

# The Polyamine Analogues PG11144 and PG11047 Inhibit Cell Proliferation and Decrease the Malignancy in LNCap-FGC Prostate Cancer Cells in Normoxia and Hypoxia

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# The Polyamine Analogues PG11144 and PG11047 Inhibit Cell Proliferation and Decrease the Malignancy in LNCap-FGC Prostate Cancer Cells in Normoxia and Hypoxia

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## Abstract

One of many new targets when looking for anti-cancer treatment is the polyamine pathway, which is essential in cell proliferation, gene regulation and cell death. A way to intervene with this pathway is to use compounds called polyamine analogues. Depletion in the intracellular polyamine pools results in decrease in cell proliferation, something that is desirable in cancer treatment. In this study the effect of two different polyamine analogues, PG11144 and PG11047 (10  $\mu$ M concentration), on the prostate cancer cell line LNCap-FGC was investigated in both normoxia (21% O<sub>2</sub>) and hypoxia (1% O<sub>2</sub>) to better mimic the actual conditions in the tumor. Tumors often contain oxygen-deprived areas, which have been reported to be involved in the development of malignancy. When the cells were cultured in hypoxia, the colony forming efficiency in soft agar increased 100-fold compared to culturing in normoxia, indicating an increase in malignancy. PG11144 or PG11047 treatment decreased the colony formation efficiency of cells grown in hypoxia. The expression of  $\beta$ -catenin, a marker of malignancy, decreased in PG11144- or PG11047-treated cells grown in normoxia and hypoxia, with PG11144 being the most effective. Treatment with either compound reduced cell proliferation, especially in normoxia. Altogether, the data show promising anticancer activities of both compounds in LNCap-FGC cells grown in normoxia and hypoxia.

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## Introduction

Prostate cancer is the most common form of cancer in Swedish men, representing 32.2% of all reported male cancer cases in 2011 (1). It is normally treated with surgery, radiation therapy or androgen ablation, and the outcome is generally good in localized tumors (2). Prostate cancer cells are initially dependent on androgens for growth and progression, but over time, the cells can develop androgen independency resulting in a more aggressive form of cancer. Among the patients with castration-resistant prostate cancer, 50-70% will develop bone metastasis, for which no effective treatment exists (3). For metastasis to occur, the epithelial cells need to change into mesenchymal cells in a process called epithelial-to-mesenchymal transition (EMT) (4). This is a well-known, essential process in normal embryonic development and in tissue repair, but it has also been shown to play an important role in cancer

progression. EMT can be induced by several extracellular factors, including growth factors such as WNT, TGF- $\beta$ , FGF, and EGF, and components of the extracellular matrix such as collagen and hyaluronic acid. Polyamines may also be involved in mediating EMT (5). The process involves down-regulation of the epithelial protein E-cadherin and up-regulation of the mesenchymal protein N-cadherin among others. It also involves up-regulation of vimentin and down-regulation of cytokeratin 18 (6-7). The mesenchymal phenotype is more invasive and is able to migrate and colonize new microenvironments (6). Once the cells reach the new site in the body they go through the reverse process of EMT, mesenchymal-to-epithelial transition (MET) and form the new tumor.

Studies have shown that EMT can be induced by a hypoxic environment (8, 9). In solid tumors the vascular system is often disorganized and variable (10). Insufficient blood supply leads to regions of hypoxia where the oxygen

consumption outweighs the oxygen supply. Low oxygen pressure decreases the proliferation of most cells, but it can also enable them to transform and become more aggressive and develop drug-resistance. Different transcription factors that help the cells to cope with the stressful environment are activated. Over-expression of the protein hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) has been shown to be involved in EMT since increasing levels of HIF-1 $\alpha$  are associated with up-regulation of vimentin expression and down-regulation of E-cadherin (9). Hypoxia also selects for apoptosis resistant cells, such as cells that lack the tumor suppressor p53 (11). As soon as the transformed cells get access to the blood stream and receive oxygen and nutrients, for example if parts of the tumor are killed by treatment, they are able to spread and colonize new areas of the body (10). Hypoxia also selects for cancer stem cells. Cancer stem cells are also able to migrate and form tumors at new sites of the body, and they can regenerate the tumor after treatment or surgery. They are similar to normal stem cells in the aspect that they have a long life span, are resistant to apoptosis, can induce angiogenesis, have ability for self-renewal and differentiation, etc. (12). It has been shown that the residues of normal stem cells, e.g. stem cell residues in the bone marrow and in the brain, are hypoxic, and as soon as the stem cells are exposed to oxygen they start to differentiate (13). The same seems to occur for cancer stem cells. Hypoxia maintains their undifferentiated state, and can also modify the gene expression, resulting in stem-ness. It is therefore of great interest to find a way to kill these cells.

One interesting approach for treatment of cancer is to target the polyamine homeostasis. Polyamines are organic polycations that exist in all, so far studied, prokaryotic and eukaryotic cells. There are three naturally occurring polyamines, the diamine putrescine, the triamine spermidine, and the tetra-amine spermine (14). The polyamines are essential for growth, differentiation and cell death (15). Their positive charge makes it possible for them to bind to negatively charged macromolecules such as

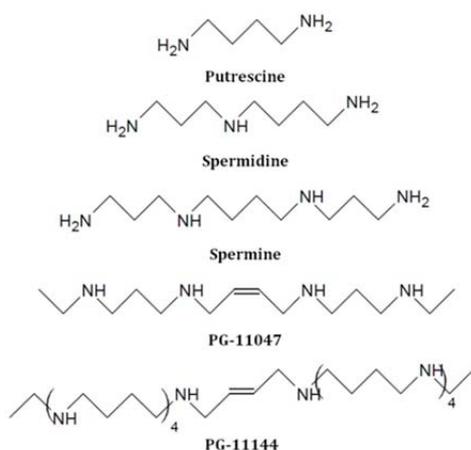
nucleic acids, phosphoproteins and phospholipids (16). They stabilize and stimulate the replication of DNA and are involved in the organization of chromatin. They regulate the activation of kinases in a number of growth-related pathways in the cell, including the Ras/MAPK pathway, one of the best-defined pathways in cell proliferation. Through these pathways polyamines regulate multiple functions, such as transcription, translation, nucleic acid synthesis, centrosome positioning in mitosis, cytokinesis, modulation of ion channels, and membrane stability (15, 17-19).

The intracellular levels of polyamines are regulated by biosynthesis, uptake/excretion, and catabolism (14, 20). Polyamines are synthesized by the enzyme ornithine decarboxylase (ODC), which converts ornithine to putrescine. This is the rate-limiting step of polyamine biosynthesis. ODC is an enzyme with a very short half-life which is e.g. synthesized upon hormone stimulation. An over-production of ODC has been observed in numerous cancers, and ODC has been defined as an oncogene (21). Putrescine can in turn be converted into spermidine and spermine by spermidine synthase and spermine synthase by addition of one or two aminopropyl groups. The aminopropyl group donor in the reaction, decarboxylated S-adenosylmethionine, is formed by decarboxylation of S-adenosylmethionine by the enzyme S-adenosylmethionine decarboxylase (22). The uptake of polyamines is mediated by active transport (14) and is dependent on the pH and the extra- and intracellular polyamine levels. Many kinds of food contain high amounts of polyamines and by changing the diet in combination with ODC inhibiting treatments, such as 2-difluoromethylornithine, it has been possible to limit the growth of prostate cancer (23).

