Akacid-medical-formulation, a novel biocidal oligoguanidine with antitumor activity reduces S-phase in prostate cancer cell lines through the Erk 1/2 mitogen-activated protein kinase pathway

HANNES NEUWIRT[1], HANSGEORG MULLER[1], ILARIA T.R. CAVARRETTA[2], MARTIN TIEFENTHALER[3], GEORG BARTSCH[2], GUENTHER KONWALINKA[1] and ZORAN CULIG[2]

[1]Division of General Internal Medicine, Department of Internal Medicine, [2]Department of Urology and [3]Division of Nephrology, Department of Internal Medicine, Innsbruck Medical University, Anichstrasse 35, A-6020 Innsbruck, Austria

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Abstract. Oligomeric guanidines are highly efficient biocides against a broad spectrum of microorganisms. However, their antitumor effects have not been studied so far. We investigated an antiproliferative effect of Akacid-medical-formulation (AMF), a member of the oligoguanidine family of biocides, against solid cancer cell lines and primary cells by measuring [^{3}H]-thymidine incorporation. Additionally, we examined cell cycle distribution in two AMF-sensitive prostate cancer cell lines (DU-145, LNCaP) using flow cytometry. Finally, the influence of AMF on cell cycle regulatory molecules and intracellular kinase cascade-related signaling molecules was assessed. We found that AMF has variable antiproliferative effects on all tested cells. In DU-145 and LNCaP cells, flow cytometric studies showed a reduction of S-phase with a maximum extent of 24 and 58%, respectively. This was associated with a decrease in expression of cyclin D1, cyclin-dependent kinases 2 and 4, while having varying effects on expression of cyclin E and p27. Additionally, reduced phosphorylation of Erk1 and Erk2 was found, whereas expression of phospho-Akt1 remained unchanged. Herein we report for the first time that AMF exerts potent antiproliferative activity against various malignant cell lines, including those of prostate. We therefore recommend further investigation of the anticancer activity of this biocidal oliguanidine.

Introduction

Polymers containing guanidine subunits have attracted substantial attention due to their high biocidal activity against a broad range of microorganisms, while showing low toxicity to humans (1). At present, guanidine-based biocidal oligomers provide a safe alternative to other common desinfectants and have found widespread application in numerous fields of human infection control (2,3). The mechanism of action of these biocides on bacteria is thought to be based on a process that causes leakage of low molecular weight cytoplasmic components (e.g. potassium ions) and activation of membrane-bound enzymes (e.g. ATPase) resulting in cytoplasmic membrane disruption (4,5). Akacid-medical-formulation (AMF) (Poly-[2-(2-ethoxy)-ethoxyethyl)-guanidinium-chloride]) is a novel oligoguanidine that is prepared by polycondensation of equimolar amounts of guanidine hydrochloride and 1,2-bis(2-aminoethoxy)ethane and exerts its biocidal activity against airborne and surface microorganisms including bacteria, viruses, fungi, and molds (6).

The antimicrobial properties of guanidine-based biocides have been extensively characterized. However, no studies with the aim of examining their possible antitumor effects have been undertaken so far. Because these biocides are known to cause not only membrane damage but also activation of proteases and kinases, we asked if AMF has an effect on human malignant cancer cells. In the present study, we tested AMF against a variety of solid cancer cells, including those derived from prostate (DU-145, PC3, LNCaP), breast (MCF-7, ZR-75-1), ovarian, colon, non-small cell lung, squamous skin cancer, melanoma, and glioblastoma.

Prostate cancer is the second most common cancer in American men after non-melanoma skin cancer. Although screening with prostate-specific antigen has led to a substantial increase in the detection of early stage diseases, prognosis of men with metastatic prostate cancer remains poor. Because of the androgen dependency of prostate cancer cells, these patients are usually treated with androgen deprivation therapy. Hormone therapy provides disease control in over 80% of cases with a median duration of 18-24 months. Eventually, therapy-resistant prostate cancer (TRPCa) develops. Chemotherapy of TRPCa has objective response rates of only 10-20% and median survival does not exceed 12 months (7). The treatment for men with TRPCa might be improved if a

Correspondence to: Dr Zoran Culig, Department of Urology, Innsbruck Medical University, Anichstrasse 35, A-6020 Innsbruck, Austria
E-mail: zoran.culig@uibk.ac.at

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survival benefit from docetaxel-based regimens as compared to mitoxantrone/prednisone is considered (8). In a recent phase III trial the combination of docetaxel and estramustine led to a 2-month improvement in median survival, as compared with mitoxantrone and prednisone (17.5 months vs. 15.6 months) (9). However, modest prolongation of survival rates was accompanied by adverse effects including thromboembolic events.

The emerge of TRPCa has been associated with androgen receptor (AR) overexpression (10,11), AR gene amplification, and mutations in the ligand-binding domain of the AR (12). Additionally, AR-coactivators such as SRC1, TIF2, ARA70 (13,14), oncogenes [e.g. Her2/NEU (15)], and cytokines like interleukin 6 (IL-6), insulin like growth factor I (IGF-I), and epidermal growth factor (EGF) (16,17) are known to enhance AR-signaling in a synergistic or, in the absence of androgens, ligand-independent manner.

Both development and progression of TRPCa have been shown to be accompanied by enhanced signaling via intracellular kinase-cascades, including the Ras/Raf/mitogen-activated protein kinase (MAPK, i.e. Erk1/2, p38), and the phosphoinositoltriphosphate-kinase (PI3K)/Akt pathway. Increased activation of MAPKs by growth factors such as EGF, transforming growth factor (TGF)-α, IGF-I/II, basic fibroblast growth factor or oncogenes (e.g. Her2/NEU) (18) correlates with higher Gleason score and tumor progression *in vivo* (19). Bakin and coworkers (20) have demonstrated a crucial role of chronic activation of MAPK signaling in the progression of LNCaP tumors by promoting hypersensitivity to sub-physiological androgen levels. Graff *et al* reported that increased Akt activity is involved in PCa progression (21) and Liao and coworkers evidenced a correlation with high Gleason scores (22). Recently, it was shown that inhibition of mammalian target of rapamycin (mTOR), a downstream effector-protein of the PI3K/Akt pathway, induced apoptosis and complete reversal of a phenotype of prostatic intra-epithelial neoplasia cells *in vivo* (23). In a study examining the requirements and activation status of Akt and MAPK signaling for the survival of a series of androgen-insensitive prostate cancer cell lines, a redundant role for both signaling pathways was reported (24).

