Anticancer activity of some polyamine derivatives on human prostate and breast cancer cell lines

Marta Szumilak1,2, Małgorzata Galadyszynska2, Kamila Dominska2, Andrzej Stanczak1 and Agnieszka Piastowska-Ciesielska2,3

INTRODUCTION

Cancer is used as a general term describing a group of approximately 120 different diseases, which can affect various parts of the body. It can be also defined as the state characterized by uncontrolled cell proliferation and normal tissues invasion (Latosinska & Latosinska, 2013). According to the Institute for Health Metrics and Evaluation (IHME) report, cancer is the second leading cause of death worldwide. Increased incidence of cancer observed in the developed countries can be attributed in part to the changes in demographic structure i.e. greater longevity of population, as well as risk factors like smoking, obesity and unhealthy diet (Fitzmaurice et al., 2015).

Prostate cancer (PCA) has the highest incidence ratio and it is the second most common cause of death in men (Siegel et al., 2016). In Poland, in 2013, about 12 162 men received a prostate cancer diagnosis and an estimated 4 281 men died of it (Dominska et al., 2012; Wojciechowska & Didkowska, 2013). When surgery is excluded, the basic method of conservative treatment in cases of advanced cancer is hormonal therapy involving the elimination of endogenous androgens and the blockade of the androgen receptor. Hormonal therapy slows down the development of cancer but it does not lead to full recovery. After an initial period of improvement, the disease progression ensues due to the development of androgen independent cancer, followed by fully hormone resistant cancer. As aggressive, poorly-differentiated high-grade PCa is currently incurable and potentially lethal, there is a need for a new treatment strategy which can be provided by newly designed anticancer medicines (Siegel et al., 2013; Jemal et al., 2011; Walczak & Carducci, 2007).

The second type of cancer cell line included in this study is derived from breast cancer, which is the most common female cancer worldwide (Wojciechowska & Didkowska, 2013; Siegel et al., 2016). In Poland, in 2013, about 17 142 women received a breast cancer diagnosis and it is estimated that 5 816 women died of the disease. Some breast cancers rapidly develop multidrug resistance to chemotherapy medicines which results in therapeutic failure (Li et al., 2015). Aforementioned data indicate that the search for novel, effective and less toxic anticancer agents is very important goal for contemporary medicine (Ma & Adjei, 2009).

Our quest for potential anticancer agents is focused on symmetrical polyamine derivatives with bicyclic terminal moieties designed according to bisintercalators’ structural requirements (Szulawska-Mroczek et al., 2013; Szumilak et al., 2010). Bisintercalators are able to interact reversibly with double stranded (dsDNA) by simultaneous insertion of two chromophores usually tethered by a polyamine linker. It results in higher DNA affinity and sequence selectivity in comparison to corresponding monointercalating agents (Braña et al., 2001; Lorente et al., 2004; Tse & Boger 2004). In addition, a positive correlation between cytotoxic potency and the strength of reversible DNA binding for bisintercalators has been observed (Tahe & Hegazy, 2013).

Our previous studies involving synthesis and biological in vitro evaluation of polyamine derivatives with various bicyclic moieties, revealed that polyamine derivatives with quinoline 3a and chromane 5a scaffolds (Fig. 1) are the most promising entities exhibiting antiproliferative activity toward a highly aggressive melanoma cell line A375 (Szulawska-Mroczek et al., 2013; Szumilak et al., 2010). Although the chemical structure of 5a was formerly known as a chelating agent (Trathnigg et al., 1985), we have obtained it by another route and assessed its...
antiproliferative activity together with other chromone/chromane derivatives designed as potential bisintercalators (Szulawska-Mroczek et al., 2013).

Taking into consideration that prostate cancer in men and breast cancer in women belong to the most frequently registered malignant cancers (Siegel et al., 2016), we decided to evaluate the influence of 3a and 5a on well-described prostate and breast cancer cell lines that are commonly used as models of drug susceptibility: PC3, DU145 and MCF7 (Sampson et al., 2013; Ellum et al., 2014; Li et al., 2015; Ming et al., 2015). The aforementioned choice can be supported by studies reporting that quinoline ring system is used in many anticancer agents (Burns et al., 2002; Deady et al., 1997; Hansch & Verma, 2007; Li et al., 2016; Rescifina et al., 2014) and chromone derivatives possess promising anticancer activity toward breast cancer (Rawat et al., 2016) and leukaemia (Nawrot-Modranka et al., 2006) or prevent progression to a metastatic phenotype for human prostate cancer (Xu et al., 2010).

