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The effects of drugs on osteoblast-associated tumour cell lines

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Erklärung

Ich erkläre an Eides statt, dass ich die hier vorliegende Arbeit selbst verfasst und nur die angegebene Literatur verwendet habe.

Wien, September 2008

Acknowledgment

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Abstract

Osteosarcoma (OS) and prostate cancer are two types of cancer that both affect bone because osteosarcoma itself develops in bone and prostate cancer cells mainly metastasize to bone and cause significant mortality and morbidity in men with advanced disease.
Today, many therapeutical options are available to treat OS and prostate cancer.
Current optimal treatment for osteosarcoma consists of multi-agent chemotherapy and aggressive surgical resection. Suramin, a polysulfonated naphthylurea compound, has been widely used for antitrypanosomal treatment. Its anticancer activity was later identified and suramin has been introduced into clinical trials for various forms of cancer.

Lysyl Oxidase (LOX) is the key enzyme that controls collagen and elastin maturation by catalyzing inter-and intamolecular cross-link formation. In the last few years it was found out that the LOX propeptide, which is released during extracellular proteolytic processing of pro-lysyl oxidase possesses anti-cancer activity.
It was shown that suramin drastically upregulated LOX expression and induced phenotype reversion of transformed NIH3T3 cells but today few data exist that show effects of suramin in osteosarcoma cells.
The clinical manifestation of prostate cancer varies from indolent tumours, requiring little or no treatment to aggressive cancers which require radical therapies. Today, metastatic prostate cancer is generally treated by hormone therapy.

Epigenetic events are defined as alterations in gene expression without changes in the DNA coding sequence. DNA methylation by DNA methyltransferases (DNMT´s) and histone modification are two mechanisms that are integral to epigenetic transcriptional control. Promoter hypermethylation of cancer-related genes is found in nearly all tumours, including prostate cancers. It has been shown that the combination of DNMT inhibitors induces apoptosis, differentiation and/or cell growth arrest in different cancer cell lines. In prostate cancer, a number of studies have reported reductions in cell proliferation and apoptosis in cell lines treated with histone-deacetylase (HDAC) inhibitors alone but little is known about the effects of combination therapy.

Aim of this thesis was to study the effects of suramin, the DNMT inhibitor 5-AZAC and the HDAC inhibitor valproate on different osteoblast-associated tumour cell lines, taking into special consideration the effects on osteoblastic differentiation and LOX expression.

Human (MG-63 and U-2 OS) and rat (UMR-106) OS cell lines as well as human prostate metastatic cell lines (DU-145, PC-3 and LNCaP) were seeded and treated with or without suramin and HDAC and DNMT inhibitors, respectively. Thereafter the effects on cell

multiplication and differentiation (alkaline-phosphatase activity and specific marker genes) and LOX were studied.

Suramin inhibited cell multiplication in all osteosarcoma cell lines tested and increased genes of the osteoblastic phenotype like alkaline phosphatase activity, OCN, OPN and COL1A1 in the human OS cell lines. Comparable effects were found with HDAC and DNMT inhibitors in human prostate cancer cells: attenuation of cell multiplication and increase of differentiation as indicated by upregulation of Runx2 by these drugs, at least in combined treatment. In all cell lines treated with suramin and HDAC and DNMT inhibitors LOX mRNA expression was increased significantly.

Our results support recent data from the literature that LOX could be a regulator for reversion of tumourigenic phenotypes in OS and prostate cancer cells.

Zusammenfassung

Knochenkrebs (Osteosarkome) und Prostatakrebs sind Krebsarten, die beide den Knochen betreffen, da sich Osteosarkome im Knochen entwickeln und Prostatakrebszellen in den Knochen und in die Lymphknoten metastasieren können. Es stehen heute viele Möglichkeiten zur Behandlung der beiden Krebsarten zur Verfügung, wobei es bei Osteosarkomen zum Einsatz von Chemotherapie und der chirurgischen Entfernung der betroffenen Areale kommt. Suramin ist ein poly-sulfonierter Naphthylharnstoff, der bis heute bei der Behandlung von diversen parasitären Erkrakungen, wie der afrikanischen Schlafkrankheit, zum Einsatz kommt. In Versuchen erkannte man, dass die Substanz eine anti-tumoröse Wirkung besitzt und Suramin wurde in klinische Studien bei verschiedensten Formen von Krebs aufgenommen.

Lysyl Oxidase (LOX) ist das Schlüssel-Enzym bei der Bildung von Kollagen und Elastin. In den letzten Jahren wurde bekannt, dass das Propeptid, welches bei der Spaltung der Pro-Lysyl-Oxidase entsteht, diverse Tumore in ihrem Wachstum hindert. Es wurde gezeigt, dass Suramin die LOX -Expression in Tumorzellen unterdrückt, aber nur wenige Daten existieren über die Wirkung der Substanz auf Osteosarkome.
Die klinische Manifestation von Prostatakrebs variiert von nicht schmerzhaften Tumoren, die wenig oder keine Behandlung benötigen, bis hin zu agressiven Tumoren, die nur durch radikale Therapien bekämpft werden können.

Epigenetische Veränderungen sind definiert als Veränderungen der Genexpression ohne Veränderungen der DNA- kodierenden Sequenzen. Promotor CpG Hypermethylierung und Histon-Hypoacetylierung, katalysiert von der DNA-Methyltransferase (DNMT) und Histon Deacetylase (HDAC), werden mit transkriptioneller Repression in einigen Krebsarten assoziiert. Studien berichten über einen Rückgang der Zell-Proliferation und der Apoptose in Prostatakrebs-Zellinien, die mit einem HDAC Inhibitor behandelt wurden**,** aber wenig ist bekannt über die Effekte einer Kombinationstherapie.

Ziel dieser Arbeit war es nun, die Effekte von Suramin, dem DNMT Inhibitor 5-AZAC und dem HDAC Inhibitor Valproat auf verschiedene Osteoblasten-assoziierte Tumor-Zelllinien zu untersuchen. Ein besonderer Schwerpunkt lag dabei auf der Wirkung der Substanzen auf die osteoblastäre Differenzierung und die LOX-Expression.

Humane (MG-63 und U-2 OS) und tierische (UMR-106) Osteosarkom-Zelllinien, sowie humane, metastasierende Prostatakrebs-Zelllinien (DU-145, PC-3 und LNCaP) wurden ausgestreut und einerseits mit Suramin, andererseits mit HDAC bzw. DNMT Inhibitoren behandelt. Anschließend wurden die Effekte dieser Substanzen auf die Zellvermehrung und

Differenzierung (Messung der alkalischen Phosphatase und verschiedener Osteoblasten-spezifischer Marker-Gene) und auf die LOX-Expression untersucht.

Suramin verhinderte die Zellvermehrung in allen untersuchten Osteosarkom-Zelllinien und erhöhte die Aktivität der alkalischen Phosphatase und die Expression von Osteoblast-spezifischen Markern wie Osteocalcin, OPN und COL1A1 in den humanen Zellen. Vergleichbare Effekte wurden auch in den mit HDAC und DNMT Inhibitoren behandelten Prostatakrebs-Zellen gefunden: Rückgang der Zellvermehrung und Anstieg der Differenzierung durch die Erhöhung von Runx2 – zumindest bei der Behandlung der Zellen mit beiden Substanzen. In allen untersuchten Zelllinien, die mit Suramin oder den HDAC bzw. DNMT Inhibitoren behandelt wurden kam es zu einem signifikanten Anstieg der LOX mRNA Expression.

Unsere Ergebnisse bestätigen Daten aus der Literatur, wonach LOX eine Änderung des Phänotyps in Osteosarkomen und Prostatakrebszellen hervorrufen könnte.

Contents

1. Introduction

1.1. Bone

Bone is a specialized connective tissue that makes up, together with cartilage, the skeletal system. These tissues serve three functions: 1. *mechanical*, as support and site of muscle attachment for locomotion; 2. *protective*, for vital organs and bone marrow; and 3. *metabolic*, as a reserve of ions, especially calcium and phosphate, for the maintenance of serum homeostasis. Anatomically, two types of bones can be distinguished: flat bones (skull bones, scapula, mandible, and ileum) and long bones (tibia, femur, humerus) (Baron R, 2003).
Flat bones undergo a process of intramembraneous ossification, whereas long bones increase in length via the endochondral ossification (Kronenberg HM, 2003).
In intramembranous ossification, a group of mesenchymal cells, under the influence of local growth factors and under the regulation of the transcription factors Runx2 and Osterix (Baron R, 2003) condense and directly differentiate into preosteoblasts and then into bone-forming osteoblasts (Kronenberg HM, 2003). These cells synthesize a bone matrix where the collagen fibers are not preferentially oriented but appear as irregular bundles and where the osteocytes are large and extremely numerous. The calcification is delayed and does not proceed in an orderly fashion but in irregularly distributed patches. This type of bone is also called woven bone (Baron R, 2003).
In endochondral ossification mesenchymal cells proliferate and differentiate into prechondroblasts and then into chondroblasts that form a cartilage template that is replaced by osteoblasts, forming mature bone (Kronenberg HM, 2003).
External examination of a long bone shows two wider extremities, the epiphyses, a more or less cylindrical tube in the middle, the diaphysis, and a developmental zone between them, the metaphysis. In a growing long bone, the epiphysis and the metaphysis, which originate from two independent ossification centers, are separated by a layer of cartilage, the epiphyseal cartilage (also called growth plate). The external part of the bones is formed by a layer of calcified tissue, the cortex (compact bone), which, in the diaphysis, encloses the medullary cavity where the hematopoietic bone marrow is housed. Toward the metaphysis and the epiphysis, the cortex becomes progressively thinner, and the internal space is filled with a network of calcified trabeculae. This is the cancellous bone, also named trabecular bone. Cortical and trabecular bone are made of the same cells and the same matrix elements, but

there are structural and functional differences. The primary structural difference is quantitative: 80–90% of the volume of compact bone is calcified, whereas only 15–25% of the trabecular bone is calcified. The result is that 70–85% of the interface with soft tissues is at the endosteal bone surface, which leads to the functional difference: the cortical bone fulfills mainly a mechanical and protective function and the trabecular bone a metabolic function. Bone is formed by collagen fibers (type I, 90% of the total protein), and noncollagenous proteins. Spindle- or plate-shaped crystals of hydroxyapatite [$3Ca_3(PO4)_2(OH)_2$] are found on the collagen fibers, within them, and in the ground substance. They tend to be oriented in the same direction as the collagen fibers.
The preferential orientation of the collagen fibers alternates in adult bone from layer to layer, giving to this bone a typical lamellar structure. The activity of bone cells is performed along the surfaces of bone and it results in bone remodeling, a process involved in bone growth and turnover (Baron R, 2003). This remodeling requires the actions of osteoclasts, that remove bone and osteoblasts, that replace it (Hadjidakis DJ, 2006). In the normal adult skeleton bone formation occurs for the most part only where bone resorption has previously occurred. The complete remodeling cycle at each microscopic site takes about 3–6 months (Baron R, 2003).

1.1.1. Bone cells

Osteoblasts

The osteoblast is the cell responsible for the production of the matrix constituents, the collagen and the ground substance, that is primarily composed of glycoproteins and proteoglycans. It originates from a local mesenchymal stem cell (bone marrow stromal stem cell or connective tissue mesenchymal stem cell) under the influence of local growth factors and differentiation factors such as fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), and Wnt proteins, and requires the transcription factors Runx2 and Osterix. These precursors undergo proliferation and differentiate into preosteoblasts and then into mature osteoblasts. Osteoblasts do not appear or function individually but are always arranged in clusters along the bone surface. They are found lining the layer of bone matrix that they are producing, before it is calcified. Gap junctions are often found between the osteoblasts. Their plasma membrane is characteristically rich in alkaline phosphatase (ALP). Osteoblasts express steroid receptors for estrogens and vitamin D3 as well as several adhesion molecules

(integrins) and receptors for cytokines. They also express cytokines in their membrane, and in particular, colony-stimulating factor 1 (CSF-1) and RANKL, which can activate osteoclastogenesis in a local, paracrine manner. Osteoblasts also secrete osteoprotegerin, a decoy RANK receptor capable of inhibiting osteoclast formation (Baron R., 2003).
Osteocalcin (OCN) expression is upregulated by vitamin D in human and in rat osteoblasts (Mahonen A, 1990), but downregulated in mouse osteoblasts where it is increased by T3 (Varga F, 2003). It is exclusively limited to cells of the osteoblastic lineage (Nakase T, 1994) and serves as a phenotypic marker for mature osteoblasts (Yeung F, 2002).
Toward the end of the secreting period, the osteoblast becomes either a lining cell or an osteocyte.

Osteocytes

Osteocytes are found embedded deep within the bone in small osteocytic lacunae. They were originally bone-forming cells (osteoblasts), which became trapped in the bone matrix that they produced and later became calcified (Baron R, 2003). Until today, their function is not clearly defined, but they seem to play key roles in the response of bone to mechanical loading, and in the initiation of remodeling activity at specific sites within bone (Russel R.G, 2006). These cells have numerous and long cell processes rich in microfilaments, which are in contact with cell processes from other osteocytes or with processes from the cells lining the bone surface. These processes are organized during the formation of the matrix and before its calcification and form a network of thin canaliculi permeating the entire bone matrix. The morphology of younger and older osteocytes differs. A young osteocyte has most of the ultrastructural characteristics of the osteoblast from which it was derived, except that there has been a decrease in cell volume and in the importance of the organelles involved in protein synthesis. An older osteocyte, that is situated deeper within the calcified bone, shows these decreases further accentuated and there is an accumulation of glycogen in the cytoplasm. These cells have been shown to be able to synthesize new bone matrix at the surface of the osteocytic lacunae, which can subsequently calcify. The fate of the osteocytes is to be phagocytized and digested, together with the other components of bone, during osteoclastic bone resorption (Baron R, 2003).

Lining cells

Lining cells originate from osteoblasts when their activity declines. They are thought to regulate the movement of phosphate and calcium into and out of the bone. They are assumed to play an important role in the coupling of bone resorption and formation. These cells also clean up the resorption pits after the osteoclast withdrawal (Everts V, 2002).

Osteoclasts

The osteoclast, a giant multinucleated cell that derives from cells in the mononuclear/phagocytic lineage, is the cell responsible for bone resorption. It develops from a mononucleated osteoclast-progenitor cell, that is in contact with osteoblasts and bone matrix and differentiates into a pre-osteoclast and then to a mature osteoclast (Klaushofer K, 1994). It is usually found in contact with a calcified bone surface and within a lacuna (Howship's lacunae) that is the result of its own resorptive activity. The most prominent features of the osteoclast are the deep foldings of the plasma membrane in the area facing the bone matrix: the ruffled border in the center is surrounded by a ring of actin that serves to attach the cell to the bone surface. The basolateral plasma membrane is expressing RANK, the receptor for RANKL, and the macrophage-colony stimulating factor (M-CSF) receptor, both of which are responsible for osteoclast differentiation, as well as the calcitonin receptor, capable of inactivating rapidly the osteoclast (Baron R, 2003). These inactivated post-osteoclasts are characterized by the the appearance of vesicles with dark granular content (Klaushofer K, 1989).

1.2. Osteoblast-like tumour cell-lines

Osteosarcoma (OS) is the most frequent malignant tumour of the bone and exhibits a peak in manifestation during the second and the third decade of life (Trieb K., 2003). The basic definition of osteosarcoma is the excessive production of pathological osteoid (Benayahu D, 2001). Patients with metastatic disease at diagnosis have only a 20% survival rate (Walters DK, 2008). Primary chemotherapy for high-grade bone sarcomas often involves intensive,

multivalent regimens, and few secondary chemotherapy options are available to treat refractory or relapsed disease (Schuetze SM, 2007).

A second type of cancer, that most commonly metastasizes to bone and causes significant mortality and morbidity in men with advanced disease, is prostate cancer. Recent autopsy data suggests that greater than 80% of men who die of prostate cancer have skeletal metastasis in the bone (Bubendorf L, 2000; Hall CL, 2006). Despite its common occurrence, the molecular mechanisms responsible for prostate cancer growth, androgen-independent progression and acquisition of bone metastatic potential are poorly characterized.

Studies show that bone matrix proteins like OCN and OPN are expressed at high levels in bone metastatic prostate cancer specimens (Brown LF, 1994; Curatolo C, 1992, Waltregny D, 1998) and androgen-independent bone metastatic prostate cancer cell lines**.** There seems to be a remarkable parallelism in the temporal expression of bone matrix proteins by prostate cancer cells and osteoblasts (Yeung F, 2002).

