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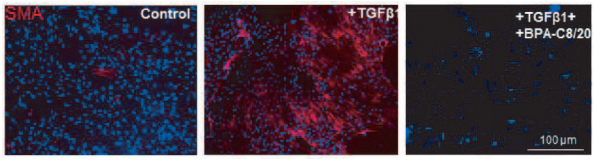

Don't start! (But do go on) TGF-β1 stimulates the conversion of fibroblasts to smooth muscle α-actin (SMA)-positive myofibroblasts. The polyamine BPA-C8 inhibits this conversion, but has no effect on cells that already express SMA. It also reduces the occurrence of an extracellular matrix around the activated fibroblasts. BPA-C8 could thus interfere with fibroblasts' effect on tumor progression.

WILEY-VCH

DOI: 10.1002/cbic.201402087

Synthetic Polyamine BPA-C8 Inhibits TGF-β1-Mediated Conversion of Human Dermal Fibroblast to Myofibroblasts and Establishment of Galectin-1-Rich Extracellular Matrix in Vitro

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Cancer-associated fibroblasts (CAFs) play a role in the progression of malignant tumors. They are formed by conversion of fibroblasts to smooth muscle α-actin-positive (SMA-positive) myofibroblasts. Polyamines are known to change the arrangement of the actin cytoskeleton by binding to the anionic actin. We tested the effect of the synthetic polyamine BPA-C8 on the transition of human dermal fibroblasts to myofibroblasts induced either by TGF-β1 alone or by TGF-β1 together with adhesion/growth-regulatory galectin-1. Pre-existing CAFs, myofibroblasts from pancreatitis, and rat smooth muscle cells were also exposed to BPA-C8. BPA-C8 impaired myofibroblast formation from activated fibroblasts, but it had no effect on cells already expressing SMA. BPA-C8 also reduced the occurrence of an extracellular matrix around the activated fibroblasts. The reported data thus extend current insights into polyamine activity, adding interference with tumor progression to the tumor-promoting processes warranting study.

Introduction

The level of complexity within human tumors can be likened to an ecosystem.[1] As has been established in culture systems, cancer cells can thrive in vivo in microenvironments suited to support cell proliferation, locally invasive properties, and propensity for distant metastasis.[2] Moreover, such a so-called niche maintains functionality of cancer stem cells.[3] On the cellular level, a variety of cell types, especially inflammatory cells and cancer-associated fibroblasts (CAFs), are counted among the major constituents that shape the growth-favoring surroundings of tumor cells through the action of a series of secreted factors, such as cytokines, chemokines or growth factors, together with the extracellular matrix.[4] CAFs are myofibroblast-like cells characterized by presence of smooth muscle α-actin (SMA).[5] Rather similar cells are also present in healing wounds, where they are involved in wound contraction, and in fibrotic tissues or organs.[6,7]

Literature data indicate that CAFs can arise from local fibroblasts, pericytes, endothelial cells, and macrophages, and also probably by epithelial to mesenchymal transition from cancer cells.[4,8,9]

Transforming growth factor-β1 (TGF-β1) has been identified as a molecular switch for the generation of myofibroblasts as CAFs from normal fibroblasts in vitro.[10] Another molecular mediator, the endogenous adhesion/growth-regulatory lectin galectin-1 (Gal-1), with activity stronger than those of galectins-3, -4, and -7,[11,12] is able to enhance the effect of TGF-β1 on fibroblasts.[13] CAFs are strong activators of growth and migration for many types of cancer cells.[5] We have previously demonstrated that CAFs isolated from human basal carcinomas of the skin and squamous cell (head and neck) carcinomas have a significantly different gene expression profile from normal fibroblasts, including upregulated transcription of genes coding for protumoral factors such as interleukin-6.[14–16] CAFs are able to modify the in vitro characteristics of normal epithelial cells to make them similar to cancer cells and to influence the phenotype of breast cancer cells of an established line.[17–19]

As one of many biochemical parameters, the production of polyamines is significantly increased in malignant tumors of

