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Final Draft
of the original manuscript:

Guenther, S.; Ruhe, C.; Derikito, M.G.; Boese, G.; Sauer, H.; Wartenberg, M.:

Polyphenols prevent cell shedding from mouse mammary cancer spheroids and inhibit cancer cell invasion in confrontation cultures derived from embryonic stem cells

In: Cancer Letters (2007) Elsevier

DOI: 10.1016/j.canlet.2006.09.014

Polyphenolspreventcellsheddingfrommousemammarycancerspheroi dsandinhibit cancercellinvasioninconfrontationculturesderivedfromembryonic stemcells

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Keywords: multicellulartumorspheroids,embryonicstemcells,reactiveoxygenspeci es, matrixmetalloproteinase,metastasis

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Abstract

The prognosis of cancer disease is worsened upon shedding of tumor cells from the primary tumor, which escape to the bloodstream and form metastases at distant sites within the body. Inhibition of cell shedding from the primary tumor could therefore be exploited to avoid metastasis and delay the progession of the cancer disease. In the present study we investigated the effects of the polyphenols resveratrol, baicalein, epicatechin, epigallocatechin and polyphenon 60 on cell shedding from multicellular tumor spheroids of the murine mammacarcinoma cell line 4T1, cell invasion into embryonic stem cell-derived tissues, generation of reactive oxygen species (ROS) and expression of matrix metalloproteinase 9 (MMP-9). With increasing tumor spheroid growth MMP-9 expression was upregulated and cells detached from tumor spheroids and formed subspheroids that displayed pronounced ROS generation. Upon incubation with polyphenols tumor growth was arrested and cell shedding was totally abolished. Polyphenol treatment decreased ROS generation and downregulated MMP-9 expression. Furthermore, polyphenols significantly inhibited invasion of tumor cells into embryonic stem cell-derived, vascularized tissues. Our data suggest that polyphenons inhibit cell shedding and invasion by their antioxidative capacity and downregulation of MMP-9 expression.

Introduction

Tumor metastasis is a multistep process by which a subset or individual cancer cells disseminate from a primary tumor to distant secondary organs or tissues. The process of metastasis follows a distinct sequence of events which are not yet known in detail. For a tumor cell to evade from the primary tumor and form a metastase at a distinct site within the body, numerous interactions with the extracellular matrix (ECM), growth factors and cytokines associated with the ECM, basement membranes, endothelial cell lining of the vasculature, blood cells in the circulation, and the microenvironment at the secondary site are required [1]. A primordial role in tumor metastasis has been attributed to MMPs which display altered expression in different human cancers. Generally, elevated expression of MMPs in primary tumors and/or metastases is associated with cancer progression, poor prognosis and shorter survival times. Especially, high serum levels of MMP-9 are found in melanoma patients displaying secondary metastasis. Plasma concentration and activity of MMP-2 and -9 have been recently shown to be elevated in patients with breast disease, breast cancer and at risk of developing breast cancer [2]. In an experimental model for spontaneous metastasis of rat mammary carcinoma, serum and plasma levels of MMP-9 were associated with the development of metastases in the lung and lymph nodes [3]. Conversely treatment of cancer patients as well as laboratory animals with MMP inhibitors have been demonstrated to reduce cancer metastasis in numerous studies.

The mechanisms by which cancer cells escape from the primary tumor are currently not well known due to limited technical possibilities to visualize and experimentally modulate this process. MMPs are presumably critically involved in degradation of intra-tumorous basement membranes, thereby loosening cell-cell contacts which facilitates migration and intravasation of tumor cells into the blood or lymphatic vasculature. The entrance of a primary tumor into the metastatic state may be regulated by ROS which are robustly generated in rapidly growing tumor cells [4] and are involved in tumor cell cycle regulation

[5, 6]. ROS are likewise involved in the regulation of MMP expression. This is especially important in malignant breast cancer, where estrogen metabolism is altered, favoring the accumulation of hydroxyestradiols, which can generate free radicals. These reactive species can activate matrix metalloproteinases (MMPs), which in turn can hydrolyze the proteins of the extracellular matrix (ECM) that act as a barrier to tumor cell passage [7]. Consequently, downregulation of several MMPs was observed upon overexpression of gamma-glutamylcysteinesynthetase (gamma-GCSh) cDNA which encodes a rate-limiting enzyme in the biosynthesis of glutathione (GSH), a major physiological redox regulator [8], and antioxidant treatment of mammary tumor-bearing mice suppressed tumor growth and inhibitedMMPactivity[9].

The present study was undertaken to evaluate the potency of flavonoids baicalein, epicatechin, epigallocatechin, polyphenon 60 and resveratrol to inhibit cell shedding from multicellular mammary tumor spheroids and and tumor cell invasion into confrontation cultures of tumor spheroids and vascularized tissues derived from ES cells. Flavonoids are a family of polyphenolic compounds found ubiquitously in fruits and vegetables as well as in food products and beverages derived from plants [10]. Resveratrol, a natural polyphenolic phytoalexin found in seeds, grapes (Vitis vinifera) and in some medicinal plants is a potent dietary antioxidant which plays an important role in protecting against pathological events of oxidativediseases,suchascardiovasculardiseasesandcancer[11-13].

Confrontationculturesoftumorspheroidswith ES cellsareideallysuitedforstudies oftumor-inducedangiogenesisandtumorcellintravasationandhavebeenpreviouslyutilized byustoinvestigatetheanti-angiogeniceffectsofplantingredientsusedintraditionalchinese medicine [14]. It is shown that growing 4T1 tumor spheroids upregulate MMP-9 expression and shed highly ROS-generating tumor cells from their surface. Spheroid growth, tumor cell intravasation and migration is abolished in the presence of polyphenols presumably due to theircapacitytoactasanti-oxidants.

Materials and Methods

Materials

Baicalein, resveratrol, epicatechin, epigallocatechin and polyphenon 60 were provided by Sigma (Taufkirchen, Germany) and used in concentrations of 1, 10 and 100 µM. NS-398 was observed from Calbiochem (Bad Soden, Germany). Stock solutions were prepared from DMSO solutions of the substances. The final concentration of DMSO in cell culture media did not exceed 0.1 %. The MMP-2 / MMP-9 inhibitor V ((4-(4-(Methanesulfonamido)phenoxy)phenylsulfonyl)methylthiirane (SB-3CT)) was obtained from Calbiochem and used in a final concentration of 1 µM.

Evaluation of tumor spheroid growth

Diameters were determined from tumor spheroids which remained untreated or were treated with polyphenols as described above. The growth of tumor spheroids was evaluated every 24 h with a standard microscope. Approximately 200 spheroids were used in each experiment.

ES cell culture

The ES cell line CGR8 was cultured in feeder-free cell culture on gelatine-coated cell culture dishes in Iscove's medium (Biochrom, Berlin, Germany). Medium was supplemented with 20% heat-inactivated FCS (Sigma, Taufkirchen, Germany), 2 mM glutamine (Biochrom), 100 µM ß-mercaptoethanol (Sigma), 1% nonessential amino acids stock solution (Biochrom), LIF 10^3 units/ml (Chemicon, Hampshire, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (both Biochrom) in a humidified environment containing 5% CO_2 at 37° C and passaged every 2 to 3 days. At day 0 of differentiation, adherent cells were enzymatically dissociated using 0.2% trypsin and 0.05% EDTA in PBS (Gibco) and seeded at a density of $1 \cdot 10^7$ cells/ml in siliconized spinner flasks (Integra Biosciences, Fernwald, Germany) containing 100 ml of

Iscove's medium supplemented with the same additives as described above without LIF supplement. After 24 h, 150 ml of medium was added to give a final volume of 250 ml. The spinner flask medium was stirred at 20-22.5 rpm using a stirrer system (Integra Biosciences), and 150 ml of cell culture medium was exchanged every day.

Spinner-Culture Technique for Cultivation of multicellular tumor spheroids

The mouse mammary tumor cell line 4T1 was used throughout the study. The cell line was grown routinely in 5% CO_2/humidified air at 37° C with Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FCS (Gibco), 2 mM glutamine (Biochrom), 100 µM ß-mercaptoethanol (Sigma), 2 mM nonessential amino acids stock solution (Biochrom), 100 Units/ml penicillin and 100 µg/ml streptomycin (both Biochrom). Spheroids were grown from single cells. Cell monolayers were dissociated enzymatically with 0.2% trypsin/0.05% EDTA (Gibco) and seeded in 250 ml spinner flasks as described previously [15]. For the experiments, tumor spheroids with mean diameters of approximately 350-450 µm were used.

Generation of confrontation cultures

For the generation of confrontation cultures, multicellular tumor spheroids and embryoid bodies were removed from spinner flasks. One embryoid body (5 to 6 days old) and one tumor spheroid (4-5 days old) were inoculated to a 40 µl drop of mixed culture medium (50% spheroid medium, 50% embryoid body medium) placed onto the lid of a 10 cm Petri culture dish. After adding 20 to 30 drops to the lid of the Petri dish, the lid was turned around and placed on the Petri dish, which was filled with 10 ml of sterile PBS. Within 48 h, embryoid bodies and tumor spheroids closely attached within the hanging drops and were then subsequently used for incubation experiments with the polyphenolic agents.

