcactctttgcacttg	I
MT1F	ccactgcttcttcgcttctc	caggtgcaggagacacca	I, II, IV,
MT1G	ctagtctcgcctcgggttg	gcatttgcactctttgcact	I, II, IV,
MT1X	cttctccttgcctcgaaatg	acaggcacaggagccaac	I
MT2A	ctagccgcctcttcagca	gcaggtgcaggagtcacc	I
KLF6	aaagctcccacttgaaagca	ccttcccatgagcatctgtaa	II, IV,
MCM2	gccaagatgtacagtgacctga	gatgtgccgcaccgtaat	I
MCM5	ccttgtccggtaccctgtc	gatgcggctcagcatctc	I

More detailed material and method information is described in original publications (I-IV).

RESULTS

1. High-throughput screening of 4910 known drugs and compounds (I)

Drugs that are already in use are ready to be taken rapidly into preclinical and clinical trials, since the pharmacological and toxicological profiles are known. Therefore, a high-throughput screening (HTS) with a library of 4910 compounds was utilized to identify novel cancer selective antineoplastic agents. The screens were done in at least two concentrations in four prostate cancer cells (VCaP, LNCaP, DU-145 and PC-3) and in two non-tumourigenic prostate epithelial cells (RWPE-1 and EP156T). VCaP cell line was used as a model for TMPRSS2-ERG fusion gene positive, androgen receptor (AR) expressing and androgen sensitive cell line. The other prostate cancer cell lines used were TMPRSS2-ERG fusion gene negative. LnCaP was used as a model for androgen sensitive, AR expressing prostate cancer and PC-3 and DU145 cell lines were used to model androgen independent prostate cancer without androgen receptor expression. Non-malignant immortalized RWPE-1 and EP156T cells were used as models for normal prostate epithelium. The results of prostate cancer cells and non-tumourigenic prostate epithelial cells were compared to identify the selectivity of the drugs for prostate cancer cells.

Many of the hit compounds were identified from different libraries, such as taxol, vinblastine sulfate, staurosporine, disulfiram, mitoxantrone, colcichine, celastrol and actinomycin D. Thus, the results supported the reliability and functionality of the HTS approach. Several compounds, including many of the currently used chemotherapeutic agents were identified to inhibit prostate cancer cell growth in the screen (I: Fig. 1). However, all the antineoplastic compounds that are currently used in the clinics or studied in clinical trials affected equally cancer and control cells at the same concentrations. Only four compounds were cancer-selective: aldehyde dehydrogenase (ALDH) inhibitor disulfiram, antibiotic ionophore monensin, fungicide thiram and histone deasetylase (HDAC) inhibitor tricostatin A (TSA) (I: Fig. 2, Table 3). The hit rate for the compounds in the screen was at least three standard deviations from the mean (I: Fig. 2). In addition to the screened cell lines, the EC_{50} values of the cancer-selective hits disulfiram, monensin and thiram were validated in LNCaP C4-2 and DuCaP prostate cancer cells and in primary prostate epithelial cells, PrEC (I: Fig. 3, Table 3). The EC_{50} value of TSA was identified earlier in another approach in which HDAC inhibitors were identified to selectively target ERG positive prostate cancers (Björkman et al. 2008). All three compounds were validated to inhibit most effectively the growth of ERG positive prostate cancer. Disulfiram and monensin were selected for further mechanistic studies to identify the molecular basis of the cancer selectivity of the compounds.

Table 3. EC_{50} values (nM) for thiram, disulfiram and monensin in various prostate epithelial cells.

cell line	thiram	disulfiram	monensin
VCaP	95	95	35
DuCaP	70	60	10
LNCaP	220	170	90
LNCaP C4-2	240	100	40
PC-3	> 1 000	> 1 000	> 1 000
DU 145	> 10 000	> 10 000	> 10 000
RWPE-1	> 10 000	> 10 000	> 10 000
EP156T	> 10 000	> 10 000	> 1000
PrEC	> 10 000	> 10 000	> 1000