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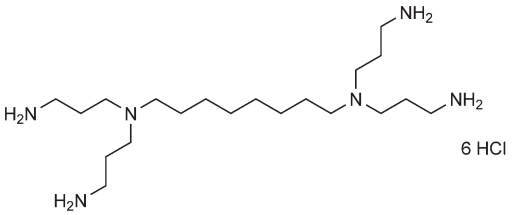
different nature. 3T3 fibroblasts, transfected with cDNA for ornithine decarboxylase, thereby increasing the production of polyamines, acquired properties of transformed cells. Correspondingly, deactivation of this enzyme reduced the malignant potential of cancer cells.[20,21] On the molecular level, polycations, including polyamines of different nature and structure, become engaged in F-actin cytoskeleton formation, namely by bundling F-actin fibers.[22,23] These substances then even shift the actin status to building F-actin lamellipodia in 3T3 fibroblasts.[24] The toxic plant-derived polyamine pavetamine has a destructive effect on the actin cytoskeletons of cardiomyocytes, leading to heart failure in ruminants.[25] Conversely, inhibition of natural polyamine synthesis by blocking arginase improved wound healing, accompanied by a high incidence of myofibroblasts in granulation tissue in mice.[26]

Given this context and the described process of transformation into a salient tumor-promoting factor in the microenvironment, the question of the impact of polyamines on formation of myofibroblasts is addressed here. We tested the effect of the synthetic polyamine BPA-C8 on the level of generation of



BPA-C8 hexahydrochloride

myofibroblasts from normal human dermal fibroblasts (HFs) either with TGF-β1 alone or with the combination of TGF-β1 and Gal-1. The results obtained were compared with data from experiments performed with CAFs isolated from a squamous cell (head and neck) carcinoma, myofibroblasts from chronic pancreatitis, and smooth muscle cells from rat aorta, all exposed to BPA-C8, thereby also providing information on BPA-C8's effects on cell viability.

Results and Discussion

Number of cells and Ki67 positivity in cultured HFs

BPA-C8 reduced the number of HFs when it was applied to fibroblast cultures at a concentration of 100 μM (Figures 1A and 2A). At 20 μM, no notable effect was seen. TGF-β1 alone or combined with Gal-1 had a strong stimulatory effect on fibroblast growth. In this experimental setting, both tested concentrations of the polyamine—that is, 20 μM and 100 μM—significantly reduced the number of HFs stimulated by TGF-β1 with or without Gal-1. On comparing these data with levels of the proliferation marker Ki67, the proportion of cells with positive nuclei was fairly constant under all experimental conditions (Figures 1B and 2B).

Influence of BPA-C8 on conversion of HFs to myofibroblasts

To set the baseline, all primary HF cultures were immuno-cytochemically monitored for SMA presence; no more than five SMA-positive cells were found on the coverslips (Figure 1B). Supplementing the culture medium with TGF-β1 resulted in the expected formation of myofibroblasts from the HFs. This process was further enhanced by adding Gal-1. The experimental conditions were thus set for the assays, and both concentrations of BPA-C8—that is, 20 μM and 100 μM—completely precluded the occurrence of SMA-positive cells.

Influence of BPA-C8 on the establishment of extracellular matrix fibers rich in Gal-1 and tenascin

As a biochemical aspect of TGF-β1 activity, we next tested the effect of the polyamine on production of an extracellular matrix, characterized by presence of tenascin and Gal-1. Immunofluorescence analysis revealed that HFs produced only negligible quantities of extracellular matrix in relation to cells exposed to TGF-β1, both without and with Gal-1. Both Gal-1 and tenascin were detected in this meshwork on probing for a matrix-presented effector and a typical proteoglycan.

The matrix generation was conspicuously sensitive to the presence of BPA-C8 (Figure 1C).

Evaluation of the levels of senescence and the ratios between viable and dead cells

Application of TGF-β1 together with Gal-1 to fibroblast cultures increased the number of senescence-associated β-galactosidase-positive cells in sites with high cell density. When the medium also contained BPA-C8, the extent of positivity was reduced (Figure 3). It is also apparent that more dead cells were detected in this case than in that of the BPA-C8-free cultures.

