Antiproliferative Activity of Derivatives of Ouabain, Digoxin and Proscillaridin A in Human MCF-7 and MDA-MB-231 Breast Cancer Cells

Katarzyna Winnicka,*a Krzysztof Bielawski,b Anna Bielawska,b and Arkadiusz Surażyński\b

*Department of Pharmaceutical Technology, Faculty of Pharmacy, Medical University of Białystok, Kilinskiiego 1, 15–089 Białystok, Poland; and \bDepartment of Medicinal Chemistry and Drug Technology, Faculty of Pharmacy, Medical University of Białystok, Mickiewicza 2, 15–089 Białystok, Poland.

Received December 18, 2007; accepted March 13, 2008; published online March 17, 2008

Three derivatives of ouabain, digoxin and proscillaridin A containing the carboxylic group instead of the lactone moiety were synthesized and examined for cytotoxicity in human breast cancer cells. Evaluation of the cytotoxicity of these compounds employing an MTT assay and inhibition of [3H]thymidine incorporation into DNA in both MCF-7 and MDA-MB-231 breast cancer cells demonstrated that compound 3, the most active of the series, proved to be only slightly less potent than proscillaridin A. We evaluated the effects of these compounds 1—3 on change in intracellular Ca\textsuperscript{2+}, appearance of apoptosis, inhibition of DNA topoisomerase I and II, and the activity of caspase-3 in breast cancer cells. These studies indicate that the increase in potency for 3 may be related, in part, to an activation of caspase-3, increasing free calcium concentration and topoisomerase II inhibition. All these data emphasize the potential usefulness of these derivatives of cardiac glycosides as anticancer agents.

Key words cardiac glycoside; cytotoxicity; breast cancer cell; DNA topoisomerase

Plants have been a rich source of bioactive glycosides. The Digitalis family, in particular, has produced a number of steroidal glycosides which have been utilized medicinally in the treatment of cardiac diseases for centuries. Epidemiological data along with reports based on \textit{in vitro} and \textit{in vivo} demonstration of steroidal glycosides mediated anticancer activity, further emphasize the possibility of developing this class of compound as antitumor agents.\textsuperscript{11} For example, observation of the altered morphology of breast cancer cells from women on digoxin when mastectomated for breast cancer showed that they had more benign characteristics than cancer cells from control patients not on digitalis.\textsuperscript{12} Five years after the mastectomy, cancer recurrence rates in patients not taking digitalis increased by 9.6-fold, as compared with patients who were treated with digitalis.\textsuperscript{21} In a 20-year follow-up Stenkvist has reported that the death rate from breast carcinoma (excluding other causes of death and confounding factors) was 6% among patients on digitalis, compared with 34% among patients not on digitalis.\textsuperscript{22}

Digitalis compounds can be classified as cardenolides, compounds identified originally in plants, and bufadienolides, identified in toad venom. They share a common general structure: \textit{i.e.}, a steroid nucleus with a lactone ring at C-17 and a hydroxyl group at C-14. The 5-membered- and 6-membered lactone rings, in cardenolides and bufadienolides, respectively, are considered the most essential functional group of these substances. Bufadienolides were found to induce apoptosis in human leukemia cells and have antiproliferative and immunosuppressive activity on T-cells.\textsuperscript{5–9} Furthermore, several studies have demonstrated that derivatives of ouabain and ouabain at high (μM) concentrations also induce apoptosis \textit{via} activation of caspase-3, early cytochrome C release from mitochondria and generation of reactive oxygen species (ROS) in prostate cell lines PC-3, LNCaP and DU145.\textsuperscript{10–15} In addition, it was recently shown that lung cancer cells can be sensitized to Apo2L/TRAIL-induced apoptosis by combined treatment with different cardiac glycosides.\textsuperscript{16} The potential use of cardiac glycosides for the treatment of cancer was abandoned because of the inherent high toxicity of these compounds. Their use can lead to death from cardiac arrhythmia and disturbances of atrio-ventricular contraction, as well as other less serious side effects (\textit{e.g.}, gastrointestinal disorders, neurological effects, anorexia). The studies on the structure–activity relationship revealed that lactone in position 17β and hydroxyl in position 14 are crucial for the cardiac activity of cardiotonic steroids.\textsuperscript{17–19} Therefore, we found it interesting to investigate whether derivatives of ouabain, digoxin and proscillaridin A without a lactone ring show the therapeutic potential in human breast cancer cells.