There is a strong correlation between polyamine accumulation and cancer (24, 25). High levels of polyamines can be observed in all hyperproliferative cells, but the high levels are extra prominent in cancer cells. This is due to a frequent dysfunction of the polyamine metabolism in most cancers. In normal cells, the

metabolic pathways are tightly regulated and polyamine levels remain low in non-proliferating cells and is stimulated when normal cells have to grow e.g. in tissue repair processes. It has also been suggested that increased levels of polyamines in cancer cells can be involved in EMT and contribute to malignancy (26). Targeting the polyamine functions and metabolic pathways has therefore been of great interest when searching for new cancer treatments. One way is to use structurally similar compounds called polyamine analogues. The analogues are supposed to use the pathways and mechanisms originated for the natural polyamines without substituting their function (27). They also inhibit the biosynthesis and uptake of the naturally occurring polyamines and enhance the activity of the catabolic enzymes. Taken together this should deplete the polyamine pools in the cells resulting in growth-inhibition and cell death. It is also important that the analogues are more toxic for cancer cells than for normal cells.

In this study the effects of two different polyamine analogues, 3,8,13,18,23,28,33,43,48-deca-aza-(trans-25)-pentacontene (PG11144, previously known as SL11144 and CGC11144) and [<sup>1</sup>N,<sup>12</sup>N]bis(ethyl)-cis-6,7-dehydrospermine (PG11047, previously known as SL11047 and CGC11047) (Fig. 1) on the androgen-dependent prostate cancer cell line LNCap-FGC were investigated. PG11047 is a bisethyl spermine



**Figure 1.** The molecular structures of the naturally occurring polyamines putrescine, spermidine and spermine, and the two polyamine analogues PG11047 and PG11144. (Ref. John Stegmayr)

analogue that competes with the natural polyamines and inhibits their normal functions (28). It is taken up by the polyamine transporter system and accumulates in the cell where it depletes the intracellular levels of all polyamines by feedback inhibition of enzymes involved in the polyamine biosynthesis. It also activates the enzymes spermidine/spermine *N*<sup>1</sup>-acetyltransferase (SSAT) and spermine oxidase (SMO), which catalyzes the catabolism of polyamines. The depletion of the intracellular polyamine pools results in decreased proliferation. It has also been suggested that it can induce MET and reduce the number of cancer stem cells in human breast cancer cell lines (29, 30). The compound is in phase II clinical trials for prostate cancer. PG11144 is a *trans*-decamine which has been shown to have high anti-proliferative properties and to cause apoptosis in both prostate and breast cancer cells (31, 32). It reduces the levels of all the three polyamines in the cell both by inhibiting polyamine biosynthetic enzymes and by increasing the activity of SSAT. PG11144 has been shown to inhibit lysine-specific demethylase-1 (LSD-1), an enzyme that is often highly expressed in cancer (33, 34). LSD-1 catalyzes the demethylation of histone 3 at lysine 4, a process that leads to epigenetic silencing of tumor suppressor genes. The catalytic domain of LSD-1 is more than 60 % similar to the catalytic domain of SMO, and PG11144 is structurally similar to the lysine tail of the histone. This makes it possible for the analogue to bind to and inhibit the enzyme.

The aim of this study was to investigate how PG11144 and PG11047 effected cell proliferation of LNCap-FGC androgen dependent prostate cancer cells, to see if they caused cell death and to see if they had any impact on the malignancy of the cells by looking at the stem cell population, the colony forming efficiency in soft agar, and EMT versus MET markers. Of importance was also to investigate how the effects differed between hypoxia (1% O<sub>2</sub>) and normoxia (21% O<sub>2</sub>). LNCap-FGC prostate cancer cells were cultured in either normoxia or hypoxia in absence or presence of

either PG11144 or PG11047. The results show that treatment with PG11144 was more effective than treatment with PG11047 in both normoxia and hypoxia, but the effect was more prominent in normoxia. The colony forming efficiency of cells grown in normoxia was very low and colonies were only found in control. When cells were grown in hypoxia, the colony forming efficiency was greatly increased. Cultivation in hypoxia increased the expression of the EMT marker vimentin, but surprisingly it also increased the expression of the MET marker cytokeratin 18. However, both PG11144 and PG11047 treatment of cells in hypoxia resulted in a decreased colony formation. Treatment with both compounds reduced the expression  $\beta$ -catenin. Thus, both PG11144 and PG11047 proved to be inhibitory in both normoxia and hypoxia. This is the second report of an effect of the compounds on prostate cancer cells in hypoxia (35).

## Material and methods

### Materials

RPMI-1640 medium was purchased from Biochrome, Berlin, Germany. Fetal bovine serum (FBS), non-essential amino acids, penicillin/streptomycin, Nonidet NP-40, NuSieve® GTG® agarose and phosphate-buffered saline (PBS) tablets were purchased from VWR, Lund, Sweden. RPMI-1640 Medium Dutch Modification, Accutase® solution, propidium iodide (PI), TWEEN® 20, ribonuclease A, 2-mercaptoethanol, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), bovine serum albumin (BSA), agar, and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich, Stockholm, Sweden. Trypsin was purchased from OneMed Sverige AB, Gothenburg, Sweden. Tissue culture plastics were purchased from Nunc, Roskilde, Denmark. NuPAGE® 4-12% Bis-Tris 1.0 mm 10 well gels, NuPAGE® MOPS SDS Running Buffer (20X), NuPAGE® Antioxidant, MagicMark™ XP Western Standard, SeeBlue® Pre-Stained Standard (1X) and iBlot® Gel Transfer Stacks (Nitrocellulose, Regular), and

AlexaFluor® 594 were purchased from Invitrogen Corporation, Carlsbad, CA, USA. Enhanced Chemiluminescence (ECL) Advanced Western Blotting Detection kit was purchased from GE Healthcare, Buckinghamshire, UK. Polyclonal swine anti-rabbit and goat anti-mouse immunoglobulins/HRP for Western Blot were purchased from Dako, Glostrup, Denmark. MEBM Basal medium (CC-3151 and CC-4136 kit) were purchased from Lonza, Basel, Switzerland. B27 supplement was purchased from Life Technologies, Carlsbad, CA, USA. Basic fibroblast growth factor (bFGF) was purchased from R & D systems, Minneapolis, Minnesota, USA. Dimethyl sulfoxide (DMSO) was purchased from Merck KGaA, Darmstadt, Germany. Primary antibodies used for Western Blot against human H3K4me2, LSD-1, and active  $\beta$ -catenin were purchased from Millipore, Bedford, MA, USA. Primary antibodies against human  $\beta$ -actin, GAPDH, poly (ADP) polymerase, caspase 3, vimentin, E-cadherin, and cytokeratin 18 were purchased from Abcam, Cambridge, UK. Primary antibodies against Bcl-2 and p53 used for Western Blot, and antibodies against human CD24 and CD44, and Isotype used for stem cell flow cytometry were purchased from BD Biosciences, Stockholm, Sweden. Anti- $\gamma$ -H2AX antibody was purchased from Cell Signaling Technology, Inc, Boston, MA, USA. Antibody against human HIF-1 $\alpha$  was purchased from Novus Biologicals, Littleton, CO, USA. Antibody against human CD29 used for stem cell flow cytometry was purchased from Nordic BioSite. Bromophenol blue was purchased from ICN Biomedicals, INC, Irvine, CA, USA.