2. Disulfiram inhibits prostate cancer growth *in vitro* and *in vivo* (I)

Disulfiram is an aldehyde dehydrogenase inhibitor that has been used for decades as a FDA-approved drug for alcoholism treatment. The validation results indicated that disulfiram inhibited prostate cancer cell growth *in vitro* and *in vivo*. Disulfiram altered genes involved in oxidative stress induction such as metallothioneins and DNA replication such as minichromosome maintenance (MCM) genes. No significant difference in androgen receptor (AR) expression was observed at early time points. However, a reduced expression of androgen regulated ERG oncogene mRNA was seen (I: Fig. 3A). Moreover, VCaP xenograft tumour growth was reduced by 40% in response to disulfiram treatment (I: Fig. 5A). Since the xenograft growth was not completely blocked, disulfiram effect was tested in combination with chelating agents such as copper or zinc *in vitro*. The results indicated that $CuCl_2$ sensitized VCaP cells to disulfiram-induced cell death already after 6 hours whereas $ZnCl_2$ did not potentiate disulfiram effect on cell viability in VCaP cells. In addition, disulfiram increased metallothionein expressions were further induced with disulfiram-copper combination. Metallothioneins are intracellular proteins that regulate zinc and copper availability, detoxify toxic metals, and protect cells against oxidative stress (Vasak 2011). Moreover, disulfiram altered gene expression profile similarly as oxidative stress inducer 12,13-EODE (Leukotoxin B). Thus, the results suggested that induction of oxidative stress is one of the key mechanisms of disulfiram reduced growth of prostate cancer.

3. Monensin reduces prostate cancer cell viability by inducing apoptosis, reducing androgen receptor signalling and increasing oxidative stress (II)

Monensin was identified as a selective reducer of prostate cancer cell growth in HTS (I). Monensin is an antibiotic drug isolated from Streptomyces cinnamonensis. It is used extensively as an antibiotic and growth promoting agent in beef and dairy cattle. In the second publication, the mechanism of monensin was studied in more detail. The results indicated that monensin reduced prostate cancer cell growth by inducing

apoptosis in VCaP and LNCaP cells but not in non-malignant RWPE-1 and EP156T cells. Moreover, monensin reduced androgen receptor (AR) mRNA and protein levels as well as decreased the mRNA levels of oncogenes ERG and MYC, which are highly expressed in VCaP prostate cancer cells. Furthermore, monensin elevated intracellular oxidative stress evidenced by increased generation of intracellular reactive oxygen species (ROS) and induction of a transcriptional profile characteristic of oxidative stress response (II: Fig. 4A). Interestingly, no monensin induced ROS or oxidative stress transcriptional profile was detected in non-malignant prostate epithelial cells. These results suggest that oxidative stress induction describes the cancer-selectiveness of monensin.

In addition, monensin induced DNA damage in VCaP prostate cancer cells (II: Fig. 4B). Moreover, the antiproliferative effect of monensin was potentiated by combinatorial treatment with the antiandrogens and antagonized by antioxidant vitamin C (II: Fig. 5B). Thus, the results suggest that reduction of AR signalling and induction of oxidative stress are the key cancer-selective mechanisms of monensin reduced cell growth in prostate cancer. The results were also confirmed by Connectivity Map analysis: oxidative stress inducer terfenadine had agonistic effects whereas antioxidative androgen precursor progesterone had antagonistic effects to monensin.

Interestingly, both disulfiram and monensin reduced ALDH activity, which is a marker for cancer stem cells (CSC). Thus, the results suggested that these prostate cancer inhibitory compounds have also effect on prostate cancer stem cells. Moreover, progesterone that had antagonistic effect to monensin is known to be highly expressed in mammary stem cells (Joshi et al. 2010). Taken together, monensin induced oxidative stress and reduced androgen receptor signalling were the key mechanisms of monensin reduced cell growth in prostate cancer cells.

4. Monensin reduces prostate cancer cell migration and cancer stem cell population (III)

Monensin induced apoptosis and reduced the level of prostate CSC marker ALDH (II). Thus, the effect of monensin on prostate CSC homeostasis as well as cell motility and differentiation was studied in the third publication (III). Moreover, to gain more insight into monensin altered signalling pathways and steroid quantities, a pathway activity assay as well as steroid profiling of monensin exposed prostate cancer cells was performed.