Tumour cells produce many factors including growth factors and cytokines (PTHrP, ET-1, BMPs, others...) that stimulate either ostoclast activity leading to osteolytic lesions or osteoblast activity generating osteosclerotic bone metastases (Peyruchaud O, 2007). Osteoclasts differentiate from bone marrow mononuclear cells through the action of receptor activator of NFkB ligand (RANKL). RANKL is expressed on mature osteoblasts and the receptor, RANK, is expressed on osteoclast precursors. The activity of RANKL is controlled by a secreted antagonist, osteoprotegerin (OPG), that prevents RANK/RANKL interactions and therefore osteoclastogenesis (Kostenuik PJ, 2005; Hall CL, 2006) The majority of cancers produce areas of osteolytic lesions when they metastasize to bone (Hall CL, 2006; Roodman GD, 2004). Prostate cancer is unique in that, in addition an osteolytic component, skeletal metastases are characterized by osteoblastic lesions (Keller E.T, 2001; Hall CL, 2006). Growth factors released from resorbed bone matrix or throughout osteoblastic bone formation sustain tumor growth. Therefore, bone metastases are the site of vicious cycles wherein tumor growth and bone metabolism sustain each other (Peyruchaud O, 2007) and where the metastasis of cancer cells to bone alters bone architecture and mineral homeostasis (Clines GA, 2008).

In vitro studies of osteoblast behaviour utilize either osteoblast-like cells derived from animal and human bones (Beresford JN, 1984**;** Beresford JN, 1986) or osteogenic OS cell lines of animal or human origin (Rodan GA, 1988). Human OS cells can contribute to our understanding of osteoblast function because they initially represent clonal populations derived from specific stages of the osteoblast lineage (Clover J, 1994).

MG-63, human OS cells, are considered to show a number of features typical of an undifferentiated osteoblast phenotype. This includes the synthesis of collagen types 1 and 3, a low basal expression of ALP which is increased following 1,25-dihydroxyvitamin D_3 (1,25 $(OH)_2$ D_3) administration, and production of osteocalcin (OCN) in the presence of 1, 25 $(OH)_2$ D_3 (Lajeunesse D, 1990; Franceschi RT, 1985). These cells have been used as an experimental model to study a variety of different osteoblast functions such as adhesion (Franceschi RT, 1985; Dedhar S, 1987; Heino J, 1989), extracellular matrix synthesis (Bassols A, 1988**,** Franceschi RT, 1988), ALP activity (Boyan BD, 1989; Franceschi RT, 1985; Franchesci RT, 1990) and OCN production (Lajeunesse D, 1990).

Another human OS cell line is U-2 OS, which was isolated from a moderately differentiated sarcoma of the tibia of a young girl. A cell line of animal origin is UMR-106. This cell line is a clonal derivative of a transplantable rat OS that had been induced by injection of radiophosphorous. The cells are responsive to parathyroid-hormone (PTH), prostaglandins and bone resorbing steroids. U-2 OS and UMR-106 cells offer an epithelial phenotype (ATCC).

To study prostate-carcinoma behaviour three human prostate cancer cell lines are commonly used. PC-3 cells are adherent cells that show an epithelial phenotype. They were initiated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old male Caucasian (ATCC). This cells share with mature osteoblasts the unique feature of synthesizing and depositing a large amount of OCN. It is considered a highly aggressive androgen-independent cell line which expresses neither androgen-receptor nor PSA and it does not require androgen for growth or survival. When injected intracardially in arythmic mouse, PC-3 cells have high propensity to metastasize to bone (Yeung F, 2002). DU-145, that show also an epithelial phenotype, were isolated from a lesion in the brain of a 69 year old patient with metastatic carcinoma of the prostate and a 3 year history of lymphocytic leukemia. LNCaP clone FGC cells are adherent, single cells and loosely attached clusters with an epithelial phenotype. The cells were isolated from a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year-old Caucasian male (ATCC). The LNCaP cell line is the only human prostate cell line established with functional androgen receptors and PSA (prostate specific antigen) expression (Horoszewicz JS, 1983).

1.3. Lysyl Oxidase (LOX)

Activated LOX was discovered by Pinnell and Martin in 1960 and it is the key enzyme that controls collagen and elastin maturation (Kagan HM, 1991). It is the most studied member of a family that includes other four structurally-related isoenzymes named LOX-like (LOXL)1, LOXL2, LOXL3 and LOXL4, whose specific substrates remain unclear (Molnar J, 2003). The gene encoding LOX has already been cloned, facilitating investigations of the regulation of expression of the enzyme in response to diverse stimuli and in numerous disease states. Transforming growth factor-β, platelet-derived growth factor, angiotensin 2, retinoic acid, fibroblast growth factor, altered serum conditions and stress are among the effectors or conditions that regulate LOX expression (Smith-Mungo LI, 1998). The LOX enzyme is localized within fibroblasts, chondrocytes, smooth muscle cells and in a variety of non-fibroblastic cells, including endothelial, basal, biliary epithelial and glomerular epithelial cells (Iwasaki H, 1979).

LOX catalyzes the oxidative deamination of peptidyl-lysine and hydroxylysine to peptidyl-a-aminoadipic-d-semialdehyde. These aldehydes lead to a spontaneous condensation forming inter- and intra-chain cross-links into collagen and elastin (Kagan HM 1991; Kagan HM, 2003; Palamakumbura AH, 2004) .

The process of cross-linking itself implies both, enzymatic catalysis, and a series of condensation reactions, to form intra and intermolecular cross-links. At present, seven major naturally occurring collagen cross-links have been established. They are dehydro-dihydroxylysinonorleucine (deH-DHLNL), dehydro-histidinohydroxymerodesmosine (deH-HHMD), histidinohydroxylysinonorleucine (HHL), pyridinoline (Pyr), deoxy-pyridinoline (d-Pyr), pyrrole (Prl) and deoxy-pyrrole (d-Prl).

The patterns of cross-linking and their molecular distributions vary from tissue to tissue. These patterns seem to be rather tissue - than collagen type specific, and probably are related to the physiological function of distinct tissues (Knott L, 1998).

The lathyrogen ß-amino-propio-nitril (ßAPN) inhibits the collagen cross linking by blocking LOX and recently it was shown that ßAPN treatment decreased the two main cross-links Pyr and deH-DHLNL (Turecek C, 2008). Also the pro-atherogenic risk factor homocysteine downregulates LOX expression (Rodriguez C, 2008). In homocysteinemia increased serum levels of this amino-acid are suggested to inhibit LOX irreversibly, impairing subsequent cross-linking of collagen (Liu G, 1997).

The importance of normal cross-link formation for healthy tissue is displayed by a number of genetically inherited diseases. In Ehlers-Danlos syndrome (EDS) type VI, which is characterized by connective tissue dysfunctions such as kyphoskoliosis or ruptures of the eye and the aorta, the level of hydroxylation is reduced to 17% of normal bone, due to a deficiency in LOX enzyme activity (Acil Y, 1995).
Conversely, overhydroxylation, as a consequence of retarded triple helix formation, can be observed in different forms of osteogenesis imperfecta (Lehmann HW, 1995).
It has traditionally been assumed that the role of LOX is restricted to the oxidation of peptidyl lysine in extracellular collagen and elastin substrates, resulting in the deposition of insoluble fibrous deposits of these proteins (Smith-Mungo LI, 1998) but in the last few years it was found out that the LOX propeptide, which is released during extracellular proteolytic processing of pro-lysyl oxidase possesses anti-cancer activity (Palamakumbura AH, 2004).
The *RAS* gene family encodes small GTPase proteins that transduce signals that promote growh and survival from membrane-bound receptor tyrosine kinases (Downward J, 2003; Wu M., 2007). Mutated Ras proteins have been detected in 25% of lung cancers and in up to 85% of pancreatic cancers (Almoguera C, 1988; Hruban RH, 1993; Wu M, 2007). The findings that LOX is downregulated in these *ras*-transformed cells and in many tumour cell lines (Palamakumbura AH, 2004; Contente S, 1990) as well as in human cancers (Kuivaniemi H, 1986; Krzyzosiak WJ, 1992; Hämäläinen ER, 1995; Ren C, 1998) show that LOX has a second, well-defined function. Spontaneous reversion of cancers or induced phenotypic reversion is accompanied by increased LOX expression (Hajnal A, 1993) and the suggested function as a tumour suppressor was directly shown by transfection of antisense LOX, which triggers transformation of normal rat fibroblasts and reversion of stable phenotypic revertants of *ras*- transformed NIH3T3. This tumour suppressor function was recently attributed to the 18-kDa propeptide (Ren C, 1998; Palamakumbura AH, 2004)**,** which is processed from the secreted 50 kDa proenzyme by the procollagen C-proteinase BMP-1 (bone morphogenic protein-1) (Kessler E, 1996). The larger fission product is the active enzyme responsible for the initiation of the cross-links. Reduced LOX mRNA levels were found in experimental and human prostate cancer (Ren C,1998) and recently, by means of methylation-sensitive representational difference analysis, LOX silencing was found by DNA-methylation in human gastric, colon, lung, and ovarian cancer cells (Kaneda A, 2004). More recently it was found that in human lung and pancreatic cancer cells that carry N-*ras* and K-*ras* mutations, respectively, the tumour suppressing part of LOX strongly inhibits *ras* signalling and NF-κB activity, and Bcl-2 was identified as an essential target of propeptide–mediated inhibition of

the transformed phenotype (Wu M, 2007). Furthermore it was shown that the LOX propeptide functions as a tumour suppressor in breast cancer cells both in vitro and in vivo, and reverts the invasive phenotype of Her-2/neu-driven breast cancer (Min C, 2007). All these findings support the relationship between LOX and tumourigenicity.

1.4. Osteocalcin (OCN)

OCN, a phenotypic marker for mature osteoblasts (Yeung F, 2002), is the main non-collagenous protein of the bone matrix and its expression is highly regulated by many local and humoral factors. It possesses several features of a hormone and is a cell-specific molecule that is synthetisized as a prepro-molecule and secreted in the general circulation (Hauschka PV, 1989; Price PA, 1989; Lee NK, 2007).
Because of their exquisite cell-specific expression OCN genes have been intensively studied to identify osteoblast-specific transcription factors and to define molecular bases of bone physiology (Harada S, 2003). Clinically OCN is routinely used as a serum marker for bone formation (Delmas PD, 1986) and it also has an importance as a marker for malignancies such as prostate carcinoma. Patients with bone metastases due to prostate cancer have higher levels of OCN and ALP than people that are lymph-node negative. So, bone turnover markers, including OCN represent a new diagnostic tool in prostate cancer (Hegele A, 2007). OCN also plays a role as a marker for leukemia and it was shown that OCN is expressed in neoplastic stem cells in hematological malignancies (Wihlidal P, 2006).
OCN expression is regulated by 1,25 D_3 but this regulation depends strictly on the species. In human and rat osteoblasts OCN is upregulated by 1,25 D_3 (Mahonen A, 1990) but this observation could not be found in mouse osteoblasts, where OCN is upregulated by triiodothyronine (T3) (Varga F, 2003). OCN-deficient mice have a higher bone mass and improved bone functional quality (Ducy P, 1996-1), and histomorphometric studies done before and after ovariectomy showed that the absence of OCN leads to an increase in bone formation in mice without impairing bone resorption (Ducy P, 1996-2).
Recently, it was shown that OCN also plays a key role in the interaction between energy and bone metabolism. Mice that lack the protein tyrosine phosphatase OST-PTP are hypoglycemic and are protected from obesity and glucose intolerance because of an increase in beta-cell proliferation, insulin secretion, and insulin sensitivity. In contrast, mice that lack

the osteoblast-secreted molecule OCN display decreased beta-cell proliferation, glucose intolerance, and insulin resistance (Lee NK, 2007).

1.5. Runx2

Runx2 is a member of the Runx family of nuclear transcription factors and was first termed PEBP2α, CBFα, AML or OSF (Ito Y, 1999; Adya N, 2000; Karsenty G, 2000). The Runx family consists of three members (Runx1, Runx2 and Runx3) and one characteristic feature is the runt domain, a highly conserved DNA binding and protein-protein interaction motif (Kamachi Y, 1990).

The members of the Runx family localize in the nuclei to punctuate foci that are involved in transcriptional control and associate with the subnuclear scaffold designated as the nuclear matrix (Zeng C, 1997; Zeng C, 1998; Tang L, 1999; Stein GS, 2000). Runx1 and Runx2 contain a C-terminal nuclear –matrix-targeting signal (NMTS) that is necessary for directing these proteins to intranuclear foci (Bidwell JP, 1994; Zeng C, 1997; Stein GS, 2000). Runx2 is expressed in the early pluripotent mesenchymal cell prior to expression of the osteoblast phenotype and increases during osteoblast differentiation (Komori T, 1997; Banerjee C, 2001). The role of Runx2 as a master regulator of osteoblast differentiation has been proved by the targeted deletion of the Runx2 gene in mice (Otto F, 1997; Komori T, 2002). $Runx2^{-/-}$ mice die shortly after birth (Otto F, 1997) and few, if any, differentiated osteoblasts can be found in these mice (Komori T, 2000). The protein has long been regarded as a bone-specific transcription factor, where it regulates bone specific and related genes, but a few years ago it has also been reported in metastatic mammary epithelial cells but not in normal human mammary cells**.** It was proposed that this expression of Runx2 is ectopic and that the expression in these metastatic cancer cells may explain the osteoblastic phenotype of human breast cancer cells (Barnes GL, 2003), as well as prostate cancer cells (Yeung F, 2002) that metatstasized to the bone (Barnes GL, 2003). But later it was found out that the protein is also expressed in mammary epithelial cell lines derived from normal mammary gland, non metatstatic mammary cancer cells and primary mammary epithelial cells (Inman CK, 2003).

1.6. The influence of drugs on osteoblastic-like cancer cells

1.6.1. The effects of suramin on human and rat OS cells

OS is the most frequent malignant tumour of the bone and affects people in the second and the third decade of life (Benayahu D, 2001). The survival rate for patients with metastatic disease lies only at about a 20% (Walters DK, 2008).

Suramin is a polysulfonated naphthylurea compound that has been widely used for the treatment of trypanosomiasis (sleeping sickness) and onchocerciasis (Hawking F, 1978). It possesses six sulfonic groups that are attached directly to aromatic rings. The molecule was originally synthesized in 1916 based on the observation that trypan red and trypan blue exhibited trypanocidal activity (La Rocca RV, 1990-2). Its anticancer activity was later identified and suramin has been introduced into clinical trials for various forms of cancer (La Rocca RV, 1990-1; La Rocca RV, 1990-2; Garcia-Schürmann JM, 1999; Small EJ, 2000; Song S, 2001). This agent has shown some promise in phase 2 clinical trials in the management of hormone-refractory human prostate cancer (Thalmann GN, 1996) and high doses of suramin inhibit growth of malignant histiocytomas *in vitro* (Abdiu A, 1999).

Tumour cell differentiation depends on growth factor autocrine loops (Franchi A, 1998; Girnita L, 2000) that play an important role in malignant bone tumour development (Mason IJ, 1994; Yamaguchi K, 2006) Fibroblast growth factor (FGF)-2 autocrine loop controls human OS phenotyping and differentiation (Bodo M, 2002).

Suramin forms strong complexes with many proteins, including growth factors and their receptors. Recently it was shown that FGF-1 binds to suramin with high affinity in the nanomolar range which prevents interaction with its receptors (Kathir KM, 2006). Suramin triggers enterocyte-like differentiation of the human colic adenocarcinoma cell clone HT29-D4 and IGF-I secreted by the cells themselves, stimulates proliferation of HT29-D4 cells via an autocrine mechanism. Blockade of this stimulation by suramin or by a specific monoclonal antibody inhibits cell growth, glucose uptake and triggers the process of enterocytic differentiation (Baghdiguian S, 1992).

As already discussed above, blocking of the autocrine loop of growth factors seems not to be the sole task of suramin on OS cells (Benini S, 2001). This blocking could explain the up regulation of ALP and OCN (Bodo M, 2002) and down regulation of cell multiplication, which could be influenced by decrease of Telomerase activity (Trieb K, 2003). The substance

drastically upregulated LOX expression and induced phenotype reversion of transformed NIH3T3 that, as already mentioned above, is mediated by the LOX propeptide domain (Palamakumbura AH, 2004).

1.6.2. The effects of valproate and 5`-aza- 2-`deoxycytidine (5-AZAC) on human prostate cancer cell-lines

Prostate cancer is an increasing prevalent health problem among males (Emami KH, 2007). Despite its common occurrence, the molecular mechanisms responsible for prostate cancer growth, androgen-independent progression and acquisition of bone metastatic potential are poorly characterized. This kind of cancer most commonly metastasizes to lymph node and bone and causes significant mortality and morbidity in men with advanced disease (Yeung F, 2002). Studies show that bone matrix proteins like OCN and OPN are expressed at high levels in bone metastatic prostate cancer specimens (Curatolo C, 1992; Brown LF, 1994; Waltregny D, 1998) and androgen-independent bone metastatic prostate cancer cell lines**.** There seems to be a remarkable parallelism in the temporal expression of bone matrix proteins by prostate cancer cells and osteoblasts (Yeung F, 2002).

Metastatic prostate cancer is generally treated by homone therapy (Huggins JP, 1994) and when prostate cancer progresses to an androgen-independent state, non of the proposed therapeutic regimens has provided evidence for objective tumor response or a significant survival benefit (Thalmann GN, 1996). However, the clinical manifestation of this disease varies greatly, from indolent tumors, requiring little or no treatment, to those aggressive cancers which require radical therapies.