In this study we show that the biocidal oligoguanidine AMF dose-dependently inhibits proliferation of the prostate cancer cell lines DU-145 and LNCaP. Analysis of cell-cycle distribution by flow cytometry illustrates an S-phase reduction of 24 and 58%, respectively. We report a substantial influence of AMF on cyclins and cyclin dependent kinases (cdks), and the Erk 1/2 signaling pathway.

Materials and methods

Chemicals. AMF was kindly provided by Geopharma (Vienna, Austria). A 10-mM stock solution was prepared in Dulbecco's phosphate-buffered saline (PBS 1X, PAA Laboratories GmbH, Pasching, Austria) and stored protected from light at 4˚C. Stability was checked regularly (every 2 months) by [^{3}H]-thymidine incorporation assay using newly diluted stock solutions (10-mM) as controls.

For Western blot analysis, the following antibodies were purchased: monoclonal anti-cdk 2 and cdk 4 antibodies from Biosource International (Camarillo, CA). Monoclonal anti-cyclin E and anti-p27 antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Monoclonal anti-phospho-p44/p42 antibody, that recognizes both phospho-p44 and phospho-p42, and polyclonal anti-phospho-Akt1 antibody were purchased from Cell Signaling Technology (Beverly, MA). Polyclonal anti-p44, anti-p42, and anti-Akt1 antibodies were products of Santa Cruz Biotechnologies. Monoclonal anti-cyclin D1 antibody was from LabVision-Neomarkers (Fremont, CA). Monoclonal anti-ß-actin antibody was purchased from Chemicon International (Temecula, CA). Monoclonal anti-mouse, anti-rabbit, and anti-goat secondary antibodies were products of Molecular Probes (Leiden, The Netherlands).

Cell lines. All cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained according to their recommendations: DU-145, PC3, LNCaP, and HRT-18 in RPMI-1640 (PAA Laboratories) with 10% fetal calf serum (FCS) and 1% glutamine; OVCAR-3, A-431 in MEM (Gibco, Paisley, UK) with 10% FCS and 1% glutamine. MCF-7, ZR-75-1, and CACO-2 were grown in MEM with 10% FCS, 1% glutamine, and 1% non-essential amino acids (NEAA) (Gibco). A-549 were maintained in DMEM - Low Glucose (PAA Laboratories) with 10% FCS and 1% glutamine, SK-28 and SK-37 in DMEM - Low Glucose with 10% FCS, 1% glutamine, and 1% NEAA. U-373 and T98 were grown in MEM with 10% FCS, 1% glutamine, 1% NEAA, and 1% sodiumpyruvate (Gibco). Primary immortalized prostate epithelial (156T, 153T) and smooth muscle (PM) cells were cultured in either MCDB-153 supplemented with 1% FCS, 25 mg bovine pituitary extract, 0.1 ng/ml EGF, and 1 nM R-1881 (PM) or MCDB-131 containing 15% horse-serum, 10 mM HEPES, 1% NEAA, 10 mg/ml insulin, 30 mg/ml transferrin, 50 μg/ml sodium selenite, 1 mM estradiol, 0.1 mM dexamethasone, 500 ng/ml bFGF, and 100 ng/ml EGF (156T, 153T). All media were supplemented with 1% pencillin/streptomycin/amphothericin B (Gibco) at end concentrations of 100 U/ml penicillin, 10 μg/ml streptomycin, and 0.25 μg/ml amphothericin B.

[^{3}H]-thymidine incorporation assay. Cells (156T, 153T, DU-145, LNCaP, PC3, MCF-7, ZR-75-1, OVCAR-3, A-431, A-549, SK-28, SK-37, CACO-2, and HRT-18 seeded at a density of 1x10^4/well, PM, U-373, and T98-G seeded at a density of 0.5x10^4/well) were incubated with increasing concentrations of AMF (0.3-100 μM) for 48 h in 96-well plates and 50 μl [^{3}H]-thymidine (2 μCi) per well was added. After a 16-h incubation, cells were frozen at -18˚C in order to brake the membranes. After thawing, DNA was harvested on fibreglass filters and incubated in small vials with 1 ml of scintillation fluid (Opti-Fluor, Packard Bioscience Company, NL). Radioactivity was quantified using a liquid scintillation counter (Wallac 1410, Pharmacia, Uppsala, Sweden).

In order to analyse the time course of AMF effect, DU-145 and LNCaP cells were incubated for 72 and 96 h. To explore effects of short exposures to AMF, these cell lines were incubated for 30 min, 1, 2, 4, 8, 12, 24, and 48 h. After washing twice with RPMI, cells were maintained for 48 h followed by [^{3}H]-thymidine staining and quantification.

Cell cycle analysis. DU-145 and LNCaP ($1x10^5$ cells/well) were exposed to 1, 2.5, and 5 μM of AMF in 6-well, flat-bottomed plates for 48 h. Cells were collected, washed with PBS, and stained with propidium jodide using CycleTest Plus, DNA Reagent Kit (Becton Dickinson, San Jose, CA). Cell cycle status was analysed on a Becton Dickinson Flow Cytometer (FACS Calibur). Gating strategies using FL3-W channel were applied in order to exclude doublets from the analysis.