In our work, we have synthesized three derivatives of ouabain, digoxin and proscillaridin A containing the carboxylic group instead of the lactone moiety (Fig. 1). We evaluated the effects of these compounds 1—3 on the proliferation, cytotoxicity, change in intracellular Ca\textsuperscript{2+}, inhibition of DNA topoisomerase I and II, and appearance of apoptosis in the estrogen dependent breast cancer cell line MCF-7 and estrogen independent breast cancer MDA-MB-231 cell line.

MATERIALS AND METHODS

Materials

Digoxin, ouabain, proscillaridin A, ethidium bromide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Fura-2/AM, EGTA, acridine orange, ethidium bromide, alkaline phosphatase-labeled anti-mouse IgG antibody, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate reagent (BCIP/NBT), controlled process serum replacement Type 1 (CPSR1), Nonidet P-40 and Protein A-Sepharose were provided by Sigma Aldrich (St. Louis, U.S.A.), as were most other chemicals and buffers used. Antibody against human caspase-3 was purchased from Calbiochem (U.S.A.). Apoptest-FITC was a product of DakoCytomation (Denmark). Stock cultures of breast cancer MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection (U.S.A.). Dulbecco’s minimal essential medium (DMEM) and foetal bovine serum (FBS) used in cell culture were products of Gibco (U.S.A.). Gluta-
Ouabain (0.57 g, 0.78 mmol) was suspended in a mixture of biologicals (U.S.A.). [3H]Thymidine (6.7 Ci/mmol) was purchased from Topogen (U.S.A.). Amide and molecular weight standards were received from Bio-Rad Laboratories (U.S.A.). Topoisomerase I and II, supercoiled pHOT1 DNA, supercoiled pRYG DNA, etoposide, camptothecin were purchased from Topogen (U.S.A.).

Chemistry

The structures of all the compounds were confirmed by 1H- and 13C-NMR spectra recorded on Bruker AC 200F (Germany) apparatus (1H-200 MHz and 13C-50 MHz) in DMSO-d6. Infrared spectra were recorded on a Perkin Elmer Spectrum 100 (U.S.A.) as KBr pellets (4000—450 cm−1). Melting points were determined on Büchi 535 melting-point (Germany) apparatus and were uncorrected. Elemental analysis of C, H, N, was performed on a Perkin Elmer 240 analyser (U.S.A.) and satisfactory results within ±0.4% of calculated values were obtained. Chemical shifts are expressed in δ value (ppm). Multiplicity of resonance peaks are indicated as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m).


Ouabain (0.57 g, 0.78 mmol) was suspended in a mixture of 2 ml of THF and 2 ml of 1 M LiOH. The reaction was allowed to stir at room temperature until no starting ouabain could be detected by TLC (CHCl3: MeOH 5:1). The basic mixture was acidified to pH 4 with 2 M HCl and diluted with acetone. The precipitated acid was filtered and dried in vacuo at room temperature to afford 0.37 g of 1. Yield=79%, mp 195 °C; 1H-NMR (DMSO-d6) δ (ppm): 0.79 (s, 3H), 1.12 (d, J = 1.5 Hz, 1H); 1.41 (dd, J = 5.3, 9.0 Hz, 1H), 1.30 (m, 1H), 1.53 (m, 1H), 3.57 (m, 1H), 3.96 (brs, 1H), 4.18 (m, 1H), 4.34 (d, J = 10.5 Hz, 1H), 4.37 (d, J = 10.5 Hz, 1H), 4.69 (s, 1H), 4.80 (brm, 1H), 4.84 (d, J = 19.1 Hz, 1H), 4.91 (d, J = 19.1 Hz, 1H), 5.89 (s, 1H), 12.08 (s, 1H); 13C-NMR (DMSO-d6) δ (ppm): 17.11, 17.62, 22.96, 26.07, 32.30, 33.20, 34.91, 35.37, 38.94, 46.58, 47.37, 48.28, 48.76, 49.45, 62.57, 63.61, 66.70, 68.17, 69.22, 70.56, 70.68, 72.18, 73.54, 83.30, 97.08, 115.96, 172.35, 175.15; IR (KBr) cm−1: 3100—3500, 2980, 2880, 1793, 1710, 1645, 1633, 1451, 1422, 1413, 1399, 1335, 1097, 1059, 1019. Anal. Calcd for C36H36O10: C, 75.79; H, 7.69. Found: C, 75.82; H, 7.71.

Zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). 20) Confluencing to the method of Carmichael using 3-(4,5-dimethylthiophen-2-yl)-2,5-diphenyltetrazolium bromide (MTT). 20) Confluencing to the method of Carmichael using 3-(4,5-dimethylthiourea such cells were used for the assays.