Prostate cancer, like all other cancers, develops and progresses as a consequence of an accumulation of genetic changes (Bott SR, 2005). Recently, common variants on human chromosome 8q24 were found to be associated with prostate cancer risk (Yeager M, 2007). Epigenetic alterations, mainly promoter hypermethylation of cancer-related genes, are found in nearly all tumours and in prostate cancer they were suggested as biomarkers (Costa VL, 2007). Epigenetic events are defined as alterations in gene expression without changes in the DNA coding sequence, that are heritable through cell division (Waddington CH, 1969). DNA methylation and histone modification are two mechanisms that are integral to epigenetic transcriptional control. DNA methylation describes the addition of a methyl group to the cytosine residue of a CpG dinucleotide by DNA methyltransferases (DNMTs) (Walton TJ,

2008). It is found in the genomes of different organisms including both prokaryotes and eukaryotes. In prokaryotes, DNA methylation occurs on cytosine and adenine bases (Wilson GG, 1991) but in multicellular eukaryotes methylation seems to be confined to cytosine bases and is associated with a repressed chromatin state and the inhibition of gene expression (Bird AP, 1999). DNA methyltransferases (DNMTs) are important regulators of gene transcription and because they play a role in carcinogenesis they have been intensively studied in the last few years (Yu N, 2008). The methyltransferases DNMT3a and DNMT3b are mainly responsible for introducing cytosine methylation at unmethylated CpG sites, whereas the methyltransferase DNMT1 copies pre-existing methylation patterns onto the new DNA strand during DNA replication. The DNA methyltransferase, DNMT2, shows weak DNA methyltransferase activity in vitro (Hermann A, 2003), but targeted deletion of the DNMT2 gene in embryonic stem cells causes no detectable effect on global DNA methylation. So, this enzyme seems to have little involvement in setting DNA methylation patterns (Okano M, 1998). DNMT3L is a DNMT-related protein and does not contain DNA methyltransferase activity, but physically associates with DNMT3a and DNMT3b and modulates their catalytic activity (Suetake I, 2004).

In the mammalian genome, methylation takes place only at cytosine bases that are located 5′ to a guanosine in a CpG dinucleotide. This dinucleotide is actually underrepresented in much of the genome, but short regions of 0.5–4 kb in length, known as CpG islands (Bird A, 2002)**,** that occur in the promoter region of approximately 60% of genes (Gardiner-Garden M, 1987)**,** are rich in CpG content (Bird A, 2002). Most CpG islands are found in the proximal promoter regions of almost half of the genes in the mammalian genome and are, generally, unmethylated in normal cells. In cancer, however, the hypermethylation of these promoter regions is now the most well-categorized epigenetic change to occur in tumours. It is found in virtually every type of human neoplasm and is associated with the inappropriate transcriptional silencing of genes (Baylin SB, 2000).

As already mentioned above, inappropriate DNA methylation is thought to be important in the development of a majority of cancers, including prostate cancers (Walton TJ, 2008) and today, over 30 genes have been shown to be epigenetically silenced by hypermethylation in prostate cancer, including genes controlling cell cycle progression, tumor cell invasion, DNA repair, and hormonal response (Li LC, 2005). Estrogen receptor β (ERβ) is one such hormonal response gene known to be hypermethylated in prostate cancer (Li LC, 2000) and upregulation of ERβ following co-treatment may sensitize prostate cancer cells to subsequent treatment with conventional estrogens (Walton TJ, 2008). ERβ contains a CpG island within

its promoter (Li LC, 2000) and has been shown to be re-expressed in prostate cancer cell-lines treated with DNMT inhibitors such as 5`-aza- 2`-deoxycytidine (5-AZAC) (Lau KM, 2000; (Sasaki M, 2002).

Another mechanism of epigenetic transcriptional control results from histone modifications. Histones form the core around which double-stranded DNA is coiled to form a nucleosome. They are subject to a number of post-tranlational protein modifications, such as acetylation, methylation, phosphorylation and ubiquination (Zhang Y, 2001). Histone acetylation is associated with transcription activation, whereas deacetylation is associated with transcriptional silencing. Dysregulated epigenetic control of gene expression by histone modification is thought to be important in the development of a number of cancers, including prostate cancer (Li LC, 2005). Both processes, histone modification and DNA methylation are linked, although they are two independent processes (Li E, 2002). DNMT`s, for example, recruit histone-deacetylases (HDACs) leading to histone deacetylation by removing the acetyl group from the histone (Jamaluddin MD, 2007) and transcriptional repression (Fuks F, 2001; Robertson KD, 2000). And methylated DNA binding proteins can recruit HDAC`s to the site of DNA methylation (Nan X, 1998; Jones PA, 2002**).** Histone deacetylation leads to chromatin condensation and inhibits the access of regulatory proteins to the promoter (Jamaluddin MD, 2007). It has been shown that the combination of HDAC and DNMT inhibitors induces apoptosis, differentiation and/or cell growth arrest in human lung, breast, thoracic, leukemia and colon cancer cell lines (Zhu WG, 2003). In prostate cancer, a number of studies have reported reductions in cell proliferation and apoptosis in cell lines treated with HDAC inhibitors alone (Thelen P, 2004; Fronsdal K, 2005), but little is known about the effects of combined treatment.

1.7. Aims of the work

Osteosarcoma and prostate cancer are two types of cancer that target different groups of people. As osteosarcoma is the most common malignant primary bone tumor in children and adolescents, prostate cancer is one of the most diagnosed and potentially devastating cancers in men, throughout the world.

One fact that both types of cancer have in common is that they both affect bone because osteosarcoma itself develops in bone and prostate cancer cells metastasize to bone and lymph nodes and cause significant mortality and morbidity in men with advanced disease.

Today, many therapeutical options are available to treat osteosarcoma and prostate cancer. Current optimal treatment for osteosarcoma consists of multi-agent chemotherapy and aggressive surgical resection of all sites of disease involvement. The current national and international cooperative trial for patients with newly diagnosed osteosarcoma builds upon the backbone of cisplatin, doxorubicin, and methotrexate. Suramin has not yet been tried in patients with osteosarcoma and little is known about its in vitro effects on osteosarcoma cells. The clinical manifestation of prostate cancer varies from indolent tumours, requiring little or no treatment to aggressive cancers which require radical therapies.

Epigenetic alterations are defined as alterations in gene expression without changes in the DNA coding sequence and in cancer promoter hypermethylation of cancer-related genes can be found. DNA methylation and histone modification are two mechanisms that are integral to epigenetic transcriptional control. A number of studies have reported reductions in cell proliferation and apoptosis in cancer cell lines treated with histone-deacetylases (HDACs) inhibitors, but little is known about the effects of combination therapy of DNA methyltransferases (DNMTs) and HDACs inhibitors in cancer cell lines.

Lysyl Oxidase (LOX) is an enzyme that is responsible for the collagen cross-linking in bone and it was found that the LOX propeptide, which is released during extracellular proteolytic processing of pro-lysyl oxidase, has some anti-cancer activity.

Aim of this thesis was to study the effects of Suramin, the DNMT inhibitor 5´-aza-2´-deoxycytidine and the HDAC inhibitor valproate on different osteoblast-associated tumour cell lines, taking into special consideration the effects on osteoblastic differentiation and LOX expression, as LOX is downregulated in human cancers. Therefore, osteoblast–specific genes were analysed to show if the three mentioned drugs cause a differentiation of the cells.

2. Results and Discussion of published and unpublished data

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Lysyl oxidase (LOX) mRNA expression and genes of the differentiated osteoblastic phenotype are upregulated in human osteosarcoma cells by suramin ☆

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Abstract

It is well known that suramin influences proliferation and differentiation of tumour cells. To study whether and how suramin effects osteosarcoma (OS) cells, proliferation, differentiation, LOX mRNA expression and telomerase activity (TA) was analysed in the human MG-63 and U-2 OS, and the rat UMR-106 OS cell lines. Data show that suramin inhibited proliferation in the human cell lines and upregulated alkaline phosphatase activity. TA was attenuated in the human cells while in UMR-106 it was not changed. In UMR-106 suramin had no influence on osteocalcin and LOX expression, in the human cells however, both genes were upregulated.

Keywords: Osteosarcoma cells; Suramin; Lysyl oxidase; Osteocalcin; Telomerase activity

1. Introduction

Activated LOX is the key enzyme that controls collagen and elastin maturation [1]. It catalyzes the oxidative deamination of peptidyl-lysine and hydroxylysine to peptidyl-a-aminoadipic-d-semialdehyde in elastin and collagen chains. The consequent aldehydes lead to a spontaneous condensation forming inter- and intra-chain cross-links in these important extracellular matrix components. An additional function of this protein was suggested by the findings that LOX is downregulated in *ras*-transformed cells and in many tumour cell lines [2,3] as well as in human cancers [4–7]. Spontaneous reversion of cancers or induced phenotypic reversion is accompanied by increased LOX expression [8]. The function as a tumour suppressor was directly shown by transfection of anti-sense LOX, which triggers transformation of normal rat fibroblasts and reversion of stable phenotypic revertants of *ras*-transformed NIH3T3. This tumour suppressor function was recently attributed to the 18-kDa propeptide [3,5],

☆ *Significance of the results:* We show that suramin increased differentiation of osteosarcoma cells that could be mediated in part by upregulation of lysyl oxidase. In osteosarcoma cells telomerase activity increased during culture, while suramin decreased TA compared to controls.

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which is processed from the secreted 50-kDa proenzyme by the procollagen C-proteinase bone morphogenic protein-1 [9]. The larger fission product is the active enzyme responsible for the initiation of the cross-links. However, recently it was found that LOX itself activates the transcription activity of human collagen III promoter, possibly by the involvement of the Ku antigen [10].

OS is the most frequent malignant tumour of the bone and exhibits a peak in manifestation during the second and the third decade of life. Primary chemotherapy for high-grade bone sarcomas often involves intensive, multivalent regimens, and few secondary chemotherapy options are available to treat refractory or relapsed disease [11]. Suramin was initially introduced for the treatment of parasitic infections [12]. Its anti-cancer activity was later identified and suramin has been introduced into clinical trials for various forms of cancer [13–17]. It was shown that high doses of suramin inhibit growth of malignant histiocytomas *in vitro* [18]. The substance drastically upregulated LOX expression and induced phenotype reversion of transformed NIH3T3 that, as already mentioned above, is mediated by the LOX propeptide domain [3]. Also, tumour cell differentiation depends on growth factor autocrine loops [19,20] that play an important role in malignant bone tumour development [21,22]. Fibroblast growth factor (FGF)-2 autocrine loop controls human OS phenotyping and differentiation [23]. In human colon cancer blockade of IGF-I stimulated HT29-D4 cell proliferation by suramin resulted in inhibition of cell growth and triggered the process of enterocytic differentiation [24]. Suramin forms strong complexes with many proteins, including growth factors and their receptors. Recently it was shown that FGF-1 binds to suramin with high affinity in the nanomolar range which prevents interaction with its receptors [25]. Although, as discussed, blocking of the autocrine loop of growth factors seems not to be the sole task of suramin on OS cells [26]. This blocking could explain the up regulation of ALP and OCN [23] and down regulation of cell multiplication, which could be influenced by decrease of TA [27].

We were interested in the effects of suramin on OS cells and whether the effects of this drug could also be observed in different OS cell types. This is described for different forms of cancer cells and may in part be due to increased expression of LOX [12,17,26].

2. Materials and methods

2.1. Cell culture

MG-63, U-2 OS und UMR-106 cells were purchased from American Type Culture Collection. MG-63 cells (CRL-1427), a human OS cell line, offer a fibroblast-like morphology whereas U-2 OS cells (HTB-96) and the rat OS cell line UMR-106 feature an epithelial phenotype. The human (MG-63 and U-2 OS) cells were grown in αMEM (α-minimum essential medium; Biochrom) containing 5% fetal calf serum (FCS; Biochrom), supplemented with 4.5 g/l L-glucose, 50 μg/ml ascorbic acid (Sigma) and 10 μg/ml gentamycin (Sigma). UMR-106 cells were grown in DMEM (Dulbecco's minimal essential medium; Sigma) containing 10% FCS, 4 mM L-glutamine (Sigma), 4.5 g/l L-glucose (Sigma) and 10 μg/ml gentamycin.

2.2. Cell multiplication and ALP activity

Cells were plated in 24-well plates at a density of 20,000 cells/cm^2 and grown in the medium as described above. Cells were treated with 150 μM suramin for 6 days. After 3 and 6 days the media were removed and the cells were washed twice with PBS and frozen at −20 °C. During subsequent thawing, 500 μl Hoechst 33258 solution (5 mg/ml, 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.1 mM EDTA) were added to each well. The amount of DNA was quantitated by measuring the fluorescence in a multiwell fluorometer (excitation 355/emission 460; Tecan). DNA was estimated using a standard curve prepared from calf thymus DNA (Roche).

Dose dependence of suramin on cell multiplication was estimated after 6 days of treatment with 200 μM, 300 μM, 500 μM and 1000 μM. The amount of DNA was measured as described above.

Before measuring ALP activity in the same multiwells, the plates were frozen again. After thawing, the cells were incubated for 20 min with 500 μl 20 mM *p*-nitro-phenylphosphate in 100 mM diethanolamine and 10 mM $MgCl_2$ at pH 9.5. The reaction was stopped with 0.1 M EDTA in 1 M NaOH. The absorption was measured at 405/490 nm in a microplate reader. ALP activity was estimated using a standard curve prepared from calf intestinal ALP (Roche).

2.3. RNA isolation and LOX mRNA expression

For the examination of the LOX mRNA expression, cells were plated in 6-well plates at a density of 20,000 cells/cm^2. After 24 h of culture, cells were treated with 100 μM suramin for 3 and 6 days and thereafter total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The first-strand cDNA was synthesized from 1 μg of total RNA by means of AMV reverse transcriptase (Invitrogen). Analyses were

performed by real-time PCR using SYBR Green (Roche Diagnostics). Specific primers for the coding sequence of human LOX were as follows: upper primer, 5′-ACT GCA CAC ACA CAG GGA TTG-3′ and lower primer 5′-GCC TTC TAA TAC GGT GAA ATT G-3′. For the amplification of rat LOX in UMR-106 cells the sequence of the primers were: upper primer, 5′-GAA TTA GCA GTG TTT CAA TTC TGT GGG-3′ and lower primer, 5′-GTT AGA TGT TTG GCC CTT CCT CAG-3′. mRNA expression of GAPDH was used for normalization. RTQ-PCR was carried out using a Rotor-Gene real-time system (Corbett Research) using 2.5 μM of specific primers: 15 s denaturation at 95 °C temperature, followed by 45 cycles with 58 °C annealing temperature for 15 s and 72 °C extension temperature for 20 s. PCR cycles were followed by the melting curves from 72 °C to 95 °C with a temperature transition rate of 0.5 °C/s. For quantification of the RT-PCR a linear amplification was assumed. All PCR's were performed as triplicate. To analyse if the effects of suramin on LOX are dose dependent we isolated RNA from U-2 OS cells that were plated at a density of 20,000 cells/cm^2 and treated 24 h after seeding with 50 μM, 100 μM and 300 μM suramin for 3 days. Analysis of the specific cDNA was performed as described above by RT-PCR.

2.4. OCN mRNA expression

After mRNA isolation and reverse transcription was done, as described above, the obtained cDNA was subjected to PCR amplification with a real-time cycler. Human OCN mRNA expression was analysed by the human specific TaqMan Gene Expression System (Applied Biosystems). TaqMan GAPDH probes were used as a housekeeping gene, amplified in the same tube. All PCR's were performed as triplicate. An amplification program (as suggested by Applied Biosystems) with 60 °C annealing temperature was used for 60 cycles. Quantification was achieved using the comparative threshold cycle method according to the manufacturer's protocol.

For analysis of the mRNA from the rat UMR-106 cells rat specific OCN primers were designed and analysed by real-time PCR using SYBR Green (Roche Diagnostics) and GAPDH was used for normalization. The primers were 5′-TGA CAA AGC CTT CAT GTC CAA-3′ and 5′-GTC CGC TAG CTC GTC ACA-AT-3′. Denaturation temperature was 95 °C for 10 s, and the annealing temperature was 60 °C for 15 s, followed by an extension temperature of 72 °C for 20 s. For quantification of the RT-PCR a linear amplification was assumed. All PCR's were performed as triplicate.

2.5. Effects of suramin on other bone differentiation markers

To investigate if suramin has an effect on other differentiation markers (besides OCN) we isolated mRNA of human U-2 OS cells that were plated in 24-well plates at a density of 20,000 cells/cm^2 and treated with suramin for 2 days. After reverse transcription human collagen (COL1A1) and human osteopontin (SPP1, abbreviated OPN) mRNA expression was analysed by the human specific TaqMan Gene Expression System (Applied Biosystems). TaqMan GAPDH probes were used as a housekeeping gene, amplified in the same tube. For quantification of the RT-PCR a linear amplification was assumed. All PCR's were performed as triplicate.

