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Comparative effects of selected plant polyphenols, gallic acid and epigallocatechin gallate, on matrix metalloproteinases activity in multidrug resistant MCF7/DOX breast cancer cells*

Anna Nowakowska[1,2] and Jolanta Tarasiuk[1,2*]

[1]Department of Biochemistry, Faculty of Biology, University of Szczecin, Szczecin, Poland; [2]Molecular Biology and Biotechnology Center, Faculty of Biology, University of Szczecin, Szczecin, Poland

The aim of the study was to investigate the effect of selected polyphenols: gallic acid (GA) and epigallocatechin gallate (EGCG) on matrix metalloproteinase (MMP-2 and MMP-9) activity in multidrug resistant (MDR) human breast adenocarcinoma cells: MCF7/DOX cells and obtained recently in our laboratory MCF7/DOX$_{500}$ cells by the permanent selection of MCF7/DOX cells with 500 nM doxorubicin (DOX). The activity of MMP-2 and MMP-9 and the effect of studied polyphenols on these matrix proteases were examined by gelatin zymography assays. We have found that the activity of MMP-2 and MMP-9 significantly increased in resistant MCF7/DOX and MCF7/DOX$_{500}$ cells whereas they were not detected in sensitive MCF7 cells. It was also observed that GA (30, 60, 100 and 120 μM) and EGCG (5, 10 and 20 μM) caused a comparable concentration-dependent inhibition of MMP-2 and MMP-9 activity in MCF7/DOX and MCF7/DOX$_{500}$ cells. Control experiments confirmed that examined compounds in these ranges of concentration did not affect the cell growth of MCF7/DOX and MCF7/DOX$_{500}$ sublines (80-100% of control cell growth was observed in the presence of studied polyphenols).

Key words: phenolic compounds, gallic acid, epigallocatechin gallate, matrix metalloproteinases, human breast cancer MCF7 cells, multidrug resistance MDR

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INTRODUCTION

Multidrug resistance (MDR) constitutes the major problem in cancer chemotherapy. Tumour cells become resistant to a wide array of chemotherapeutic agents, structurally diverse and having different mechanisms of action (Borst *et al*., 2007; Baguley, 2010). The occurrence of MDR is conferred by multiple mechanisms, especially it is associated with the overexpression of membrane transporters (e.g. P-glycoprotein, P-gp; MRP1; BCRP/MXR1) responsible for the active ATP-dependent efflux of drugs out of resistant cells (Sharom, 2008). Moreover, in the case of many clinical tumours, it is observed that resistant tumour cells have higher invasive and metastatic properties than cells sensitive to chemotherapy (Osmak *et al*., 1999; Molinari *et al*., 2005, Colone *et al*., 2008). Among cellular factors involved in the development of the metastatic potential of MDR tumour cells are matrix metalloproteinases (MMPs) (Yang *et al*., 2003; Li *et al*. 2007; Karroum *et al*., 2010). It is proposed that several signal transduction pathways play a crucial role in the regulation of gene expression involved simultaneously in multidrug resistance (e.g. *Mdr1* encoding P-glycoprotein) and metastasis (e.g. *MMPs* genes) of tumour cells (Liang *et al*., 2004; Raguz *et al*., 2004; Işeri *et al*., 2010; Lu *et al*., 2012).

A huge effort is performed on the search of agents able to reduce the metastatic potential of MDR tumour cells. Recently, increasing interest of many investigators is focused on the use of dietary polyphenols in cancer prevention and chemotherapy (Kampa *et al*., 2007; Afzal *et al*., 2015). It was demonstrated that they are able to interrupt cellular signalling, mainly NF-κB and AP1 pathways, by scavenging reactive oxygen species (ROS) responsible for their activation (Park & Dong, 2003; Ramos, 2008; Sen *et al*., 2010).

Among polyphenol compounds exhibiting antitumour properties in *in vitro* studies are gallic acid (GA) (Raina *et al*., 2008; Kaur *et al*., 2009, Ho *et al*., 2010; Lu *et al*., 2010) and epigallocatechin gallate (EGCG) (Farabegoli *et al*., 2010, Chen *et al*., 2011; Lecumberri *et al*., 2013; Afzal *et al*., 2015). However, although there are some interesting data demonstrating their activity against tumour cells, up to now little is known about their properties towards MDR breast cancer cells.