Influence of BPA-C8 on smooth muscle cells from the aorta of the rat, CAFs isolated from squamous cell carcinoma, and myofibroblasts from chronic pancreatitis

Smooth muscle cells (genuinely positive for SMA), CAFs, and myofibroblasts were not sensitive to exposure to BPA-C8. Clearly, no effect on the expression of SMA was observed in these cell types after culture in the presence of the polyamine at the two concentrations (20 and 50 μM) tested (Figure 4). Establishment of an extracellular matrix rich in fibronectin was also not sensitive to polyamine treatment (Figure 4B, C).

The key finding of this study is the observation that the polyamine BPA-C8, when added to the culture medium of TGF-β1-exposed human dermal fibroblasts, disrupts the program of myofibroblast formation. This effect appears to be accompanied by a reduction of cell viability, with indications of dose dependency. A decrease in cell number was observed in control fibroblasts exposed to 100 μM of the polyamine, whereas even as little as 20 μM led to this effect in TGF-β1-treated cells. This activity of BPA-C8 does not appear to be related to suppression of proliferative activity, because the presence of the

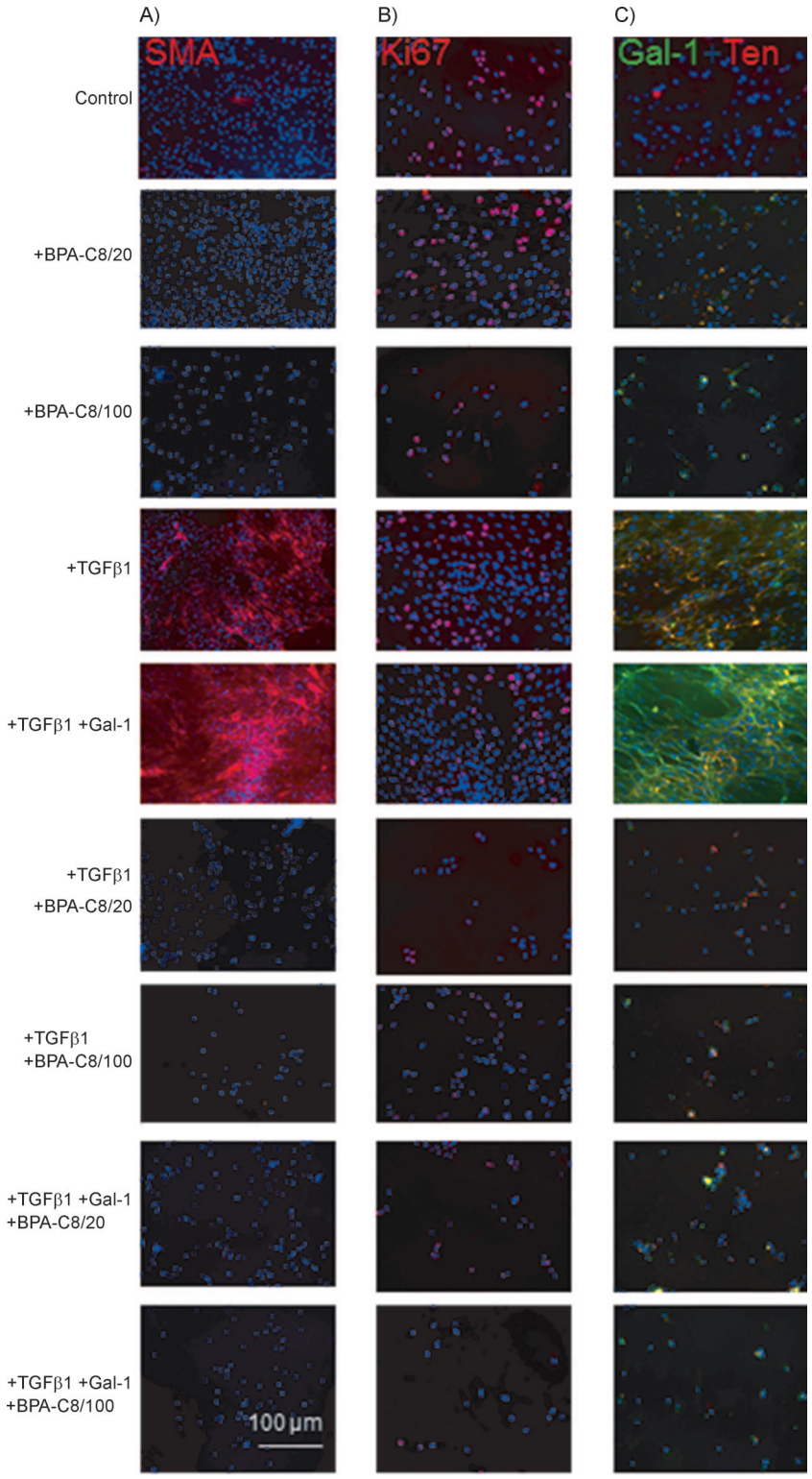


Figure 1. Immunocytochemical detection of A) α-smooth muscle actin (SMA, red signal), B) the proliferation marker Ki67 (red signal) and C) galectin-1 (Gal-1, green signal) and tenascin (Ten, red signal) in normal human fibroblasts (control) and in fibroblasts exposed both to BPA-C8 at concentrations of 20 μM (BPA-C8/20) and 100 μM (BPA-C8/100) and to TGF-β1, either alone or in combination with Gal-1. Nuclei were counterstained with DAPI (blue signal). Illustrations document that TGF-β1, both alone and together with Gal-1, stimulated conversion of fibroblasts into myofibroblasts (A). This process was blocked by both concentrations of BPA-C8 (A). The level of proliferation was markedly decreased by the high concentration of BPA-C8 (100 μM) under all experimental conditions, and also by the 20 μM concentration when used in combination with TGF-β1 (B). Production of tenascin and Gal-1 was increased by TGF-β1, a process impaired by BPA-C8 (C).