Confocal Laser Scanning Microscopy

Fluorescence recordings were performed by means of a confocal laser scanning setup (LSM 510, Carl Zeiss, Jena, Germany) connected to an inverted microscope (Axiovert 200M, Zeiss). The confocal setup was equipped with a 5mW helium/neon laser, with single excitation of 633 nm (excitation of Cy5), a 0.5 mW helium/neon laser, with single excitation of 543 nm (excitation of Cy3), and an argon laser, with single excitation of 488 nm. Emission was recorded using the long pass filter sets LP570, RG665, and LP515, respectively. A 5x or 10x numerical aperture objective was used.

Long-Term Labelling of Multicellular Tumor Spheroids

To discriminate tumor spheroids grown in confrontation culture from embryoid bodies, tumor spheroids were labelled with the long-term cell tracker dye 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes, Eugene, OR). In brief, tumor spheroids were incubated for 1 h in DMEM that contained 10 µM CMFDA (stock solution 10 mM, dissolved in DMSO). Spheroids were then washed and incubated for further 24 hours in bacteriological Petri dishes. Subsequently, the labelled tumor spheroids were used in co-culture experiments. Stable CMF fluorescence was observed for more than 5 days of tumor spheroid culture [16]. Fluorescence excitation was performed by the 488 nm line of an argon-ion laser of the confocal setup. Emission was recorded using a long pass LP515-nm filter set.

Determination of intracellular ROS levels

Intracellular ROS levels were measured using the fluorescence dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes, Eugene, OR USA), which is a nonpolar compound that is converted into a non-fluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. H_2DCF is membrane impermeable and rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF)

in the presence of intracellular ROS. For the experiments, multicellular tumor spheroids were incubated for 48 h with the tested polyphenols. Subsequently they were immersed in E1 medium containing NaCl (135 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgCl_2$ (1 mM), glucose (10 mM), HEPES (10 mM), (pH 7.4 at 23°C), and 20 µM H_2DCFDA dissolved in DMSO was added. The intracellular DCF fluorescence (corrected for background fluorescence) was evaluated in 5 min intervals over 30 minutes.

Dead cell staining of multicellular tumor spheroids

To assess the cytotoxicity of polyphenols, dead cell staining was performed by the use of the dead cell stain Sytox®green (Molecular Probes, Eugene, OR). In brief, tumor spheroids treated with polyphenols were incubated for 30 min in 10 ml cell culture medium containing 0.5 µM Sytox®green, which is cell membrane impermeable and intensively stains the cell nuclei of dead cells with compromised cell membranes after intercalation into DNA. Sytox®green fluorescence was excited by an argon-ion laser of the confocal setup. Emission was recorded using a 515-nm longpass filter set.

Calculation of surface roughness

Cells shedding from the surface of multicellular tumor spheroids increases surface roughness to higher values. The surface roughness parameter was determined by division of the diameter of the tumor spheroid through the circumference of the tumor spheroid. A perfectly even surface would result in a surface parameter = 1. Rough surfaces arising from cell shedding accordingly result in higher values.

Immunohistochemistry for flow cytometry

Immunohistochemistry was performed with disaggregated tumor spheroids (trypsin/EDTA treatment for 20 min at 37°C). The Intrastain® Kit (Dako Cytomation, Dako, Hamburg,

Germany) was used according to the protocols supplied by the manufacturer. As primary antibodies the polyclonal anti-mouse Ki-67 (Abcam, Cambrigde, UK) (dilution 1:50) was used. Incubation with primary antibody was performed for 30 min at room temperature. As secondary antibody an Cy3-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany) was used. An incubation at room temperature for 30 minutes in the dark followed. FACS analysis was performed by the use of a FC500 Flow Cytometer (Beckman Coulter, Krefeld, Germany).

Isolation of RNA

All isolation steps were performed at room temperature. A 100 µl cell pellet of multicellular tumor spheroids was transferred to 2 ml tubes and centrifuged at 80 *g* for 2 min. After removal of the supernatant 1000 µl Trizol (Invitrogen) was added and the pellet was homogenized. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and quantitative real time PCR gene expression profiles of MMP-2 and MMP-9 were monitored by RT-PCR and quantified with the means of real-time PCR. Isolated total RNA was reverse-transcribed using random hexamere-primers by use of MMLV RT (Invitrogen, Germany). cDNA aliquots corresponding to 50 ng total RNA were semi-quantitatively analyzed using sense and antisense primers of the respective genes and analyzed with the real time PCR (Applied Biosystems 7500) with the SYBR®-Green Method (Qiagen). The used sequence for sense and antisense primers are listed below. Real time PCR was performed in the presence of sense and antisense primers for the housekeeping gene α-polymerase. The relative gene expression of the examined genes was normalized to the expression of the housekeeping gene via the ΔΔCt-method.

mMMP9:	sense:5´-CGTCGTGATCCCCACTTACT-3´

	antisense:5´-AACACACAGGGTTTGCCTTC-3´

Statistical analysis

Student´s t test for unpaired data was applied as appropriate. A value of $P < 0.05$ was considered significant.

Results

Tumor cell shedding and formation of subspheroids from multicellular 4T1 mammary breast cancer cells

The present study was undertaken to investigate mechanisms of metastasis in multicellular tumor spheroids grown from 4T1 mouse mammary cancer cells. 4T1 cells resemble in their growth characteristics and invasiveness to that of stage IV breast cancer cells in humans. Cell shedding from tumor spheroids has been previously attributed to metastatic potential [17]. Primary tumors from 4T1 metastasize as early as 2 weeks after inoculation to lungs, liver, bone and brain [18]. To correlate the occurrence of the metastatic state of 4T1 tumor spheroids with spheroid size, tumor spheroid diameters were monitored over cell culture time. During the first 7 days in cell culture tumor spheroids displayed an exponential growth characteristics which subsequently passed over into a growth plateau. With the onset of the plateau phase of tumor spheroid growth the roughness of the tumor spheroid surface increased and single cells shedded into the cell culture medium and formed subspheroids (see Fig. 1). Gaussian distribution of 4T1 spheroid diameters at different time of cell culture indicated an appearance of small sub-spheroids from day 8 on which resulted in a two-peaked histogram of mother spheroids and subspheroids during prolonged cell culture (Fig. 2).

Generation of reactive oxygen species in 4T1 tumor spheroids

The metastatic switch of tumors may be related to the redox state of specific tumor cell subsets. We therefore investigated intracellular ROS levels at different times of cell culture in mother spheroids, detaching cells at the periphery of the mother spheroids and in free subspheroids after detachment from the mother spheroid (Fig. 3A, n = …). It was observed that intracellular ROS levels did not change in mother spheroids during the time course of cell culture. Conversely, significantly elevated ROS levels were observed in detaching cells and subspheroids, suggesting that cell shedding is related to increased ROS generation in cells at the periphery of large 4T1 multicellular tumor spheroids. To investigate the source of ROS generated in 4T1 tumor spheroids (day 8-9 of cell culture), the tissues were preincubated for … min with the NADPH oxidase inhibitors DPI (25 µM) and apocynin (10 µM) as well as with the cyclooxygenase-2 (COX-2) inhibitor NS-398 (25µM) (Fig. 3B, n= 4). Preincubation with NADPH oxidase inhibitors resulted in a significant decrease in DCF fluorescence, whereas no effect was observed with the NS-398, which indicates that ROS generation in 4T1 multicellular tumor spheroids is mainly mediated via NADPH oxidase activity.

Expression of MMPs during the growth of 4T1 multicellular tumor spheroids

MMPs have been attributed to play a major role in tumor metastasis [19]. Among the different MMPs, MMP-2 and MMP-9 are of primordial significance for the onset of the metastatic cascade and tumor-induced angiogenesis. To investigate the involvement of MMP-2 and MMP-9 in cell shedding of 4T1 multicellular tumor spheroids, gene expression studies were performed (Fig. 4, n = …). A continuous increase in *MMP-9* expression occurred with increasing culture time of 4T1 multicellular tumor spheroids (see Fig. 4), whereas no change in *MMP-2* expression was observed (data not shown), which indicates that MMP-9 may be responsible for the process of cell shedding from multicellular tumor spheroids.

Effect of polyphenol treatment on cell proliferation in 4T1 multicellular tumor spheroids

Polyphenols have been demonstrated to interfere with the progression of cancer either by exerting direct anti-proliferative effects on tumor cells and/or by interfering with the metastatic cascade [20]. In the present study the anti-proliferative effects of baicalein (1, 10, 100 µM), resveratrol (1, 10, 100 µM), polyphenon 60 (10 µM), epicatechin (10 µM) and epigallocatechin (10 µM) was investigated. All applied polyphenols inhibited tumor spheroid growth, when applied during 5 days of tumor spheroid culture (day 5 - day 10) (Fig. 5 *A*; n = 3). None of the substances exerted direct toxic effects as evaluated by Sytox green dead cell staining (data not shown). However, when the expression of the proliferation marker Ki-67 was assessed by FACS analysis, it was apparent that treatment of 4T1 multicellular tumor spheroids significantly decreased the number of Ki-67 positive cells from 45 ± 1.34% in the untreated control to 11 ± 9.05 % when tumor spheroids were treated with 100 µM baicalein and 8.2 ± 1.33% when spheroids were treated with 100 µM resveratrol (Fig. 5 *B*; n = 3).