2.6. Protein extraction for TA

Cells were plated in 6-well plates at a density of 20,000 cells/cm^2 and treated with 100 μM suramin 24 h after plating. On each of the following 6 days proteins were isolated. Therefore cells were washed and scraped with phosphate buffered saline (PBS). Cells were centrifuged for 5 min at 500*g* and extracted with 300 μl lysis-buffer (0.5% CHAPS 10 mM Tris–Cl, pH 8.0, 1 mM EDTA,) containing RNase inhibitor. After an incubation period of 30 min at 4 °C the extracts were centrifuged for 30 min at 12,000 rpm and then the supernatant was stored at 4 °C. The protein concentration was measured using Coomassie blue (Pierce).

2.7. Real-time quantitative (RTQ)-trap assay for estimation of TA [28]

In a total volume of 20 μl containing the FastStart SYBR Green Master Mix (Roche Diagnostics) about 0.2 μg proteins, 50 pM TS primer (5′-AAT CCG TCG AAG AGT T-3′) and 50 pM ACX primer (5′-GCG CGG CTT ACT AAC C-3′) were incubated at room temperature for 20 min and then the PCR was started and held at 95 °C for 10 min, followed by a 45-cycle amplification (95 °C for 20 s, 50 °C for 30 s and 72 °C for 90 s). TA in the OS cell lines was calculated based on the threshold cycle (CT) and normalized to the protein concentration. All samples were run as triplicate.

2.8. Statistical analysis

Statistical significance was tested by ANOVA (posthoc test: Fisher) or *t*-test using Prism 4.0 (GraphPad Software Inc., CA, USA).

3. Results

3.1. Cell multiplication and ALP activity

Firstly, the effects of suramin on cell growth and viability of the OS cell lines were studied. Suramin decreased cell multiplication as measured by the amount of DNA in the human OS cell lines significantly after 6 days of treatment (Fig. 1). In the rat OS cells we observed a trend, however, the decrease was statistically not significant

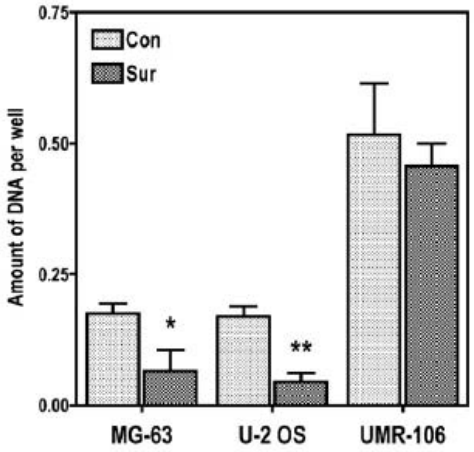

Fig. 1. Effects of suramin on DNA amount of OS cell lines after 6 days of culture. After treatment with suramin (150 μM), Hoechst dye was added (1 μg/ml) and the fluorescence of the incorporated dye was measured at 360/460 nm. Bars represent means ± SD. $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$ (control vs suramin treatment).

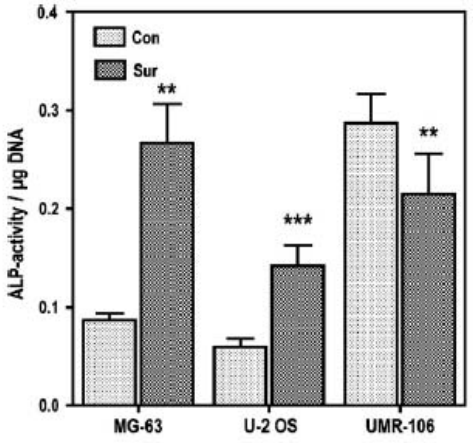

Fig. 3. ALP activity in OS cells after 6 days of treatment with suramin (150 μM) was analysed with *p*-nitro-phenylphosphate (2.5 mg/ml). Absorption was measured at 405/492 nm and compared to a standard curve. Bars represent means ± SD. $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.001$ (control vs suramin treatment).

(Fig. 1). We further tested the maximum concentration of suramin, which can be used to treat OS cells. Fig. 2 shows that concentrations higher than 200 μM resulted in a significant decrease of cell multiplication in rat OS cells. Therefore, we decided to use concentrations lower than 200 μM for further experiments. Secondly, the effects of suramin on ALP activity on the three different OS cell lines were tested. After 3 days of culture ALP activity was increased by suramin in the human but not in the rat OS cells, where suramin decreased the ALP activity significantly (Fig. 3). This indicates that treatment with suramin increased the differentiation of human OS cells but not of the rat OS cells.

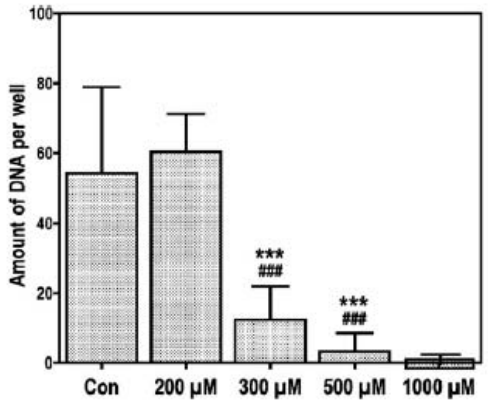

Fig. 2. Effects of suramin on DNA amount of rat OS cells after 6 days of culture treated with 200 μM, 300 μM, 500 μM and 1000 μM suramin. Hoechst dye was added (1 μg/ml) and the fluorescence of the incorporated dye was measured at 360/460 nm. Bars represent means ± SD. $^{***}P \leqslant 0.001$ (300 μM, 500 μM suramin vs control). $^{\#\#\#}P \leqslant 0.001$ (300 μM, 500 μM vs 200 μM suramin). No significant difference was found between control and 200 μM suramin treatment as well as between the higher suramin concentrations (300 μM, 500 μM and 1000 μM).

3.2. LOX mRNA expression

The results of real-time PCR analysis showed that suramin increased LOX expression at the mRNA level in the human OS cell lines MG-63 and U-2 OS (Fig. 4). This increase was statistically significant after three days of culture. In rat UMR-106 cells suramin had no effect on LOX expression (data not shown). We further tested whether the increase of LOX expression in human OS cells was dose dependent. Fig. 5 shows that already a concentration of 50 μM suramin resulted in a significant increase of LOX mRNA expression. Although, there was no significant difference between 50 μM and 100 μM treatment, the significant increase of LOX mRNA expression of the 300 μM treated cells indicates a dose dependence.

3.3. OCN mRNA expression

ALP activity (Fig. 3) indicates that suramin could influence osteoblast differentiation. Therefore, we looked for OCN expression, an indicator of differentiated osteoblasts. As shown in Fig. 6, only in MG-63 cells suramin statistically significant stimulated OCN mRNA expression 11-fold. In U-2 OS (Fig. 6) and UMR-106 we found

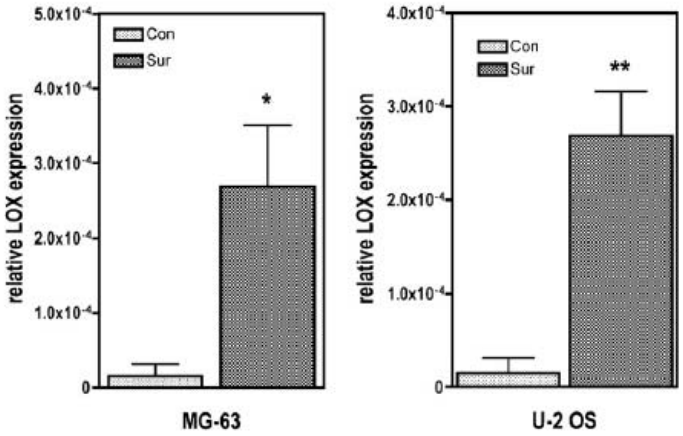

Fig. 4. Relative LOX-expression in MG-63 and U-2OS cells after 3 days of treatment with suramin (100 μM). The total RNA amount of suramin treated cells and the control group was isolated using TRIzol reagent and the reverse transcription was done. Analysis of the specific cDNA was performed with a real-time PCR using SYBR Green and the expression was normalized to GAPDH. Bars represent means ± SD. $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$ (control vs suramin treatment).

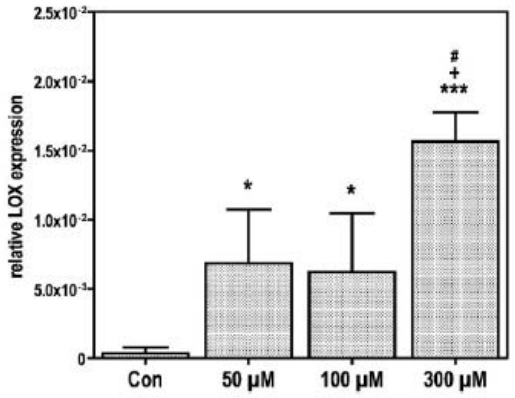

Fig. 5. Dose dependency of suramin on LOX expression. RNA from U-2 OS cells that were plated at a density of 20,000 cells/cm^2 and treated with 50 μM, 100 μM and 300 μM suramin for 3 days was isolated using TRIzol. Analysis of the specific cDNA was performed by real-time PCR using SYBR Green (Roche Diagnostics) and human LOX primers as described above. mRNA expression of human GAPDH was used for normalization. Bars represent means ± SD. $^{*}P \leqslant 0.05$ (control vs 50 μM and 100 μM suramin treatment). $^{***}P \leqslant 0.001$ (control vs 300 μM suramin treatment); $^{+}P \leqslant 0.05$ (50–300 μM suramin treatment); $^{\#}P \leqslant 0.05$ (100–300 μM suramin treatment).

no statistically significant differences. The absence of a significant increase of the differentiation marker OCN in U-2 OS cells let us look for changes in the expression of other osteoblast-characteristic genes like COL1A1 and OPN. Fig. 7 shows that suramin treatment of U-2 OS cells increased both genes significantly.

3.4. Effects on telomerase activity

Downregulation of cell multiplication in OS cells could be mediated by decreasing TA. We used RTQ-trap assay to study TA in treated and untreated OS cells. Effects of suramin on TA varied depending on the cell line (Fig. 8). In MG-63 cells during the culture time, TA increased significantly beginning with day 3 (decrease of the CT-values, Fig. 8A) while in suramin treated cells TA did not change. In U-2 OS cells, however, TA increased in both controls and suramin treated cells (decrease of the CT-values, Fig. 8B) but in the suramin treated cells the increase was significantly lower; the difference between treated and untreated cells was less pronounced as found in MG-63. In the rat OS cell line UMR-106 we could not find neither a difference nor a trend for any change (not shown).

4. Discussion

Our investigations targeted on phenotype regulation of OS cells in culture by evaluating multiplication and differentiation under the control of suramin that is a known inhibitor of cell multiplication of OS [26,27,29] and other transformed cells [3,30]. Our results confirm previous data, however, added new information about this relationship. As already described [27], suramin downregulates cell multiplication of human OS cells and decreases TA, although, this was not a general fact, it depended on the type of the OS cells. In both human OS cell lines, there was a signifi-

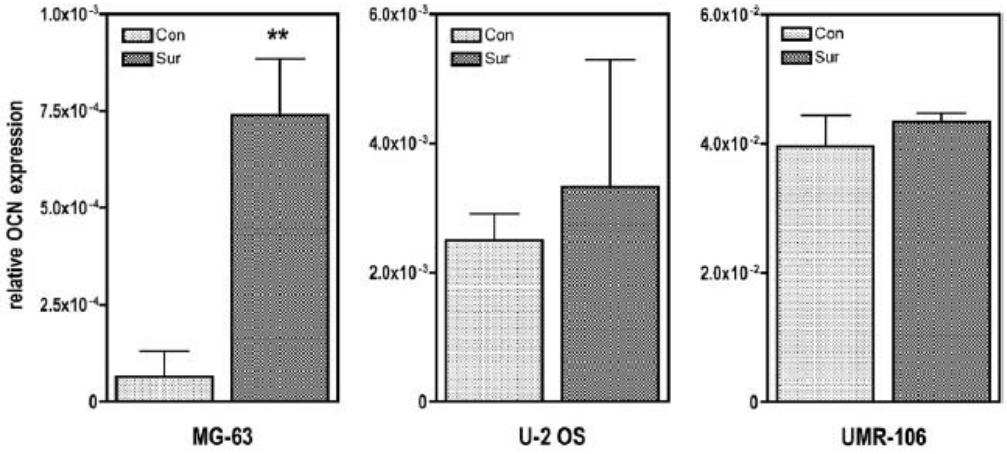

Fig. 6. Relative OCN-expression in MG-63, U-2 OS and UMR-106 cells after 2 days of treatment with suramin (100 μM). The total RNA amount of suramin treated cells and the control group was isolated using TRIzol reagent and the reverse transcription was done. The PCR was performed with a real-time cycler using TaqMan Gene Expression Master Mix and TaqMan system primer (MG-63 and U-2 OS) and SYBR Green (UMR-106). The expression was normalized to GAPDH. Bars represent means ± SD. $^{**}P \leqslant 0.01$ (control vs suramin treatment).

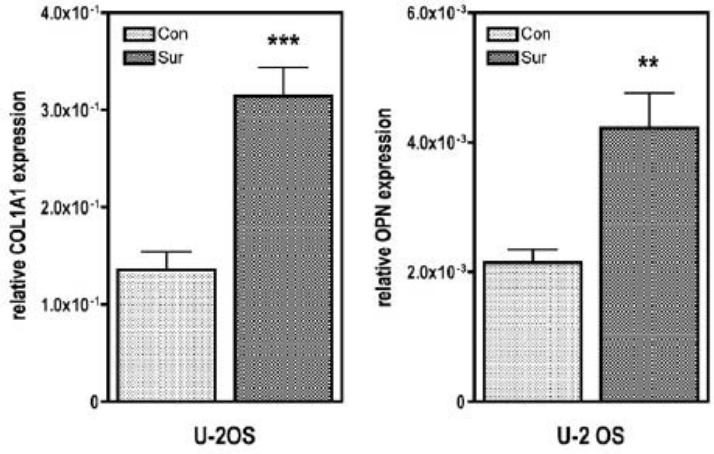

Fig. 7. Relative COL1A1 and OPN expression in human U-2 OS cells after 2 days of treatment with suramin (100 μM). The total RNA amount of suramin treated cells and the control group was isolated using TRIzol reagent and the reverse transcription was done. The PCR was performed with a real-time cycler using TaqMan Gene Expression Master Mix and TaqMan system primer. The expression was normalized to GAPDH. Bars represent means ± SD. $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.001$ (control vs suramin treatment).

cant downregulation of cell multiplication, in the rat OS cells there was no significant change neither relating to proliferation nor to TA. However, a concentration of 300 μM suramin or more resulted in a considerable downregulation of cell multiplication indicating a toxic effect. This suggests that in the studied human OS cells an indirect mechanism could be responsible for the significant reduction of the cell numbers found after 6 days of treatment. From our data we cannot deduce whether the attenuation of cell multiplication results from downregulation of proliferation or increase of cell death, possibly by increasing the rate of apoptosis [31]; however, the decrease of TA could be responsible for both [32]. Decrease of cell multiplication in isolated osteoblasts is associated with enhanced differentiation as shown by an increased ALP activity and

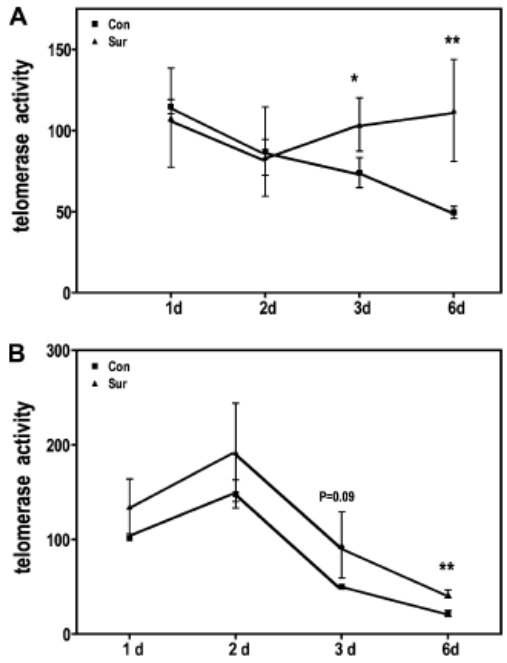

Fig. 8. Telomerase activity (TA) in human OS cell lines MG-63 (A) and U-2 OS (B). After protein extraction TA was determined by means of real-time quantitative (RTQ)-trap assay [28]. TA in the OS cell lines was calculated based on the threshold cycle (CT) and normalized to the protein concentration. Suramin prevented upregulation (no change of the CT-values) in MG-63 cells and decreased TA (increase of the CT-values) in both human OS cells compared to controls. $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$ (control vs suramin treatment).

increased expression of genes of the mature osteoblastic phenotype like OCN [33–36]. In agreement to the cell multiplication and TA measurements, suramin consistently increased ALP activity in the human but not in the rat OS cells. Moreover, suramin decreased the ALP activity in the latter cells. This indicates that in the UMR-106 cells there is a decoupling between proliferation and differentiation which is well known for tumour cells [37] and cannot be altered by suramin. The second well-defined marker of osteoblastic differentiation, OCN, behaved comparable: up regulation by suramin in both human OS cell lines, although not significantly in U-2 OS, but not in the rat UMR-106 cells. Suramin acts by inhibiting the autocrine loop of growth factors [19,21,23,26,29]. This could explain the upregulation of OCN by suramin in human OS cells, because most investigated growth factors decrease OCN expression in osteoblasts [38,39]. Again, our findings point out that suramin acts differently in the studied cell lines.