Western blot analysis. DU-145 and LNCaP cells were incubated with 3 different concentrations of AMF (1, 2.5, 5 μM) for 48 h in medium containing 3% steroid-free FCS and 1% glutamine. Cells were then collected and washed twice with PBS. In all experiments, whole cell extracts were used. For this purpose, samples were resuspended in lysis buffer [20 mM NaH_2PO_4, 1 mM EDTA, 10% glycerol, 0.1 nM PMSF, 0.5 nM NaF, 0.5% Protease Inhibitor Cocktail Sett III (Calbiochem, Darmstadt, Germany) Phosphatase Inhibitor Cocktail 2 (Sigma, St. Louis, MO)] and stored for 30 min on ice. Protein amount was determined according to Bradford (25). For electrophoresis, aliquots were diluted in NuPAGE LDS sample buffer (Invitrogen, Leek, The Netherlands) according to the manufacturer's instructions. After sonication and boiling for 10 min at 70˚C, lysates were loaded onto 4-12% Bis-Tris gels and run for 1.5 h at 150 V with NuPAGE running buffer. The proteins were then transferred to nitrocellulose membranes (Invitrogen) with the Xcell blot module for 1.5 h at 30 V with NuPAGE transfer buffer. After the transfer, membranes were washed once with PBS for 5 min, blocked for 1 h at room temperature using blocking buffer (LiCor Biosciences, Lincoln, NE), and incubated with the respective primary antibody overnight at 4˚C. After four washes with TBST, the membranes were incubated with the respective secondary antibody for 1 h light-protected at room temperature and then washed four times with TBST and once with TBS. The antibodies were diluted as follows: anti-cdk 2 1:100, anti-cdk 4 1:100, anti-cyclin E 1:100, anti-p27 1:50, anti-ß-actin 1:8000, anti-cyclin D1 1:200, anti-phospho-Erk1/2 1:1000, anti-Erk1 and -Erk2 1:2000, anti-phospho-Akt1 1:1000, and anti-Akt1 1:100. Secondary anti-mouse, -rabbit and -goat antibodies were diluted 1:5000. Western blots were developed using Odyssey Infrared Imaging System (LiCor Biosciences, Lincoln, NE). As a control for equal protein loading, Bradford test and Western blot for ß-actin were performed.

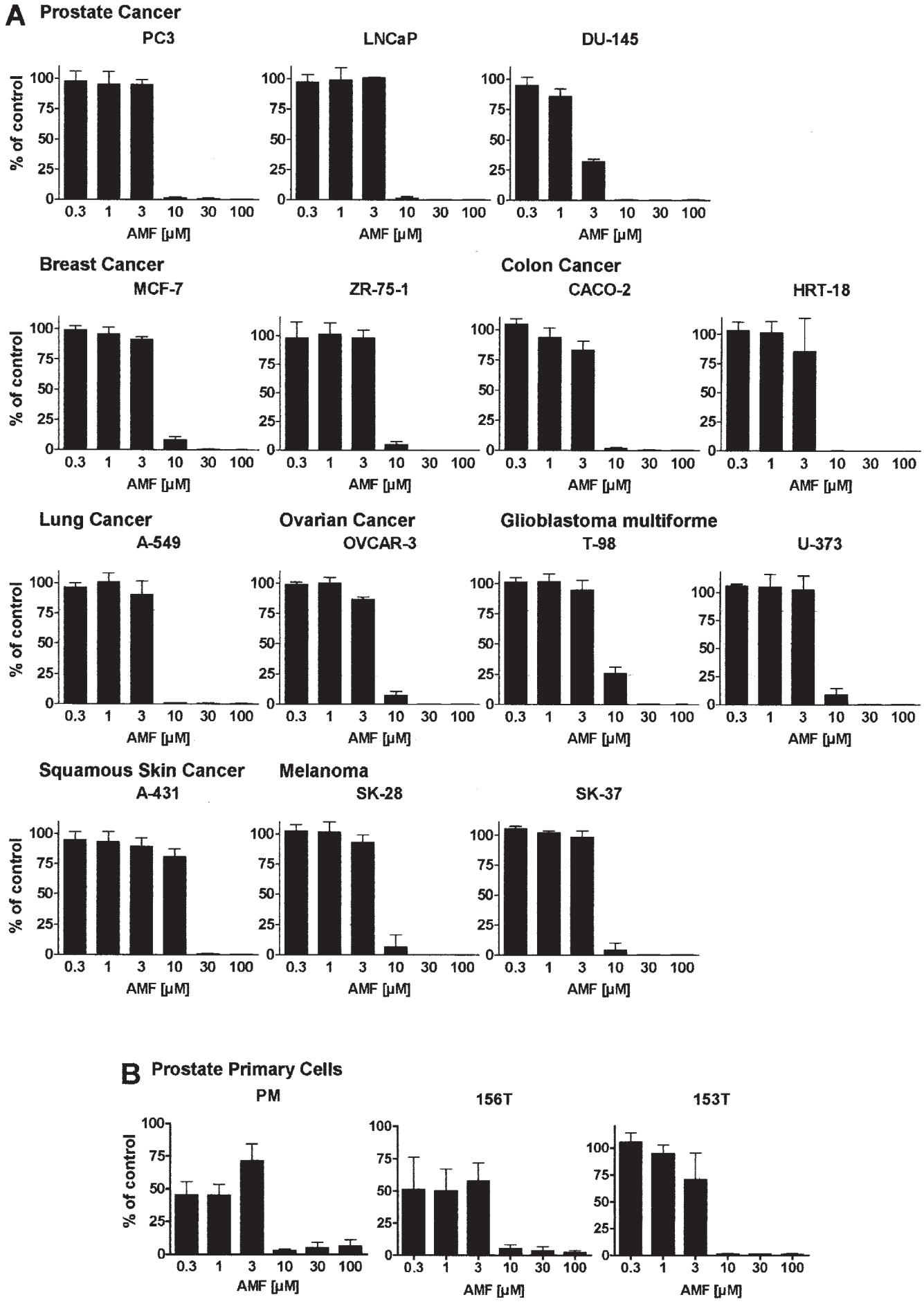
To study short-time effects of 5 μM of AMF on phosphorylation of signal transduction molecules, DU-145 and LNCaP cells were incubated with 5 μM of AMF for 5 and 15 min prior to Western blot analysis. To exclude effects of FCS on phosphorylation, the same short exposure experiments we performed under serum-free conditions.

Statistical analyses. All statistical analysis were performed using SPSS 12.0 (SPPS, Chicago, IL) and Graphpad Prism 4.0 (Graphpad Software, San Diego, CA). To analyze [^{3}H]-thymidine assays, Student's t-tests were performed. Welch's Correction was applied when variances were statistically significantly different. Dunnett-T-Test was performed to compare untreated controls with treated cells in Western blots and flow cytometric experiments. P-values <0.05 were defined as statistically significant.