MATERIALS AND METHODS

Examined compounds. \(N,N'-(\text{piperazine-1,4-diyl-diprop}-\text{ane-3,1-diylbis(4-aminomethylamine-3-carboxamide) and 5a N,N'[\text{methylimino}]-\text{dipropionate-3,1-diylbis(3-aminomethy}-\text{lene)chroman-2,4-dione}]} \) (Szumilak et al., 2010; Trathnigg et al., 1985; Szulawska-Mroczek et al., 2013).

Cell culture. Metastatic human prostate adenocarcinoma cell line PC3 (American Type Culture Collection, ATCC® CRL-1435®) and mammary gland adenocarcinoma cell line MCF7 (European Collection of Cell Culture, ECACC® 86012803) were maintained in RPMI1640 medium. Human carcinoma cell line DU145 (American Type Culture Collection, ATCC® HTB145®) was maintained in DMEM. Both media were supplemented with 10% (v/v) heat-inactivated Foetal Bovine Serum (FBS) (Thermo Fisher Scientific Inc./Life technologies). The cells were cultured at 37°C under a humidified atmosphere with 5% CO₂. Before each experiment, the cells were deprived of serum for 24 h.

Cell viability – mitochondrial metabolic activity. Cell viability was estimated on the basis of mitochondrial metabolic activity using WST1 (disodium mono-[43- (4-iodophenyl)-2H-tetrazol]-3-im-5-yl benzene-1,3-disulfonate]) assay as described elsewhere (Piastowska-Giesielska et al., 2013). PC3, DU145 and MCF7 cells were seeded on 96-well plates at a density of \(1 \times 10^4 \) per well. After 24 h, the culture medium was replaced by an experimental one with desired concentrations of compounds and treated for 24 or 48 h. Following incubation, 10 µl of WST1 reagent was added and plate was incubated for further 4 h. The spectrophotometric absorbance of each well was measured at 450 nm using ELX808IU plate reader (BioTek). The same procedure was repeated 14 days after the initial dissolution of compounds. Relative cell viability (%) was expressed as a percentage relative to untreated control cells. IC₅₀ – concentration leading to 50% reduction in cell viability, compared to untreated control, was determined from the sigmoidal curve obtained by plotting the percentages of cell viability relative to the control versus logarithmic concentration of compounds using a non-linear regression analysis (Saleh et al., 2015). On the basis of WST1 results, effective concentrations of 3a and 5a (for each cell line) were chosen for use in the remaining experimental assays.

Cell damage – Lactate Dehydrogenase (LDH) Leakage Assay. Cytotoxic potential of polyamine derivatives was measured using Cytotoxicity Detection Kit PLUS (LDH) (Roche) which allows the activity of lactate dehydrogenase released from damaged cells to be measured. Briefly, cells grown in 96-well plates at a density of \(7 \times 10^4 \) per well were treated with compounds 3a or 5a for 48 h. After incubation, LDH assay was performed according to the manufacturer’s protocol. The absorbance of each well was measured at 490 nm and 690 nm as a reference, using ELX808IU plate reader (BioTek). LDH leakage was calculated using the following function: LDH leakage (%) = \(100 \times (\text{CS–BC})/\text{(NDC–BC)}\). CS, BC and NDC refer to absorption of the culture supernatant, the background control and the undamaged control, respectively.

Mitochondrial membrane potential. Changes in mitochondrial membrane potential were determined with Muse™ Mitopotential Kit (Merk Millipore) which measures the accumulation of dye within the inner membrane of intact mitochondria. Briefly, cells grown in 6-well plates (at a density of \(2.5 \times 10^5 \) per well) were treated with 3a or 5a. After 48 h of incubation, cells were collected by trypsinization, resuspended in media and counted. All samples were prepared according to the manufacturer’s protocol and measured on Muse™ Cell Analyzer (Merk Millipore) according to manufacturer’s instruction, standardized to control probes.