compound is not able to affect the occurrence of Ki67-positive (proliferating) cells significantly. With regard to cellular targets, the actin monomer is an anionic protein, and its polymerization is prone to be disturbed by polycations.[27] Introducing a synthetic polyamine into this system can therefore inhibit the formation of SMA-based fibers, a process that can be fatal for the cell. In addition, targeting actin (and other polyanions) might account for further biochemical consequences, a reduction in the extent of generation of extracellular matrix. The production of this molecular meshwork (with strong positivity for Gal-1 and tenascin, forming a scaffold suited to the presentation of mediators such as lectins) by CAFs appears to be relevant for tumor progression.[28,29] With regard to at Gal-1, the best-studied member of this lectin family,[12,30] contact of T-cells to (thymic) stromal cells, a source of the lectin, or Matrigel loaded with Gal-1 triggers the apoptotic elimination of the defense cells.[31] The induction of T-cell apoptosis, in a caspase-3-dependent manner, by Gal-1 from tumor-associated stroma obtained from a patient with cutaneous T-cell lymphoma required p56lck/ZAP70 tyrosine kinases.[32] Similarly, Gal-1 presentation in skin can at least contribute to the accumulation of $CD4^+$ $CD7^-$ T-cells (resistant to Gal-1-dependent apoptosis induction through CD7 ligation) during progression of the Sézary syndrome,[33] Gal-1 presence showed a negative correlation to $CD45^+$ lymphocytes in laryngeal squamous cell carcinomas,[34] and stromal (not tumor) presence of a galectin was an unfavorable prognostic marker in breast cancer.[35]

Exploiting new insights from tumor biology to devise innovative means to set limits to tumor progression is the driving force