### Cell culturing

All the experiments were performed on the human prostate cancer cell line LNCap-FGC, which was purchased from American Tissue Culture Collection (Manassas, VA, USA). The cells were grown as a monolayer culture. The cells cultured under normoxic conditions (21% O<sub>2</sub>) were grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 1 mM

non-essential amino acids, and kept in a humidified incubator with 5% CO<sub>2</sub> in normal air at 37°C. The cells were passaged once a week by harvesting with trypsin followed by reseeding at a density of 27 000 cells/cm<sup>2</sup>. Cells cultured under hypoxic conditions (1% O<sub>2</sub>) were grown in RPMI-1640 Dutch Modification medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM non-essential amino acids (hypoxia medium). These cells were kept in a humidified Whitley H35 hypoxiastation (Don Whitley Scientific, West Yorkshire, UK), with 5% CO<sub>2</sub>, 1% O<sub>2</sub>, and 94% N<sub>2</sub> at 37°C.

For the experiments, cells were seeded at a density of 20 000 cells/cm<sup>2</sup> unless otherwise stated. The cells were harvested using trypsin solution (0.05% in 1 mM EDTA) unless otherwise stated. The compounds were added 24 hours after seeding in all the experiments. The compounds were added to concentrations of 10 µM if not stated differently.

Stock solutions of 4 mM of both PG11144 and PG11047 in PBS were used in the experiments. Both the compounds were provided by Progen Pharmaceuticals (Palo Alto, CA, USA). The solutions were stored at -20 °C.

### **Dose-response assay**

Cells were harvested by trypsinization, counted in a hemocytometer and thereafter re-suspended to a final concentration of 33 000 cells/ml. The cell suspension was added to 96-well plates in aliquots of 180 µl, containing 6000 cells, per well. The cells were incubated under normoxic conditions for 24 hours. PG11144 and PG11047 were diluted in sterile PBS and added in aliquots of 20 µl to obtain final concentrations of 0.01, 0.1, 0.5, 1, 5, 10, 50, and 100 µM. Sterile PBS was used as control. Cells seeded for culturing in hypoxia were placed under hypoxic conditions and the medium was replaced with hypoxia medium prior to drug addition. The cytotoxicity was investigated after 48 and 72 hours of treatment. Twenty µl MTT solution (5 mg/ml in PBS) was added to each well followed by incubation for one hour in normoxia and 2 hours in hypoxia. The medium was removed and

100 µl DMSO was added to each well. The plates were put on a shaker for 10 minutes before the absorbance was measured at 540 nm with a Labsystems iEMS Reader MF (Labsystems Oy, Helsinki, Finland) and the software DeltaSoft II v. 4.14 (Biometallics Inc., Princeton, NJ, USA).

### **Cell proliferation assay**

Four hundred thousand cells were seeded in 5 ml of medium in a 25 cm<sup>2</sup> tissue culture flask. PG11144 or PG11047 was added after 24 hours of incubation to final concentrations of 10 µM. The cells seeded for culturing in hypoxia were placed under hypoxic conditions after 24 hours. The medium was changed to hypoxia medium, before addition of compound.

The attached cells were harvested and collected by trypsinization 24 hours after seeding, and after 24, 48, and 72 hours of treatment. The cells were counted in a hemocytometer and total cell number determined.

### **Flow cytometric analysis of cell death and cell cycle phase distribution**

Cells were harvested using trypsin 24 hours after seeding and after 24, 48, and 72 hours of treatment with 10 µM PG11144 or PG11047 in normoxia and hypoxia. Both detached (floating in the medium) and attached cells were collected, pelleted and re-suspended in ice cold 70% ethanol. The cells were stored at -20°C until further analysis.

The cells were washed once with 10 ml PBS and thereafter pelleted and re-suspended in PBS with 100 µg/ml propidium iodide, 0.6% NP-40, and 100 µg/ml ribonuclease-A. The samples were kept at 4°C over night. The samples were analyzed using a BD Accuri C6 flow cytometer, (BD Biosciences, San Jose, CA, USA). The cell cycle phase distribution and sub-G<sub>1</sub> fraction were evaluated using the MultiCycle® software, (Phoenix Flow Systems, CA, USA).

### **Western Blot analysis**

Cells were harvested by trypsinization after 48 and 72 hours of treatment in normoxia or hypoxia. Only attached cells were collected. The

cells were counted and thereafter pelleted. The pellets were dried and stored at -80°C until further analysis.

The pellets were suspended in sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate, 5%  $\beta$ -mercaptoethanol) with addition of 0.1% NP-40, 5000 cells/ $\mu$ l. The samples were sonicated for 2x10 seconds, and boiled for 6 minutes. The samples were stored at -20°C until further use. Pre-cast polyacrylamide gels were loaded with aliquots equivalent to 75000 cells per well. The gel electrophoresis was performed in a XCell Surelock™ Mini-Cell Electrophoresis system (Invitrogen). The gels were run at 150 V for 90 minutes and they were thereafter blotted using an iBlot Dry Blotting System (Invitrogen). The nitrocellulose membranes were washed in PBS with 0.05% TWEEN® 20 (PBT) for 15 minutes and then stored at -20°C until antibody staining. Prior to staining, the membranes were washed 4x15 minutes in 50 ml PBT and then blocked with 10 ml PBT with 50 mg/ml dry milk for 1-2 hours at room temperature. For analysis of phosphorylated proteins the membranes were blocked with 10 ml PBS with 50 mg/ml bovine serum albumin. They were thereafter stained with primary antibodies against H3K4me2, LSD-1,  $\beta$ -actin, GAPDH, PARP, caspase 3, Bcl-2, p53,  $\beta$ -catenin,  $\gamma$ H2Ax, vimentin, E-cadherin, cytokeratin 18, and HIF-1 $\alpha$  diluted in 5 ml PBT with 50 mg/ml dry milk or bovine serum albumin and incubated for one hour at room temperature or overnight at 4°C. The membranes were washed with PBT once again and thereafter incubated with secondary antibody, diluted in 5 ml PBT, for one hour in room temperature. The membranes were washed with PBT followed by exposure to ECL solution. To detect the protein bands, the ChemiDoc XRS system (Bio-Rad Inc., Hercules, CA, USA) was used.

#### **Colony forming efficiency in soft agar**

The cells were harvested using trypsin and counted after 72 hours of treatment with either 10  $\mu$ M PG11144 or PG11047, in either hypoxia or normoxia. MEBM basal medium containing hydrocortisone, insulin, epidermal growth factor

(all from CC-4136 kit), B27 supplement, bFGF (20 ng/ml), penicillin (50 U/ml), and streptomycin (50  $\mu$ g/ml) was heated to 42°C. Agarose was added to the medium to a final concentration of 0.4%, followed by immediate addition of cells to a concentration of 1000 cells/ml. Five hundred  $\mu$ l was added to the inner wells of a poly-HEMA-coated 48-well plate (200  $\mu$ l of 5 mg/ml poly-HEMA in 96% ethanol was added to the wells in advance and the wells were left in a 37°C incubator until the ethanol had evaporated and only a thin poly-HEMA-film was left in the wells). One ml PBS was added to the outer wells to minimize evaporation in the agarose-containing wells. The plates were wrapped in saran wrap, to further minimize evaporation, and incubated in 5% CO<sub>2</sub> in air at 37 °C for 14 days. The colonies were counted using an inverted phase contrast microscope.

#### **Cancer stem cell flow cytometry**

The cells were rinsed with PBS and incubated with Accutase® for 10 min after 72 hours of treatment with 10  $\mu$ M of either PG11144 or PG11047. The incubation with Accutase® was stopped by addition of ice-cold PBS+1% FBS. Two hundred thousand cells from each treatment were transferred to test tubes kept on ice. The tubes were centrifuged at 600 x g at 4°C for 5 min. The supernatant was discarded and the cells were washed with 5 ml ice-cold PBS+1% FBS followed by centrifugation at 600 x g, 4°C for 5 min. The supernatant was discarded and antibodies against human CD44 and CD133 (+AlexaFluor® 594) or CD29 and CD24 in 100  $\mu$ l (1:100) were added to each tube followed by incubation on ice on a shaker for 15 minutes. The cells were thereafter washed with 5 ml PBS+1% FBS and centrifuged, twice. The supernatant was discarded and the pellets were vortexed with 1 ml PBS+1% FBS. The samples were analyzed using BD Accuri C6 flow cytometer, and analyzed with the BD CFlow® software.