The results indicated that monensin reduced the activities of several cancer signalling pathways. The most significant decrease was seen in the activity of NF-κB pathway. The Connectivity Map results also showed that monensin has agonistic effects to NF-κB inhibitor and oxidative stress inducer niclosamide (Jin et al. 2010). NF-κB is involved in maintaining cellular antioxidant defence capacity. Moreover, inhibition of NF-κB induces oxidative stress and reduces tumourigenesis, metastasis and cancer stem cell potential in prostate cancer (Meng et al. 2010, Sarkar et al. 2008, Gloire,

Legrand-Poels & Piette 2006, Blum et al. 2009, Gluschnaider et al. 2010). Interestingly, a significant difference was observed in the NF-κB activity in VCaP and LNCaP cells; VCaP cells showed ten times higher basal NF-κB activity. This was in accordance with previous results showing that NF-κB is activated by TMPRSS2-ERG fusion isoforms in prostate cancer (Wang et al. 2010).

In addition to reduced ALDH activity, monensin reduced the $CD44^+/CD24^-$ cells in VCaP and LNCaP prostate cancer cells. $CD44^+/CD24^-$ cells are known to have stem cell characteristics and correlate with the cell ability to migrate in prostate cancer (Klarmann et al. 2009). Moreover, monensin induced epithelial cell differentiation shown as induction of E-cadherin mRNA and protein expression. Monensin also reduced migration of cultured prostate cancer cells in scratch wound assay. Hence, the results indicated that in addition to reduced CSC characteristics, monensin also induces cell differentiation and reduces cell migration in cultured prostate cancer cells. Interestingly, it has been suggested that CSCs have a controlled redox balance system including high ALDH and CD44 expression which protects from oxidative stress. The results confirm that oxidative stress inducer monensin reduces prostate CSCs potentially by targeting the key redox balancing genes of prostate CSCs.

Furthermore, the steroid profiling indicated that monensin increases the levels of oxidative stress inducing steroids 7-ketocholesterol and aldosterone and reduces androgen precursors progesterone and pregnenolone in cultured prostate cancer cells (II: Fig. 6). The previous Connectivity Map results also indicated that monensin has antagonistic effects to progesterone. The previous results showing a decrease in androgen signalling in response to monensin exposure also support the steroid profiling results. Taken together, the results suggest that impairing the redox control, which has a crucial role in cancer cells enabling survival under high intracellular ROS, is a potent way to target prostate cancer cells and also prostate CSCs.

5. Salinomycin induces oxidative stress and reduces prostate cancer stem cell population (IV)

Interestingly, salinomycin that shares a similar chemical structure with monensin, has recently been shown to reduce cancer stem cells in breast cancer cells (Gupta et al. 2009). However, the detailed mechanism of action of salinomycin was not clear, nor had the effect of salinomycin in prostate cancer cells been studied before. Since our results indicated that monensin induced oxidative stress in prostate cancer cells, we hypothesized that also salinomycin could act as an oxidative stress inducer in cultured prostate cancer cells. Thus we studied the salinomycin effect on prostate cancer cell viability. The mechanism of salinomycin action was studied in several ways utilizing gene expression studies, ROS and pathway analyses, androgen signalling studies as well as steroid profiling. Interestingly, the results indicated that salinomycin indeed reduced prostate cancer cell growth. However, salinomycin was not as effective as monensin; the EC_{50} value of salinomycin (380 nM) was ten times higher than the EC_{50} value previously identified for monensin (35 nM) in VCaP cells.

In addition, induction of ROS as well as gene expression signature characteristics of oxidative stress induction was seen in response to salinomycin exposure in prostate cancer cells (III: Fig. 4). Moreover, salinomycin reduced the expression of key prostate cancer oncogenes such as AR, MYC and ERG. Furthermore, the steroid profiling results indicated that similarly to monensin, also salinomycin induced oxidative stress by inducing steroids 7-ketocholesterol and aldosterone and reduced progesterone and pregnenolone levels. The reduction of the levels of progesterone was also confirmed with Connectivity Map; salinomycin showed antagonistic effect to progesterone. Moreover, salinomycin reduced ALDH and NF-κB activities and the population of prostate CSCs shown as reduction of $CD44^+$ cells in cultured prostate cancer cells. In addition, a decrease in cell migration was seen in response to salinomycin exposure whereas addition of antioxidant vitamin C rescued the cell motility inhibited by salinomycin. Salinomycin also induced cell differentiation in prostate cancer cells. In conclusion, salinomycin showed cancer selectivity by inducing oxidative stress and reducing characteristics of CSCs in prostate cancer cells.