Results

Effect of AMF on cellular proliferation. We examined the antiproliferative activity of AMF ($3x10^{-7}$-10^{-4} M) on immortalized prostate primary muscle and epithelial cells and various solid cancer cell lines, including those of prostate, breast, melanoma, brain, colon, ovary, lung, and skin (Fig. 1A and B). This screening was performed using the [^{3}H]-thymidine incorporation assay after a 48-h exposure to AMF. The squamous skin cancer cell line (A-431), which is known to be insensitive to therapeutic concentrations of topotecan, cisplatin, etoposide, and paclitaxel (26), was found to be less sensitive with a significant reduction of incorporation after treatment with 30 μM of AMF. Treatment with 10 μM AMF significantly reduced proliferation of breast (MCF-7, ZR-75-1), intestinal (CACO-2, HRT-18), lung (A-549), ovarian (OVCAR-3), prostate (PC-3, LNCaP), melanoma (SK-28, SK-37), and glioblastoma cells (U-373, T98). The androgen-insensitive AR-negative human prostate cancer cells DU-145, which are derived from a brain metastasis, were most sensitive. There was a significant inhibition of proliferation at a 3-fold lower concentration (3 μM) than in other tested cells. In order to compare the influence of AMF on malignant and benign cells, we also tested the antiproliferative effect on the benign prostatic cell line BPH-1, which showed a similar sensitivity as PC-3 and LNCaP cells (data not shown). Comparing the response to 3 μM of AMF of malignant DU-145 cells and primary cells, the latter were about 25% less sensitive ($p=0.034$). Interestingly, these cells could not be totally inhibited even with the highest concentrations of AMF (10-100 μM) (Fig. 1B).

[^{3}H]-thymidine-incorporation assays were carried out after long (48-96-h) and short (30-min - 48-h) incubation periods to analyse the time-dependency of AMF effects in prostate cancer cell lines DU-145 and LNCaP. Experiments after an exposure time of 48 h revealed a similar antiproliferative activity as that observed in initial screening assays, while incubation for longer time periods (72, 96 h) did not lead to additional effects in inhibition of proliferation (Fig. 1C). To further investigate the time course of AMF-mediated antiproliferative activity on DU-145 and LNCaP, cells were exposed to AMF for a period between 30 min and 48 h, washed twice, and maintained for another 48 h. In DU-145 cells, a short exposure time (30-min - 4-h) yielded a time- and dose-dependent antiproliferative effect. After 30-min, 1-, 2-, and 4-h incubation with 10 μM of AMF, [^{3}H]-thymidine uptake was ~75, 55, 35, and 12% of control, respectively. Treatment longer than 4 h had no additional antiproliferative effect, meaning that 4 h were sufficient to cause a reduction in proliferation to the similar extent as observed after 48 h. In LNCaP cells, exposure times from 4 to 48 h caused a time- and dose-dependent inhibition of [^{3}H]-thymidine incorporation. After 4-, 8-, 12-, and 24-h incubation with 10 μM of AMF, we measured 80, 65, 50, and 25% of control values, respectively. Comparing DU-145 and LNCaP cells, we clearly show a time- and dose-dependent effect of AMF in both cell lines (Fig. 1C). While cell growth is rapidly inhibited in DU-145 cells (30 min - 4 h), growth of LNCaP cells is slowed down after longer incubation periods (4-48-h).


A Prostate Cancer
PC3
LNCaP
DU-145
Breast Cancer
MCF-7
ZR-75-1
Colon Cancer
CACO-2
HRT-18
Lung Cancer
A-549
Ovarian Cancer
OVCAR-3
Glioblastoma multiforme
T-98
U-373
Squamous Skin Cancer
A-431
Melanoma
SK-28
SK-37
% of control
AMF [µM]
B Prostate Primary Cells
PM
156T
153T