Effect of polyphenol treatment on cell shedding from 4T1 multicellular tumor spheroids

Escape from the primary tumor is a prerequisite for the process of metastasis. Hence, the inhibition of cell shedding from the tumor could be clinically exploited to inhibit the incidence of metastatic cancer. In the present study the effects of polyphenols on cell shedding from 4T1 multicellular tumor spheroids was investigated as the ratio of the diameter and the circumference of the tumor spheroid. An ideally even tumor spheroid should receive a roughness index of 1.0. It was apparent that all applied polyphenols significantly decreased the roughness index from 1.28 ± 0.13 in the control sample to 1.09 ± 0.05 in the sample treated with 100 µM baicalein, 1.11 ± 0.09 in the sample treated with 100 µM epicatechin, 1.12 ± 0.01 in the sample treated with 100 µM resveratrol, and 1.08 ± 0.06 in the sample treated with 100 µM polyphenon 60 (n = 3). Comparable results were achieved with the

MMP-9 inhibitor SB-3CT which decreased cell roughness to 1.13 $\pm$ 0.06 when applied in a concentration of 10 µM.

Besides decreasing cell shedding from tumor spheroids and increased tendency of tumor spheroids to agglomerate was observed (see Fig. 6, n = 3), which suggests that polyphenols increase cell adhesiveness, thereby inhibiting cell escape from the primary tumor spheroid.

Effects of polyphenols on ROS generation in 4T1 multicellular tumor spheroids.

According to the working hypothesis of the present study, cell shedding and escape from the primary tumor spheroid is mediated by increased MMP-9 expression which could be regulated by ROS generated within peripheral cell layers and shedding cells. Consequently, it should be hypothesized that treatment with polyphenols inhibited ROS generation due to their properties to act as antioxidants. To verify this assumption tumor spheroids were loaded with the redox-sensitive dye H_2DCFDA and ROS generation was assessed either in the absence or presence of either 10 µM epicatechin, 10 µM baicalein, 10 µM resveratrol, or 10 µM polyphenon 60 (Fig. 7, n =3). It was apparent that all applied substances significantly inhibited ROS generation, suggesting that they are acting as antioxidants.

Effects of polyphenols on the expression of MMP-9

MMP-9 expression has been previously shown to be regulated by ROS [21]. Consequently, it should be expected that down regulation of *MMP-9* expression can be achieved by pre-treatment with polyphenols. To investigate this issue 4T1 tumor spheroids were treated for 5 days with either 10 µM epicatechin, 10 µM baicalein, 10 µM resveratrol, or 10 µM polyphenon 60 and mRNA expression of *MMP-9* was monitored (Fig. 8, n=3). As expected pretreatment with polyphenols resulted in significant down regulation of *MMP-9* mRNA

expression, which explains the inhibitory effect of polyphenols on cell shedding from tumor spheroids.

Inhibition of cell invasion in confrontation cultures by polyphenols

To exert metastatic potential, tumor cells have to own migration and invasion properties. Inhibition of cell migration should therefore abolish metastasis formation. To investigate cell migration and invasion we made use of confrontation cultures of tissues derived from ES cells (embryoid bodies) and 4T1 tumor cells, labelled with the long term cell tracker CMFDA. Following 4 days of confrontation culture cells migrated from the tumor spheroid towards the embryoid body and invaded into the depth of the tissue. In contrast cell migration and invasion was significantly inhibited in the presence of polyphenols as well as in the presence of the MMP-9 inhibitor SB-3CT (not shown), which strongly suggests that the effects of polyphenols on cell migration and invasion into embryoid bodies is due to inhibition of MMP expression (Fig. 9, n=3).

Discussion

Metastasis is the primary cause of mortality associated with cancer. Molecular mechanisms leading to metastatic spread are poorly studied. To get a better understanding of this process, we examined cell shedding and invasiveness of 4T1 breast cancer cells using multicellular tumor spheroids and confrontation cultures consisting of multicellular tumor spheroids and ES cell-derived embryoid bodies. The cell line 4T1 is known to be highly metastatic and invasive [22], and is therefore ideally suited to investigate the metastatic cascade. The in vitro model of multicellular tumor spheroid allows to study very early events within the metastatic cascade, e.g. the onset of cell shedding from a three dimensional tumor tissue. It furthermore allows to investigate physiological properties and gene expression patterns of the shed cells

and sub-spheroids in comparison to the mother-spheroid, free of side effects and impurities arising from interactions with other cells and tissues.

Our working hypothesis was, to analyze the mechanisms of cell detachment from multicellular tumor spheroids as a measure of metastasis and the effects of polyphenols on this process. It was observed that cell shedding from 4T1 tumor spheroids was closely associated with increased ROS generation in shedding cells and growing subspheroids, suggesting that redox changes are required for the process of experimental metastasis, possibly by upregulation of enzymes, e.g. MMPs, involved in the degradation of the extracellular matrix. In line with this notion upregulation of MMP-9 expression occurred in 4T1 tumor spheroids during prolonged cell culture and size, whereas no change in the expression of MMP-2 was observed. MMP-9 expression has been previously shown to be regulated by ROS [23]. It was therefore hypothesized that cell shedding was mediated by MMP-9 activity, thereby degrading the extracellular matrix and allowing cells to escape from the tumor spheroid. Since MMP-9 expression is regulated by ROS, it should be assumed that cell shedding is inhibited upon preincubation with free radicals, i.e. by administration polyphenols, which have previously reported to act as potent anti-oxidants [24]. In addition or associated with their anti-oxidant properties, green tea polyphenols have been shown to exert cancer chemopreventive and therapeutic potential against various cancer sites in animal tumor bioassay systems and in some human epidemiologic studies [25,26]. The mechanisms of cancer prevention by polyphenols remain, however, controversial. Previous study have demonstrated that resveratrol inhibits cell cycle progression at different stages [27,28], which has been likewise reported upon treatment of cancer cells with flavonoids [29, 30]. In the present study it was shown that polyphenols significantly down regulated the expression of the proliferation marker Ki-67, thereby inhibiting tumor growth. However, no overall toxicity was observed as evaluated by dead cell staining. This suggests that cells are entering a quiescent state and suggests that inhibition of tumor growth with polyphenols in patients

would require a lifelong treatment with the compounds. Although it is generally accepted that polyphenols exert anti-cancer effects at least partially by inhibiting tumor cell invasiveness, the molecular mechanisms underlying these effects remain to be elaborated. The data of the present study demonstrate that incubation with polyphenols downregulated the expression of MMP-9 and consequently inhibited cell shedding from the surface of tumor spheroids. Incubation with polyphenols as well as with the synthetic MMP-9 inhibitor SB-3CT resulted in smoothing of the tumor spheroid surface and increased the tendency for cell aggregation which likely hinders cell escape from the multicellular tissue. Since SB-3CT exerted comparable effects, we concluded that cell shedding was indeed mediated by MMP-9 activity. Besides the escape from the primary tumor, metastasis is accompanied by increased tissue invasiveness and infiltration of tumor cells. To investigate this issue we used the confrontation culture model consisting of multicellular tumor spheroids and embryoid bodies previously applied by us to study tumor-induced angiogenesis [31, 32]. It was clearly shown that in control confrontation culture tumor cells rapidly invaded the embryoid body tissue which was significantly inhibited in the presence of polyphenols.

In summary the data of the present study demonstrate that polyphenols occurring naturally in green tea, vegetables and fruits significantly inhibit escape of tumor cells from multicellular tumor spheroids, presumably by downregulation of ROS generation and MMP-9 expression. Furthermore, polyphenol treatment results in cell cycle arrest of treated cancer cells and inhibits cell migration and invasion into non-tumorous tissues derived from ES cells. The latter may hinder the tumor cells to intravasate, enter the patient´s bloodstream and form metastases at distant places within the body.

Acknowledgments

This work was supported by the Karl and Veronika Carstens Foundation and the Federal Institute for Risk Assessment (BfR).

References

[1] E. I. Deryugina and J. P. Quigley, Matrix metalloproteinases and tumor metastasis, Cancer Metastasis Rev. 25 (2006) 9-34.

[2] S. B. Somiari, C. D. Shriver, C. Heckman, C. Olsen, H. Hu, R. Jordan, C. Arciero, S. Russell, G. Garguilo, J. Hooke, R. I. Somiari, Plasma concentration and activity of matrix metalloproteinase 2 and 9 in patients with breast disease, breast cancer and at risk of developing breast cancer, Cancer Lett. 233 (2006) 98-107.