Cell cycle analysis. The cell cycle distribution analysis was performed with Guava® easyCyte (Merk Millipore) using FlowCellec™ Bivariate Cell Cycle Kit for G2/M Analysis kit (Merk Millipore). Cells were cultured on cell-cycle dish at a density of \(1 \times 10^6 \) for 24 h, after which they were treated with 3a (20 µM for all cell lines) and 5a (5 µM for MCF7, 10 µM for PC3 and 25 µM for DU145) for 48 h. After incubation, cells were harvested and counted. Samples were prepared according to the manufacturer protocol and then measured on Guava® easyCyte (Merk Millipore).

Quantification of apoptosis. Apoptosis was examined using Muse™ Annexin V & Dead cell Kit (Merk
Millipore). Cells were cultured on 6-well plates at a density of $2.5 \times 10^5$ for 24 h, after which they were treated with compound 3a or 5a for 48 h. After treatment, cells were collected and incubated with Annexin V and 7-aminoactinomycin D (7AAD), a dead cell marker, for 20 min at room temperature in the dark. All samples were measured using Muse™ Cell Analyzer (Merck Millipore).

**Statistical analysis.** Results were expressed as means of results from a minimum of three independent experiments with similar patterns. IC$_{50}$ was estimated from the sigmoidal curve obtained by plotting the percentages of cell viability relative to the control against the logarithmic concentration of compounds using non-linear regression analysis. Statistical analysis was performed using one-way ANOVA. All calculations were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, California, USA). A p-value below 0.05 was considered statistically significant. All experiments were performed as three independent repetitions.

**RESULTS**

Compounds’ stability in cell culture media

The influence on cancer cell viability of 3a and 5a immediately after initial dissolution (T0) and after 14 days (T14) was compared to determine the compounds’ stability. A decrease in cell viability after treatment with 5a was denoted after both incubation times (24 and 48 h) for T0 and T14 as well. However, 5a at 50 µM, decreased the cell viability by 50% in PC-3 cells at T0 (24 h incubation), but only by 31% at T14. Similar results were observed after 48 h of incubation (data not shown). Furthermore, the ability of compound 3a to inhibit cancer cell viability fell 14 days after dissolution. For example, after 24 h of incubation with compound 3a at 25 µM, a 45% decrease in cell viability was observed at T0, but only 21% at T14. No significant difference between the results of 24 and 48 h of incubation for each time point was noticed. Therefore, both compounds were dissolved immediately before each experiment and a 48 hr incubation period was used.

**Influence of compounds on cancer cells – determination of IC$_{50}$.** Water-soluble tetrazolium (WST1) was used to assess the influence of compounds 3a and 5a on cell viability of prostate and breast cancer cells (measured via mitochondrial metabolic activity). The assay is based on WST1, a highly sensitive tetrazolium that produces soluble formazan via the NADPH oxidation reduction in mitochondria. The amount of formazan dye yielded, directly correlates to the number of metabolically-active live cells in the culture (Xiong et al., 2015). As before, in the first step, a concentration response course
analysis was performed to determine the compounds’ concentration required to inhibit the growth of cancer cells by 50% ($IC_{50}$) after 48 h of incubation (Szumilak et al., 2010). Compounds 3a and 5a were tested in a wide range of concentrations from 5 µM to 90 µM. Treatment of prostate and breast cancer cells with examined compounds resulted in concentration-dependent inhibition of cell mitochondrial activity which corresponded to cell viability (Fig. 2). $IC_{50}$ values for compound 3a were found to be 23.70 µM for PC3, 26.64 µM for DU145 and 18.54 µM for MCF-7. Chromane derivative 5a exhibited a lower inhibitory activity than the quinoline one which is illustrated by following the $IC_{50}$ values: 36.19 µM, 49.20 µM and 21.39 µM for PC3, DU145 and MCF7, respectively.