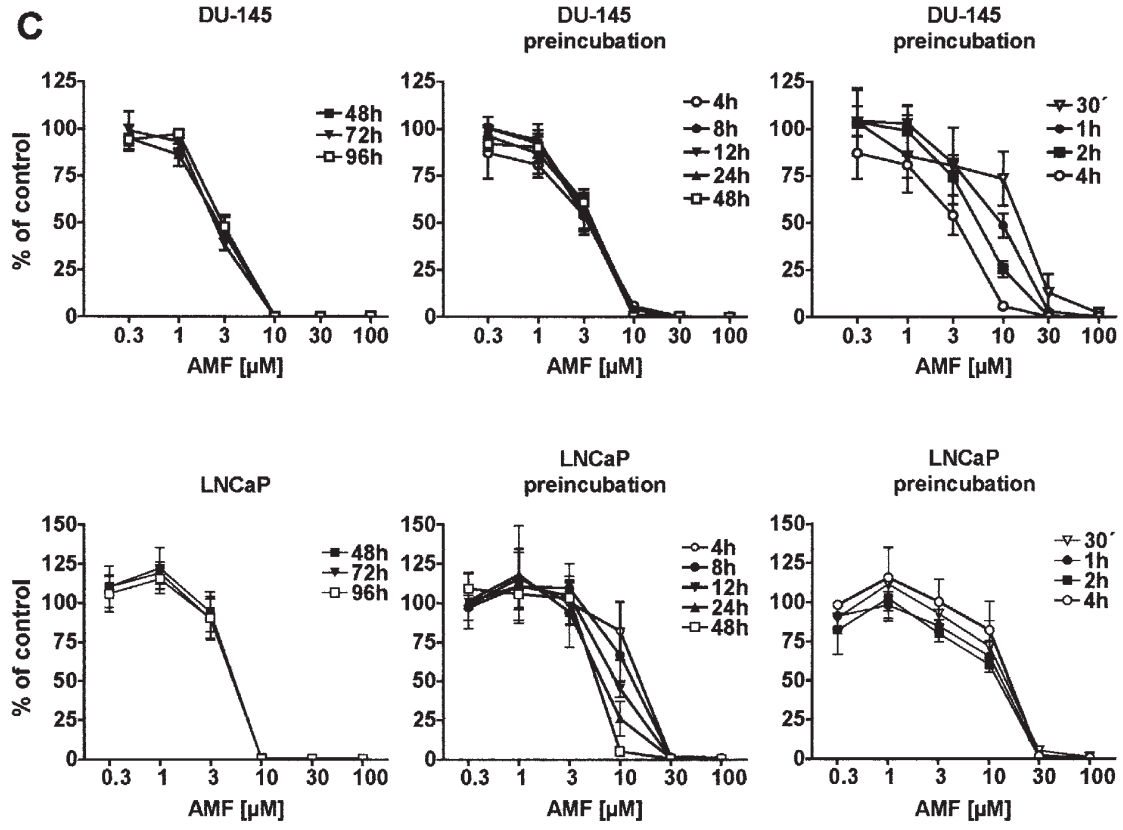


Figure 1. Effect of Akacid on the growth of various tumor cell lines *in vitro*. A variety of tumor cell lines (A), including those derived from prostate (LNCaP, PC3, DU-145), breast (MCF-7, ZR-75-1), glioblastoma multiforme (T-98, U-373), intestinum (CACO-2, HRT-18), lung (A-549), ovary (OVCAR-3), melanoma (SK-28, SK-37) and squamous skin cancer (A-431) and primary prostate epithelial and smooth muscle cells (PM, 156T, 153T) (B) were treated with increasing concentrations of Akacid ($3x10^{-7}$-10^{-4} M) for 48 h and proliferation was measured by [^{3}H]-thymidine incorporation assay. Results represent the mean ± SD of at least three independent experiments carried out in triplicates. In (C), the time-dependence of the effect of Akacid on DU-145 and LNCaP cells is shown. Cells were either incubated for 48-96 h with of Akacid (B, first plot) or preincubated for 30 min to 48 h and then maintained for 48 h in the recommended medium. Finally, [^{3}H]-thymidine assay was performed. Results represent the mean ± SD of at least three experiments in triplicates.

Effect of AMF on cell cycle distribution and expression of cell-cycle regulatory molecules. In order to investigate the effect of AMF on cell cycle distribution in DU-145 and LNCaP cells, flow cytometric analysis using propidium iodide staining was performed after a 48-h exposure to 1, 2.5, and 5 μM of AMF. We show that AMF leads to an S-phase reduction in a dose-dependent manner (Fig. 2). In DU-145 cells, the percentage of cells in S-phase decreased at 2.5 and 5 μM AMF to 80.83±3.74% ($p<0.0001$) and 76.63±5.95% of control ($p<0.0001$), respectively. LNCaP cells were more sensitive. Treatment with 1, 2.5, and 5 μM of AMF reduced S-phases to 89.13±4.16%, 38.1±32.64% ($p<0.0001$), and 42.5±18.46% ($p<0.0001$), respectively.

To elucidate the molecular mechanisms of AMF-mediated inhibition of DU-145 and LNCaP cell proliferation, we investigated its possible effects on the expression of cell-cycle regulatory molecules. Cells were incubated with increasing concentrations of AMF (1-5 μM) for 48 h, collected, lysated, and subsequently analysed for expression of cdk 2, cdk 4, cyclins E and D1, and p27 proteins (Fig. 3).

We show that both cdk 2 and cdk 4 expression are significantly reduced at a concentration of 5 μM of AMF (DU-145: cdk 2: 48.84±14.02% of control, $p<0.0001$; cdk 4: 51.41±17.48% of control, $p<0.0001$) (LNCaP: cdk 2: 46.82±12.79% of control, $p<0.0001$; cdk 4: 33.14±12.85% of control, $p<0.0001$). In DU-145 cells, cyclin E expression was 70.57±5.59% at 5 μM of AMF ($p=0.0162$), when compared to control cells, while in LNCaP cells we found an unexpected significant ($p<0.0001$) increase to 181.52±9.01 and 161.82±21.42% of control after treatment with 2.5 and 5 μM of AMF, respectively. In both cell lines, a significant reduction was found for cyclin D1 expression. After 48-h incubation with 1, 2.5, and 5 μM of AMF, we quantified a mean of 81.04±1.27% ($p=0.0025$), 60.46±12.38% ($p<0.0001$), and 40.56±6.33% of control ($p<0.0001$) in DU-145 cells. LNCaP cells similary responded to AMF treament showing a reduction of 88.01±20.43% (n.s.), 91.63±21.25% (n.s.), and 54.57±15.08% ($p<0.0001$) of control at 1, 2.5, and 5 μM of AMF, respectively. Expression of the cell-cycle inhibitor p27 did not significantly differ from that in control DU-145 cells, whereas we found a substantial increase in LNCaP cells. Treatment with 2.5 and 5 μM of AMF yielded an increase to 175.34±36.91% ($p=0.0013$) and 176.89±34.4% of control ($p=0.0011$), respectively. This finding could at least partially explain the results of cell cycle analysis in LNCaP cells.

Effect of AMF on signaling pathways. The two major kinase-cascade related signaling pathways that are involved in

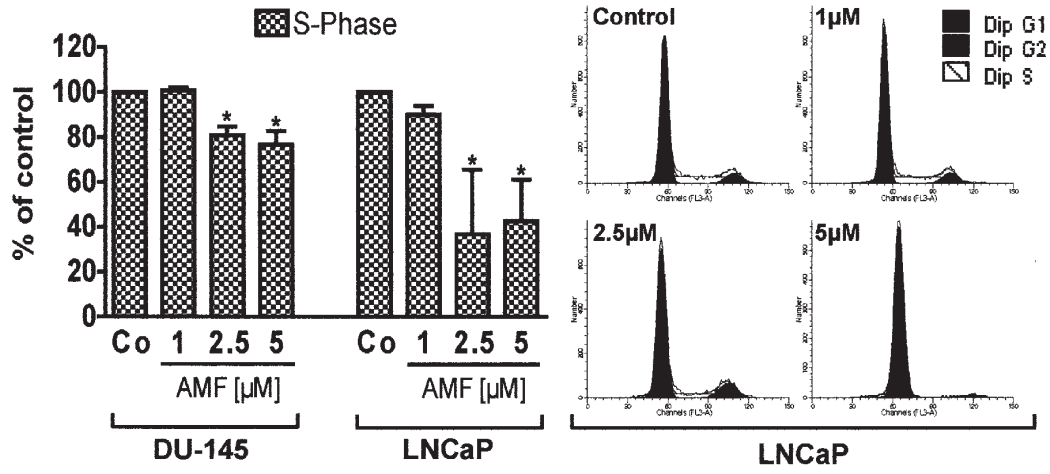


Figure 2. Effect of Akacid on cell cycle distribution (S-phase) in DU-145 and LNCaP cells. After incubation with 1, 2.5, and 5 μM of Akacid for 48 h, cells were collected, propidium jodide staining was performed, and cell cycle distribution was analysed using flow cytometry. Results represent means ± SD of five independent experiments performed in duplicates.