#### **Statistics**

A two-tailed, unpaired student's t-test was used to evaluate the significance.

## Results

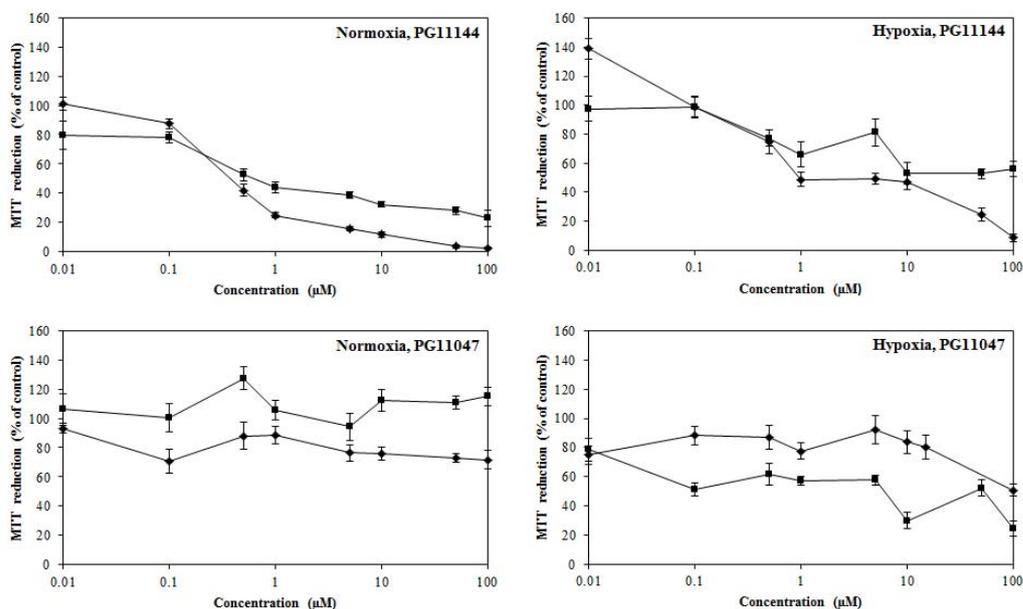
### The dose-response relationship of PG11144 and PG11047 measured by the MTT assay

To investigate if the basal toxicity of PG11144 and PG11047 in normoxia and hypoxia are dose dependent the MTT assay was used. The yellow soluble MTT salt is reduced to blue formazan crystals by Complex I in the electron transport chain in the mitochondria (36). The absorbance of the dissolved crystals at 540 nm, is proportional to the magnitude of the MTT reduction and is therefore said to represent the mitochondrial activity and the number of viable cells in the culture. Treatment with increasing concentrations of PG11144, ranging from 0.01  $\mu\text{M}$  to 100  $\mu\text{M}$ , resulted in a decrease of MTT reduction compared to control after both 48 and 72 hours of treatment, in both normoxia and hypoxia (Fig. 2). Treatment with PG11047 resulted in less toxicity. There was a small decrease of the MTT reduction compared to control after treatment in hypoxia but the treatment did not seem to have any prominent

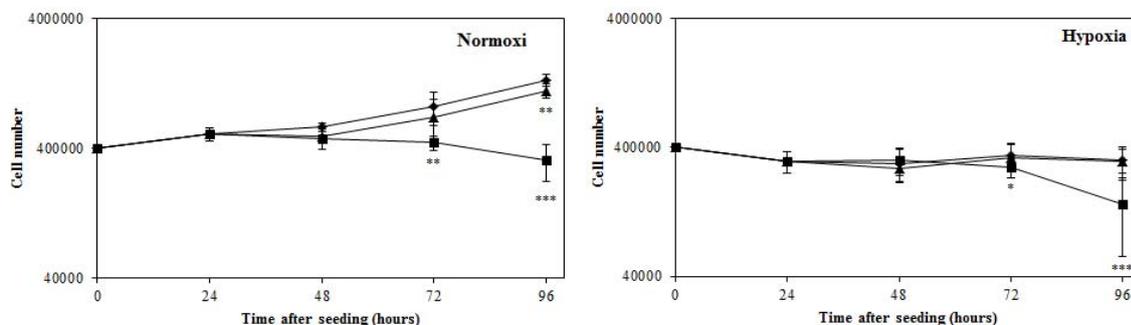
effect in normoxia. The concentration used in the future experiments, 10  $\mu\text{M}$ , was based on these results.

### The effects of PG11144 and PG11047 treatment on cell proliferation

The effects of PG11144 and PG11047 on the cell proliferation were investigated by counting cells in a hemocytometer after trypsinization. The cells were treated with 10  $\mu\text{M}$  of either PG11144 or PG11047, or grown in the absence of the drugs (Fig. 3). Treatment with PG11144 decreased the cell number to a greater extent than treatment with PG11047 in both normoxia and hypoxia. PG11144 was most effective in normoxia with a significant decrease in cell number compared to control after both 48 ( $P \leq 0.01$ ) and 72 hours ( $P \leq 0.001$ ). After 48 hours of treatment the cell number was 53% of control and after 72 hours it was 24% of control. The effect in hypoxia was less prominent, though yet significant ( $P \leq 0.01$ ) after both 48 and 72 hours of treatment. PG11047 treatment decreased the number of cells significantly compared to control



**Figure 2.** MTT reduction in LNCap-FGC prostate cancer cells treated with PG11144 or PG11047 in normoxia (21%  $\text{O}_2$ ) or hypoxia (1%  $\text{O}_2$ ). The cells were seeded in 96-well plates and the drugs were added after 24 hours to the final concentrations shown in the figure. After 48 ( $\bullet$ ) and 72 hours ( $\blacklozenge$ ) of treatment the mitochondrial activity was determined using the MTT assay. The results are presented as the mean value of the MTT reduction in percent of control and the bars represent the standard error ( $n=12$  from two independent experiments).



**Figure 3.** The effects of 10  $\mu$ M PG11144 or PG11047 on cell proliferation of LNCap-FGC prostate cancer cells incubated in normoxia and hypoxia. The cells were seeded at a density of 20000 cells/cm<sup>2</sup> and the drugs were added 24 hours after seeding to a final concentration of 10  $\mu$ M. The cell number was determined using a hemocytometer. The results are presented as the mean cell number and the bars represent the standard deviation (n=6 from two independent experiments).  $\blacklozenge$ , Control;  $\blacksquare$ , PG11144;  $\blacktriangle$ , PG11047. \*,  $P \leq 0.05$ , \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ , compared to control.