Effect of compounds on cancer cell lactate dehydrogenase leakage and mitochondrial membrane potential

The effect of 3a and 5a on lactate dehydrogenase activity in all cancer cell lines was determined by LDH assay. Results of LDH leakage after treatment with 3a and 5a at various concentrations are shown in Fig. 3. LDH leakage significantly increased after treatment with 3a as compared to control ($p<0.05$). It depended on the compound’s concentration and cancer type. The highest level of LDH leakage was observed in PC3 and DU145 prostate cancer cell lines following treatment with compound 3a at 30 µM ($p<0.05$) (Fig. 3A, B). In case of compound 5a, LDH leakage was observed only for DU145 at the concentration of 50 µM (data not shown) and MCF7 at all tested concentrations (Fig. 3D). The toxicity of compounds 3a and 5a was further evaluated by microphotographs, which demonstrated that cells treated with 3a decreased in size and density and exhibited increased cellular damage (Fig. 2A-C, lower panel). In addition, π significantly higher number of PC3 cells with depolarized mitochondria was observed after their exposure to 3a (at indicated concentrations), as compared to non-treated cells (Table 1). This observation was not confirmed for DU145 and MCF7 cell lines. Compound 5a did not induce morphological changes in any tested cell line (Fig. 2A–C).

### Table 1. The effect of polyamine derivative 3a containing quinoline moiety on depolarization of the mitochondrial membranes in PC3 cells. Results are presented as the percentage of cells compared to untreated cells.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Live (LR)</th>
<th>Depolarized/Live (LL)</th>
<th>Depolarized/Dead (UL)</th>
<th>Dead (UR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µM</td>
<td>59.66 ± 1.59</td>
<td>52.87 ± 3.64</td>
<td>52.91 ± 2.04</td>
<td>204.8 ± 2.61</td>
</tr>
<tr>
<td>25 µM</td>
<td>10.93 ± 0.65</td>
<td>11.37 ± 1.46</td>
<td>16.52 ± 0.50</td>
<td>30.31 ± 1.66</td>
</tr>
<tr>
<td>30 µM</td>
<td>29.19 ± 1.12</td>
<td>35.61 ± 2.39</td>
<td>30.31 ± 1.66</td>
<td>59.66 ± 1.59</td>
</tr>
</tbody>
</table>

Influence of compounds on cell cycle and apoptosis of cancer cells

To gain insight into the cytotoxic mechanism of action of compounds 3a and 5a, their influence on the cell cycle was assessed. DNA analysis based on PI-based staining of DNA content and Anti-phospho-Histone H3 (Ser10) antibody was used to discriminate and measure the percentage of cells in each cell cycle phase (G1, S, G2 and M). The cell cycle analysis confirmed that cell treatment with 3a resulted in cell cycle perturbation for all cancer cell lines. In untreated cells, a predominant number of cancer cells accumulated in the G1 phase. Compound 3a caused a reduction in the number of cells in the G1 phase and induced a cell population shift to the S phase of the cell cycle. This effect was clearly visible in DU145 cells treated with 3a at 20 µM (Fig. 4B). A discreet accumulation of cells in the G2 phase was also observed.

In turn, PC-3 and MCF-7 cells exposed to the chromane derivative 5a at the concentration of 10 µM and 5 µM, respectively, started to accumulate in the M phase (Table 2).

Programmed cell death can be initiated by several pathways. To determine whether the antiproliferative effect of both compounds can trigger cell apoptosis, DNA was stained with Annexin V and 7AAD. Apoptosis was induced in all cancer cells exposed to compound 3a at a concentration in the range from 20 µM to 30 µM. However, the greatest increase in the amount of early apoptotic cells (LR) was observed in prostate cancer (Fig. 5). The exposure of prostate and breast cancer cells to compound 5a did not induce apoptosis in any significant manner (data not shown).