Therefore, the aim of this study was to investigate the effect of selected polyphenols on matrix metalloproteinase (MMP-2 and MMP-9) activity in multidrug resistant (MDR) human breast adenocarcinoma cells: MCF7/DOX cells and obtained recently in our laboratory MCF7/DOX$_{500}$ cells by the permanent selection of MCF7/DOX cells with 500 nM doxorubicin (DOX).

MATERIALS AND METHODS

Chemicals. Anti-P-glycoprotein (P-gp) monoclonal antibody were purchased from Becton Dickinson. Bromophenol blue, calcium chloride ($CaCl_2$), doxorubicin (DOX), epigallocatechin gallate (EGCG), gallic acid (GA), gelatine from porcine skin type A, sodium chloride (NaCl), sodium dodecyl sulphate (SDS) and Triton X-100 were provided by Sigma-Aldrich. Tris-Base was obtained from Calbiochem, Merck-Millipore. Acrylamide, ammonium peroxodisulfate, Coomassie brilliant blue, N,N'-methylenediacrylamide and N,N,N',N'-

✉e-mail: tarasiuk@univ.szczecin.pl

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Abbreviations: DOX, doxorubicin; EGCG, epigallocatechin gallate; GA, gallic acid; MCF7, human breast adenocarcinoma cell line; MDR, multidrug resistance; MMP, matrix metalloproteinase; P-gp, P-glycoprotein

tetramethylethylenediamine were purchased from Merck and glycerol was obtained from Fluka.

Cell culture. The MCF7 human breast adenocarcinoma cell line and its resistant subline MCF7/DOX were kindly provided by Institut Bergonié (Bordeaux, France). MCF7/DOX_{500} subline was obtained in our laboratory by the permanent selection of MCF7/DOX cells with 500 nM DOX. The cells were grown in DMEM (Sigma-Aldrich) medium supplemented with 2 mM L-glutamine and 10% foetal bovine serum (FBS) (Gibco Limited), 0.02 mg/ml calf insulin (Sigma-Aldrich), at 37°C in a humified atmosphere of 95% air and 5% CO_2. MCF7/DOX_{500} were cultured in the presence of 500 nM DOX. All cultures (MCF7, MCF7/DOX and MDCF7/DOX_{500}) were initiated at a density of 4×10^4 cells/ml and grew for 168 h to reach the cell confluence. Thereafter, culture medium was removed, cells were washed with PBS and incubated in culture medium without FBS in the presence of GA (30–120 μM) or EGCG (5–20 μM), respectively for 24 h and analyzed for MMP-2 and MMP-9 activity.

Mesurement of P-glycoprotein level. Cells were harvested and washed with phosphate-buffered saline (PBS). Thereafter, 5×10^4 cells were incubated with 2 μl phycoerythrin (PE)-conjugated anti-P-gp monoclonal antibody (Becton Dickinson) for 30 min in the dark at room temperature. Then, cells were washed with PBS containing 0.1% sodium azide, fixed with 150 μl of 1% paraformaldehyde and analyzed by flow cytometry (FACSCalibur, Becton Dickinson). The measurements were conducted within the FL-2 fluorescence channel (bandpass filter $\lambda = 585 \pm 21$ nm) after excitation with the argon-ion laser ($\lambda = 488$ nm). For each experimental point, a fluorescence signal of 5×10^3 events was measured.