[3] M. Nakajima, D. R. Welch, D. M. Wynn, T. Tsuruo, G. L. Nicolson, Serum and plasma M(r) 92,000 progelatinase levels correlate with spontaneous metastasis of rat 13762NF mammary adenocarcinoma, Cancer Res. 53 (1993) 5802-5807.

[4] T. P. Szatrowski and C. F. Nathan, Production of large amounts of hydrogen peroxide by human tumor cells, Cancer Res. 51 (1991) 794-798.

[5] H. Sauer, B. Klimm, J. Hescheler, M. Wartenberg, Activation of p90RSK and growth stimulation of multicellular tumor spheroids are dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP, FASEB J. 15 (2001) 2539-2541.

[6] M. Wartenberg, H. Diedershagen, J. Hescheler, H. Sauer, Growth stimulation versus induction of cell quiescence by hydrogen peroxide in prostate tumor spheroids is encoded by the duration of the Ca(2+) response, J. Biol. Chem. 274 (1999) 27759-27767.

[7] B. Paquette, M. Bisson, C. Baptiste, H. Therriault, R. Lemay, A. M. Cantin, Invasiveness of breast cancer cells MDA-MB-231 through extracellular matrix is increased by the estradiol metabolite 4-hydroxyestradiol, Int. J. Cancer 113 (2005) 706-711.

[8] N.Savaraj,Y.Wei,H.Unate,P.M.Liu,C.J.Wu,M.Wangpaichitr ,D.Xia,H.J.Xu, S. X. Hu, K. M. Tien, Redox regulation of matrix metalloproteinase gene family in smallcelllungcancercells,FreeRadic.Res.39 (2005)373-381.

[9] M. W. Roomi, V. Ivanov, T. Kalinovsky, A. Niedzwiecki, M. Rath, In vivo and in vitro antitumor effect of ascorbic acid, lysine, proline, arginine, and green tea extract onhumanfibrosarcomacellsHT-1080,Med.Oncol.23 (2006)105-111.

[10] S. W. Lee, G. S. Song, C. H. Kwon, Y. K. Kim, Beneficial effect of flavonoid baicalein in cisplatin-induced cell death of human glioma cells, Neurosci. Lett. 382 (2005)71-75.

[11] M. Lopez-Velez, F. Martinez-Martinez, C. Valle-Ribes, The study of phenolic compounds as natural antioxidants in wine, Crit Rev. Food Sci. Nutr. 43 (2003)233-244.

[12] E. Ignatowicz and W. Baer-Dubowska, Resveratrol, a natural chemopreventive agent againstdegenerativediseases,Pol.J.Pharmacol.53 (2001)557-569.

[13] S. Ulrich, F. Wolter, J. M. Stein, Molecular mechanisms of the chemopreventive effects of resveratrol and its analogs in carcinogenesis, Mol. Nutr. Food Res. 49 (2005)452-461.

[14] M. Wartenberg, P. Budde, M. De Marees, F. Grunheck, S. Y. Tsang, Y. Huang, Z. Y. Chen, J. Hescheler, H. Sauer, Inhibition of tumor-induced angiogenesis and matrix-metalloproteinase expression in confrontation cultures of embryoid bodies and tumor spheroids by plant ingredients used in traditional chinese medicine, Lab Invest 83 (2003) 87-98.

[15] M. Wartenberg, F. Donmez, F. C. Ling, H. Acker, J. Hescheler, H. Sauer, Tumor-induced angiogenesis studied in confrontation cultures of multicellular tumor spheroids and embryoid bodies grown from pluripotent embryonic stem cells, FASEB J. 15 (2001) 995-1005.

[16] M. Wartenberg, P. Budde, M. De Marees, F. Grunheck, S. Y. Tsang, Y. Huang, Z. Y. Chen, J. Hescheler, H. Sauer, Inhibition of tumor-induced angiogenesis and matrix-metalloproteinase expression in confrontation cultures of embryoid bodies and tumor spheroids by plant ingredients used in traditional chinese medicine, Lab Invest 83 (2003) 87-98.

[17] K. Sakata, S. Okada, H. Majima, N. Suzuki, Cell shedding from x-irradiated multicellular spheroids of human lung carcinomas, Strahlenther. Onkol. 167 (1991) 723-725.

[18] G. H. Heppner, F. R. Miller, P. M. Shekhar, Nontransgenic models of breast cancer, Breast Cancer Res. 2 (2000) 331-334.

[19] E. I. Deryugina and J. P. Quigley, Matrix metalloproteinases and tumor metastasis, Cancer Metastasis Rev. 25 (2006) 9-34.

[20] S. K. Katiyar, Matrix metalloproteinases in cancer metastasis: molecular targets for prostate cancer prevention by green tea polyphenols and grape seed proanthocyanidins,Endocr.MetabImmune.Disord.DrugTargets.6 (2006)17-24.

[21] P.Spallarossa,P.Altieri,S.Garibaldi,G.Ghigliotti,C.Barisione, V.Manca,P.Fabbi, A.Ballestrero,C.Brunelli,A.Barsotti,Matrixmetalloproteinase-2and-9areinduced differently by doxorubicin in H9c2 cells: The role of MAP kinases and NAD(P)H oxidase,Cardiovasc.Res.69 (2006)736-745.

[22] C.J.AslaksonandF.R.Miller,Selectiveeventsinthemetastaticprocessdefinedby analysis of the sequential dissemination of subpopulations of a mouse mammary tumor,CancerRes.52 (1992)1399-1405.

[23] P.Spallarossa,P.Altieri,S.Garibaldi,G.Ghigliotti,C.Barisione, V.Manca,P.Fabbi, A.Ballestrero,C.Brunelli,A.Barsotti,Matrixmetalloproteinase-2and-9areinduced differently by doxorubicin in H9c2 cells: The role of MAP kinases and NAD(P)H oxidase,Cardiovasc.Res.69 (2006)736-745.

[24] S.Sang,Z.Hou,J.D.Lambert,C.S.Yang,Redoxproperties ofteapolyphenolsand relatedbiologicalactivities,Antioxid.Redox.Signal.7 (2005)1704-1714.

[25] N. Khan, F. Afaq, M. Saleem, N. Ahmad, H. Mukhtar, Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate, Cancer Res. 66 (2006)2500-2505.

[26] I.C.ArtsandP.C.Hollman,Polyphenolsanddiseaseriskinepidemiologicstudies, Am.J.Clin.Nutr.81 (2005)317S-325S.

[27] Y. C. Liang, S. H. Tsai, L. Chen, S. Y. Lin-Shiau, J. K. Lin, Resveratrol-induced G2 arrest through the inhibition of CDK7 and p34CDC2 kinases in colon carcinoma HT29 cells, Biochem. Pharmacol. 65 (2003) 1053-1060.

[28] N. Ahmad, V. M. Adhami, F. Afaq, D. K. Feyes, H. Mukhtar, Resveratrol causes WAF-1/p21-mediated G(1)-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells, Clin. Cancer Res. 7 (2001) 1466-1473.

[29] S. Gupta, N. Ahmad, A. L. Nieminen, H. Mukhtar, Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells, Toxicol. Appl. Pharmacol. 164 (2000) 82-90.

[30] C. S. Yang, J. M. Landau, M. T. Huang, H. L. Newmark, Inhibition of carcinogenesis by dietary polyphenolic compounds, Annu. Rev. Nutr. 21 (2001) 381-406.

[31] M. Wartenberg, P. Budde, M. De Marees, F. Grunheck, S. Y. Tsang, Y. Huang, Z. Y. Chen, J. Hescheler, H. Sauer, Inhibition of tumor-induced angiogenesis and matrix-metalloproteinase expression in confrontation cultures of embryoid bodies and tumor spheroids by plant ingredients used in traditional chinese medicine, Lab Invest 83 (2003) 87-98.

[32] M. Wartenberg, F. Donmez, F. C. Ling, H. Acker, J. Hescheler, H. Sauer, Tumor-induced angiogenesis studied in confrontation cultures of multicellular tumor spheroids and embryoid bodies grown from pluripotent embryonic stem cells, FASEB J. 15 (2001) 995-1005.

Legends to Figures

Fig. 1. Growth kinetics of multicellular 4T1 breast cancer spheroids and development of subspheroids. Note, that from day 8 on subspheroids develop, which arise from single cells detaching from the periphery of the mother spheroids. * $P < 0.05$, significantly different from tumor spheroids on day 1 of cell culture.

Fig. 2. Gaussian size distribution of multicellular 4T1 breast cancer spheroids during prolonged cell culture. The images show representative tumor spheroids on day 4, day 6 and day 9 of cell culture. In 9-day-old tumor spheroids single cells and subspheroids are detaching which results in a two-peaked Gaussian size distribution during prolonged tumor spheroid culture (day 10, 12).

Fig. 3. Generation of ROS in mother 4T1 breast cancer spheroids as well as in spheroids detaching from the surface of mother spheroids and freely floating subspheroids (A). Source of ROS generated in 4T1 tumor spheroids (B). The tumor spheroids were treated with the NADPH oxidase inhibitors DPI (25 µM) and apocynin (10 µM) as well as with the COX-2 inhibitor NS-398. Note, that in the presence of NADPH oxidase inhibitors ROS generation was significantly inhibited. * $P < 0.05$, significantly different from ROS generation in mother spheroids.