### DISCUSSION

The search for new compounds targeting breast and prostate cancer is extremely important, due to their high incidence rates (Siegel et al., 2016; Wojciechowska et al., 2010). Compounds 3a and 5a were tested in a wide range of concentrations from 5 µM to 90 µM. Treatment of prostate and breast cancer cells with examined compounds resulted in concentration-dependent inhibition of cell mitochondrial activity which corresponded to cell viability (Fig. 2). $IC_{50}$ values for compound 3a were found to be 23.70 µM for PC3, 26.64 µM for DU145 and 18.54 µM for MCF-7. Chromane derivative 5a exhibited a lower inhibitory activity than the quinoline one which is illustrated by following the $IC_{50}$ values: 36.19 µM, 49.20 µM and 21.39 µM for PC3, DU145 and MCF7, respectively.

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Polyamine derivatives as potential anticancer compounds

As it was previously demonstrated, polyamine derivatives structurally related to known bisintercalators containing the quinoline 3a and chromane 5a moieties as terminal scaffolds, exhibit antiproliferative activity on a human melanoma cell line A375 (Szumilak et al., 2010), but the influence of these compounds on breast and prostate cancer has not been studied yet.

The results of current screening revealed significant differences in the anticancer activity of the examined compounds depending on a cancer cell line. Compound 5a was found to exhibit antiproliferative activity at a lower concentration in MCF7 (21 μM) in comparison to PC3 and DU145 cell lines (36 μM and 49 μM, respectively) (Fig. 2). However, it had no significant influence on the lactate dehydrogenase leakage or mitochondrial membrane depolarization which may suggest its limited anticancer properties. Our present findings also confirmed that the compound 3a exhibited anticancer activity against prostate and breast cancer cells. Inhibition of mitochondrial activity was directly connected with the increase of the compound concentration. Estimated IC50 values for PC3, DU145 and MCF7 cancer cells ranged from 18 μM to 27 μM (Fig. 2). Furthermore, in contrast to the chromane derivative 5a, the quinoline one (3a) caused changes in cell membrane integrity, as evidenced by increased lactate dehydrogenase leakage which correlated with mitochondrial membrane depolarization in the PC3 line (Table 1, Fig. 3).

To expand our knowledge about possible cytotoxic mechanism of action of the tested compounds, their effect on the cell cycle progression in breast and prostate cancer cells was examined. Compound 5a applied at the concentration approaching IC50 was found to have no statistically significant influence on changes in the prostate cancer cell cycle. In the examined prostate cell line, compound 5a was not able to decrease the number of cells in the G1, G2 or M phases, but an accumulation of cells in the S phase of the cell cycle was observed. However, when applied at the concentration needed for cell viability reduction to 60% (10 μM), compound 5a was responsible for changes in the number of PC3 cells in the G1, S, G2/M phases, and the increase of cell number in the G2 and M phases of the cell cycle (Table 2).

Compound 3a strongly affected changes in the cell cycle progression. Most of the cells from untreated PC3, DU145 and MCF7 lines accumulated in the G1 phase. In contrast, compound 3a induced a noticeable reduction in the number of cells in the G1 phase and the
displacement of cells in the S and G2/M phases. The exposure of DU145 cells to 3a resulted in 3a led to predominant distribution of cells in the S phase. However, the treatment of PC3 and MCF7 cells with 3a led to significant inhibition of cell cycle progression which is induction of cancer cell apoptosis. In case of the examined compounds, this effect is not unequivocal. Compound 5a was unable to significantly inhibit cell cycle, whereas the quinoline derivative exhibited a rather cytostatic than cytotoxic effect, whereas the quinoline derivative 3a changed the cell membrane integrity, as well as inhibited the growth of prostate and breast cancer by apoptosis. In addition, it has caused inhibition of cell cycle progression which suggests its potential application in the cancer therapy.

Conflicts of interests
The authors declare that they have no competing interests.

Acknowledgments

REFERENCES

CONCLUSION
The work presented here describes anticancer activity of polyamine derivatives with quinoline 3a and chromone 5a scaffolds, on human prostate and breast cancer cell lines. The observed differences in antiproliferative activity of the examined compounds were significant and depended on the cancer cell line. Bischromone derivative 5a exhibited a rather cytostatic than cytotoxic effect, whereas the quinoline derivative 3a changed the cell membrane integrity, as well as inhibited the growth of prostate and breast cancer by apoptosis. In addition, it has caused inhibition of cell cycle progression which suggests its potential application in the cancer therapy.