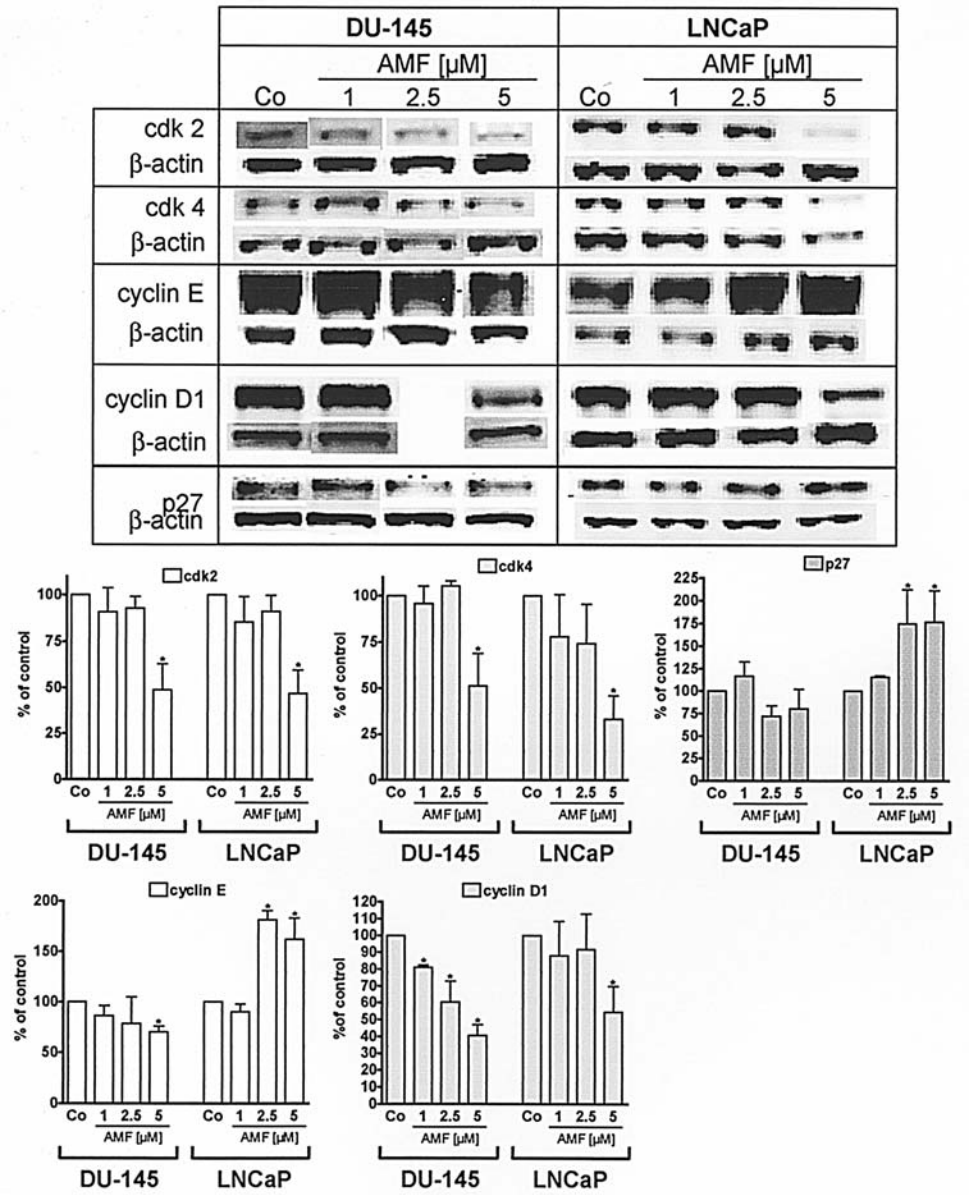


Figure 3. Effect of Akacid on expression of cell cycle regulatory molecules in androgen-independent DU-145 cells and androgen-dependent LNCaP cells. Cells were incubated with 1, 2.5, and 5 μM of Akacid for 48 h. Cell lysates were harvested and probed for protein expression of cdk 2, cdk 4, cyclin E, cyclin D1, and p27. The amount of protein was normalized to levels of ß-actin. Means ± SD of three (cdk 2, cyclin D1, cyclin E), four (p27) or five (cdk 4) experiments are shown.

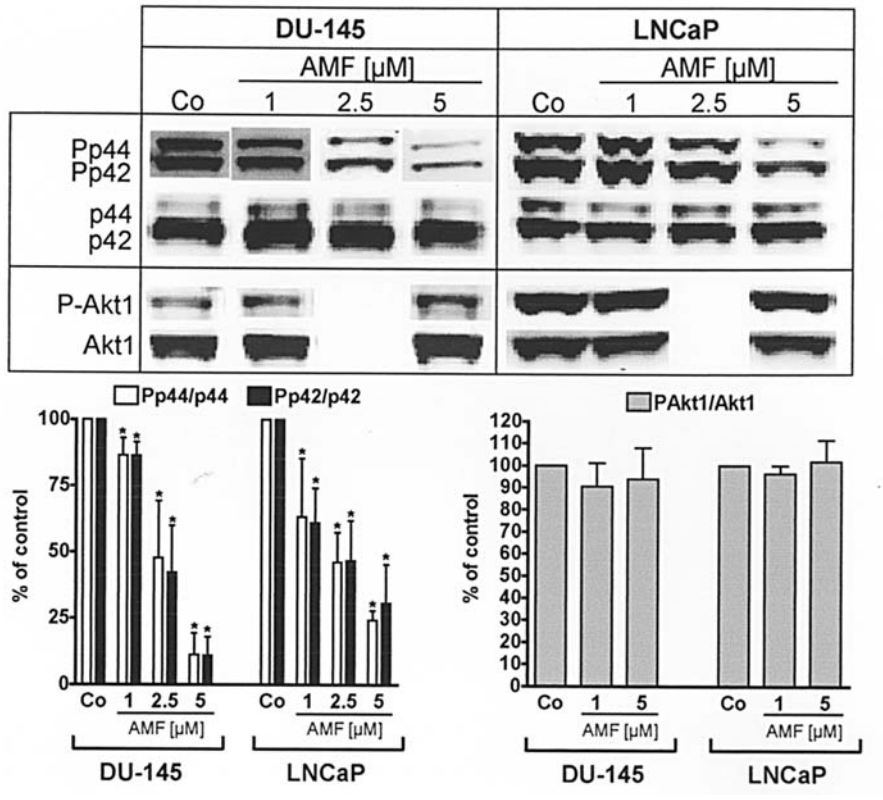


Figure 4. Effect of Akacid on expression of signaling pathways in DU-145 and LNCaP cells. Cells were cultured with 1, 2.5 and 5 μM of Akacid. After 48 h of treatment, cell lysates were made, and probed with antibodies for Pp44/Pp42, p44/p42, PAkt1, and Akt1. Results represent the mean ± SD of three (p44, p42) or four (Akt1) independent experiments.