Fig. 4. Expression of *MMP-9* during the growth of 4T1 multicellular tumor spheroids. mRNA expression of *MMP-9* was assessed by real time RT-PCR analysis using mPolr2A as house-keeping gene. Note, that *MMP-9* mRNA expression increased with the size and cell culture time of 4T1 tumor spheroids. * $P < 0.05$, significantly different from *MMP-9* mRNA expression on day 1 of cell culture.

Fig. 5. Effects of polyphenols on tumor spheroid size (A) and expression of the proliferation marker Ki-67 (B). 4T1 multicellular tumor spheroids were treated from day 5 to day 10 of cell culture with different concentrations as indicated of baicalein, resveratrol, polyphenon 60, epicatechin and epigallocatechin and spheroid diameters were determined on day 10. Polyphenol treatment resulted in reduced growth of tumor spheroids as well as decreased expression of Ki-67. * $P<0.05$, significantly different from the control.

Fig. 6. Effects of baicalein and resveratrol on cell shedding from multicellular 4T1 tumor spheroids. Tumor spheroids were treated from day 4 to day 10 with either 10 µM baicalein or 10 µM resveratrol and transmission images were taken. Shown are representative tumor spheroids. Note, that treatment with polyphenols increased the tendency of tumor spheroids to aggregate. The bar represents 200 µm.

Fig. 7. Effects of polyphenols on ROS generation in multicellular 4T1 tumor spheroids. Tumor spheroids were loaded with the redox-sensitive dye H_2DCF-DA and the fluorescence of oxidized DCF was monitored over time. ROS generation was significantly reduced in tumor spheroids treated with either resveratrol (10 µM), baicalein (10 µM), epicatechin (10 µM) or polyphenon 60 (10 µM), indicating that the polyphenols exert antioxidative capacity. *$P<0.05$, significantly different from the untreated control.

Fig. 8. Effects of polyphenols on the expression of *MMP-9*. 4T1 tumor spheroids were treated for 5 days with either 10 µM epicatechin, 10 µM baicalein, 10 µM resveratrol, or 10 µM polyphenon 60 and mRNA expression of *MMP-9* was monitored. *MMP-9* expression was significantly downregulated upon treatment of tumor spheroids with polyphenols. * $P<0.05$, significantly different from the untreated control.

Fig.9. Inhibition of cell invasion in confrontation cultures of ES cells and 4T1 multicellular tumor spheroids upon treatment with either baicalein (10 µM), epicatechin (10 µM), epigallocatechin (10 µM), polyphenon 60 (10 µM) or resveratrol (10 µM). 4T1 tumor spheroids were labelled with the long term cell tracker CMFDA to discriminate tumor cells. Within 4 days green fluorescent tumor cells invaded the ES cell-derived tissue in the untreated control, whereas cell invasion was largely absent in confrontation cultures treated with polyphenols. Shown are representative overlay images between a fluorescence image (green) and a transmission image. The bars represent 100µm.

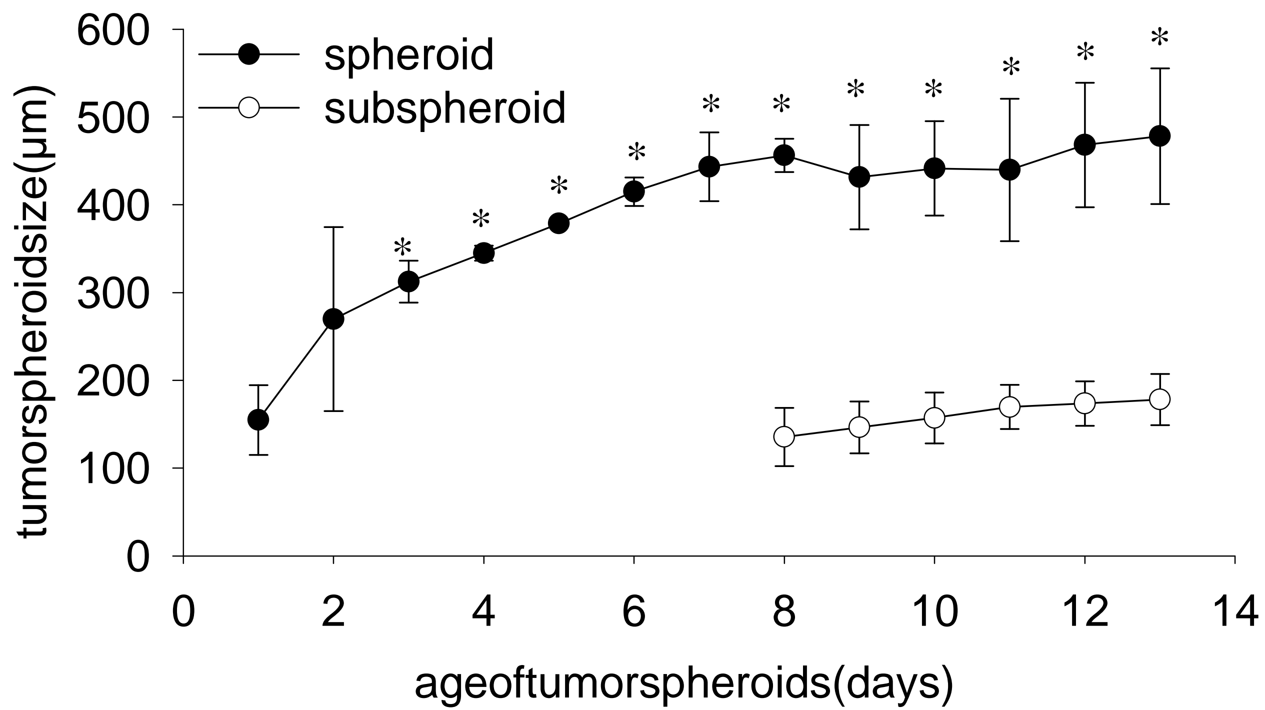
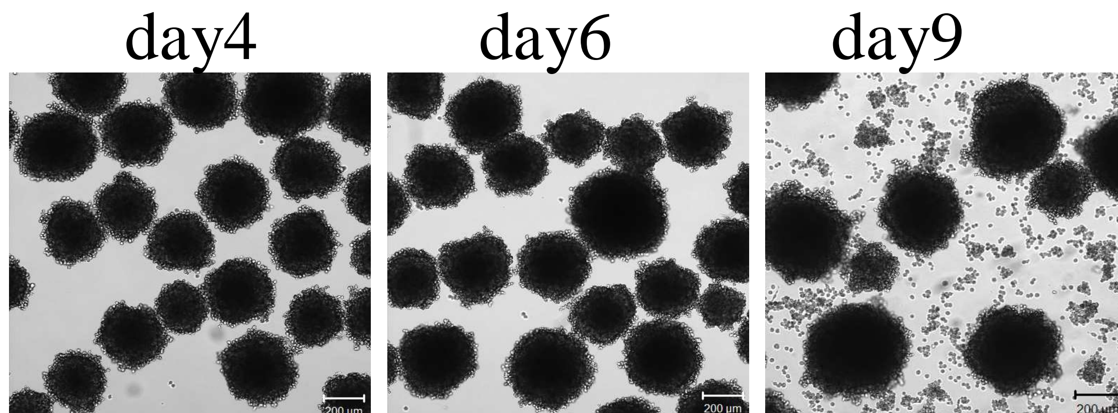


Figure1



A day4

B day8

C day10

D day12

numberoftumorspheroids(%)

tumorspheroidsize(µm)