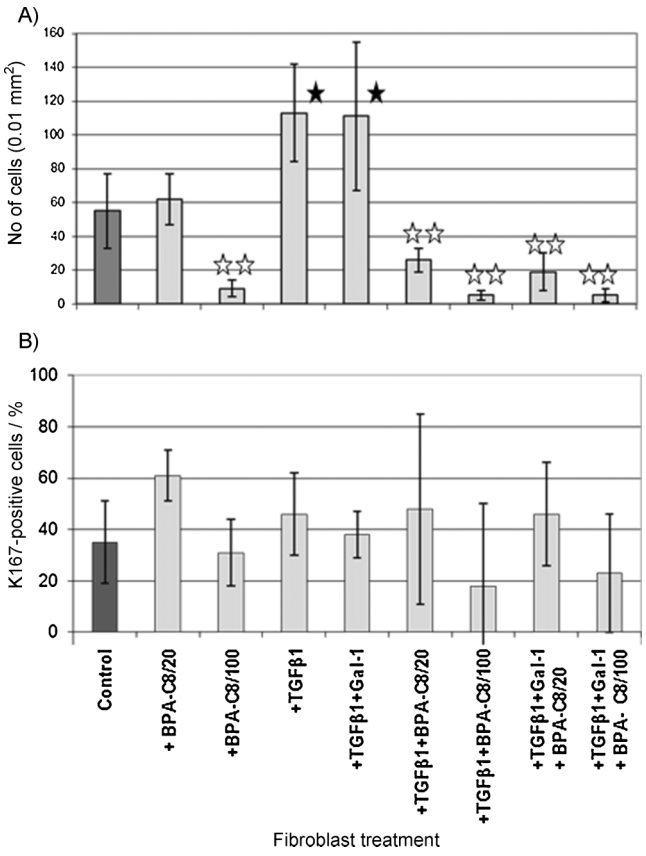


Figure 2. A) TGF-β1 (alone or in combination with Gal-1) increased the cell number, whereas BPA-C8/100 significantly reduced this parameter. The stimulatory effect of TGF-β1 was neutralized by BPA-C8 at both tested concentrations. B) Percentages of marker-positive (Ki67) cells under all tested conditions. Experimental measurements significantly higher than those for the control (gray column) with $p < 0.05$ are each marked with a black asterisk, and those significantly lower than those for the control with $p < 0.01$ by two open asterisks. All data were processed by use of Student's paired t-test after examination of 50 view fields in technical triplicate. For abbreviations, see the legend to Figure 1.

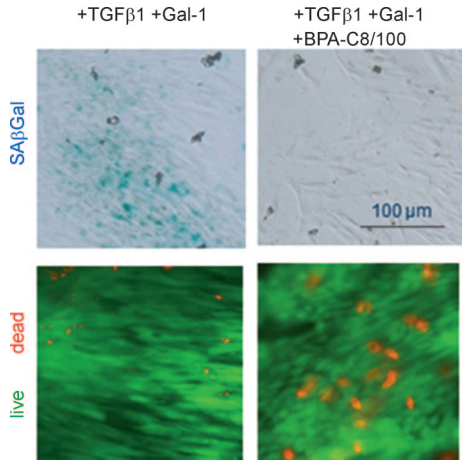


Figure 3. Cytochemical detection of senescence-associated β-galactosidase (SAβGal, blue signal) revealed higher levels in cultures of stimulated cells than in cells additionally exposed to 100 µM of BPA-C8 (top). The ratio of dead cells (red/orange signal) to viable cells (green) was shifted by BPA-C8 in the direction of dead cells (bottom).

behind therapeutic advances.[36] Although a mainstream approach of the pharmaceutical industry is focused predominantly on attacking cancer cells, the risk of invasion and spread, with contributions from the microenvironment, should also be taken into consideration.[37] Along these lines, targeting CAFs or their interaction with cancer cells involving the surrounding matrix with presented effectors can open a promising route. Our control observation that BPA-C8 did not affect the SMA status of smooth muscle cells allays immediate concerns for a negative impact on vessels. It appears warranted to envision further applicability tests on hypertrophic/keloid scars.

Conclusions

A challenge for synthetic chemistry is to develop new reagents to interfere with the effect of CAFs on tumor progression, an increasingly documented factor in this process. The polyamine BPA-C8 has a strong inhibitory effect on the transformation of fibroblasts into myofibroblasts. It also inhibits production of extracellular matrix. On the other hand, it has only negligible effect on SMA-positive cells, such as smooth muscle cells or pre-existing myofibroblasts from chronically inflamed tissue.

