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

Marta Szumilak1,*, Malgorzata Galdysznyska2, Kamila Dominska2, Andrzej Stanczak1 and Agnieszka Piastowska-Ciesielska1,2,3,3a

1Department of Hospital Pharmacy, Faculty of Pharmacy, Medical University of Lodz, Łódź, Poland; 2Department of Comparative Endocrinology, Medical University of Lodz, Łódź, Poland; 3Laboratory of Cell Cultures and Genomic Analysis, Medical University of Lodz, Łódź, Poland

The aim of this study was to expand our knowledge about anticancer activity of some polyamine derivatives with quinoline or chromane as terminal moieties. Tested compounds were evaluated in vitro towards metastatic human prostate adenocarcinoma (PC3), human carcinoma (DU145) and mammary gland adenocarcinoma (MCF7) cell lines. Cell viability was estimated on the basis of mitochondrial metabolic activity using water-soluble tetrazolium WST1 to establish effective concentrations of the tested compounds under experimental conditions. Cytotoxic potential of polyamine derivatives was determined by the measurement of lactate dehydrogenase activity released from damaged cells, changes in mitochondrial membrane potential, the cell cycle distribution analysis and apoptosis assay. It was revealed that the tested polyamine derivatives differed markedly in their antiproliferative activity. Bischromane derivative 5a exhibited a rather cytostatic than cytotoxic effect on the tested cells, whereas quinoline derivative 3a caused changes in cell membrane integrity, inhibited cell cycle progression, as well as induced apoptosis of prostate and breast cancer cells which suggest its potential application in cancer therapy.

Key words: polyamine derivatives, prostate cancer, breast cancer, mitochondrial potential, cell cycle, apoptosis

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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 (Branca 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 (Taher & 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 (Szuławska-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; Řehmann 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 chromane 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’-(piperazine-1,4-diyl)diprop-3-ylpropane-3,1-diyl)bis[4-aminoquinoline-3-carboxamide] 3a as hydrochloride and N,N’-[methyl(methylamino)diprop-3-1-diyl]bis[3-(aminomethylene)chroman-2,4-dione] 5a (Fig. 1) used in this study, were chosen from previously synthesized and in vitro evaluated polyamine derivatives. Their synthesis and analytical data were described earlier (Szułmilak et al., 2010; Trathnigg et al., 1985, Szuławska-Mroczek et al., 2013). Both substances were dissolved immediately before the experiment at concentrations ranging from 5 μM to 50 μM for 3a and 5 μM to 90 μM for 5a. Untreated cells were used as a control.

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® HTB81®) was maintained in DMEM. Both media were supplemented with 10% fetal 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-[4-[3-(4-iodophenyl)-2H-tetrazol]-3-ium-5-ylbenzene-1,3-disulfonate]) assay as described elsewhere (Piasowska-Giesielska et al., 2013). PC3, DU145 and MCF7 cells were seeded on 96-well plates at a density of 1 × 10⁴ 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 (BioTeck). 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 × 10⁴ 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 (BioTeck). LDH leakage was calculated using the following function: LDH leakage (%) = 100 × (CS–BC)/(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 (Mercck 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 × 10⁶ 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 (Mercck Millipore) according to manufacturer’s instruction, standardized to control probes.

Cell cycle analysis. The cell cycle distribution analysis was performed with Guava® easyCyte (Mercck Millipore) using FlowCell® Bivariate Cell Cycle Kit for G2/M Analysis kit (Mercck Millipore). Cells were cultured on cell-cycle dish at a density of 1 × 10⁶ per well, 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 (Mercck Millipore).

Quantification of apoptosis. Apoptosis was examined using Muse™ Annexin V & Dead cell Kit (Mercck
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 versus 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 PC3 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 oxidase 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
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., 2017). The exposure of prostate and breast cancer cells to compounds containing chromane moiety increases LDH activity in PC3 (A), DU145 (B) and MCF7 (C) cell lines. Polyamine derivative 5a containing chromane moiety increases LDH activity in the MCF7 (D) cell line. All data is the mean of three independent experiments (*<p<0.05; **<p<0.01; ***<p<0.001).

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, PC3 and MCF7 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).

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). IC50 values for compound 3a were found to be 23.70 µM for PC3, 26.64 µM for DU145 and 18.54 µM for MCF7. Chromane derivative 5a exhibited a lower inhibitory activity than the quinoline one (3a) which is illustrated by following the IC50 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).

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</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></td>
</tr>
<tr>
<td>25 µM</td>
<td>10.93 ± 0.65</td>
<td>11.37 ± 1.74</td>
<td>16.52 ± 0.50</td>
<td></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>0.22 ± 0.05</td>
</tr>
<tr>
<td>50 µM</td>
<td>95.86 ± 1.59</td>
<td>95.65 ± 3.45</td>
<td>107.8 ± 1.64</td>
<td>114.2 ± 4.62</td>
</tr>
</tbody>
</table>

Table 2. The effect of polyamine derivative 5a containing chromane moiety, on cell cycle distribution of PC3 cells. Results are presented as the percentage of treated cells when compared to untreated cells.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>PC3</th>
<th>DU145</th>
<th>MCF7</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µM (G1)</td>
<td>95.98 ± 2.41</td>
<td>90.98 ± 2.43</td>
<td>97.89 ± 3.0</td>
</tr>
<tr>
<td>10 µM (S)</td>
<td>88.61 ± 3.95</td>
<td>102.8 ± 2.91</td>
<td>95.55 ± 3.45</td>
</tr>
<tr>
<td>10 µM (G2)</td>
<td>138.1 ± 1.08</td>
<td>84.89 ± 3.44</td>
<td>107.8 ± 1.64</td>
</tr>
<tr>
<td>10 µM (M)</td>
<td>204.8 ± 2.61</td>
<td>47.23 ± 1.67</td>
<td>114.2 ± 4.62</td>
</tr>
</tbody>
</table>

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., 2017). The exposure of prostate and breast cancer cells to compounds containing chromane moiety increases LDH activity in PC3 (A), DU145 (B) and MCF7 (C) cell lines. All data is the mean of three independent experiments (*<p<0.05; **<p<0.01; ***<p<0.001).
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 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 the greatest accumulation of cells in the S rather than the G2/M phase. However, the treatment of PC3 and MCF7 cells with 3a led to predominant distribution of cells in the G2/M phase (Fig. 4).

The expected anticancer feature of any potential drug is induction of cell cancer apoptosis. In case of the examined compounds, this effect is not unequivocal. Compound 5a was unable to significantly induce apoptosis, while compound 3a induced programmed cell death in the prostate and breast cancer cell lines in a significant manner. As shown in Fig. 5, the greatest increase in the amount of early apoptotic cells (LR) was observed in prostate cancer. Our findings are in accordance with other studies, showing that quinoline derivatives are capable of inducing apoptosis by an intrinsic pathway which involves depolarization of mitochondria together with disruption of their membranes (Sahu et al., 2013; Saleh et al., 2015a,b).

CONCLUSION

The work presented here describes anticancer activity of polyamine derivatives with quinoline 3a and chromane 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. Bischromane 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.

Conflicts of interests

The authors declare that they have no competing interests.

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