Gelatin zymography. The samples containing FBS-free culture medium collected after 24 h-incubation of cells with GA or EGCG, respectively were centrifuged at $1500 \times g$ for 5 min at 4°C. To each sample (V=50 μl) 15 μl of a loading buffer (Tris-Base) containing 10% glycerol, 2% sodium dodecyl sulphate (SDS) and 0.05% bromophenol blue (pH 6.8) was added. Then, samples were loaded in a 7.5% polyacrylamide-SDS gel containing 0.1% gelatin and separated by electrophoresis at 110 V for 2 h at 4°C. Thereafter, gels were washed three times for 60 min with 2.5% Triton X-100 to remove SDS. Gels were then incubated for 48 h at 37°C in zymogram developing buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 200 mM NaCl, pH 7.5) and stained with 0.1% Coomassie blue in methanol: acetic acid: water (9:2:9, v:v:v) mixture for 1.5 h. Finally, gels were destained with methanol: acetic acid: water (1:1:8, v:v:v) mixture for 24 h. Proteolytic activity of MMPs was visualized as clear bands on a dark blue background. The bands were identified basing on the molecular weights of MMPs: 72 kDa and 92 kDa corresponding to MMP-2 and MMP-9, respectively. Proteolytic activity was determined using an imaging system (biostep Dark Hood DH-40/50, Germany) and a software package (TotalLab TL100 v 2006b, Nonlinear Dynamics).

Cell growth and viability. The effect of studied compounds (GA and EGCG) on cell growth and viability was assessed by counting viable cells in the presence of trypan blue (Sigma-Aldrich) using a Bürker hemocytometer.

Statistical analysis. Results are presented as the median (interquartile range) of at least 3 independent experiments. Statistical analysis of the significance of differences observed was carried out using non-parametric Mann-Whitney U-test. $P \leq 0.05$ was considered as a significant difference ($^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$) between compared groups.

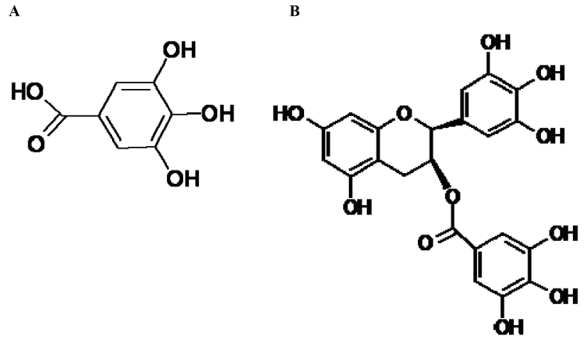

Figure 1. Structure of gallic acid (GA) (A) and epigallocatechin gallate (EGCG) (B).

RESULTS AND DISCUSSION

Metastatic tumours resistant to chemotherapy are the major cause of the clinical failure in the treatment of breast cancer patients (Smith, 2012). The degradation of the extracellular matrix by metastatic cancer cells involves several proteolytic enzymes (e.g. metalloproteinases, MMPs; cathepsins and plasminogen activation system proteins) (Sugira *et al.*, 1999; Westermarck *et al.*, 1999; Brooks *et al.*, 2010; Christensen & Shastri, 2015). Among the members of the MMPs family, gelatinase A (72 kDa type IV collagenase, MMP-2) and gelatinase B (92 kDa type IV collagenase, MMP-9) play a critical role in extracellular matrix degradation and tumour cell invasion and metastasis in breast cancer (Chabottaux & Noel, 2007; Li *et al.*, 2007; Karroum *et al.*, 2010).

In this work we studied the effect of selected polyphenols: gallic acid (GA) and epigallocatechin gallate (EGCG) (Fig. 1) on MMP-2 and MMP-9 activity in multidrug resistant (MDR) human breast adenocarcinoma cells: MCF7/DOX cells and obtained recently in our laboratory MCF7/DOX_{500} cells by the permanent selection of MCF7/DOX cells with 500 nM doxorubicin (DOX).

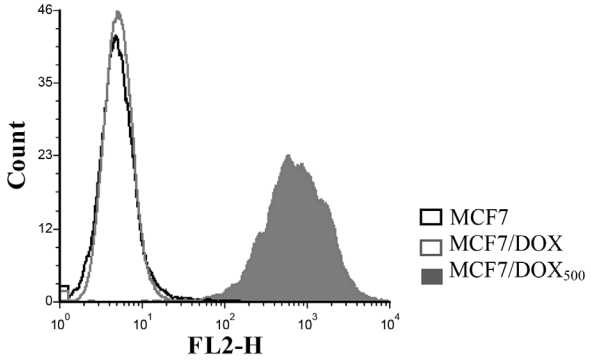

Figure 2. Anti-P-gp labelling of sensitive MCF7 and resistant MCF7/DOX and MCF7/DOX_{500} cells. The representative histograms are presented.