DISCUSSION

In this study, a high-throughput screening (HTS) of known drugs in malignant and non-malignant prostate cancer cells was utilized to identify novel prostate cancer growth inhibitors. The screening was successful: out of 4910 known drugs, drug-like molecules and small molecular compounds, four were identified to have selective antineoplastic effects. Most of the drugs that are already in the clinics or studied in clinical trials, such as docetaxel, doxorubicin, paclitaxel, mitoxantrone, suramin, camptothecin, staurosporine, ixabepilone, 17-AAG, epothilone B, MS-275, vinblastine sulfate salt and cardiac glycosides, were identified to nonselectively affect equally all cancer and control cells studied.

Only aldehyde dehydrogenase inhibitor disulfiram, antibiotic ionophore monensin, HDAC inhibitor tricostatin A (TSA) and fungicide thiram were identified as cancer selective compounds at nanomolar concentrations. Interestingly, disulfiram was recently confirmed to reduce the growth of prostate cancer cells. The results were identified in a HTS approach in which cardiac glycoside digoxin was identified as a potent inhibitor of prostate cancer cell viability and as a reducer of prostate cancer risk among digoxin users (Platz et al. 2011, Lin et al. 2011). The results indicate that our high-throughput screening shows reliability and reproducibility.

Moreover, the chemical biology HTS approach gave an excellent opportunity to gain more information about the mechanisms to target prostate cancer cells when the cancer-selectivity of the hit compounds were studied. ALDH inhibitor disulfiram is an alcohol deterrent long-term used in the clinics. In contrast, antibiotic ionophore monensin has been used as a biochemical tool to block intracellular protein transport as well as to improve muscle growth in cattle (Mollenhauer, Morre & Rowe 1990). Moreover, monensin as a Na^+/H^+ antiporter increases intracellular calcium levels, induces mitochondria swelling and lipid peroxidation as well as decreases ATP production (Mollenhauer, Morre & Rowe 1990, Donoho 1984). Previously, disulfiram and monensin have been identified to have antineoplastic effects also in other cancer models. For example, disulfiram has been shown to inhibit myeloma, leukemia, lung cancer, cervical adenocarcinoma, melanoma, neuroblastoma and colorectal cancer whereas monensin showed antitumourigenic effects in myeloma, renal cell carcinoma, colon cancer, lymphoma and leukemia (Park et al. 2003a, Park et al. 2003b, Park et al. 2003c, Park et al. 2002, Wang, McLeod & Cassidy 2003, Wickstrom et al. 2007, Yakisich et al. 2001, Sauna, Shukla & Ambudkar 2005). In leukemia, disulfiram was identified to reduce proteasome signalling pathway and NF-κB activity whereas monensin induced cell cycle arrest and apoptosis in several cancers (Lovborg et al. 2006, Park et al. 2003a, Park et al. 2003b, Park et al. 2003c, Park et al. 2002). However, disulfiram and monensin effects on prostate cancer had not been studied before.

The mechanistic results showed that disulfiram reduced prostate cancer cell growth by inducing oxidative stress and reducing DNA replication. Disulfiram was not able to completely block the xenograft growth *in vivo* suggesting that synergistic approaches to treat prostate cancer with disulfiram are needed. Interestingly, the *in vitro* results indicated that disulfiram effect was potentiated with copper, potentially due to the copper chelating ability of disulfiram. Copper levels have been reported to be higher in prostate cancer tissue tumours suggesting that copper could sensitize tumours to free radicals (Gupte, Mumper 2009). In addition, disulfiram-copper combination has identified as an inducer of oxidative stress in melanoma (Cen et al. 2002, Morrison et al. 2010). A sufficient copper level may not have been exceeded in the VCaP xenograft study. Hence, additional copper administration might potentiate the disulfiram effect also *in vivo*.