To confirm the trend of suramin to increase the osteoblastic phenotype of the OS cells we tested the change of two other genes involved in osteoblastic differentiation: COL1A1and OPN. COL1A1 is the main constitute of bone and OPN comprises approximately 2% of the non-collagenous protein in bone. OPN is an acid-rich, multidomain, phosphorylated glycoprotein and is involved in many physiological and pathological processes including cell adhesion [40], angiogenesis [41], apoptosis, inflammatory responses and tumour metastasis [42]. A lot of studies have shown that OPN plays an important role in controlling cancer progression and OPN was found to be expressed in many human cancer types [43]. High levels of OPN expression correlate with tumour invasion, progression or metastasis in multiple cancer [44]. Both proteins, COL1A1 and OPN are expressed during differentiation when proliferation ceases [37]. As mentioned above, inhibition of the autocrine loop can be responsible for these findings because growth factors usually increase cell proliferation.

Telomerase activity is critically involved in cell development, ageing and tumourigenesis. Recently, it was found that in some OS cells suramin downregulated TA [27]. We extended these observations by studying different time points of TA. Treatments of both human cell lines with suramin resulted in a significant decrease of TA (increase of CT-values) compared to controls at day 6 but the mechanism in the two human cell lines seems to be different. We clearly show that in both MG-63 and U-2 OS cells the TA was increased (decrease of CT-values) during the culture period; but although in U-2 OS cells TA increased during the culture period as well, in MG-63 cells suramin had no influence on TA. TA was found to be upregulated by some growth factors (EGF [45], IGF-I and IGBP-2 [46,47]) and downregulated by TGF-β via the SMAD-3 pathway [48,49]. We conclude from our findings that in MG-63, suramin inhibited all growth factor interactions responsible for regulation of TA. In U-2 OS, however, only some signals were blocked, while others, possibly resulting from the autocrine loop were left open. In UMR-106 as found with OCN and cell multiplication, TA did not differ between controls and treatment. The relation between TA and tumour cells is contradictory: While over expression of hTERT, the gene responsible for TA, is insuffi-

cient to cause malignant transformation [50,51], the close correlation between telomerase activation and cancer cell immortalisation from an early stage has positioned telomerase as an important target for malignancy diagnostics and therapeutic applications [32]. However, other results indicate that TA is involved in ageing as found in primary osteoblasts outgrown from donors with different ages; the cellular activity, telomere lengths, and replicative life span of osteoblastic cells gradually decrease with the advance of donor age [52]. Therefore, the relevance of TA for tumour cells and the influence of suramin on this activity remain to be clarified.

A central point of our investigation was the influence of suramin on LOX expression which indicates dose dependently a non-toxic effect. This exciting protein possesses two functions, namely collagen cross-linking and tumour suppressor activity, is downregulated in many investigated tumours. The propeptide domain of this protein induces phenotype reversion in *ras*-transformed cells [3], and the constitutive activation of *ras* proto-oncogene in NRK-49F cells is characterized by downregulation of LOX-induced tumourigenic transformation [53]. Reduced LOX mRNA levels were found in experimental and human prostate cancer [5] and azatyrosine, a possible inhibitor of tyrosine kinases blocking the signal transduction like suramin, inhibited behaviour of cancer cells [54].

Recently, by means of methylation-sensitive representational difference analysis LOX silencing was found by DNA-methylation in human gastric, colon, lung, and ovarian cancer cells [55]. More recently it was found that in human lung and pancreatic cancer cells that carry N-*ras* and K-*ras* mutations, respectively, the tumour suppressing part of LOX strongly inhibits *ras* signalling and NF-κB activity, and Bcl-2 was identified as an essential target of propeptide–mediated inhibition of the transformed phenotype [56]. Furthermore, the tumour suppressor activity of LOX propeptide reverses the invasive phenotype of Her-2/neu-driven breast cancer [57]. All these findings support the relationship between LOX and tumourigenicity.

We found that in both studied human OS cell lines suramin upregulated LOX expression dose dependently at least in U-2 OS, possibly by inhibition of the autocrine loop, which was found to control the OS phenotyping and differentiation. Recently a redundancy of autocrine loops in human OS cells was described, and inhibiting growth factor signal transduction alone seems not to be enough to block tumour reversion. Our finding that LOX is upregulated by suramin could, additionally to blocking the autocrine loop, influence OS phenotype and could support the significance of the anti-tumour activity of this drug as suggested recently [26]. In UMR-106 cells there was no upregulation of LOX expression neither in 3 days treated cells nor after 6 days indicating that there is a difference between different osteosarcomas.

In conclusion we could show that in human osteosarcoma cells suramin inhibits cell proliferation, increases differentiation as measured by OCN expression and ALP activity, increases LOX expression and decreases telomerase activity. Taking these findings together with existing evidence in the literature the observed phenomena could contribute to anti-tumour effects of suramin in human osteosarcomas.

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2.2. The effects of valproate and 5`-aza- 2`-deoxycytidine on human prostate cancer cell-lines

2.2.1 Material and Methods

Cell culture

Human PC-3, DU 145 and LNCaP cells were purchased from American Type Culture Collection. PC-3 cells (CRL-1435) were grown in DMEM (Dulbecco`s minimal essential medium; Sigma)/F-12K medium containing 10% FCS and 10 µg/ml gentamycin. DU 145 (HTB-81) and LNCaP (CRL-1740) cells were grown in αMEM (α-minimum essential medium; Biochrom, Germany) supplemented with 10% FCS (Biochrom, Germany) and 10 µg/ml gentamycin.

Cell multiplication

Experiments were performed to determine the optimal seeding concentration of the cells. Therefore, different cell numbers (250, 500, 1000, 2000, 4000, 8000, 16 000 and 32 000 cells /cm^2) were seeded into individual wells in a 48-well plate to a total volume of 500 µl.
Each experiment was performed in quadruplicate with additional wells filled with media alone. On day 5 the media were removed and the cells were washed twice with PBS and frozen at −20°C. During subsequent thawing, 500 µl Hoechst 33258 solution (10 µg/ ml, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1 mM EDTA) were added to each well. The amount of DNA was quantitated by measuring the fluorescence in a multiwell fluorometer (excitation 355/emission 460; Tecan). DNA was estimated using a standard curve prepared from calf thymus DNA (Roche).
To analyse if the DNMT inhibitor 5-AZAC and the HDAC inhibitor valproate have an effect on cell multiplication on the described cell lines 8000 cells/cm^2 were seeded in 24-well plates and treated either with 5 uM 5-AZAC or 1 mM valproate or with both drugs. Untreated cells served as a control. On day 1, 2, 3 and 7 the amount of DNA was quantitated as described above.

RNA isolation and reverse transcription

Cells were plated in 50 cm^2 plates at a density of 8,000 cells/cm^2. After 24 hours of culture, cells were treated either with 5 uM 5-AZAC or 1 mM valproate or with both drugs. Untreated cells served as a control. On day 1 and 7 total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer`s protocol. RNA was cleaned using RNeasy Mini Kit (QIAGEN). The first-strand cDNA was synthesized from 1 μg of total RNA by means of AMV reverse transcriptase (Invitrogen). Analyses were performed by real time PCR.

LOX, Runx2 and OPG mRNA expression

After mRNA isolation and reverse transcription was done, expression analyses were performed by real time PCR using TaqMan Gene Expression System (Applied Biosystems). TaqMan GAPDH probes were used as a housekeeping gene, amplified in the same tube. An amplification program (as suggested by Applied Biosystems) with 59°C annealing temperature was used for 60 cycles. For quantification of the RT-PCR a linear amplification was assumed. All PCR's were performed as triplicate

OCN mRNA expression

Epression analyses were performed by real time PCR using SYBR Green (Roche Diagnostics). Specific primers for the coding sequence of human OCN were as follows: upper primer, 5`-CAT GAG AGC CCT CAC A -3` and lower primer 5`-AGA GCG ACA CCC TAG AC -3`. mRNA expression of GAPDH was used for normalization. RTQ-PCR was carried out using a Rotor-Gene real-time system (Corbett Research) using 2.5 μM of specific primers: 15 seconds denaturation at 95°C temperature, followed by 45 cycles with 58°C annealing temperature for 15 seconds and 72°C extension temperature for 20 seconds. PCR cycles were followed by the melting curves from 72°C to 95°C with a temperature transition rate of 0.5°C/s. For quantification of the RT-PCR a linear amplification was assumed. All PCR's were performed as triplicate.

Statistical analysis

Statistical significance was tested by t-test or ANOVA (post hoc: Bonferroni) using Prism 4.0 (GraphPad Software Inc., CA, USA).

2.2.2. Results and Discussion

Effects of 5-AZAC and valproate on cell multiplication

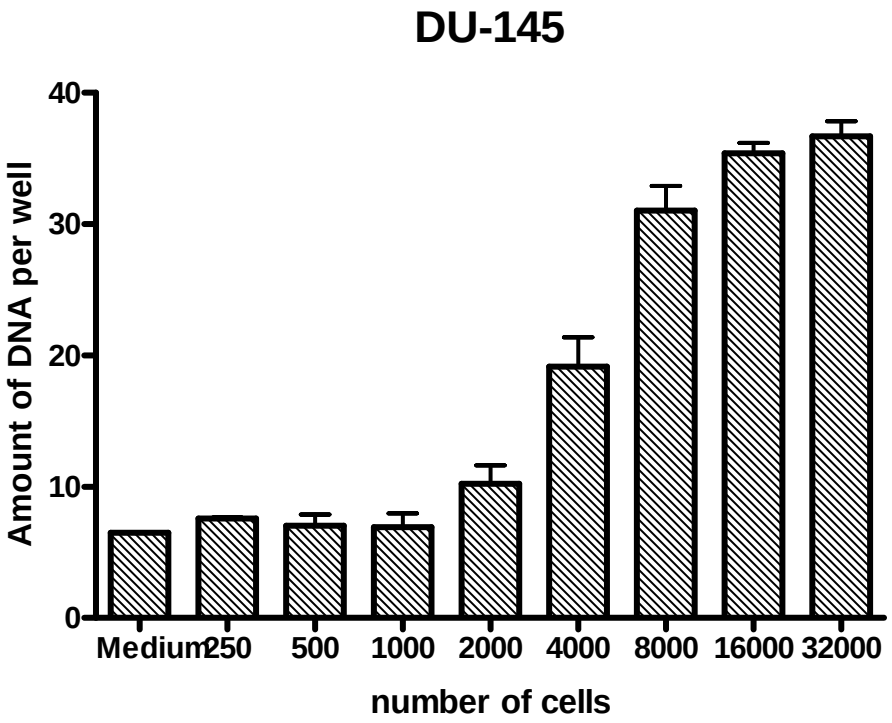
Firstly, careful optimization experiments were performed to determine the optimal seeding concentration of the three human prostate cancer cell lines DU-145, PC-3 and LNCaP. As shown in Fig.1, a seeding concentration of about 8000 cells/cm^2 for all cell lines was the optimal concentration that was associated with unrestricted cell proliferation. After 5 days of culture the amount of DNA in the three cell lines shows that the cell multiplication of DU-145 cells was higher than those of PC-3 and LNCaP cells. Therefore, DU-145 cells earlier reached confluence than the other cell lines.

Fig. 2 shows the visual appearance of the three different cell lines after 7 days of single-agent treatment with 5-AZAC or valproate, and co-treated cells. In concordance with the observations on cell multiplication, as described above, all cultures of DU-145 either untreated or treated with 5-AZAC or valproate, or with both drugs nearly reached confluence (Fig. 2A), but valproate seemed to reduce cell proliferation more than 5-AZAC or co-treated cells.

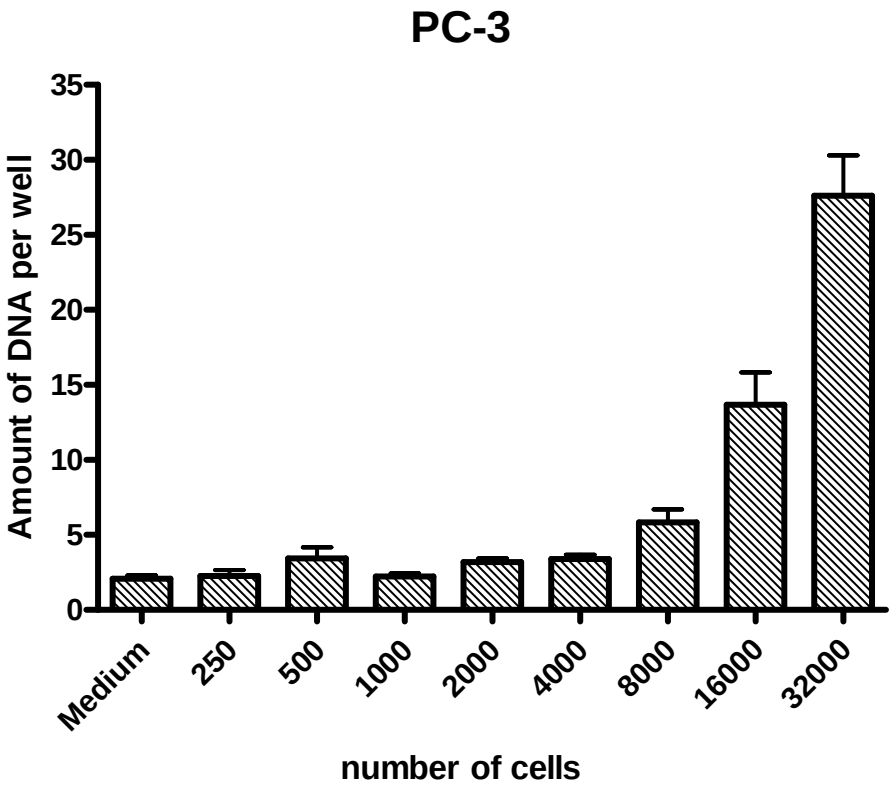
Untreated PC-3 cells (Fig.2B) were confluent and even cells treated with 5-AZAC or valproate alone nearly reached confluence. In this cell line 5-AZAC seemed to be more potent in reducing cell multiplication and co-treatment resulted in a further reduction (Fig. 2B).

LNCaP cells (Fig. 2C) were not confluent but formed a loose network of cells with contacts (untreated cells) or appeared in small groups (co-treated cells). Following 5-AZAC treatment cell proliferation was reduced and similar appearance was seen following valproate treatment. Combined treatment resulted in a further reduction in cell proliferation.

A


DU-145
Amount of DNA per well
0
10
20
30
40
Medium
250
500
1000
2000
4000
8000
16000
32000
number of cells

B


PC-3
Amount of DNA per well
0
5
10
15
20
25
30
35
Medium
250
500
1000
2000
4000
8000
16000
32000
number of cells

C

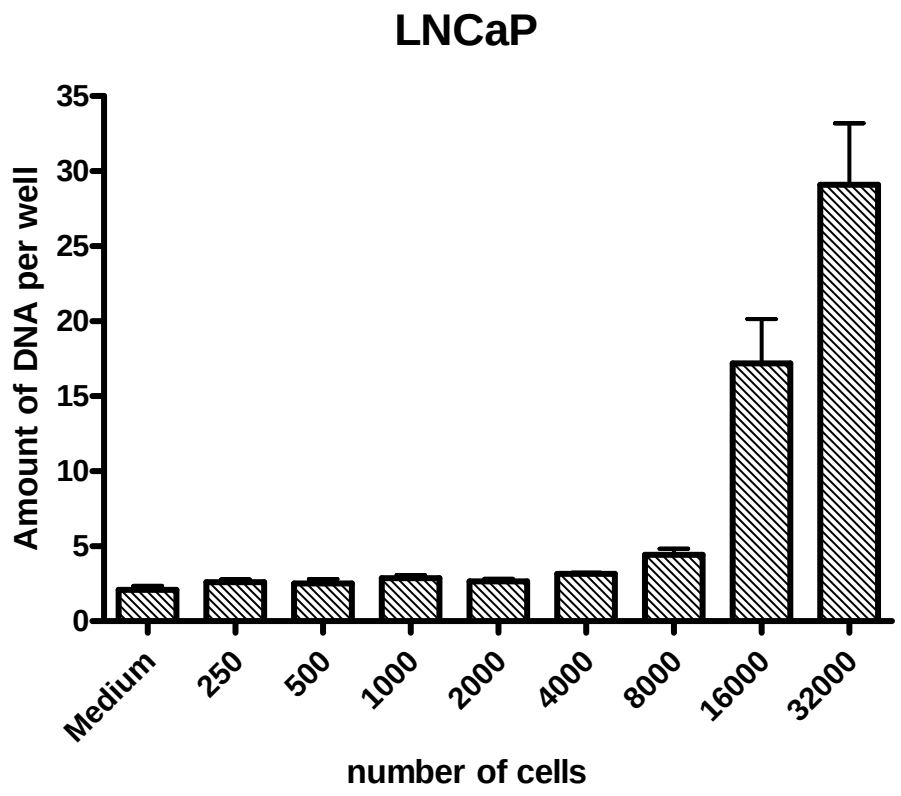
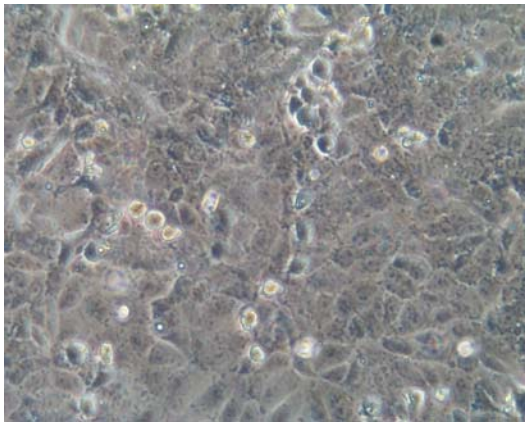
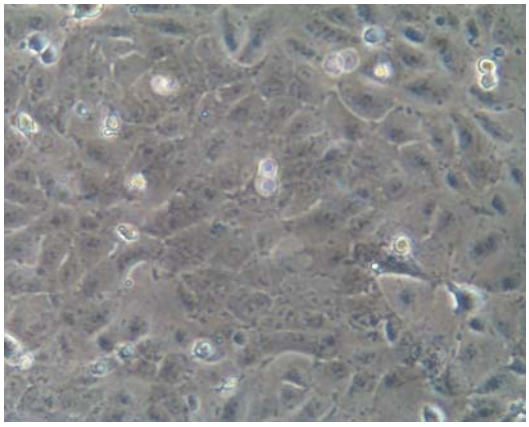


Fig.1: Effects of seeding concentration on DNA amount of DU-145 (A), PC-3 (B) and LNCaP (C) cells after 5 days of culture. Hoechst dye was added (10 µg/ml) and the fluorescence of the incorporated dye was measured at 360/460 nm.