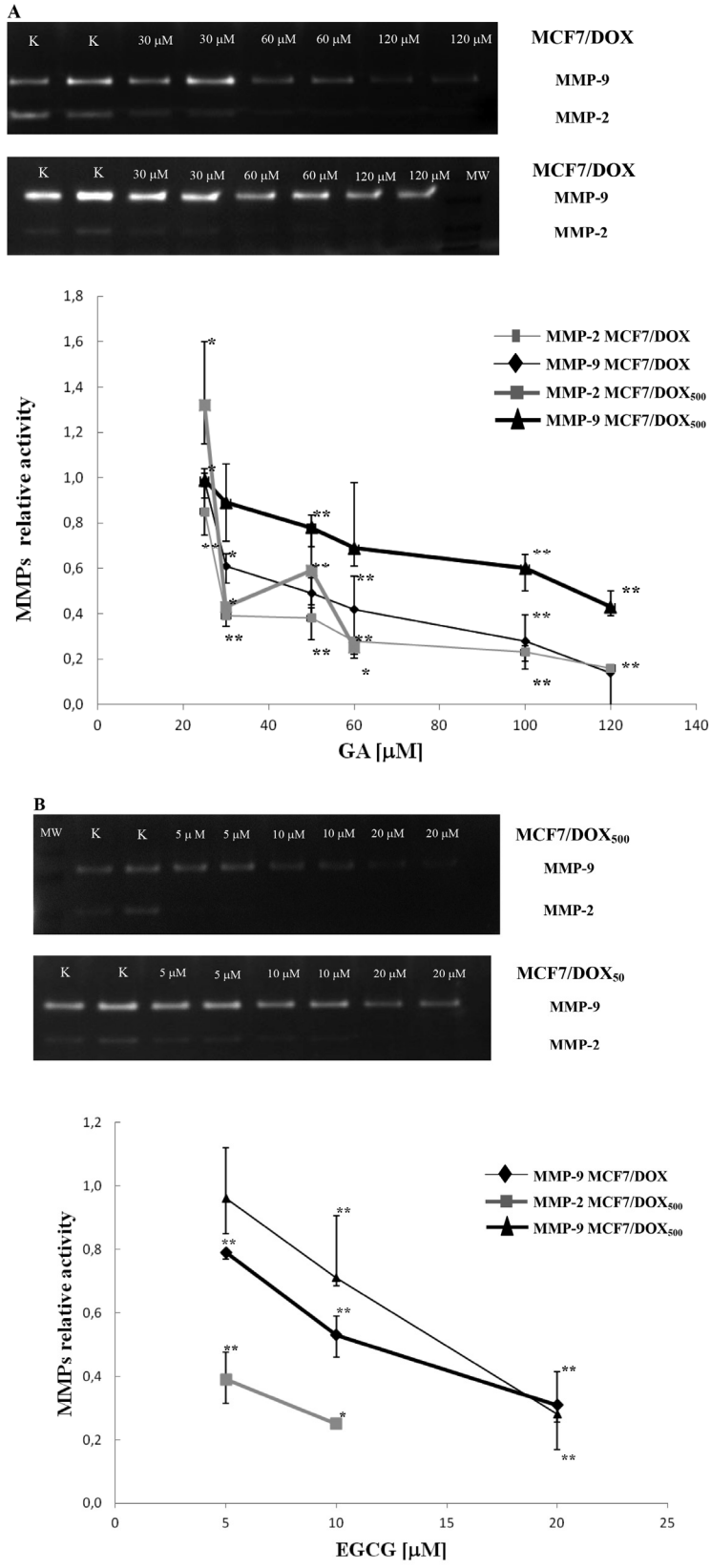

Figure 3. Effect of GA (A) and EGCG (B) on MMP-2 and MMP-9 activity in MCF7/DOX and MCF7/DOX$_{500}$ cells assessed by gelatin zymography.
Representative zymograms and MMPs relative activity are presented. The data points represent median (interquartile range) values of at least three independent experiments. $P<0.05$ was considered as a significant difference between compared groups (*$P<0.05$, **$P<0.001$, ***$P<0.0001$; non-parametric Mann-Whitney U-test).