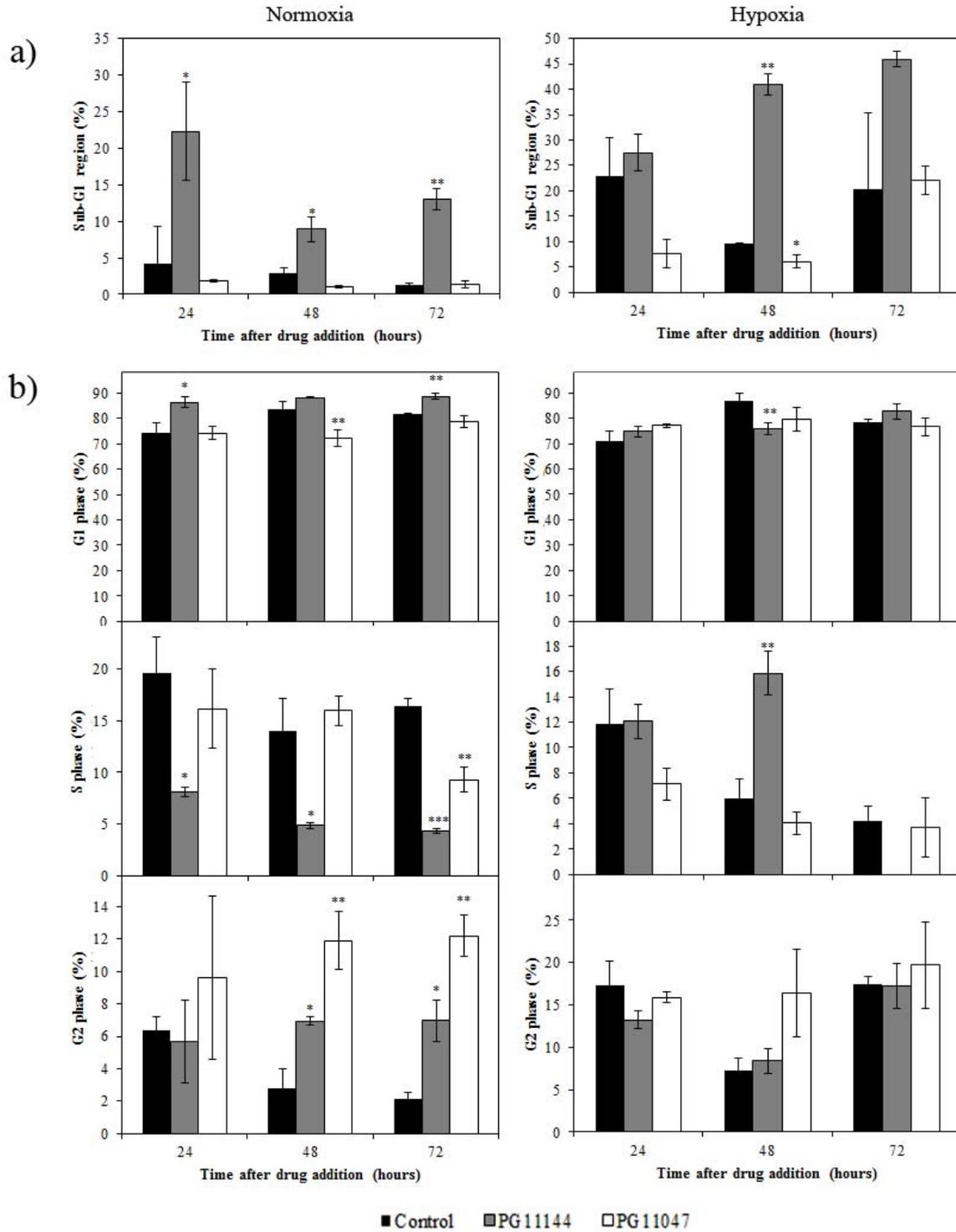
( $P \leq 0.01$ ) after 72 hours of treatment in normoxia, but no effect of the treatment was seen in hypoxia. After 72 hours of treatment in normoxia the cell number was 83% of control. The number of untreated cells did not increase over time in hypoxia.

#### Treatment with PG11144 induced cell death in both normoxia and hypoxia

Treatment with PG11144 decreased the number of cells over time, compared to control, and it was therefore of interest to investigate whether the compound caused cell death and how it affected the cell cycle phase distribution. Since the cell number did not increase over time for any of the treatments in hypoxia it was also of interest to see if this was due to decrease in proliferation or if the cells were dying at the same rate as they were dividing. This was done by analyzing DNA histograms obtained from cells treated with 10  $\mu$ M PG11144 or PG11047 for 24, 48, and 72 hours. The number of cells in the different phases of the cell cycle is presented as percentage of the total cell number (Fig. 4). Dead cells are defined as those found in the sub-G<sub>1</sub> region. PG11144 treatment significantly increased the number of dead cells in the cultures in normoxia ( $P \leq 0.01$  after 72 hours compared to

control). In hypoxia a significant increase in the sub-G<sub>1</sub> region was seen after 48 hours of treatment ( $P \leq 0.01$  compared to control), but the difference was not significant after 72 hours of treatment. Cells cultured in hypoxia showed a higher rate of cell death in control and both treatments compared with cells cultured in normoxia. In both normoxia and hypoxia cell death observed in cells treated with PG11047 was not different compared to control.

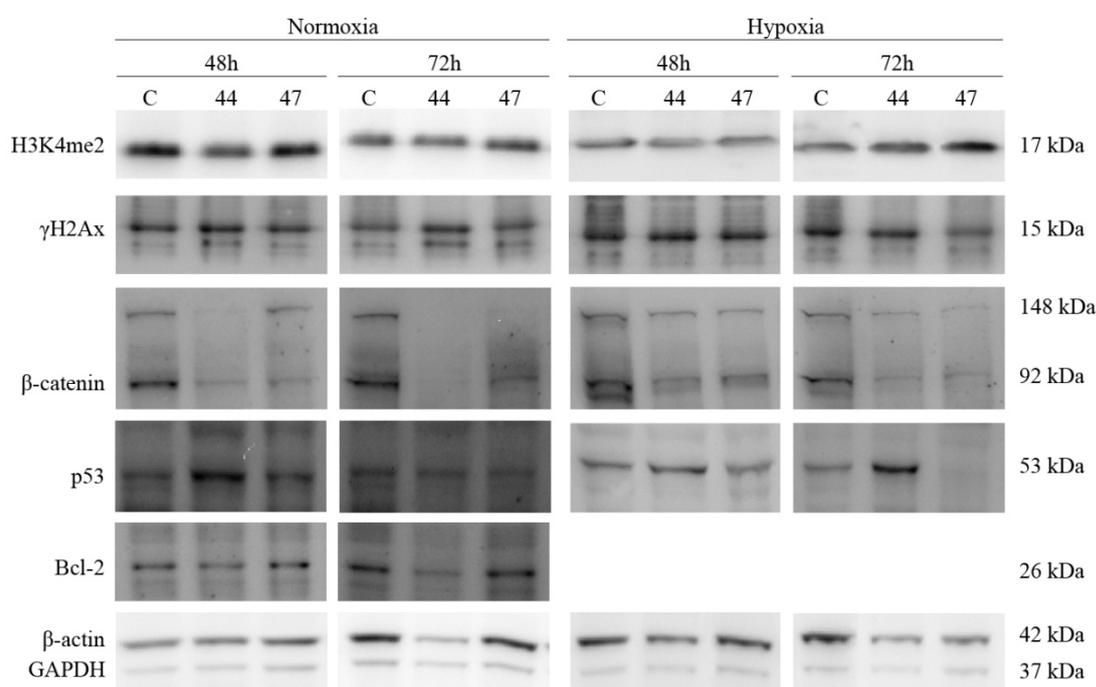
To investigate whether the observed cell death after PG11144 treatment in normoxia, and for cells cultured in hypoxia, was apoptotic, Western Blot was performed to investigate the cleavage of poly(ADP-ribose) polymerase (PARP) and the expression of caspase 3, Bcl-2, and p53. Active caspase 3, the cleavage product of procaspase 3, is a critical downstream effector molecule in the apoptotic pathway. It cleaves other specific substrates involved in apoptosis, such as PARP (31). No distinct expression of caspase 3 or PARP was observed in the LNCap cell line, independent of treatment (data not shown). Proteins of the Bcl-2 family are central in the regulation of the mitochondrial pathway in apoptosis. During apoptosis they translocate to the outer membrane of the mitochondria where they change the permeability of the membrane,