Figure2

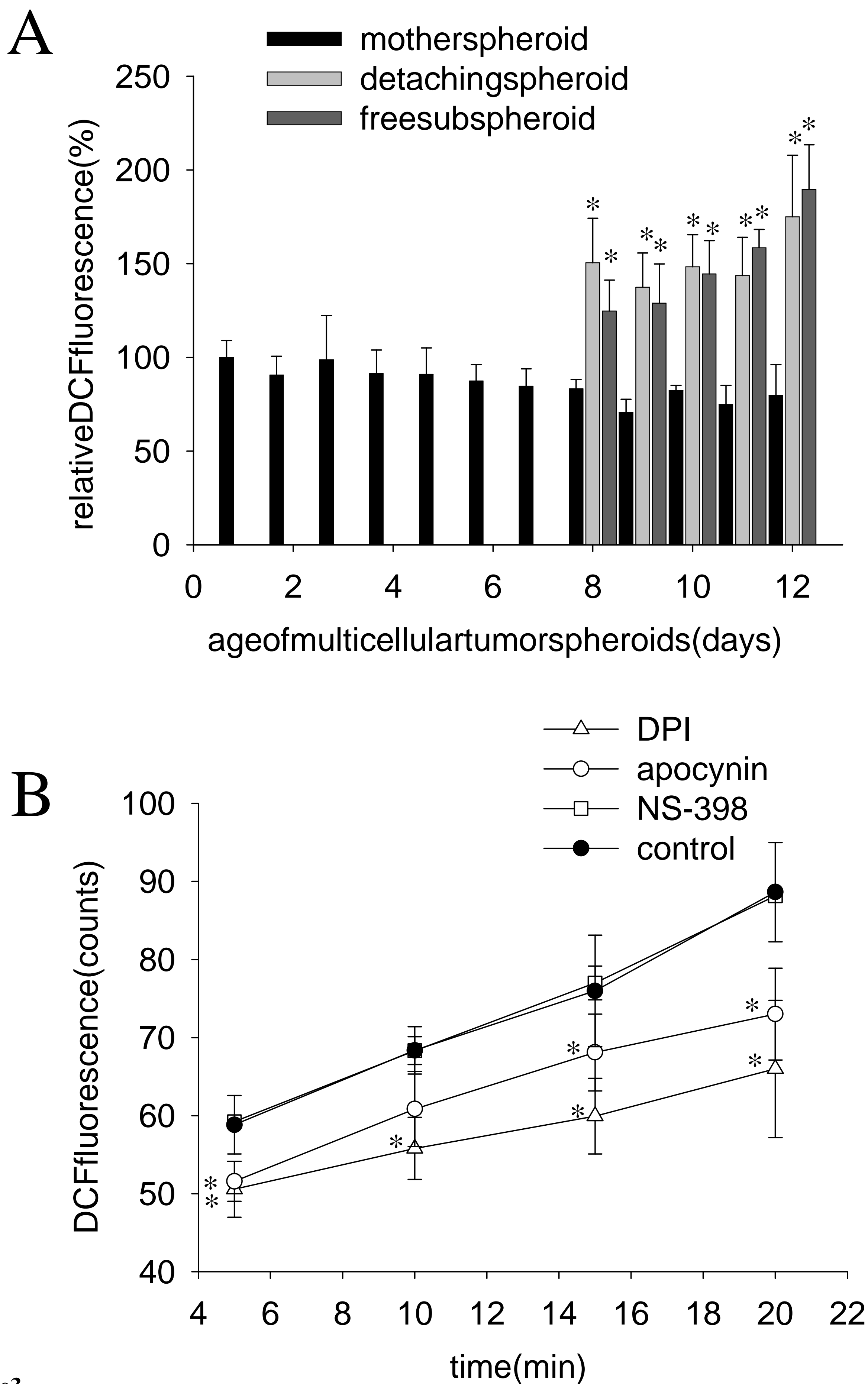


Figure 3

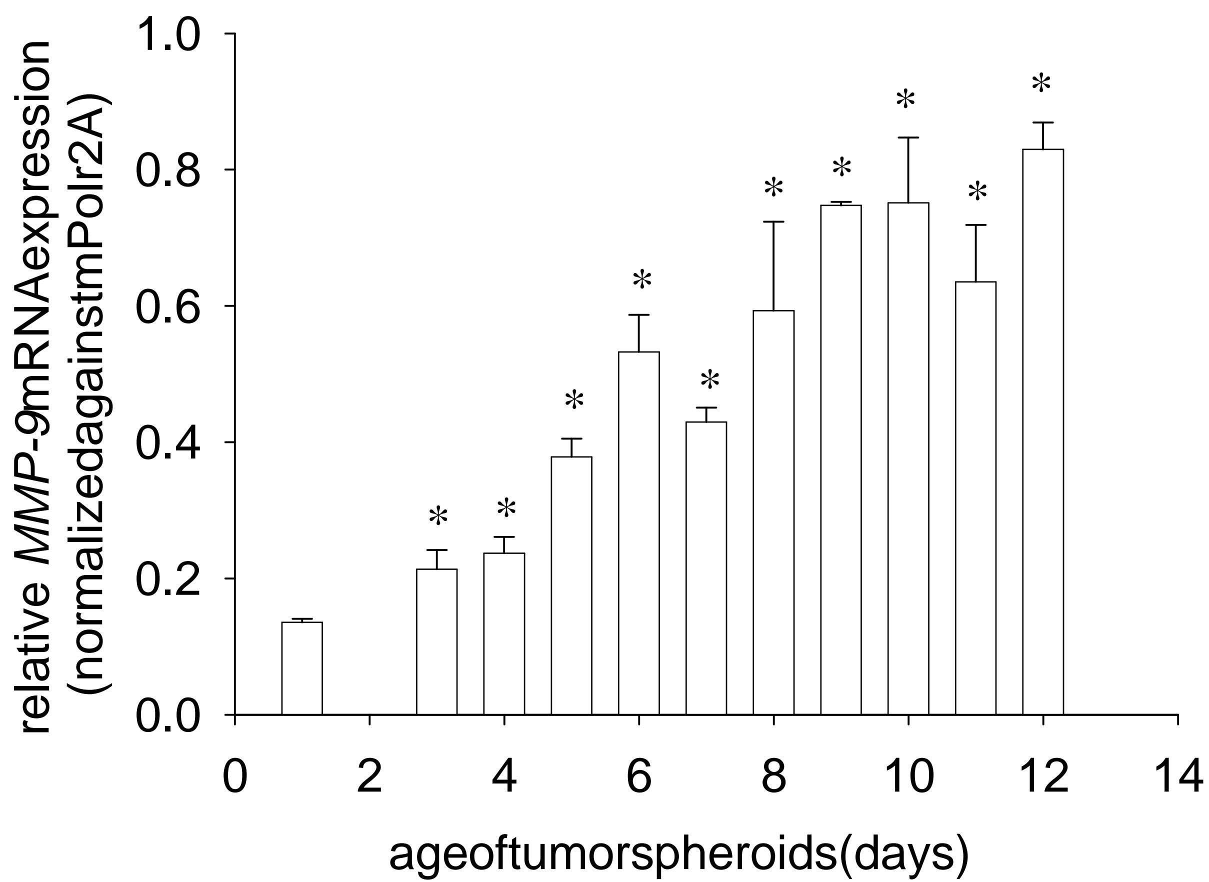


Figure4

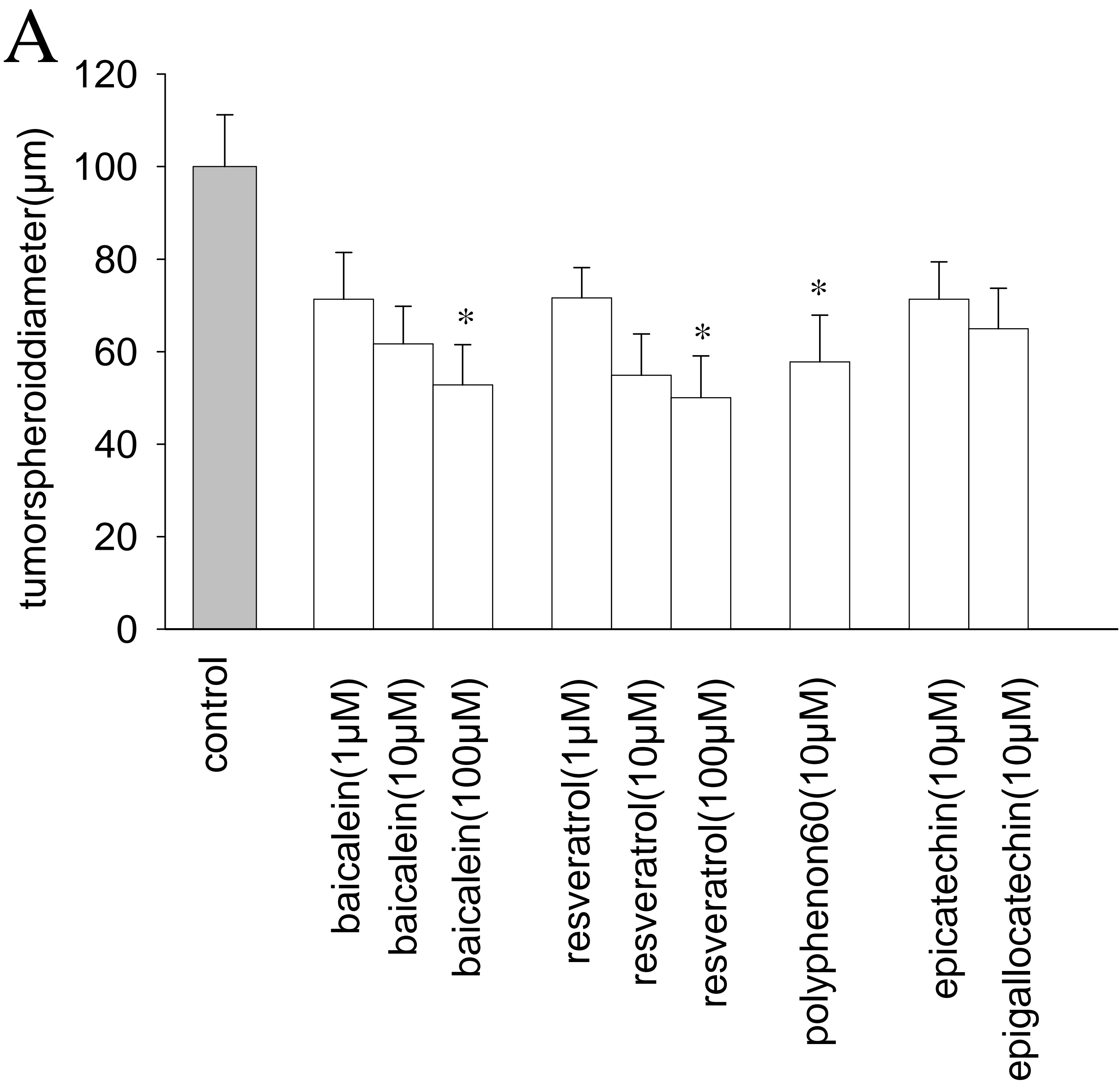