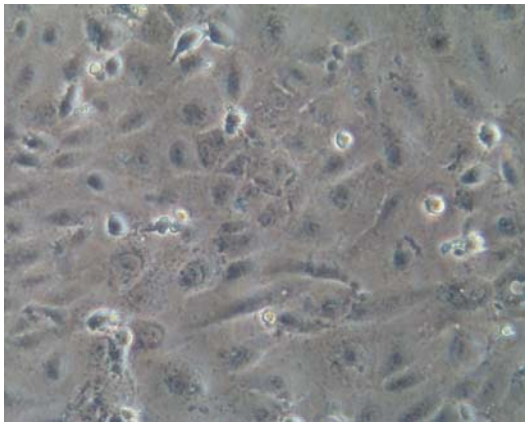
A



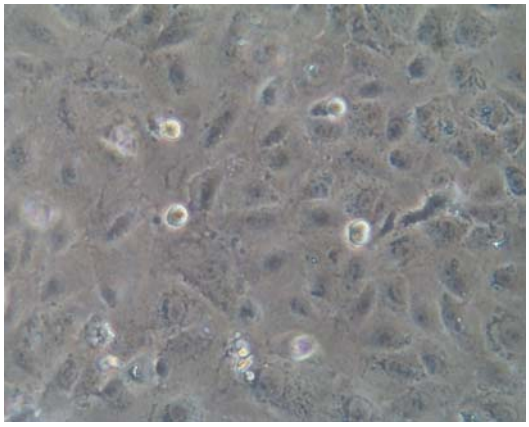
untreated cells



treatment with 5-AZAC



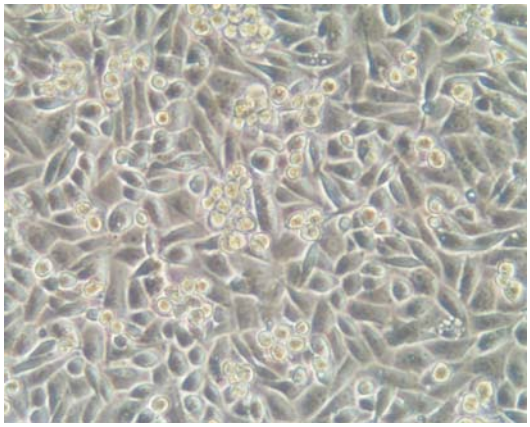
treatment with valproate



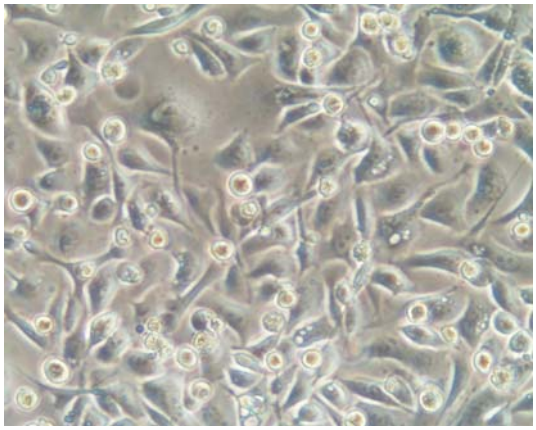
co-treated cells

Fig. 2A:
Visual appearance of DU-145 cells after 7 days of single-agent treatment with 5-AZAC and valproate, or with both drugs. Untreated, single-treated and even co-treated DU-145 cells nearly reached confluence, but valproate seemed to reduce cell proliferation more than 5-AZAC.

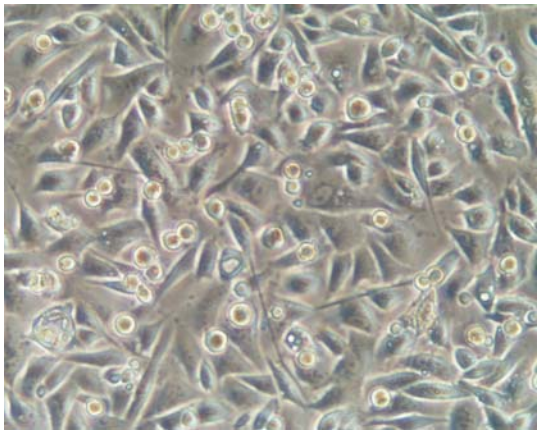
B



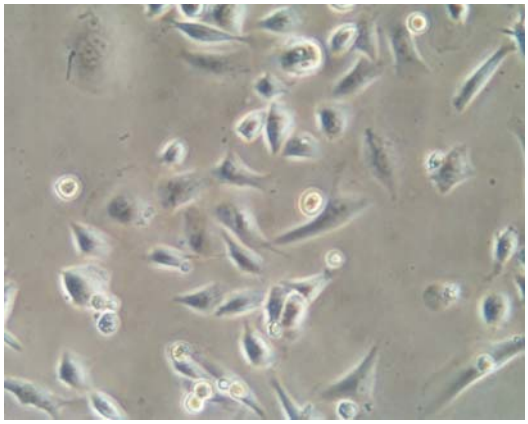
untreated cells



treatment with 5-AZAC



treatment with valproate

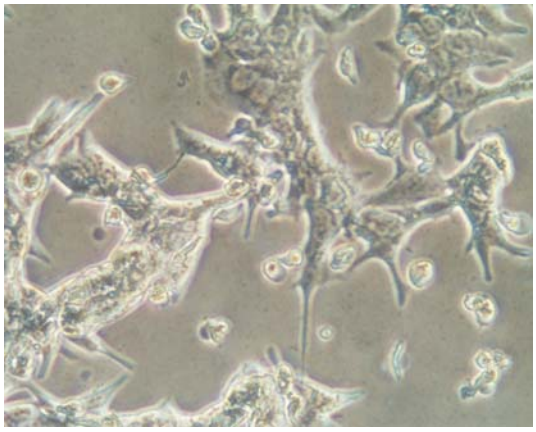


co-treated cells

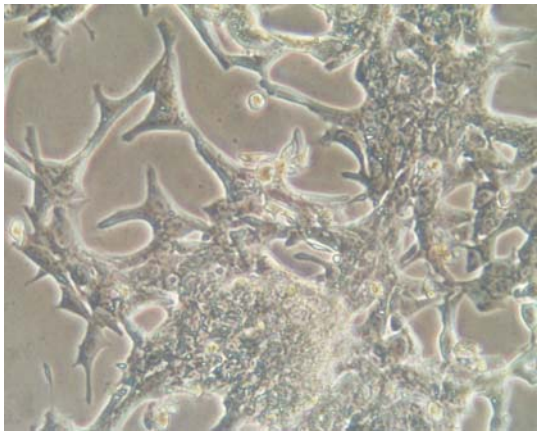
Fig. 2B:

Visual appearance of PC-3 cells cells after 7 days of single-agent treatment with 5-AZAC and valproate, or with both drugs. Untreated PC-3 cells were confluent and even cells treated with 5-AZAC or valproate alone nearly reached confluence. In this cell line 5-AZAC seemed to be more potent in reducing cell proliferation and co-treatment resulted in a further reduction.

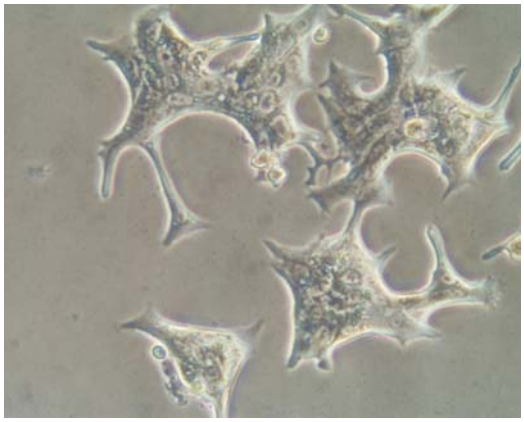
C



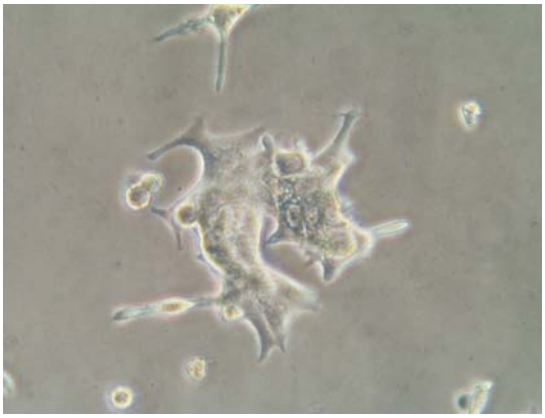
untreated cells



treatment with 5-AZAC



treatment with valproate



co-treated cells

Fig. 2C:
Visual appearance of LnCaP cells after 7 days of single-agent treatment with 5-AZAC and valproate, or with both drugs. Cells were not confluent but formed a loose network of cells with contacts (untreated cells) or appeared in small groups (co-treated cells). Following 5-AZAC treatment cell proliferation was reduced with a higher proportion of dead cells. Similar appearance was seen following valproate treatment and combined treatment resulted in a further reduction in cell proliferation.

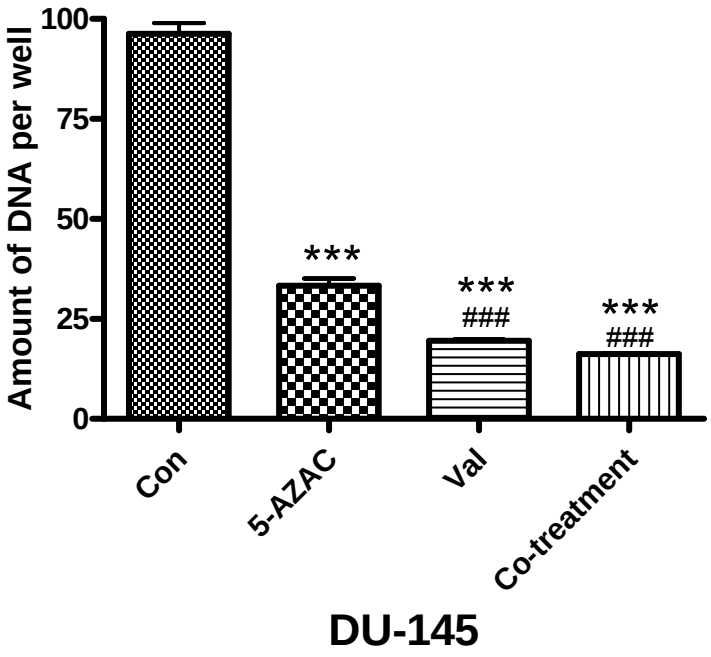
Although, in our studies, all treatments significantly reduced cell multiplication in DU-145 cells, the absolute DNA content was higher than in both other cell lines (Fig. 3A, control). Moreover, the DNA contents of the treated cultures were still conspicuously different to the other cell lines, explaining the obvious difference in the confluence (compare Fig. 3A, 3B, 3C). In the LNCaP cell lines valproate was more effective in the regulation of cell multiplication and treatment with both drugs further decreased cell number of 7 day old cultures.

There have been many approaches to understand the mechanism by which DNMT and HDAC inhibitors could cause these reductions in cell multiplication. HDAC inhibitors, like valproate, induce cell cycle arrest, differentiation and/or apoptosis of tumour cell lines in vitro (Schroeder TM, 2005) and it has been reported that the HDAC inhibitor valproate alone reduces cell proliferation in human PC-3 and LNCaP prostate cancer cells (Iacopino F, 2008). One approach is the re-activation of tumour suppressor genes by DNMT inhibitors and HDAC inhibitors. P16 ^{INK4a} is a major tumour suppressor implicated in a wide variety of human cancers (Ruas M, 1998; Sherr CK, 2000; Park YB, 2002)**.** Inactivation of p16^{INK4a} by promoter hypermethylation is common in a variety of human cancers, including osteosarcoma (Herman JG, 1995; Park YB, 2002). Another mechanism is DNA-methylation dependent downregulation of genes like P14^{ARF} that associates and stabilizes tumour suppressor genes like p53 (Liggett WH, 1996; Esteller M., 2000; Park YB, 2002**).** It was shown that the p14^{ARF} promoter was methylated in the osteosarcoma cell line U2OS and thus, osteosarcoma can be one of the tumor types showing methylation-dependent inactivation of the p14^{ARF} promoter (Park YB, 2002). So, DNMT inhibitors like 5-AZAC and HDAC inhibitors like valproate could re-express the tumour suppressor genes p16 ^{INK4a} and p14^{ARF} in human prostate cancer cell lines, which leads to reduced cell multiplication in these cells.

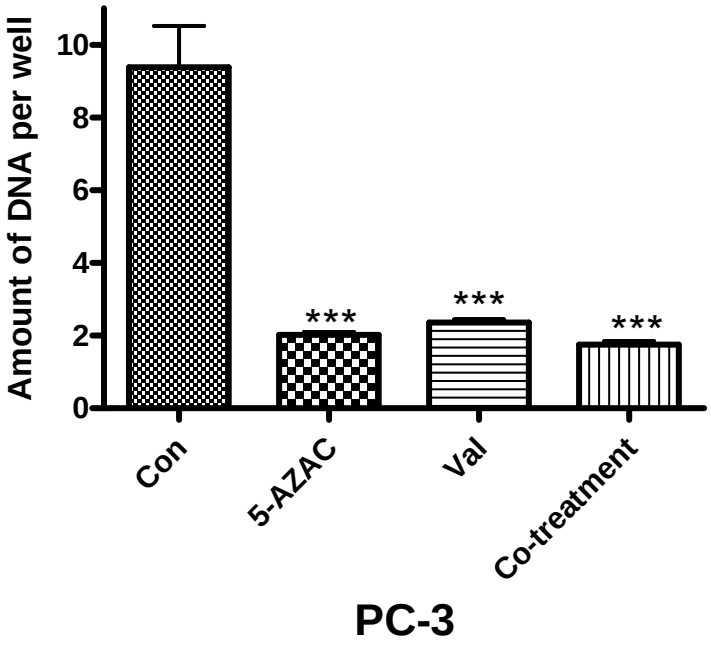
Another mechanism was demonstrated in HUVEC´s, where homocysteine, a possible inhibitor of DNMT`s, inhibits promoter-specific methylation. As a consequence, it was demonstrated that a repressor binds instead of methyl-binding-proteins downregulating transcription of cyclin A and proliferation (Jamaluddin MD, 2007).

Recently, it was shown that in renal cell carcinoma HDAC inhibitors strongly influence gene expression of cell-cycle regulating proteins, in particular, CDK2, cyclin B, cyclin D3, Rb and p21 (Jones J, 2008). The latter was shown to regulate proliferation of colon cancer cells. Downregulation of HDAC by siRNA resulted in downregulation of p21 and proliferation (Wilson AJ, 2008). This supports our findings that inhibition of HDAC by valproate downregulated cell multiplication.