The mechanistic studies showed that also monensin induced oxidative stress in prostate cancer cells. The cancer-selectiveness of monensin was due to increased level of oxidative stress in malignant but not in non-malignant cells. In contrast, antioxidant vitamin C antagonized the antiproliferative effect of monensin. Thus, the results indicated that the acute induction of oxidative stress by monensin targets prostate cancer cells since the level of oxidative stress is already higher in cancer cells compared to non-malignant cells (Khandrika et al. 2009, Yossepowitch et al. 2007, Kumar et al. 2008, Dakhova et al. 2009). Moreover, monensin reduced androgen receptor signalling and had synergistic effects with antiandrogens which suggests that induction of oxidative stress and antiandrogen response are closely linked. Androgen is known to induce adaptation to oxidative stress in prostate cancer as well as to regulate oxidative stress in rat prostate (Pinthus et al. 2007, Tam et al. 2003). This further supports the link between AR signalling and oxidative stress induction.

Furthermore, NF-κB pathway was the most significantly reduced pathway in response to monensin exposure in prostate cancer cells. Monensin also had agonistic effects to niclosamide, which is known to inactivate NF-κB and induce oxidative stress in leukemia stem cells (Jin et al. 2010). Interestingly, NF-κB induces antioxidant defence capacity as well as tumourigenesis, metastasis and cancer stem cell potential in prostate cancer (Meng et al. 2010, Sarkar et al. 2008, Gloire, Legrand-Poels & Piette 2006, Blum et al. 2009, Gluschnaider et al. 2010). In addition, NF-κB inhibitors reduce AR signalling suggesting that reduction of NF-κB activity mediates the monensin induced oxidative stress and reduction of AR signalling in prostate cancer (Zhang et al. 2009b, Jin et al. 2008). Although some natural compounds inhibiting NF-κB activity, such as curcumin, genistein and docosahexaenoic acid, have been identified to reduce prostate cancer cell viability, there are no specific NF-κB inhibitors in clinical trials for prostate cancer (Mahon et al. 2011, Hour et al. 2002, Li et al. 2005, Shaikh et al. 2008). Thus, the results reveal monensin as a potential NF-κB inhibitor and a reducer of AR signalling which leads to increase in oxidative stress in prostate cancer cells.

In addition to ALDH inhibitor disulfiram, also monensin reduced ALDH activity in prostate cancer cells. Aldehyde dehydrogenases detoxify aldehydes under oxidative conditions and ALDH activity is a marker for prostate cancer stem cells and predictor

of outcome for prostate cancer patients (Davydov, Dobaeva & Bozhkov 2004, Burger et al. 2009, van den Hoogen et al. 2010, Li et al. 2010). In addition, monensin reduced cancer stem cells in prostate cancer cell cultures shown as reduction of the fraction of $CD44^+/CD24^-$ cells. High expression of ALDH and CD44 contributes to the controlled redox balance in cancer stem cells (Kobayashi, Suda 2011, Ishimoto et al. 2011, Croker, Allan 2011). Thus, the results suggest that monensin targets prostate and prostate CSCs by impairing redox control in these cells. The hypothesis is also supported by inhibition of NF-κB which is also considered an attractive chemotherapeutic target against CSCs (Birnie et al. 2008). Interestingly, also disulfiram has recently been confirmed to reduce NF-κB activity and population of cancer stem cells in breast cancer cells (Yip et al. 2011).

Moreover, monensin induced cell differentiation and reduced cell migration in prostate cancer cells. Furthermore, monensin induced oxidative stress inducive steroids 7-ketocholesterol and aldosterone and androgen precursors progesterone and pregnenolone. Interestingly, 7-ketocholesterol oxidizes cholesterol and induces ROS, mitochondrial dysfunction and ER stress and also aldosterone is linked to induction of oxidative stress (Leonarduzzi et al. 2006, Gramajo et al. 2010, Lee et al. 2009b, Calo et al. 2010, Queisser et al. 2011, Ozacmak, Sayan 2009). Moreover, progesterone is known to have antioxidative properties and induce adult mammary stem cell expansion (Joshi et al. 2010, Ozacmak, Sayan 2009). Steroidogenic enzymes and stem cell markers are induced in castration-resistant prostate cancer and many studies have shown that steroidogenesis is inhibited by ROS (Blum et al. 2009, Pfeiffer et al. 2011, Tsai et al. 2003, Kodaman, Aten & Behrman 1994, Lee et al. 2009a, Abidi et al. 2008). Taken together, the steroid profiling results further indicate monensin as oxidative stress inducer and potential reducer of steroidogenesis in prostate cancer cells.