Figure5A

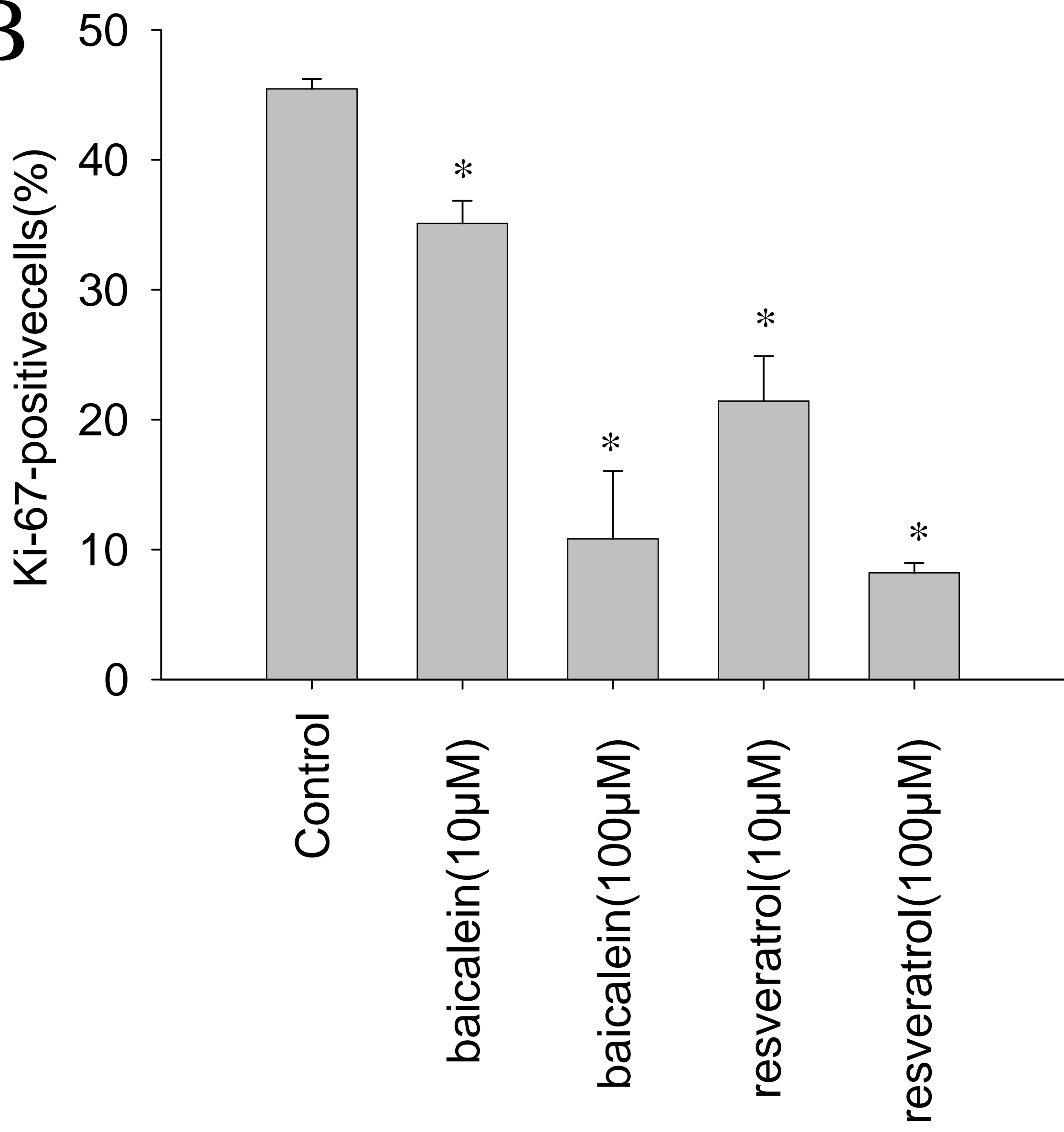


Figure5B

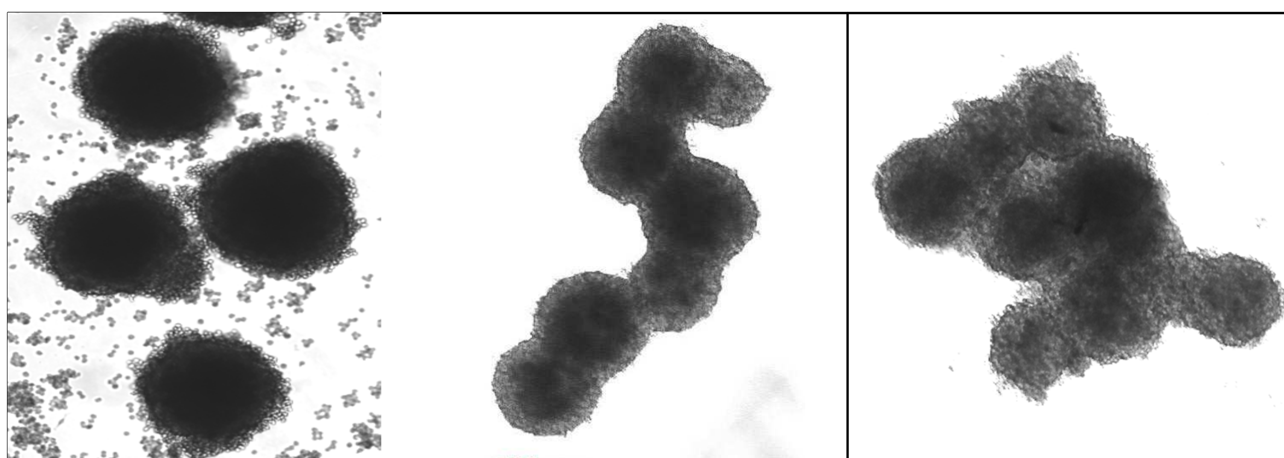


Figure6