Experimental Section

Preparation of BPA-C8 polyamine: The branched polyamine BPA-C8 was prepared by treatment of acrylonitrile with octane-1,8-diamine to afford the corresponding tetranitrile. Reduction of this with Raney nickel afforded BPA-C8 as a light yellow oil. It was converted into, and stored as, its hexahydrochloride salt, as described in detail previously.[24]

Preparation of Gal-1: Human Gal-1 was obtained by recombinant production and purified by affinity chromatography on lactosylated Sepharose 4B as crucial step, routinely followed by the removal of any lipopolysaccharide contamination.[38] Product analysis was performed 1) for purity, by one- and two-dimensional gel electrophoresis, gel filtration, and mass spectrometric fingerprinting, as well as 2) for activity, through hemagglutination, cell surface binding, and induction of anoikis.[39–42]

Preparation of cells

Primary culture of normal human fibroblasts (HFs): HFs were freshly prepared by taking advantage of free migration from small fragments of the dermis from the skin of a breast specimen obtained from the Department of Aesthetic Surgery of the 3rd Faculty of Medicine (Charles University, Prague, Czech Republic) with informed consent of the donor and the local ethical committee according to the Declaration of Helsinki. They were cultured in Dulbecco's modified Eagle's medium (DMEM, Biochrom, Berlin, Germany) supplemented with fetal bovine serum (FBS, Biochrom, Berlin, Germany, 10%) at 37 °C and under CO_2 (5%).

Primary culture of squamous cell CAFs and fibroblasts from chronic pancreatitis: CAFs were prepared from a specimen of a human laryngeal squamous cell carcinoma obtained from the Department of Otorhinolaryngology and Head and Neck Surgery of the 1st Faculty of Medicine (Charles University). A fibroblast population rich in myofibroblasts was prepared from human chronic pancreatitis of a tissue specimen received from the 1st Department of Surgery of the 1st Faculty of Medicine (Charles University) according to the

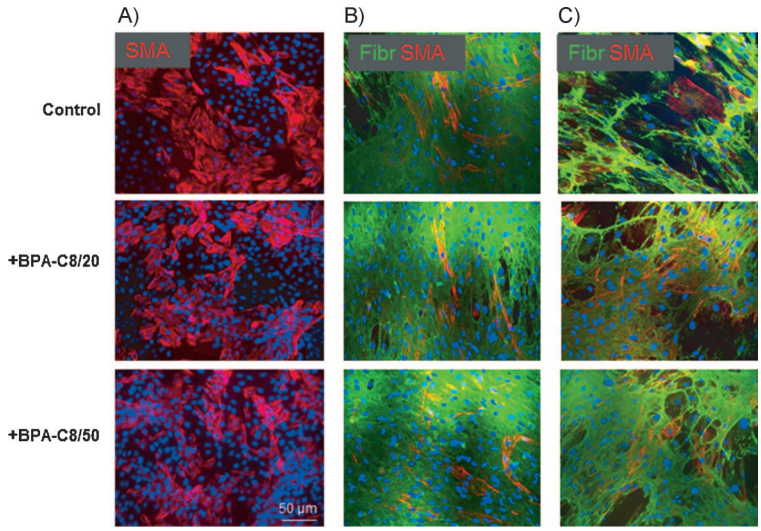


Figure 4. Immunocytochemical detection of SMA (red/orange signals) A) in rat smooth muscle cells, B) in CAFs from a squamous cell carcinoma, and C) in myofibroblasts from chronic pancreatitis, as well as of fibronectin (green signals) in cultures of CAFs (B) and myofibroblasts (C), control without the test substance and tests in the presence of 20 µM and 50 µM of BPA-C8, respectively. Nuclei were counterstained with DAPI.

ethical standard given above. Both types of cells were prepared by previously published protocols.[12,14]

Primary culture of smooth muscle cells from rat aorta: Smooth muscle cells of a degree of about 80% purity were freshly prepared and provided by Dr. Lucie Bačáková (Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic) as described,[43] and then cultured under the same conditions as used for the other cell preparations used in this study.