Interestingly, an antibiotic ionophore salinomycin that has a structure similar to monensin was also identified as a reducer of prostate cancer cell viability. However, salinomycin was not as effective as monensin in prostate cancer cells. Salinomycin has been identified as a CSC inhibitor and cell differentiation inducer in breast cancer (Gupta et al. 2009). Salinomycin showed cancer-selectivity and very similar antiproliferative mechanisms as monensin in prostate cancer cells. For example, salinomycin induced oxidative stress, reduced AR, ERG and MYC as well as ALDH and NF-κB activities, induced cell differentiation and reduced the fraction of prostate CSCs. In addition, salinomycin reduced cell migration and the anti-migratorial effect was antagonized by antioxidant vitamin C indicating that oxidative stress induction and reduction of cell migration are closely connected in prostate cancer cells. Interestingly, androgen has recently been indicated as inducer of cell migration in prostate cancer cells (Castoria et al. 2011, Ryan, Tindall 2011). Thus, the results suggest that in addition to ROS induction, the anti-migratorial effects of monensin and salinomycin are also mediated via reduced AR signalling. A very recent study confirmed the salinomycin induced oxidative stress in prostate cancer cells (Kim et al. 2011b).

Moreover, salinomycin had a similar steroid profile as monensin in prostate cancer cells; salinomycin induced 7-ketocholesterol and aldosterone and reduced the levels of

progesterone and pregnenolone. The results were particularly interesting, since in addition to reduced AR signalling, both monensin and salinomycin altered similarly the steroid profile in prostate cancer cells. Interestingly, 7-ketocholesterol is potentially an antagonistic ligand for aryl hydrocarbon receptor (AhR), which expression is elevated in prostate cancer and prostate CSCs (Blum et al. 2009, Gluschnaider et al. 2010, Savouret et al. 2001). AhR pathway protects cells against foreign chemicals and oxidative stress, increases ALDH protein expression, binds to NF-κB, induces MYC as well as reduces cell differentation (Lindros et al. 1998, Vrzal, Ulrichova & Dvorak 2004, Nebert et al. 2000, Kohle, Bock 2007, Kim et al. 2000, Dietrich, Kaina 2010). AhR also forms a complex with AR and protects prostate cancer cells during androgen ablation (Gluschnaider et al. 2010, Ohtake, Fujii-Kuriyama & Kato 2009). Thus, the results suggest that both monensin and salinomycin induced oxidative stress is mediated by reduction of AhR signalling.

As a summary, disulfiram, monensin and salinomycin showed cancer-selectivity by inducing oxidative stress in prostate cancer cells, but not in non-tumourigenic prostate epithelial cells. In addition, all the three drugs reduced the activity of CSC marker ALDH. Impairing redox balance and antioxidative capacity is an attractive novel means to target CSCs and prevent cancer cell viability and metastasis (Pani, Galeotti & Chiarugi 2010). The results of this thesis also support the hypothesis. Figure 5 summarizes the molecular mechanisms of actions for monensin and salinomycin in prostate cancer cells (III).