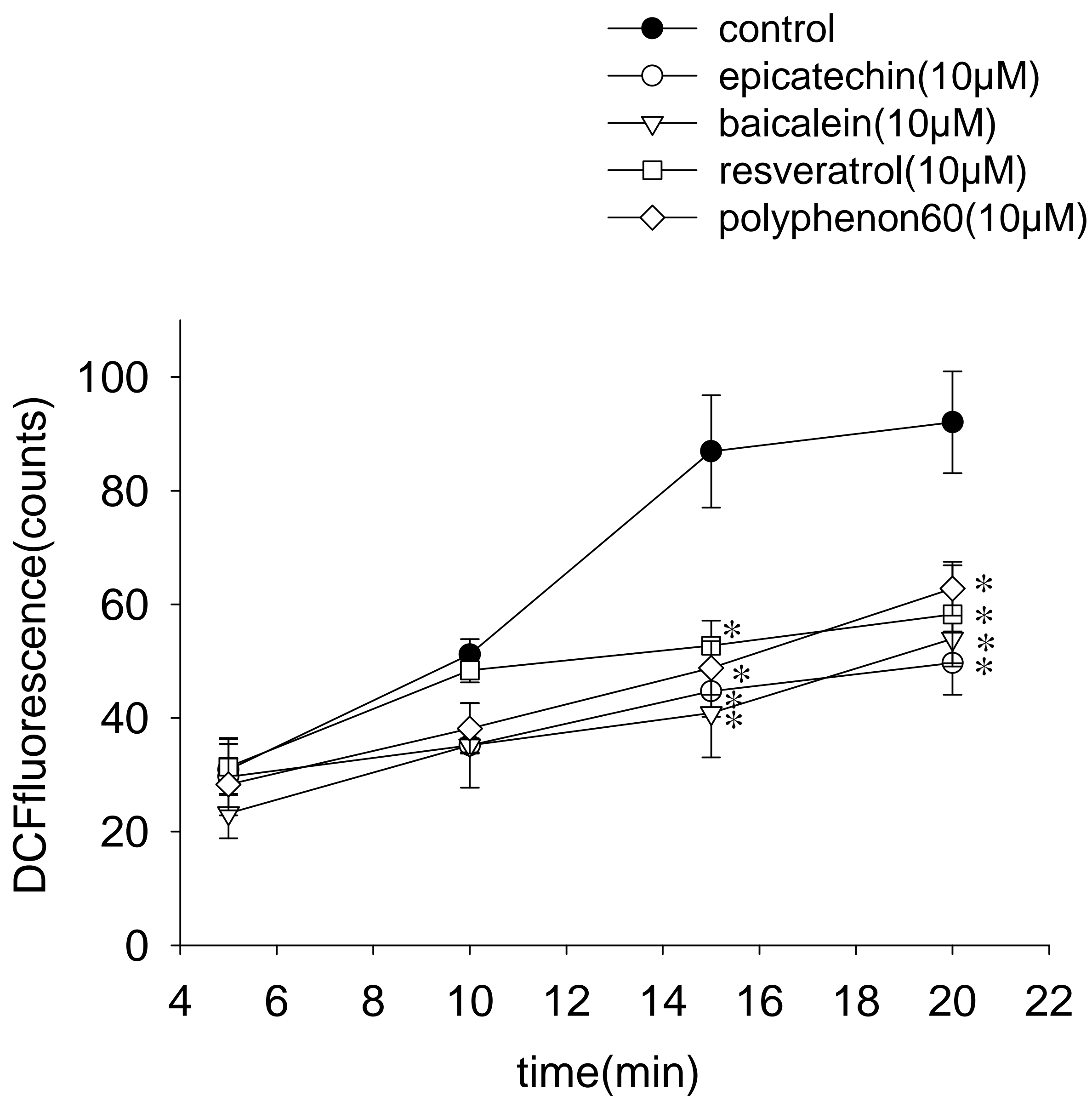
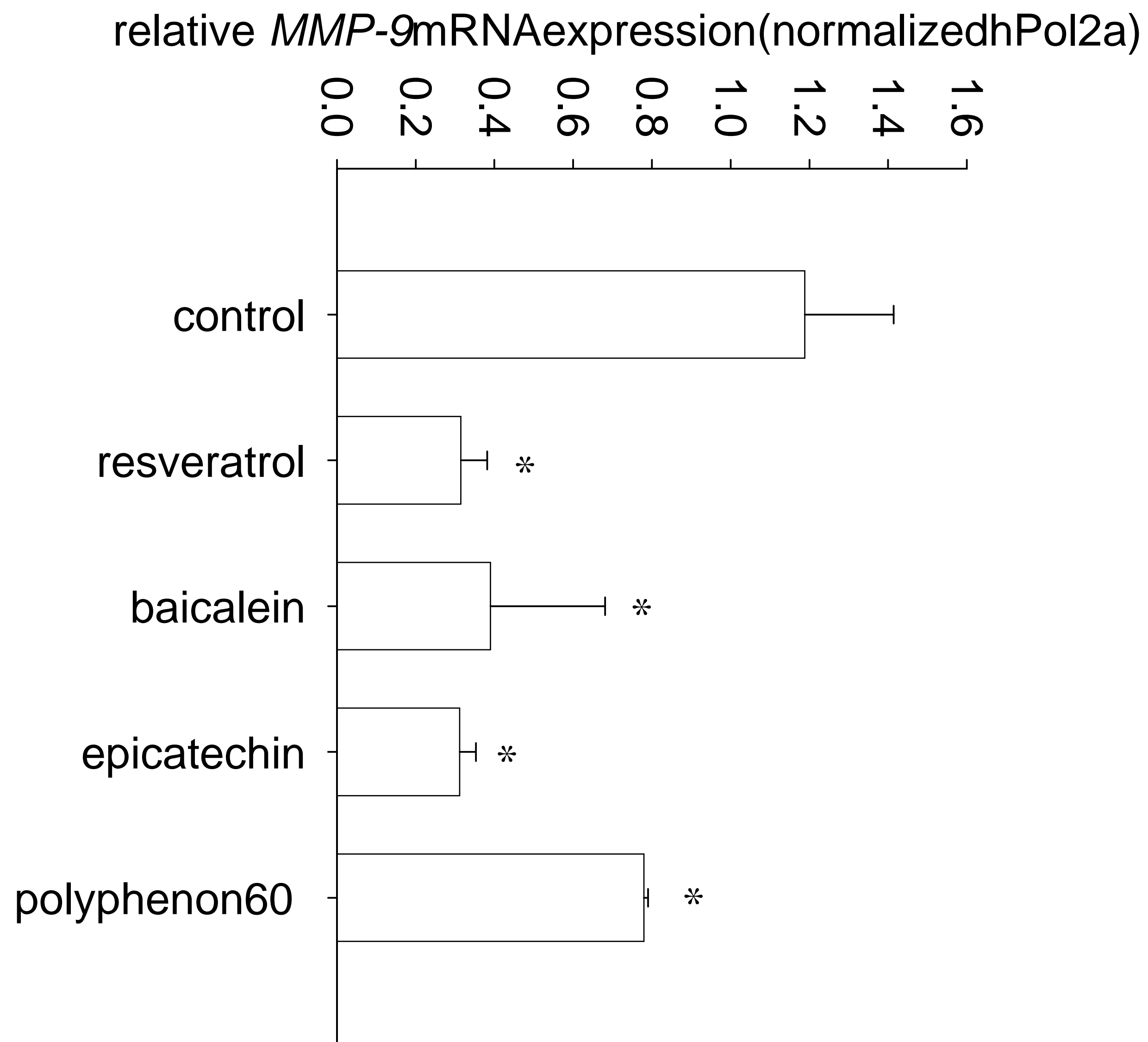


Figure7

Figure8



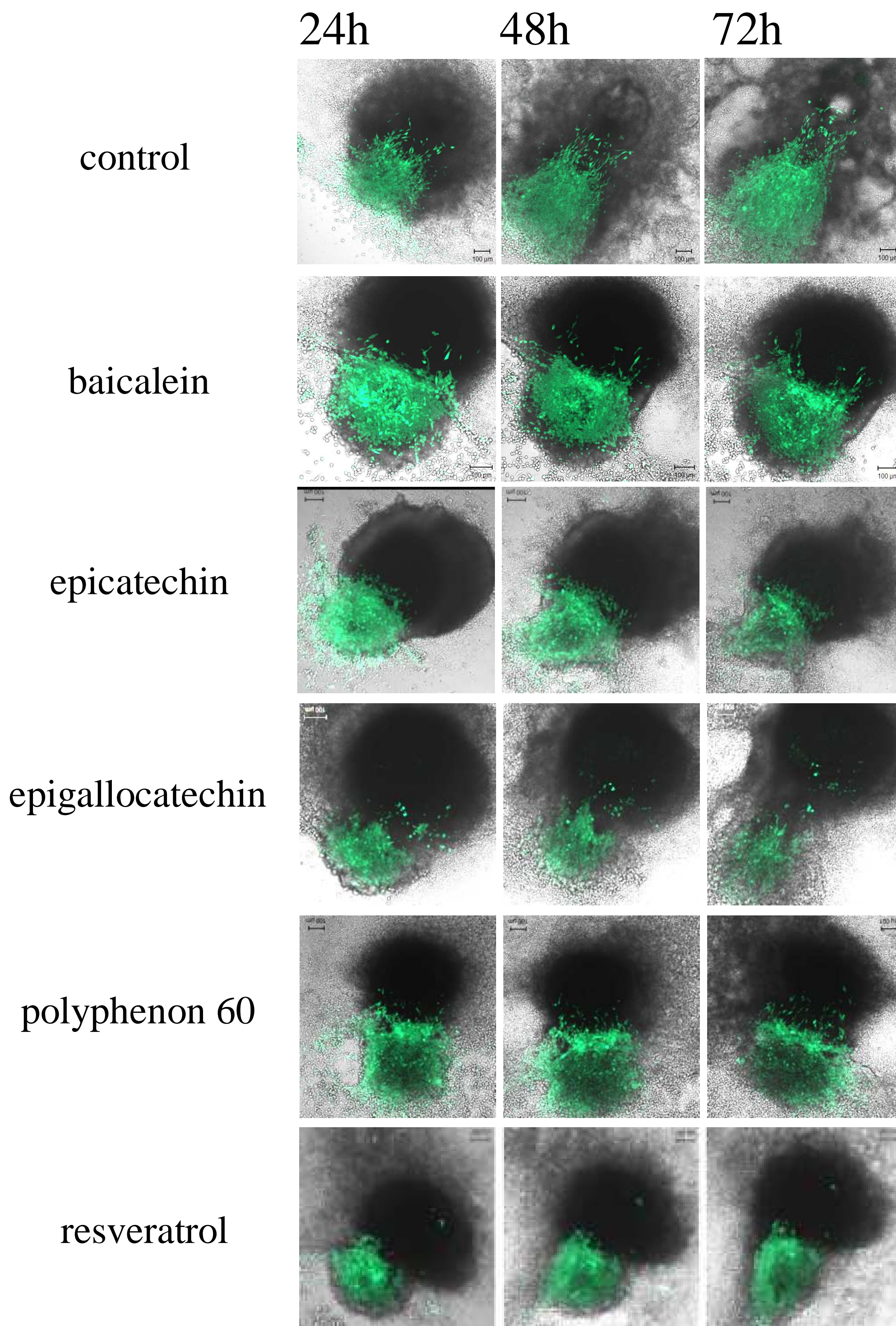


Figure9