A


Amount of DNA per well
100
75
50
25
0
***
***
###
***
###
Con
5-AZAC
Val
Co-treatment
DU-145

B


Amount of DNA per well
10
8
6
4
2
0
***
***
***
Con
5-AZAC
Val
Co-treatment
PC-3

C

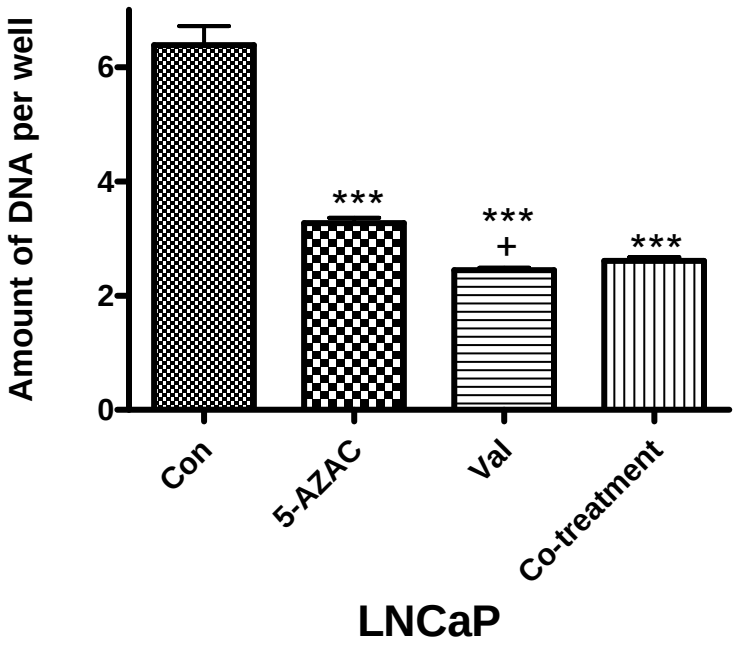


Fig. 3: Effects of 5-AZAC and valproate on DNA amount of human DU-145 (A), PC-3 (B) and LNCaP (C) cells after 7 days of treatment either with 5 uM 5-AZAC, 1 mM valproate or with both drugs. Hoechst dye was added (10 µg/ml) and the fluorescence of the incorporated dye was measured at 360/460nm.Bars represent means ± SD.
*** P≤0.001 (5-AZAC, valproate and co-treatment vs control); ### P≤0.001 (valproate, co-treatment vs 5-AZAC); + P≤0.05 (valproate vs 5-AZAC)

LOX mRNA expression

Diminished levels of LOX can be found in cancer cell lines as well as in transformed cell lines (Kuivaniemi H, 1986; Contente S, 1990; Ren C, 1998; Palamakumbura AH, 2003). Recently it has been shown that in human osteosarcoma cell lines LOX is increased by suramin and this drug also reduced cell multiplication in these cells (Buchinger B, 2008). In human primary and metastatic prostate cancer LOX expression is markedly reduced compared with benign glandular epithelium of the prostate and loss of LOX expression is associated with prostate cancer progression (Ren C, 1998)**.** Therefore we were interested in the effects of 5-AZAC and valproate on LOX expression in the three human prostate cancer cell lines and if the gene is re-expressed by this drugs.

In LNCaP cells no LOX mRNA expression could be determined (data not shown), going confirm with the report that in lymph node metastases reduced LOX mRNA can be found compared to primary prostate cancer cells (Ren C, 1998).

Human GAPDH-normalized gene expression for LOX is shown in Fig. 4 and Fig. 5.

Co-treatment increased LOX expression already on the first day of treatment in DU-145 cells (Fig. 4A). Both, 5-AZAC and valproate showed a trend towards upregulation but the difference was not statistically significant. Surprisingly, after 7 days of culture, the LOX expression in the controls increased to the level as found after 1 day of co-treatment that did not change during further culture time. The same expression was found with 5-AZAC and valproate after 7 days of treatment (Fig. 4B). At that time point all cultures reached confluence.

In the cell line of prostate metastasis from bone (PC-3) valproate significantly increased LOX expression after 1 day of treatment compared to controls (Fig. 5A). 5-AZAC did not increase LOX expression on the first day of treatment in these cell lines, but, as in DU-145 cells, a trend could be observed. Combined treatment resulted in a strong increase of LOX expression after this culture time. After 7 days of culture, as found in DU-145 cells controls strongly expressed LOX (Fig. 5B). This expression was about twice as high as all treatments and strongly significant. Both, 5-AZAC and valproate increased LOX expression compared to that found on day 1 and similarly expression of the valproate treated cells was higher than that of 5-AZAC. Compared to 1 day expression, 7 days co-treatment did not further increase LOX expression.

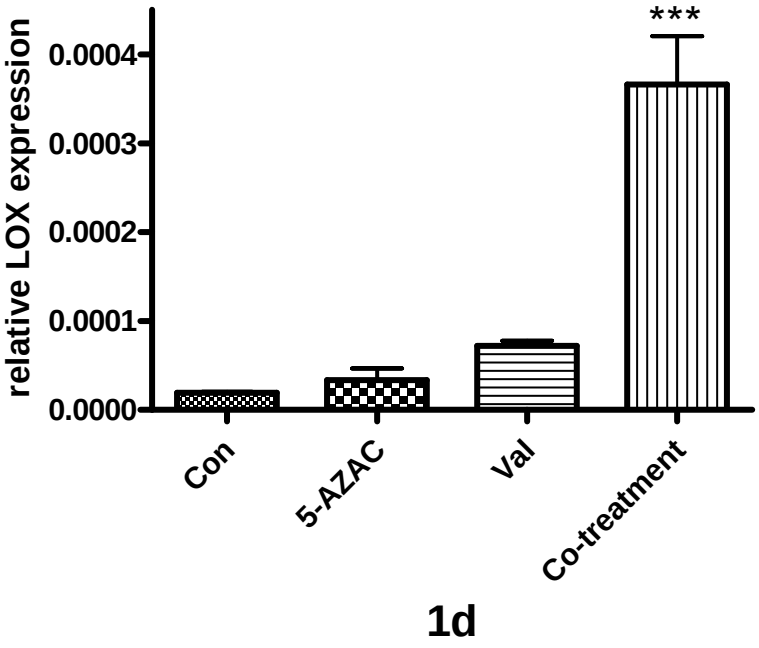
Fig. 2 suggests that for basal as well as for single-agent treatment confluence plays an important role in LOX expression. In co-treated PC-3 cells that did not reach confluence after 7 days of culture expression of LOX seems to be regulated by the drugs.

Taking these findings together valproate stimulated the expression of LOX in the two prostate cancer cell lines DU 145 and PC-3. 5-AZAC also showed some increase in LOX expression, but data were not significant. Combined treatment had an additional effect on LOX expression, already after 1 day of culture.

Analyses of the methylation status of the LOX promoter demonstrated that PC-3 cells show decrease of methylation during the culture period. This seemingly correlates with LOX expression. Treatment with 5-AZAC did not influence methylation status of the promoter. Both, valproate and combined treatment prevented methylation of CpG´s in the LOX promoter (Thaler R, unpublished results). This suggests, that valproate itself can upregulate LOX expression possibly by increase of histone acetylation.

Controversially 5-AZAC increased demethylation of promoter CpG´s of LOX in DU-145 cells already after 1 day treatment which did not change during further culture time. Similarly to PC-3 cells after 7 days of culture demethylation status reached a maximum with all treatments (Thaler R, unpublished results). Again, this suggests that methylation status depends on cell density or culture time and correlates with LOX expression. Further experiments are in progress to clarify this topic.

A



B

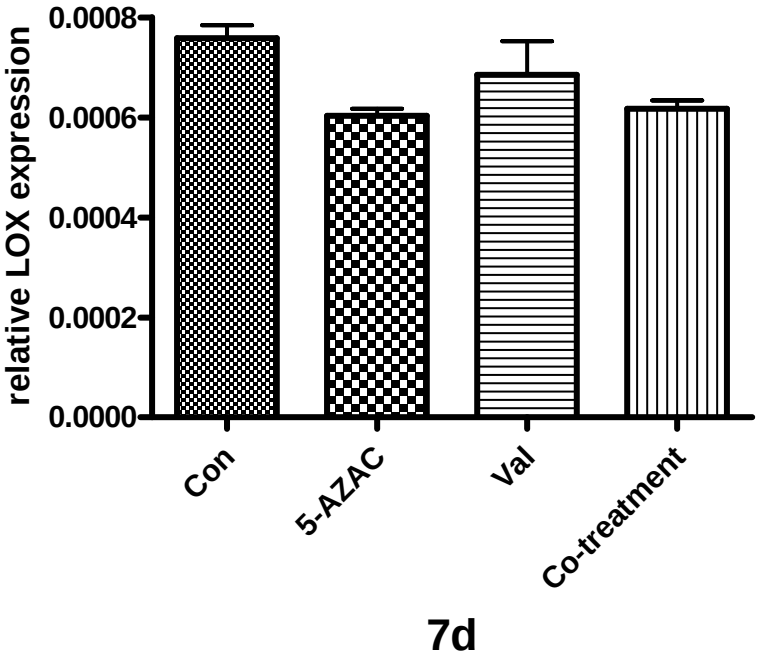
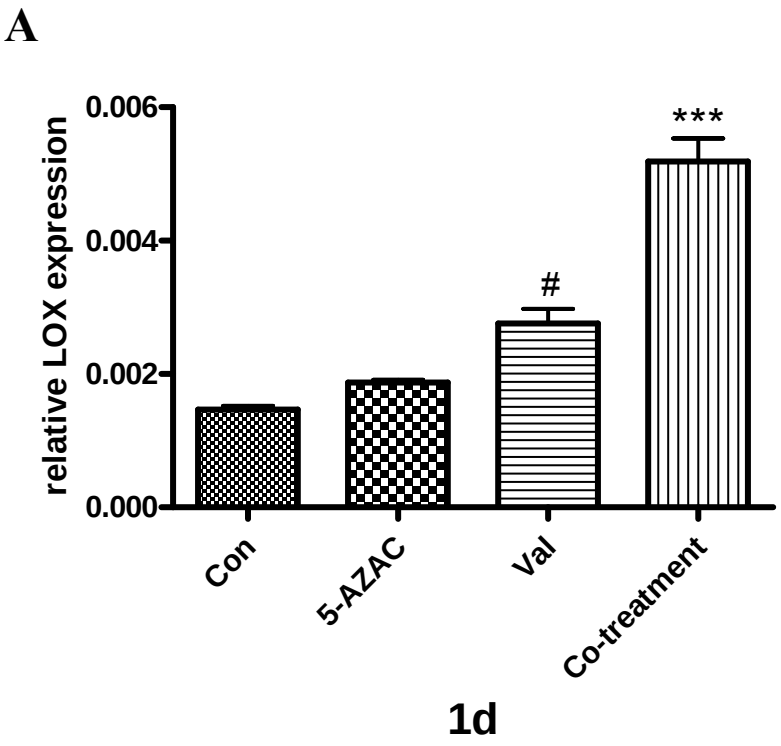


Fig. 4: Relative LOX-expression in DU-145 cells after 1 day (A) and 7 days (B) of treatment. The total RNA amount of cells treated either with 5 uM 5-AZAC, 1 mM valproate or with both drugs and the control group was isolated using TRIzol reagent. Analysis of the specific cDNA was performed with a real time PCR.
Bars represent means ± SD. *** $P \leq 0.001$ (control, 5-AZAC, valproate vs co-treatment)



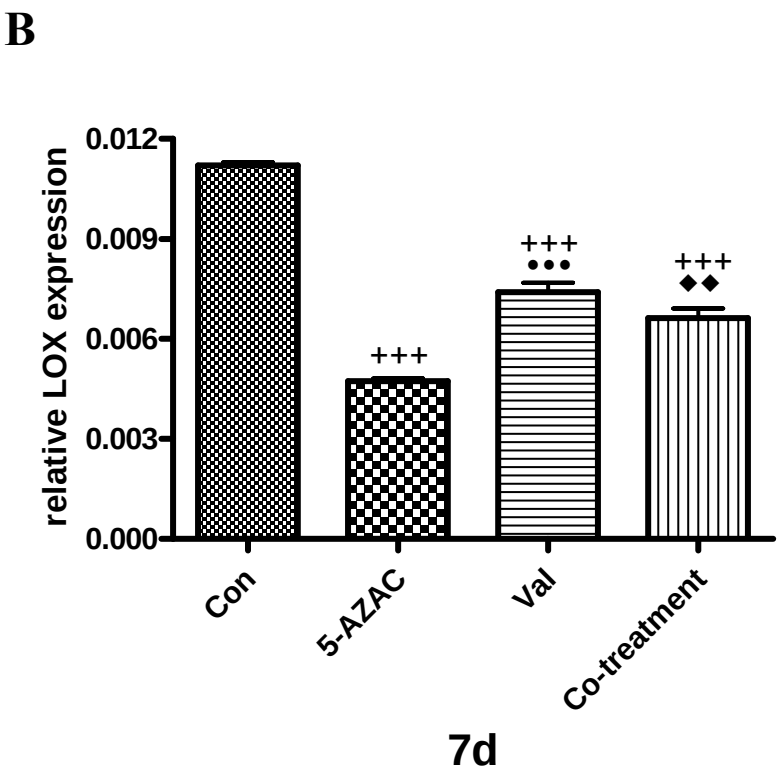


Fig. 5: Relative LOX-expression in PC-3 cells after 1 day (A) and 7 days (B) of treatment. The total RNA amount of cells treated either with 5 uM 5-AZAC, 1 mM valproate or with both drugs and the control group was isolated using TRIzol reagent. Analysis of the specific cDNA was performed with a real time PCR
Bars represent means ± SD. *** $P \leq 0.001$ (control, 5-AZAC, valproate vs co-treatment)
$^{\#}P \leq 0.05$ (valproate vs control); $^{+++}P \leq 0.001$ (5-AZAC, valproate, co-treatment vs control)
$^{\bullet\bullet\bullet}P \leq 0.001$ (valproate vs 5-AZAC); $^{\blacklozenge\blacklozenge}P \leq 0.01$ (co-treatment vs 5-AZAC)

Runx2 mRNA expression

Runx2 belongs to the runt domain transcription factor family (Ogawa E 1993; Yeung F, 2002). and plays a major role in osteoblast differentiation, maturation and bone formation (Komori T, 1997; Yeung F, 2002). This skeletal transcription factor is aberrantly expressed at high levels in breast and prostate cancers and cells that aggressively metastasize to the bone and was shown to regulate early metastatic events in these cancer types (Pratap J, 2006). Prostate cancer bone metastases express numerous proteins associated with bone cells. Specific transcription factors, including Runx2 regulate the expression of many bone-related factors in osteoblasts and the expression of Runx2 may be the molecular switch that is associated with expression of various bone-specific factors in prostate cancer (Brubaker KD, 2003)

Runx2 is expressed in the aggressive androgen-independent prostate cancer cell lines DU 145 and PC-3. In the latter cell line, Runx2 binds to the OSE2 site in the hOC promoter and regulates the transcription of OCN (Yeung F, 2002). Recent studies have identified several co-repressor proteins that bind to Runx2 to regulate gene expression. Binding sites for both histone acetylases and HDAC´s were identified on this protein (Westendorff JJ, 2006).

Our results show that valproate increased Runx2 expression in DU 145 already on the first day of treatment compared to controls (Fig.6 A). Combined treatment resulted in a further increase of Runx2 expression which was about three times as high as with valproate treatment and 6 times as high as 5-AZAC and control.

After 7 days of treatment, in confluent DU-145 cells, (Fig. 6B) all cultures reached the level of the co-treated cells found at day 1.

In 7 day old PC-3 cultures (Fig. 7B) Runx2 expression did not change in untreated cells compared to day one (Fig. 7A), while all other treatments increased mRNA expression of Runx2 significantly. Valproate increased mRNA expression about 7 times, while both 5-AZAC and co-treatment stimulated only 4 times. The difference between 5-AZAC and valproate was statistically significant.

LNCaP cells are OCN negative and do not express Runx2 (Yeung F, 2002). In concordance with this, in our studies no Runx2 expression could be detected (data not shown).

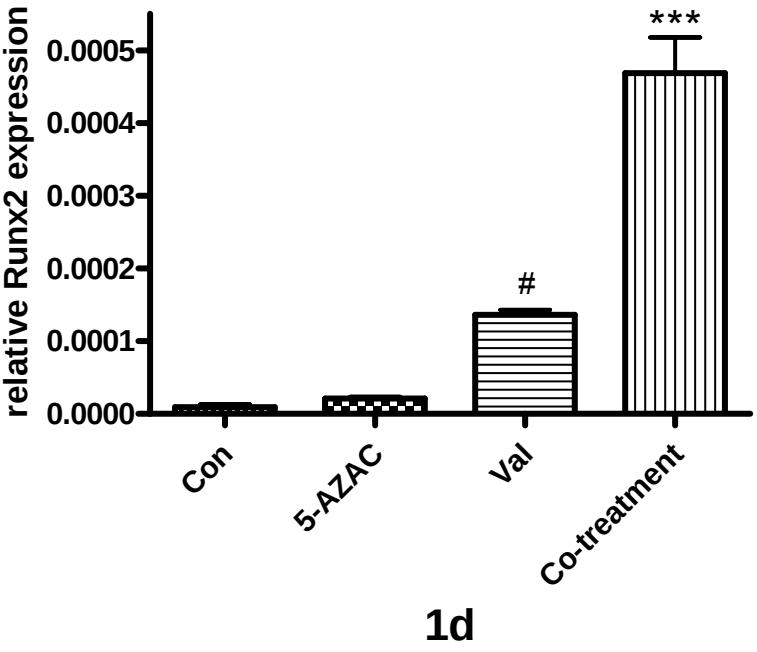
Taken together our data suggest that the HDAC inhibitor valproate has an effect on Runx2 expression in DU-145 and PC-3 cells. Recently it was described that trichostatin, an HDAC inhibitor like valproate increased, osteogenic factors like ALP, Runx2 and osteopontin. It was suggested that Twist-1, a key regulator of mesenchymal differentiation, inhibits BMP-signalling by HDAC1 recruiting to SMAD, and prevents differentiation (Hayashi M. 2007).