**Figure 4.** The effects of treatment with PG11144 and PG11047 on cell death and cell cycle phase distribution analyzed by flow cytometry. LNCap-FGC prostate cancer cells were seeded and allowed to attach for 24 hours before the compounds were added to a final concentration of 10  $\mu$ M. The cells were cultured in either normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>) and harvested at the time points shown in the figure. **a)** The sub-G<sub>1</sub> region is correlated to cell death and **b)** the percentage of cells in the G<sub>1</sub>, S and G<sub>2</sub> phases shows the cell cycle phase distribution. The results are presented as the mean values from 3 independent cultures and the bars represent the  $\pm$  SD. \*, P  $\leq$  0.05; \*\*, P  $\leq$  0.01; \*\*\*, P  $\leq$  0.001, compared to control.

which in turn mediates the release of cytochrome *c*, a protein that induces caspase activation (31). PG11144 treatment decreased the expression of Bcl-2 in normoxia after 72 hours of treatment, which indicates that apoptosis may have been induced. No decrease in Bcl-2 expression was observed in PG11047-treated cells in normoxia. Bcl-2 expression in hypoxia was not investigated. p53 is a protein that plays a key role at G<sub>1</sub>-S phase checkpoint transition (37). It regulates cell cycle progression and

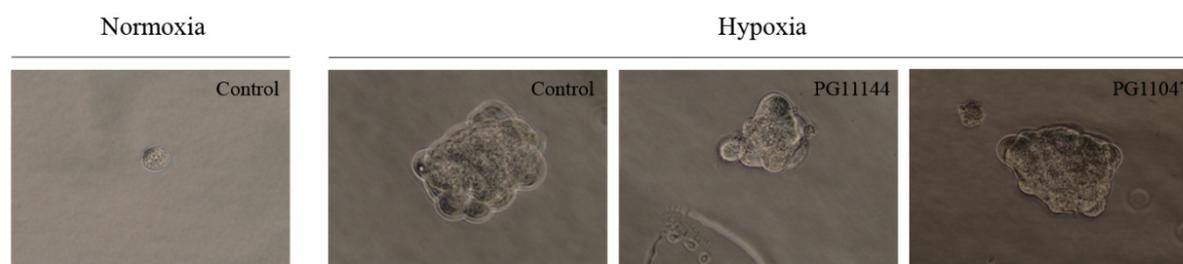
programmed cell death by monitoring DNA damage, nucleotide levels, and the status of the mitotic spindle. An increase in p53 is maintained by protein stabilization and leads either to apoptosis or G<sub>1</sub> arrest, depending on cell type. No changes in p53 levels were observed for any of the treatments in normoxia. However PG11144 treatment resulted in a higher expression of p53 in hypoxia after 72 hours of treatment, while PG11047 treatment resulted in a prominent decrease of the protein. (Fig. 5)



**Figure 5.** Western Blot analysis of protein levels in cells treated with 10 μM PG11144 (44 in the figure) or PG11047 (47 in the figure) in normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>). The compounds were added 24 hours after seeding and the cells were harvested at the time points shown in the figure. Homogenates equivalent to 75 000 cells were added to each well of the gels. The figure shows the expression of H3K4me2, γH2Ax, active β-catenin, p53, Bcl-2, β-actin, and GAPDH and the results are representatives of 3-9 independent cultures from 1-3 independent experiments.

**Table 1.** The colony forming efficiency of LNCap-FGC prostate cancer cells in soft agar. The cells, incubated in normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>) were treated with PG11144 or PG11047 at a final concentration of 10 μM 72 hours. After treatment, the cells were seeded at a density of 1000 cells/ml in soft agar. The number of colonies was determined after 14 days of incubation in normoxia. The data represents the mean ± SD from 4 independent cultures from 2 independent experiments.

Normoxia				
Control (% of cell population)	PG11144 (% of cell population)	PG11144 (% of control)	PG11047 (% of cell population)	PG11047 (% of control)
< 0.1	0	0	0	0
Hypoxia				
Control (% of cell population)	PG11144 (% of cell population)	PG11144 (% of control)	PG11047 (% of cell population)	PG11047 (% of control)
20.3 ± 4.0	8.7 ± 1.6	43.1	15.0 ± 2.7	74.0



**Figure 6.** Images of the colonies formed by LNCap-FGC cells in soft agar. The cells were treated for 72 hours with PG11144 (10 μM) or PG11047 (10 μM) in normoxia and hypoxia and were thereafter seeded in soft agar. The photos were taken after 14 days of incubation in normoxia, using an inverted phase contrast microscope. No colonies were observed after treatment with either of the compounds in normoxia.

### Cells cultured in hypoxia showed higher colony forming efficiency in soft agar compared to cells cultured in normoxia

In the soft agar assay the ability of cells to form colonies, independently of attachment to a surface, is investigated. The ability to grow and divide under these conditions is said to correlate to more aggressive cancer cells. In this experiment, cells were seeded at a low density in soft agar after 72 hours of treatment with 10 μM of PG11144 or PG11047 in either normoxia or hypoxia. After 14 days of incubation in normoxia, the colonies were counted in an inverted phase contrast microscope (Table 1). In normoxia, only very small colonies were found in control while no colonies were seen in any of the treatments. Less than 1% of the untreated cells seeded were able to form colonies. When

cells were treated in hypoxia however, the number of colonies formed was much higher: 20% of the control cell population was able to form colonies. The colonies were also bigger than the ones observed after culturing in hypoxia (Fig. 6). Both treatments decreased the number of colonies formed in hypoxia, with PG11144 being the most effective.

### Expression of EMT-/MET-associated proteins after treatment in with PG11144 or PG11047 in normoxia or hypoxia

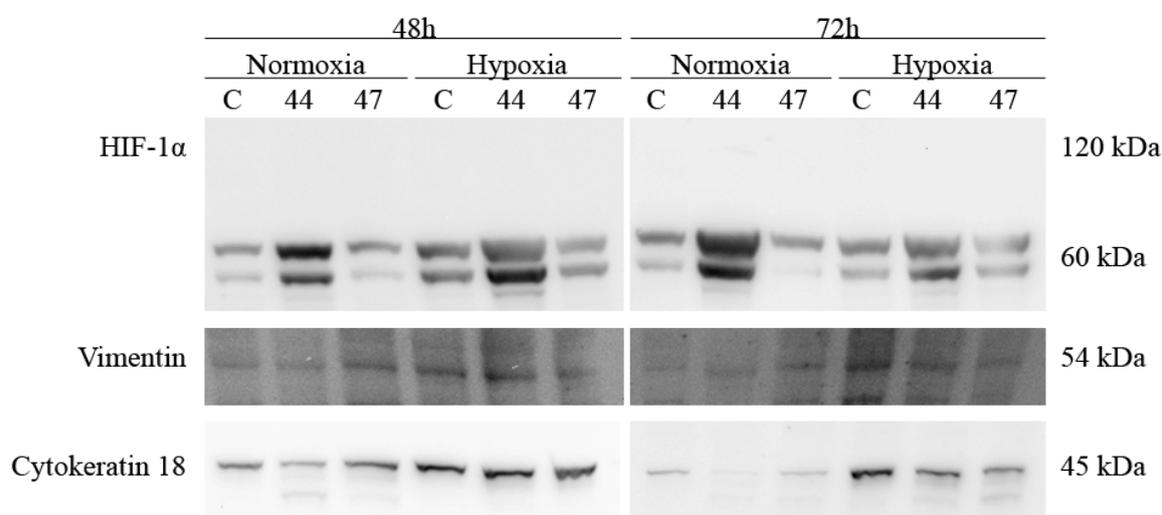
Since the number of colonies formed in soft agar increased after treatment in hypoxia it was of interest to investigate if this was due to EMT. This was done by analyzing the expression of the proteins vimentin, E-cadherin, and cytokeratin 18 by Western Blot. Vimentin is expressed by