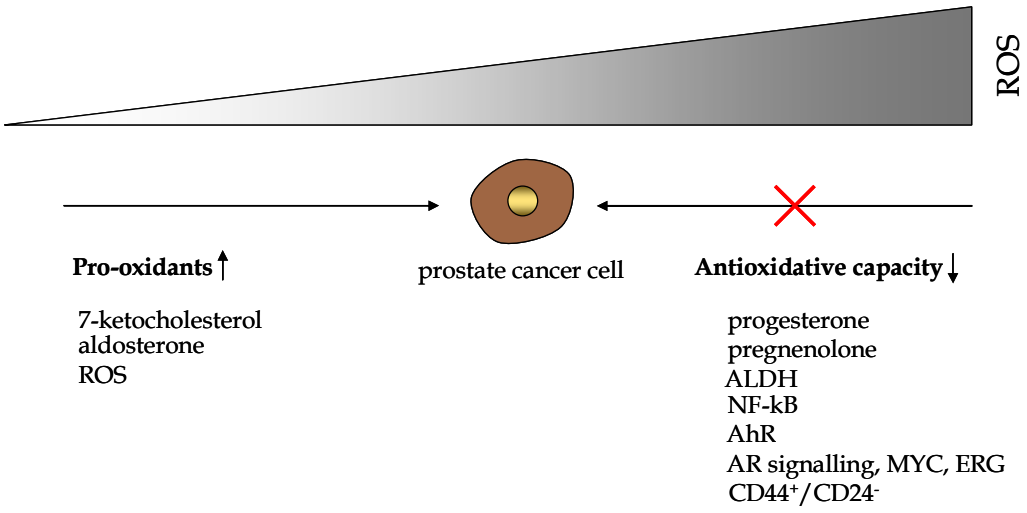

Figure 5. Monensin and salinomycin show cancer-selectivity by impairing the redox control via inducing pro-oxidants and reducing antioxidative capacity in prostate cancer cells (III).

CONCLUSIONS

The aim of this study was to utilize a chemical biology high-throughput screen to identify novel prostate cancer inhibitors and to gain novel information about prostate cancer biology. Four cancer-selective inhibitors were identified for prostate cancer: disulfiram, monensin, thiram and TSA. Nevertheless, the future aspects for utilizing the hit compounds of this screening to further preclinical and clinical tests are rather mixed; known "non-profit" drugs may not attract pharmaceutical companies to bring them to the clinical trials (Cvek, Dvorak 2008, Cvek 2011). Thus, the support for the clinical trials may need to come from other sources. However, promising results with disulfiram treatment have been reported in a case report in which disulfiram and zinc gluconate combination induced clinical remission in a melanoma patient (Brar et al. 2004). In addition, clinical trials for disulfiram in lung and liver metastases are ongoing and a Phase I clinical trial for prostate cancer patients is currently recruiting patients at Johns Hopkins University (identifier NCT01118741). The advantages of taking monensin and salinomycin to clinical trials are that they have been used as antibiotics in several animal species and thus the pharmacological and toxicological profiles have already been performed. However, in some animal species monensin and salinomycin have caused severe toxicity and accidental intoxications have occurred with salinomycin and monensin in human (salinomycin: 1 mg/kg, monensin: dose of three times the dose considered lethal for cattle) (Story, Doube 2004, Caldeira et al. 2001, Kouyoumdjian et al. 2001). Thus, a careful design of prostate cancer clinical trials with these drugs is needed.

Interestingly, the mechanistic studies indicated that in addition to induced oxidative stress, cancer stem cell characteristics were reduced in response to disulfiram, monensin and salinomycin. As a summary, the results suggest that the oxidative stress induction and reduction of antioxidative capacity are the main characteristics of the agents that reduce prostate cancer cell viability and the population of cancer stem cells. Thus, targeting antioxidative capacity is an attractive novel means to target cancer stem cells and prevent cancer cell viability and metastasis. Interestingly, the hypothesis has been confirmed also by other recent studies that have identified oxidative stress inducers as potential, novel, prostate cancer selective agents (Chen et al. 2011, Fajardo et al. 2011). In this study, the reduction of cancer stem cell characteristics was seen in prostate cancer *in vitro* models. Thus, novel approaches to study the effects of these and other potential cancer stem cell inhibiting compounds on patient cancer stem cells are needed to fully understand the heterogeneity of the tumours and the mechanisms of actions of the compounds in individual cells.

In conclusion, several novel cancer-selective prostate cancer cell inhibitors that have potential to be taken to clinical trials were identified in this study. Moreover, the study revealed important novel information about prostate cancer cell biology and the potential of targeting antioxidative defence mechanisms as novel means to selectively target prostate cancer cells as well as prostate cancer stem cells and metastases.

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