We have found by performing direct immunofluorescence assays with the use of anti-P-gp antibodies conjugated with phycoerythrin (PE) as a fluorophore that MCF7/DOX$_{500}$ is characterized by an important increase (about 100-fold) in P-gp level, whereas in the case of MCF7/DOX we observed the comparable level of this MDR transporter comparing to the sensitive MCF7 cells (Fig. 2).

The activity of MMP-2 and MMP-9 and the effect of studied polyphenols on these matrix proteases were examined by gelatin zymography assays. Interestingly, we have found that the activity of MMP-2 and MMP-9 significantly increased in resistant MCF7/DOX and MCF7/DOX$_{500}$ cells (Fig. 3) whereas they were not detected in sensitive MCF7 cells (data not presented). It was also observed that GA (30, 60, 100 and 120 μM) and EGCG (5, 10 and 20 μM) caused a concentration-dependent inhibition of MMP-2 and MMP-9 activity not only in MCF7/DOX but also in MCF7/DOX$_{500}$ cells (Fig. 3) characterized by the high level of P-gp (Fig. 2). Control experiments performed by counting viable cells in the presence of trypan blue using a Burker hemocytometer confirmed that examined compounds in these ranges of concentration did not affect the cell growth of MCF7/DOX and MCF7/DOX$_{500}$ sublines (80–100% of control cell growth was observed in the presence of 30–120 μM GA and 5–20 μM EGCG) (not presented).

The effect data of EGCG on matrix MMPs were also studied by other authors in regard to various tumours (Roomi *et al.*, 2010; Chen *et al.*, 2011), including breast cancer. Their findings are in agreement with the results obtained for this polypenolic compound in our study. It was demonstrated by Sen and coworkers (2009) that 24 h-treatment of MCF7 with 20 μM EGCG resulted in a significant decrease in the activity, mRNA expression and protein level of

MMP-2. Similar results are also found by this group for MMP-9 downregulating in human breast cancer MDA-MB-231 cells (Sen *et al.*, 2010; Sen *et al.*, 2011). The ability of EGCG to inhibit the MMP-2 and MMP-9 level was also observed in breast cancer patients undergoing radiotherapy (Zhang *et al.*, 2012). Interestingly, regarding the results of the present study, the report of Farabegoli and coworkers (2011) confirm our findings showing the ability of EGCG to reduce the activity of matrix MMPs in resistant tumour cells. They have demonstrated that EGCG downregulates MMP-2 and MMP-9 in MCF7 tamoxifen-resistant cells and inhibits their invasion.

As presented above, there is an interest of several investigators to examine the effect of EGCG on the downregulation of matrix MMPs in breast cancer, including resistant cells. In contrast, although there are some data reporting the activity of GA in matrix MMPs downregulating in various tumours (Liu *et al.*, 2011; Lo *et al.*, 2011: Chen & Chang, 2012), up to now very little is known in the literature about this activity in regard to resistant breast cancer. Nevertheless, our results showed that the ability of this polyphe polyphenolic compound in reducing the activity of MMP-2 and MMP-9 in MCF7/DOX and MCF7/DOX$_{500}$ cells, comparing the effects exerted by EGCG, is also considerable, although achievable at higher concentrations (in the range of 100 μM).

Further studies are required to investigate at the molecular level the demonstrated activity of GA and EGCG to reduce the activity of MMP-2 and MMP-9 in MDR breast cancer cells. These studies should include the investigations of their cellular signalling pathway effectors and the examination of the ability of these polyphenolic compounds to reduce the invasion and metastatic potential of resistant tumour cells.

Conflict of interest

There are no conflicts of interest to disclose.

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