cells of the mesenchymal phenotype while E-cadherin and cytokeratin 18 are expressed by cells of the epithelial phenotype. The results (Fig. 7) showed that both vimentin and cytokeratin 18 were expressed at a higher level in cells cultured in hypoxia compared with normoxia after 72 hours. The increase in cytokeratin 18 could be seen after 48 hours. No expression of E-cadherin was detected (data not shown). No clear difference in the expression of vimentin and cytokeratin 18 between the different treatments was observed.

#### Expression of HIF-1 $\alpha$ in LNCap-FGC treated with PG11144 and PG11047 in normoxia or hypoxia

Hypoxia inducible factor HIF-1 is a heterodimer consisting of two subunits HIF-1 $\alpha$  and HIF-1 $\beta$ . HIF-1 $\beta$  is expressed by cells cultured in both normoxia and hypoxia while HIF-1 $\alpha$  is rapidly degraded by the proteasomes when oxygen is present. In hypoxia, this degradation is inhibited and HIF-1 $\alpha$  can form the dimer with HIF-1 $\beta$ . HIF-1 $\alpha$  helps the cells to cope with the hypoxic

stress by activation of oxygen-regulated genes (38). It is for example involved in increasing the glycolytic capacity, regulation of pH, and glucose-uptake. Therefore it was of interest to look for expression of HIF-1 $\alpha$  to see if the cells responded to the hypoxic environment. In addition, HIF-1 $\alpha$  has also been shown to play an important part in the development of malignancy and drug resistance (38). It suppresses apoptosis in most transformed cells, it is involved in DNA-repair, it acts as a co-activator of the androgen receptor, and it is suggested to induce EMT in LNCap cells (39). The expression of HIF-1 $\alpha$  was investigated using Western Blot. No bands were detected at 120 kDa, but two distinct bands were observed at approximately 58 and 65 kDa. The expression of these products was highly increased by PG11144-treatment in both normoxia and hypoxia compared to control, with most prominent effect in normoxia. The difference between the treatments seemed to decrease in hypoxia after 72 hours of treatment (Fig. 7).



**Figure 7.** Western Blot analysis of the levels of HIF-1 $\alpha$ , vimentin and cytokeratin 18 in cells treated with 10  $\mu$ M PG11144 (44 in the figure) or PG11047 (47 in the figure) in normoxia or hypoxia. The compounds were added 24 hours after seeding and the cells were harvested at the time points given in the figure. Homogenates equivalent to 75 000 cells were loaded to each well of the gels. The results are representatives of 3-6 independent cultures from 1-2 independent experiments.

### **The effects of treatment with PG11144 and PG11047 on the surface markers CD44, CD133, CD29 and CD24**

No expression of the stem cell associated surface markers CD44, CD133 and CD24 was seen in the LNCap-FGC cells. CD29 however was expressed in both normoxia and hypoxia for all the treatments. The experiment was performed twice, each time with two independent samples. The first time no clear difference was observed between normoxia and hypoxia or between the different treatments, and the results varied considerably, but the second time the expression of CD29 increased from 56.0% for control in normoxia to 82.1% for control in hypoxia (primary data from flow cytometry not shown). The expression in PG11144-treated cells in normoxia was 30.2% and in hypoxia 66.7%. PG11047-treatment resulted in an expression of 42.8% in normoxia and 79.1% in hypoxia.

### **Treatment with PG11144 and PG11047 decreased the expression of $\beta$ -catenin**

$\beta$ -catenin is the key nuclear effector in the Wnt/ $\beta$ -catenin signaling pathway (40). Imbalance in this pathway is correlated with deregulated growth and metastasis in cancer. It is suggested that it plays a role in the activation of EMT (41, 42) and it is also a co-activator of the androgen receptor (43, 44). Therefore it was of interest to see whether PG11144 and PG11047 had any effect on the expression of  $\beta$ -catenin, and whether the expression of the protein differed in hypoxia and normoxia. This was evaluated using Western Blot. Treatment with both compounds resulted in a decrease in  $\beta$ -catenin expression in both normoxia and hypoxia, but the effect was more prominent in normoxia. The decrease could be observed already after 48 hours of treatment (Fig. 5).

### **$\gamma$ H2Ax level after PG11144 and PG11047 treatment in hypoxia and normoxia**

The phosphorylation of the histone H2Ax forming  $\gamma$ H2Ax, occurs rapidly upon DNA damage (38). However, the phosphorylation is suggested to be dependent on HIF in cells cultured in hypoxia (38). The level of  $\gamma$ H2Ax in

cells treated with PG11144 and PG11047 in hypoxia or normoxia was evaluated using Western Blot. A small increase of  $\gamma$ H2Ax expression could be seen after 72 hours in cells treated with PG11144 in normoxia, but except for that no changes in the levels of the protein could be detected. (Fig. 5)

### **Treatment with PG11144 seemed to reduce the expression of $\beta$ -actin in cells cultured in normoxia**

When beginning the Western Blot experiments,  $\beta$ -actin was chosen as a loading control. However it turned out that the levels of  $\beta$ -actin were lower in all the samples treated with PG11144 in normoxia compared to control. No such decrease was seen for the same treatment in hypoxia. GAPDH is also a loading control commonly used. The levels of GAPDH also decreased slightly after 72 hours of treatment with PG11144 in normoxia. (Fig. 5)

### **Treatment with PG11144 and PG11047 did not seem to have an impact on the demethylation of H3K4me2**

Previous studies have shown that PG11144 inhibits the enzyme LSD-1 in human colorectal cancer (33, 45). LSD-1 demethylates the histone H3K4me1/me2, which leads to silencing of tumor suppressor genes. Thus, PG11144 treatment has been shown to increase the level of H3K4me2 (33, 45). To investigate if PG11144 had the same actions in LNCap prostate cancer cells the expression of H3K4me2 in whole cell extracts and LSD-1 in nuclear extracts was investigated using Western Blot. No detectable levels of LSD-1 were seen. The levels of H3K4me2 did not seem to change in treated cells with any of the compounds (Fig. 5)

## **Discussion**

One of many new targets in the development of anti-cancer drugs is the polyamine pathway. Polyamines are essential for cell proliferation and the levels are highly up-regulated in cancer cells. One way to interfere with this pathway is

to use polyamine analogues that gain access to the cells by the natural polyamine transport system. Once inside the cells they take advantage of the self-regulatory mechanisms of the polyamine pathway, thereby depleting the levels of the naturally occurring polyamines putrescine, spermidine and spermine, which leads to decreased cell proliferation (15).