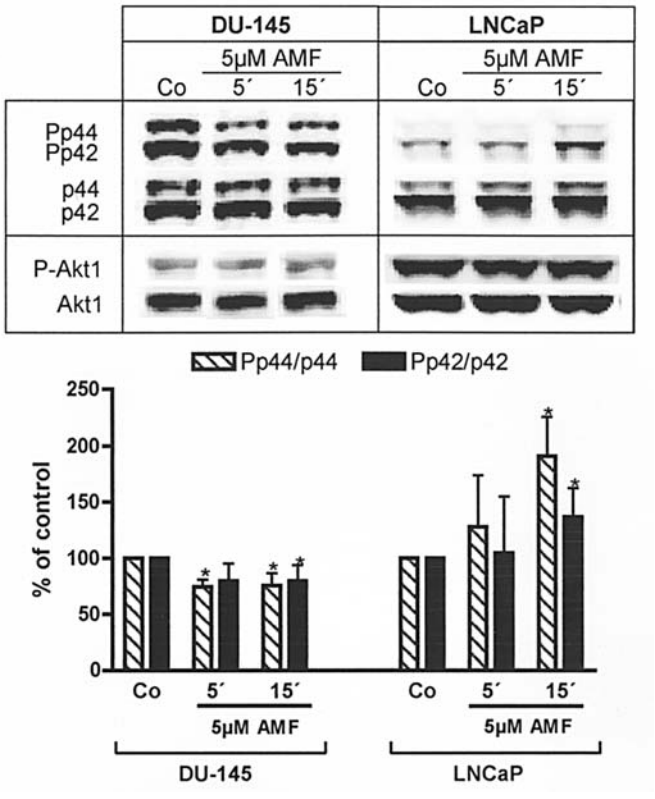


Figure 5. DU-145 and LNCaP cells were incubated with 5 μM of Akacid for 5 and 15 min. After Western blotting of cell lysates the antibodies against phospho- and non-phospho p44, p42, and Akt1 were used. Means ± SD of six (p44, p42) independent experiments are shown. Representative Western blot analyses show protein expression levels of (phospho-)MAPKs and -Akt1.

progression of prostate cancer are the Ras/Raf/MAPKs (Erk 1/2) and the PI3K/Akt pathway.

In our experiments, we found that expression of phosphorylated MAPKs (p44, p42) was reduced by AMF in a dose-dependent manner after 48 h. In DU-145 cells, 1, 2.5, and 5 μM of AMF decreased expression of phosphorylated Erk1/Erk2 to 86.61±6.37% (n.s.)/86.28±5.17% (n.s.), 47.77±21.63% (p=0.0061)/42.28±17.78% (p=0.0001), and 11.34±8.1% (p=0.0001)/11.01±7.04% of control (p<0.0001), respectively. An equal effect of AMF was observed in LNCaP cells, which showed a reduction of phospho-Erk1/Erk2 expression to 63.26±21.94% (p=0.001)/60.97±13.17% (p=0.0003), 46.23±11.11% (p<0.0001)/46.81±15.01% (p<0.0001), and 24.42±3.5% (p<0.0001)/30.78±14.51% of control (p<0.0001), respectively. Phosphorylation status of Akt1 remained unchanged after 48 h of incubation in both cell lines (Fig. 4).

To further study the influence of AMF on the phosphorylation of Erk1/2, and Akt1, DU-145 and LNCaP cells were incubated with 5 μM of AMF for 5 and 15 min (Fig. 5). Phosphorylation of Erk1 and Erk2 decreased after 5 (DU-145: Erk1: 74.43±6.58%, p=0.0009; Erk2: 79.7±15.52%, n.s.) and 15 min (DU-145 Erk1: 75.59±11.06%, p=0.001; Erk2: 79.7±13.86%, p=0.0483) while phosphorylation of Akt1 remained unchanged. To exclude possible FCS-mediated effects, the same experiments were repeated under serum-free conditions with DU-145 cells (data not shown). Phosphorylation of Erk1 and Erk2 was found to be unchanged after 5 min. However, levels significantly lower than those in experiments with FCS (Erk1: 64.89±19.59%, p=0.001; Erk2: 57.64±9.89%, p=0.0001) were measured after 15 min. We

did not find any alteration in Akt1 phosphorylation in LNCaP cells. Unexpectedly, phosphorylation status of MAPKs was increased after 5 and 15 min of treatment in that cell line.

Discussion

Guanidine-based oligomeres exert a high biocidal activity against a broad range of microorganisms while showing a low toxicity to humans (1,2). In the present study, we demonstrate for the first time the inhibitory effect of AMF, a novel biocidal oligoguanidine, on proliferation of 14 human malignant cancer cell lines, including those representing prostate, breast, intestinal, lung, skin and ovarian cancer, melanoma, and glioblastoma. Higher doses of AMF were required for inhibition of growth of A-431 cells, which are known to be resistant to therapeutic concentrations of topotecan, cisplatin, etoposide or paclitaxel (26). In a recent study, genomic rearrangements in A-431 cells, in particular amplification of the c-myc and the cyclin D1 genes and loss of the TP53 tumor suppressor gene were revealed (27).