Culture of stimulated fibroblasts with BPA-C8 polyamine: Fibroblasts were inoculated at a density of 800 cells cm^{-2} on glass coverslips placed in 6-well dishes (TPP, Trasadingen, Switzerland) and further cultured in DMEM, variously with no additions, after addition of TGF-β1 (10 ng mL^{-1}, Sigma–Aldrich), and after addition of TGF-β1 (10 ng mL^{-1}) and Gal-1 (300 ng mL^{-1}) as described.[11] BPA-C8 was tested at final concentrations of 20 µM and 100 µM as before in assays on lamellipodial growth.[24] Cultures without addition of BPA-C8 were used as control. Cells were cultured at 37 °C under CO_2 (5%) for one week; medium was changed every 48 h.

Culture of CAFs, myofibroblasts from chronic pancreatitis, and smooth muscle cells with PBA-C8 polyamine: All three types of cells were inoculated at a density of 2500 cells cm^{-2} in DMEM on glass coverslips placed in 6-well dishes. After 24 h, the medium was changed, and DMEM containing BPA-C8 (20 or 50 µM) was added. Cells kept in DMEM were cultured in parallel as control. All cells were maintained at 37 °C under CO_2 (5%) for one week; medium was changed every 48 h. Coverslips were washed with phosphate-buffered saline (Biochrom), dried, and stored at −20 °C for cytochemical processing.

Immunocytochemistry: Cells were briefly fixed with paraformaldehyde (4%, *w/v*) at pH 7.2 and washed in phosphate-buffered saline solution. The specimens were routinely processed for detection of α-smooth muscle actin (DAKO, Glostrup, Denmark), the proliferation marker Ki67 (DAKO), and tenascin (Sigma–Aldrich) with specific mouse monoclonal antibody preparations diluted as recommended by the supplier. Fibronectin presence was visualized with a specific rabbit polyclonal antibody (DAKO). Gal-1 was localized with a home-made polyclonal antibody (rigorously tested for presence of crossreactive fractions against other human galectins, which were then removed chromatographically), applied at 20 µg mL^{-1}.[28,44] Swine-anti rabbit immunoglobulin G labeled with fluorescein isothiocyanate (FITC, DAKO) and tetramethylrhodamine isothiocyanate (TRITC)-labeled goat anti-mouse immunoglobulin G (Sigma–Aldrich) were used as second-step reagents. Nuclear DNA was visualized with 4′,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich). Controls for specificity were performed by omission of the specific antibodies from processing and by their replacement with irrelevant isotypic antibodies or preimmune serum. Specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA) and then inspected with an Eclipse 90i fluorescence microscope (Nikon) equipped with filter blocks for FITC, TRITC, and DAPI and a Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany); microphotographs were analyzed with a LUCIA 5.1 computer-assisted image analysis system (Laboratory Imaging, Prague, Czech Republic).

Evaluation of levels of senescence and cell death: To determine levels of senescence and death in the culture cells, commercial staining kits (CS0030-1KT and 04511, Sigma–Aldrich) were used.

Acknowledgements

This study was supported by the Grant Agency of the Ministry of Health of the Czech Republic (No. 13488-4), by Charles University (projects PRVOUK-27-1, UNCE 204013), by a Specific University Research grant from the European Regional Development Fund BIOCEV (No. CZ.1.05/1.1.00/02.0109), and by the EC FP7 program (grant agreement no. 317297(GLYCOPHARM)). G.K. thanks the CNRS and the University of Strasbourg for postdoctoral financial support. The authors are grateful to Dr. Lucie Bačáková of the Institute of Physiology of the Academy of Sciences of the Czech Republic for her generous gift of smooth muscle cells, as well as to Marie Jindráková and Radana Kavková for their excellent technical assistance.

Keywords: actin · cancer · galectin · polycations · tenascin

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Received: March 10, 2014
Published online on May 27, 2014