In this study, the effect of the two polyamine analogues PG11144 and PG11047 on the androgen-dependent prostate cancer cell line LNCap-FGC was investigated. Since most tumors contain oxygen-deprived areas, and these areas have been associated with development of more aggressive cancer cells, it was of interest to study the effects on cells cultured in both normoxia (21% O<sub>2</sub>) and hypoxia (1% O<sub>2</sub>). Hypoxia usually results in decreased cell proliferation and therefore the hypothesis was that the compounds would be less effective under these conditions compared to normoxia, both because the time required to see any result would be longer and because the polyamine pathway is down-regulated in slowly proliferating cells (17, 18), which could make the polyamine analogues less effective. This turned out to be true for both the treatments, regarding effects on cell proliferation. Treatment with PG11047 did not have any detectable effect on the cell number in hypoxia, while PG11144 reduced the number of cells, but was still less effective than in normoxia. It would have been of interest to treat the cells for a longer time than 72 hours. Analysis of DNA histograms obtained by flow cytometry showed that treatment with PG11144 also caused cell death in LNCap-FGC cells in both hypoxia and normoxia. PG11047 decreased the number of cells in normoxia but no cell death was observed indicating that the decrease was only due to inhibition of cell proliferation. To investigate if the decrease in cell proliferation actually was due to depleted polyamine levels, the intracellular polyamine contents have to be measured in future studies.

The protein expressions of the cells were investigated using Western Blot. Initially,  $\beta$ -actin was chosen as a loading control. Unexpectedly the results showed a decrease in  $\beta$ -actin after 72

hours of treatment with PG11144 in normoxia (n=9). GAPDH was also tested as a loading control and yielded similar results as  $\beta$ -actin although not as pronounced. The turnover of actin is very slow in cells (46), thus, if the observation proves to be correct, PG11144 treatment may specifically stimulate actin breakdown, even though the mechanisms are unknown. It has also been shown that polyamine depletion results in disappearance of actin filaments in the wild-type Chinese hamster ovary cell line CHO (47). Since it was unclear whether this decrease in  $\beta$ -actin levels was due to a systematic mistake or an actual degradation of  $\beta$ -actin caused by the treatment we decided not to do any loading compensation for the other proteins studied, based on  $\beta$ -actin levels. This must be further investigated before we can draw any conclusions. The following discussion about the results obtained by Western Blot is based on the idea that the  $\beta$ -actin levels are reduced by PG11144, until proven differently.

The Western Blot data indicated that 72 hours of treatment with PG11144 in hypoxia increased the levels of p53 in LNCap-FGC cells. Since p53 inhibits growth and is involved in the induction of apoptosis (37) this increase may suggest that the increased sub-G<sub>1</sub> fraction seen when analyzing DNA histograms could be due to apoptosis. Cell death was however also observed after treatment with PG11144 in normoxia but no increase in p53 was seen. The Bcl-2 expression decreased though, which is also associated with apoptosis. In the time-laps movies obtained by holographic imaging, apoptosis could be seen in connection with mitosis (not shown). Treatment with PG11047 in hypoxia however, seemed to drastically reduce the expression of p53 compared to control. This was unexpected since a decrease in p53 levels usually is correlated with rapid proliferation and aggressive cancer cells. To further investigate the mechanisms behind this reduction it would be of interest to investigate the effect on other proteins involved in the regulation loop of p53, such as MDM2 which blocks the expression of p53 as well as promotes a rapid degradation of the protein (48, 49). Studies also shows that hypoxia selects for

cells that do not express p53 (11) but if so, this should be expected also in control cells. Deregulation of wild type p53 is not a desirable feature of a potential cancer drug. LNCap-FGC cells express wild type p53 (49).

When studying the effect of PG11144 and PG11047 on the colony forming efficiency in soft agar only a few, very small colonies were seen in the untreated cultures in normoxia. However in hypoxia, 20.3% of the seeded, untreated cell population managed to form colonies suggesting that hypoxia increased the malignancy of the cells. This supported the hypothesis that hypoxia induces EMT (8, 13) and increases the number of cancer stem cells (13). Both PG11144 and PG11047 did decrease the number of colonies formed in hypoxia. Markers for EMT/MET were further studied by Western Blot. An increase in vimentin expression was observed in control after 72 hours in hypoxia which suggest that EMT was involved in the increased colony forming efficiency. However, cytokeratin 18 also increased in hypoxia indicating the opposite. No differences in the expressions of vimentin or cytokeratin 18 between the treatments were seen. This suggests that there are other mechanisms involved as well. The effect on cancer stem cells was also studied. Four different cancer stem cell markers were studied: CD44, CD133, CD24 and CD29 (51). Only expression of CD29 was observed in this cell line as has been reported previously (51). CD29 or  $\beta 1$  integrin plays a role in cell attachment (52). In the first experiment performed no difference between hypoxia and normoxia or between the different treatments was observed. But when the study was repeated the number of cells expressing CD29 increased in hypoxia compared to normoxia and both the treatments reduced the number of CD29-expressing cells, with PG11144 being the most effective. Since this is only based on two independent samples from one experiment no conclusions can be drawn, but it might give an indication that hypoxia can increase the number of cancer stem cells and that the PG11144 and PG11047 can reduce the number in both

normoxia and hypoxia. Repeating this experiment would be necessary.

The expression of active  $\beta$ -catenin decreased after treatment with both PG11144 and PG11047, in both normoxia and hypoxia. Since  $\beta$ -catenin is said to be involved in metastasis and EMT (41) this may be an explanation to the decrease in the colony forming efficiency in soft agar and the possible decrease in the number of cancer stem cells.

HIF-1 $\alpha$  is a protein that is rapidly degraded in normoxia and the levels were therefore investigated to see if the cells actually were cultured in a hypoxic environment. Another reason to investigate HIF-1 $\alpha$ , is its involvement in the induction of EMT. No levels were seen at 120 kDa in either hypoxia or normoxia. This could mean that the environment was not hypoxic after all, but it could also be because of the time points chosen for investigation of the HIF-1 $\alpha$  expression. HIF-1 $\alpha$  is highly expressed when the cells first come in contact with hypoxia to make them cope with the stressful environment, but the level decreases over time when other factors are taking over (53). It would have been of interest to study the expression at earlier time points than 48 hours. Even though no bands showed up at 120 kDa two strong bands quickly appeared at  $\sim 60$  kDa. PG11144 treatment gave much stronger bands in both normoxia and hypoxia. The identity of these bands is unknown but there are several splice variants of HIF-1 $\alpha$  of sizes that may explain the presence of two bands around 60 kDa (54).

To conclude, both PG11144 and PG11047 showed features that are desirable for anti-cancer drugs. Treatment with PG11144 effectively inhibits cell proliferation and cause cell death in both normoxia and hypoxia and it decreases the malignancy of the cells. But there might be a risk that it is too toxic for the cells and that it inhibit functions that is not unique for cancer cells but exists in normal cells too and therefore further studies of the toxicity is required. PG11047 was less toxic than PG11144, it significantly inhibited the proliferation in normoxia but it did not cause cell death. The decreased number of

colonies formed in soft agar and the decrease in  $\beta$ -catenin expression also indicates that this compound is also able to reduce the malignancy of the cells. PG11047 could therefore be considered the most promising of the two compounds. However, PG11047 caused a down-regulation of p53 in cells cultured in hypoxia, something that is associated with rapid, uncontrolled cell proliferation. Therefore it is of great importance to find the mechanisms behind this. Since these studies were only performed *in vitro*, *in vivo* studies would also be of interest, especially to see whether the compounds can reach the hypoxic regions of the tumor or not. Since these regions lack sufficient blood supply, it requires that the concentration of the drug is high enough to be able to diffuse into these areas. Then it is important that this dose is not toxic to the rest of the body. Since cancer treatment often occurs over a long time it would also be interesting to do long-time studies with the compounds *in vitro*.

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