This could be a mechanism how valproate increased Runx2 expression and osteoblastic differentiation.

The results show, that in both cell lines co-treatment increased Runx2 expression. One main difference between the cell lines was that in DU 145 cells confluence seemed to regulate Runx2 expression; this was not the case in PC-3 bone metastatic cells, where control did not change during the culture period. However, all treatments increased Runx2 expression after 7 days of treatment, indicating that they promote osteoblastic differentiation.

Surprisingly, in this study, no OCN expression, neither in DU 145, nor in PC-3 cells could be detected. OCN expression requires an intact extracellular bone-like matrix. Unfavorable culture conditions for proper matrix formation in the cultures could be a reasonable explanation (Fig. 2). As expected, in LNCaP neither OCN, nor Runx2 was properly expressed (Yeung F, 2002).

A



B

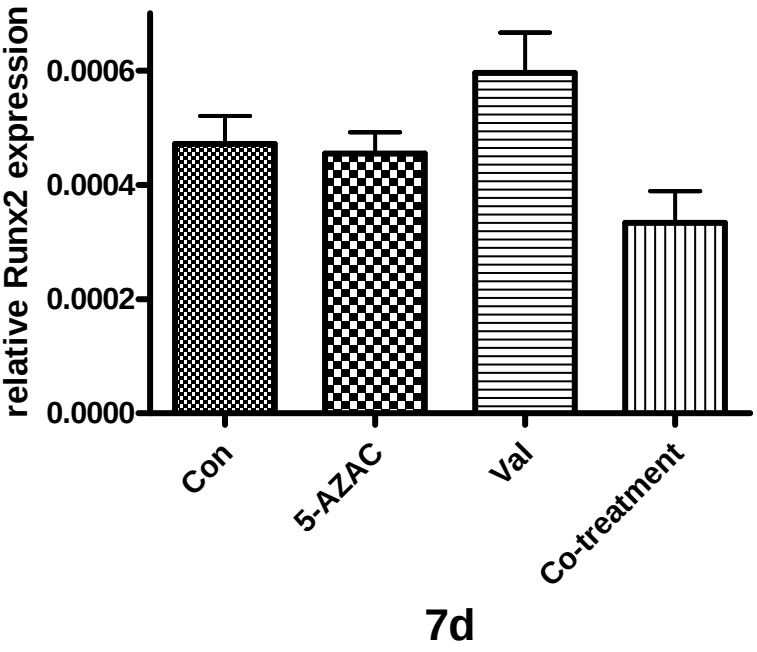
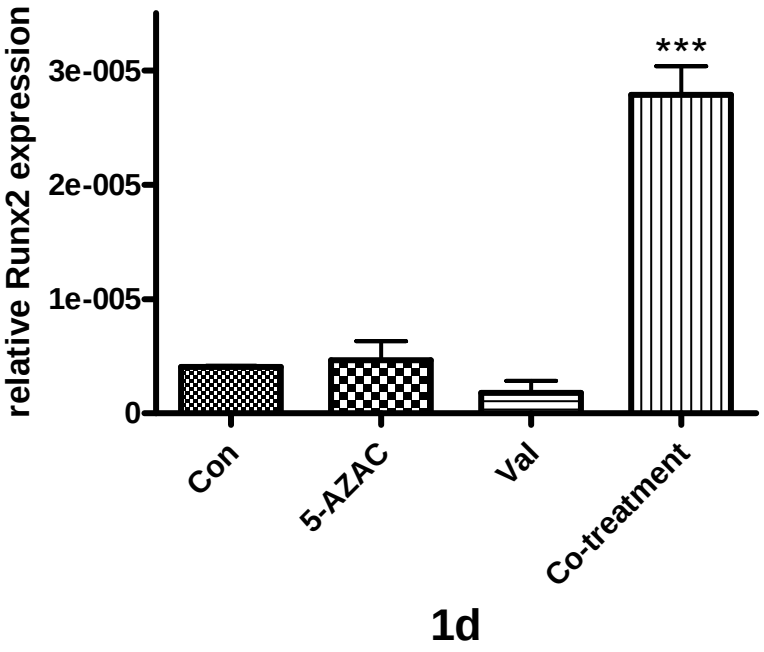


Fig. 6: Relative Runx2-expression in DU-145 cells after 1 day (A) and 7 days (B) of treatment. The total RNA amount of cells treated either with 5 uM 5-AZAC, 1 mM valproate or with both drugs and the control group was isolated using TRIzol reagent. Analysis of the specific cDNA was performed with a real time PCR.
Bars represent means ± SD.*** $P \leq 0.001$ (control, 5-AZAC, valproate vs co-treatment); $^{\#}P \leq 0.05$ (valproate vs control)

A



B

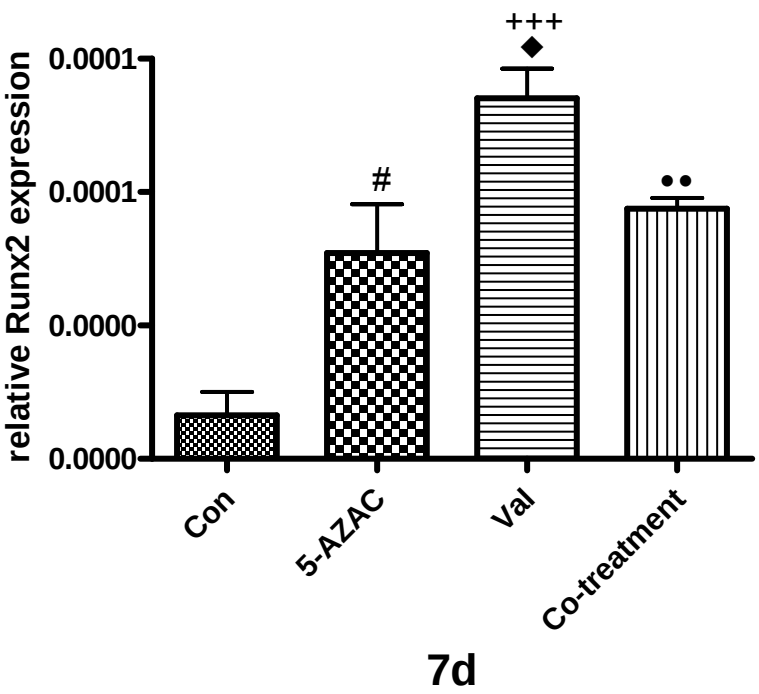


Fig. 7: Relative Runx2-expression in PC-3 cells after 1 day (A) and 7 days (B) of treatment. The total RNA amount of cells treated either with 5 uM 5-AZAC, 1 mM valproate or with both drugs and the control group was isolated using TRIzol reagent. Analysis of the specific cDNA was performed with a real time PCR .Bars represent means ± SD. *** $P \leq 0.001$ (control, 5-AZAC, valproate vs co-treatment) $^{\#}P \leq 0.05$ (5-AZAC vs control); $^{+++}P \leq 0.001$ (valproate vs control); $^{\bullet\bullet}P \leq 0.01$ (co-treatment vs control); $^{\blacklozenge}P \leq 0.05$ (valproate vs 5-AZAC)

Bone metastases of prostate cancer can result in either lytic, that means bone resorbing, or in osteoblastic, that means bone forming metastases (Peyruchaud O, 2007). Under physiological conditions the ratio between RANKL and OPG determines whether bone is formed or resorbed (Hamdy A, 2008). Therefore there is great interest whether in bone metastase the ratio of both genes directs bone formation or resorption. As the PC-3 cell line is a human prostate cancer cell metastases from the bone we were interested in the RANKL/OPG ratio to determine how the cells react under the treatment of 5-AZAC and DNMT inhibitors.
Results show that in confluent PC-3 cells 5-AZAC and valproate increased OPG expression significantly compaired to controls (Fig. 8). Surprisingly, no RANKL expression could be found. These observations, however, let us suggest that the cell line was established from a metastases with bone-forming properties.

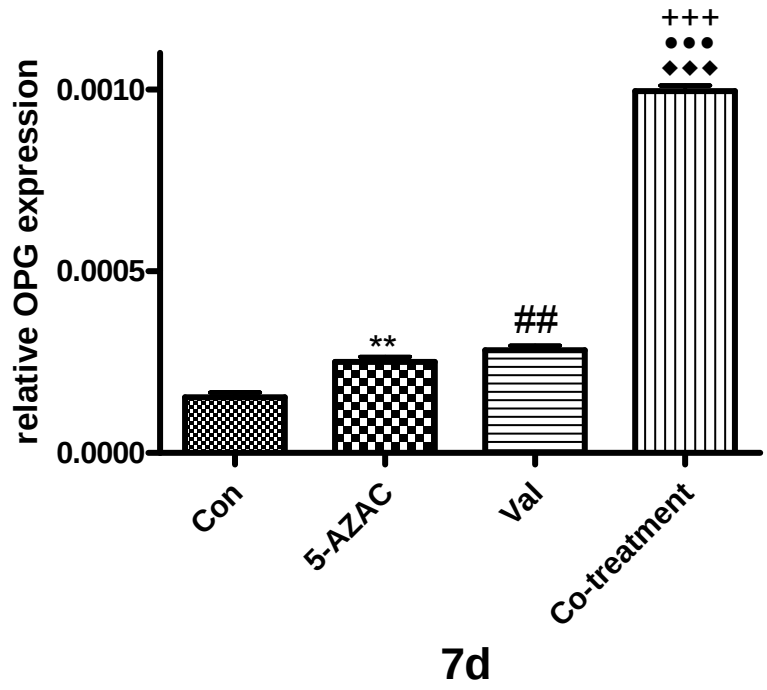


Fig.8: Relative OPG-expression in PC-3 cells after 7 days of treatment. The total RNA amount of cells treated either with 5 uM 5-AZAC, 1 mM valproate or with both drugs and the control group was isolated using TRIzol reagent. Analysis of the specific cDNA was performed with a real time PCR .Bars represent means ± SD. ** P≤0.01 (5-AZAC vs control) $^{\#\#}$P≤0.01 (valproate vs control);$^{+++}$P≤0.001 (co-treatment t vs control); $^{\bullet\bullet\bullet}$P≤0.001 (5-AZAC vs co-treatment); $^{\blacklozenge\blacklozenge\blacklozenge}$ P≤0.001 (valproate vs co-treatment)

3. Epilogue

Osteosarcoma and prostate cancer are two types of cancer that both affect bone because osteosarcoma itself develops in bone and prostate cancer cells metastasize to bone, lymph nodes and other organs and cause significant mortality and morbidity in men with advanced disease (Yeung F, 2002). Autopsy data suggest that greater than 80% of men who die of prostate cancer have skeletal metastasis, specifically in the marrow containing trabecular bone of the pelvis, femur, or vertebral bodies (Bubendorf L, 2000, Hall CL, 2006).

Tumour cell lines afford the opportunity to investigate effects of drugs on metabolic processes. Three drugs, all in use for tumour treatment, were studied. These drugs act on different levels of gene transcription: Suramin is known to affect biochemical signal-transduction, valproate, an inhibitor of histone-deacetylases and 5-AZAC, an inhibitor of DNMT`s, affect chromatin, one on the level of histone modification, the other directly on the DNA methylation. Their influence on cell multiplication and differentiation as indicators of normalization of tumour cells were investigated.

Suramin is known to interrupt the autocrine loop of signal transduction between expressed growth factors and their receptors (Hosang M, 1979). This could explain our finding of downregulation of cell multiplication, confirming previous data (Trieb K, 2003).

In addition, suramin increased ALP activity, OCN, COL1A1 and OPN expression in these cell lines. These results clearly demonstrate that suramin increased the differentiation status of the tumour cells.

A central point of our investigation was the effects of these drugs on LOX expression, as the N-terminal part of the protein is known to posses tumour suppressor (anti-cancer) activity (Kagan HM, 2003; Palamakumbura AH, 2004). This interesting protein, secreted as a precursor, is cleaved by procollagen C-proteinase (BMP-1), yielding a tumour suppressor part and an active enzyme that is responsible for collagen cross-linking (Kessler E, 1996). As a matter of fact suramin significantly and dose-dependently increased LOX mRNA expression and lead us to the speculation that this gene could be responsible for the found effects on proliferation and differentiation in OS cell lines.

Next we studied whether the “epigenetic drugs”, (HDAC and DNMT inhibitors), also influence LOX expression in tumour cell lines. For this purpose this drugs were used alone or in combination to treat three prostate cancer cell lines that were isolated from bone (PC-3), lymph-node (LNCaP) and brain (DU-145) metastases. While in LNCaP cells no LOX expression could be detected in the other two cell lines combined treatment with HDAC and

DNMT inhibitors resulted in a strong upregulation of LOX mRNA expression. Surprisingly and different to the results found with suramin in OS cells, after 7 days of culture the LOX expression increased strong and significantly, indicating that culture age or confluence effect LOX expression in the same way.

The fact that the expression of the osteoblast-specific transcription factor Runx2 goes in line with LOX expression in PC-3 and DU-145 cells suggests that LOX could also influence the differentiation to an osteoblastic phenotype like suramin.

These data let us hypothesize that these drugs could effect tumour development in a positive way. One could further speculate that a formally dedifferentiated phenotype found in bone metastases tends to ameliorate.

The findings that LOX expression parallels the improvement of cancer are supported by many in vitro and in vivo studies (Palamakumbura AH , 2004; Wu M, 2007; Min C, 2007).

These results clearly demonstrate that reversion the differentiation of cancer cells could be influenced at the biochemical as well as at the epigenetic level and recommend to strive elucidating the molecular mechanisms of this processes.

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Curr Med Chem Anticancer Agents. **3**(3):187-99

5. Index of figures

6. Index of abbreviations

ALP	Alkaline Phosphatase
5-AZAC	5`aza 2`deoxycytidin
ßAPN	ß -amino-propio-nitril
BMP	Bone morphogenic protein
DNMT	DNA methyltransferase
ERβ	Estrogen receptor β
FGF-2	Fibroblast growth factor-2
HDAC	histone deacetylase
IGF	Insulin-like growth factor
LOX	Lysyl Oxidase
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
OS	Osteosarcoma
PTH	Parathyroid hormone
RANK	Receptor activator of NFkB
RANKL	Receptor activator of NFkB ligand
TGF–β	Tumour growth factor β
T3	triiodthyroxin

7. Curriculum vitae

Curriculum vitae

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- Bone biology course at the Medical University of Vienna,
- Applied osteology research course, Barmherzige Schwestern Hospital, Vienna,

4. Scientific Meetings:

- Management of Osteoporosis, Oktober 2005, Vienna, Austria
- Autumn Meeting of the Austrian Society of Bone and Mineral Research, November 2005, Vienna, Austria
- Sixth European Congress on Clinical and Economic Aspects of Osteoporosis and Osteoarthritis March 2006, Vienna, Austria
- Management of Osteoporosis, Oktober 2006, Vienna, Austria
- International Conference on Progress in Bone and Mineral Research, November 2006, Hofburg, Vienna
- 34th European Symposium on Calcified Tissues, May 2007, Copenhagen, Denmark (poster presentation)
- 1st ECTS PhD Training Course, September 2007, Oxford, England

5. Talks:

- "Suramin upregulates lysyl oxidase (LOX) in osteosarcoma cells" at the International Conference on Progress in Bone and Mineral Research, November 2006, Hofburg, Vienna (selected for oral presentation)

6. Publications:

- B. Buchinger, S. Spitzer, H. Karlic, K. Klaushofer, F. Varga „Lysyl oxidase (LOX) mRNA expression and genes of the differentiated osteoblastic phenotype are upregulated in human osteosarcoma cells by suramin"
  Cancer Letters 2008; 265:45-54

- C. Turecek, N. Fratzl-Zelmann, M. Rumpler, B. Buchinger, S. Spitzer, R. Zöhrer, E. Durchschlag, K. Klaushofer, E.P.Paschalis, F. Varga „Collagen Cross-Linking Influences Osteoblastic Differentiation"
  Calcif. Tissue Int. 2008; 82:392-400

7. Memberships:

- European Calcified Tissue Society (ECTS)
- Austrian Society for Bone and Mineral Research