Interestingly, the human androgen-insensitive AR-negative prostate cancer cell line DU-145 was most sensitive to AMF, showing a decrease in [^{3}H]-thymidine incoporation at a concentration as low as 3 μM. Furthermore, we studied the effect of AMF on primary cells representing prostatic muscle and epithelial tissue. Our experiments revealed a lower sensitivity to AMF compared to malignant cell lines, in particular at concentrations in which the latter were totally inhibited (10-30 μM). In this context it is of interest that other authors published that antiproliferative or proapoptotic substances, that do not target a specific receptor, exert their inhibitory effect at even higher concentrations. Garikapaty and co-workers recently reported that diindolylmethane, a synthetic dimer of indole-3-carbinol, downregulates cyclin D1, cdk 4, and androgen receptor and has its IC_{50} at 50 μM (28). Another group reported that the garlic compound diallyl disulfide causes antiproliferative and proapoptotic effects on LNCaP and PC3 cells at concentrations ranging from 20 to 50 μM (29,30). Due to the fact that AMF showed a pronounced effect at lower concentrations, we focused on prostate cancer cell lines (LNCaP, DU-145). DU-145 and LNCaP cells show differences in androgen-dependence and aggressiveness. Our results demonstrate that AMF-induced growth suppression is not restricted to a single prostate cancer cell line.

Growth inhibitory effects of AMF are associated with changes in expression of cell cycle regulatory proteins. In this context, a dose-dependent reduction of cyclin D1, cdk 2, and cdk 4 by AMF may represent an underlying mechanism of the anti-tumor effect of the drug. Yim *et al* showed that, in DU-145 cells, treatment with high micromolar concentrations of a coumarin compound decursin yielded a decrease in cdk 2, cdk 4, cdk 6, and cyclin D1 expression and a reduction of percentage of cells in S-phase (31). Similar data were obtained in DU-145 cells with the antibiotic geldanamycin, a drug whose target is the chaperone heat shock protein 90 (32). At present, molecular mechanism of the geldanamycin effect, in particular requirement of functional tumor suppressor retinoblastoma, is not fully understood (33).

Cdks and cyclins play a pivotal role in controling progression through the cell cycle (34). Cdk 4 - cyclin D complexes and cdk 2 - cyclin E complexes govern the transition through the G1 phase of the cell cycle. It was shown that inhibition of cyclin D1 expression results in cell-cycle arrest, whereas moderate overexpression accelerates G1 phase progression (35-37). Cyclin D1, whose expression is increased in prostate cancer cell lines and clinical tumors, is causally related to tumorigenesis (38). *In vivo* cyclin D1 overexpression in LNCaP cells yielded faster tumor growth and insensitivity to castration (39). It was also shown that cyclin D1 expression correlates with metastatic spread of prostate cancer to bone (40).

In contrast to AMF-induced inhibition of cyclin D1 expression in both prostate cancer experimental models, cyclin E regulation in DU-145 and LNCaP is dissimilar. These results contrast with those obtained with chemopreventive agents for prostate cancer, such as vitamin E succinate, that decreases expression of both cyclins (41). However, clinical evidence for cyclin E overexpression in prostate cancer is lacking (42). Although investigation of possible interactions between AMF and the androgen signaling pathway is beyond the scope of the present study, it should be mentioned that cyclin D1 and E regulate AR activity in different ways. Cyclin D1 is known as a corepressor of the AR, whereas cyclin E potentiates receptor function in the presence of ligand (43,44). We also noted cell type-specific differences in regulation of expression of the tumor suppressor p27 by AMF. Its up-regulation was observed solely in LNCaP cells.

Although there is a number of novel approaches to improve prostate cancer treatment, tumor heterogeneity is a considerable limitation in clinical practice (45). Metastatic lesions even within the same patient show differences in morphology, immunophenotype, and genotype. One of potential strategies for prostate cancer therapy is an interference with the EGF signaling. Sgambato *et al* reported that, in prostate cancer cell lines LNCaP, DU-145, and PC-3, inhibition of the epidermal growth factor receptor (EGFR) by ZD1839 (Iressa) reduces proliferation and induces cell cycle arrest (46). The anti-proliferative effects of flutamide or irradiation were potentiated in combination with ZD1839. However, EGFR could be targeted only in a subgroup of prostate cancer patients (47). Use of drugs such as AMF that do not target a single molecule might be therefore justified.

AMF exerts its inhibitory effect at least in part through inhibition of phosphorylation of MAPK. Activation of MAPK in normal prostate epithelium, benign prostate hyperplasia, and early stage prostate cancer occurs upon paracrine stimulation by growth factors. However, in late stage prostate cancer, TGF-α (48) and EGF (49) stimulate prostate cancer cell growth in an autocrine manner. Activation of MAPK was shown to correlate with tumor progression (19). Furthermore, an increasing body of evidence indicates that MAPK signaling is involved in the regulation of cell motility, which is a prerequisite for tumor metastasis (50). Zelivianski and colleagues evinced that inhibition of MAPK signaling in TRPCa cells leads to an enhanced apoptosis rate after docetaxel treatment (51). Inhibition of MAPK signaling may thus represent a novel approach for the treatment of TRPCa. Therefore we hypothesize that AMF is a potential substance for combination with cytotoxic chemotherapeutics with other cellular targets (e.g. cisplatin, docetaxel). Our experiments,

performed after short-time AMF treatment, show a down-regulation of phospho-MAPKs in DU-145 cells while an upregulation was found in LNCaP cells. This finding in androgen-sensitive prostate cancer cells was suprising. However, it should be kept in mind that activation of MAPK in different cell types may lead to pleiotropic effects. Pinelli *et al* demonstrated that fumonisin B1 leads to an increasing MAPK phosphorylation in short-time experiments, whereas p-MAPK levels were reduced after 24 h (52). Fumonisin B1 induces apoptosis and inhibits the proliferation of several cell types, including those from kidney, liver or skin (53).

In conclusion, our data show a dose- and time-dependent antiproliferative activity of the biocidal oligoguanidine AMF on various solid cancer cell lines, with the highest potency against the prostate cancer cell lines DU-145 and LNCaP. In these cells, low micromolar concentrations of AMF reduced expression of the essential cell cycle regulatory molecules cyclin D1, cdk 2, and cdk 4 and the MAPKs Erk1/Erk2. The antiproliferative effect of AMF was confirmed by cell cycle analysis which showed a reduction of cells in S-phase to a maximum extent of 58%. Collectively these data and the fact that primary prostate cells were less sensitive compared to malignant prostate cancer cell lines may provide the rationale for further studies on the possible anticancer activity of oligomeric guanidines and AMF in particular.

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