Cells reached about 80% of confluency at day 3 and in most experiments the cells were lysed in 1 ml of 0.1 M phosphate-buffered saline (PBS), counted in hemocytometers and plated at 5×10^5 cells per well of six-well plates (Nunc) in 2 ml of growth medium (DMEM without phenol red with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin at 37 °C. Cells were cultured in Costar flasks and subconfluent cells were detached with 0.05% trypsin and 0.02% EDTA in calcium-free phosphate-buffered saline (PBS), counted in hemocytometers and plated at 5×10^5 cells per well of six-well plates (Nunc) in 2 ml of growth medium (DMEM without phenol red with 10% controlled process serum replacement Type 1 - CPSR1).

Cells reached about 80% of confluency at day 3 and in most cases such cells were used for the assays.

**Cell Viability Assay** The assay was performed according to the method of Carmichael using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). 20) Confluent cells, cultured for 24 h with various concentrations of studied compounds in six-well plates were washed three times with PBS and then incubated for 4 h in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37 °C. The medium was removed and 1 ml of 0.1 mol/l HCl in absolute isopropanol was added to detached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability of breast cancer MCF-7 and MDA-MB-231 cells cultured in DMEM with or without 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin was measured in a scintillation counter.

**DNA Synthesis Assay** To examine the effects of the drugs on cells proliferation, the cells were seeded in six-well plates and were incubated with varying concentrations of compounds 1—3 and 0.5 μCi of [3H] thymidine for 24 h and 48 h at 37 °C. After that time cell surface was rinsed three times with PBS. Then, the cells were lysed in 1 ml of 0.1 M NaOH containing 1% SDS. The cell lysate was added to 9 ml of scintillation liquid, and radioactivity incorporation into DNA was measured in a scintillation counter.

**Flow Cytometry Assessment of Annexin V Binding** Apoptosis was determined by assessment of phosphatidylserine exposure by Annexin V-FITC binding using the Annexin V-FITC staining kit (Apoptest-FITC, DakoCytomation, Denmark) according to the manufacturer’s instruction. Ungerated cells (10000) were analyzed in a flow cytometer (Beckman Coulter, U.S.A.). Annexin V binds with high affinity to phosphatidylserine and can thus be used to identify cells in all stages of programmed cell death. 21, 22) Propidium iodide exclusively stains cells with a disrupted cell membrane and can be used to identify late apoptotic and dead cells.

**Fluorescent Microscopy Assay** To assess apoptosis, cell viability was estimated 24 h after the addition of compounds 1—3. The cell suspension (250 μl) was stained with 10 μl of the dye mixture (10 μM acridine orange and 10 μM ethidium bromide), which was prepared in PBS. Acridine orange (fluorescent DNA-binding dye) intercalates into DNA, making it appear green, and binds to RNA, staining it red/orange. Ethidium bromide is only taken up by nonviable cells; its fluorescence overwhelms that of the acridine orange, making the chromatin of necrotic cells appear orange. 23) Two hundred cells per sample were examined by fluorescence microscopy, according to the following criteria: viable cells with normal nuclei (fine reticular pattern of green stain in the nucleus and red/orange granules in the cytoplasm); viable cells with apoptotic nuclei (green chromatin which is highly condensed or fragmented and uniformly stained by the acridine orange); nonviable cells with normal nuclei (bright orange chromatin with organized structure); and nonviable cells with apoptotic nuclei (bright orange chromatin which is highly condensed or fragmented).

**Immunoprecipitation** Subconfluent cells in six-well plates were washed with PBS, scraped out of the wells and centrifuged at 1000×g for 3 min. Then the cells (from six wells) were solubilized with lysis buffer containing 10 mm Tris–HCl, pH 7.4, 250 mm NaCl, 0.5% Nonidet P-40, 1 mm EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mm phenylmethylsulfonyl fluoride, at 4 °C for 10 min. The insoluble material was removed by centrifugation at 10000×g for 5 min at 4 °C. Supernatant containing 100 μg of protein was added to 100 μg of Protein A-Sepharose that had been linked to primary antibody in the following manner. Protein A-Sepharose was washed three times with lysis buffer and 100 μl of suspension containing about 100 μg of beads was incubated for 1 h at 4 °C with 20 μl of primary antibody. Then, the conjugate was incubated for 1 h at 4 °C with shaking. Immunoprecipitate was washed four times with lysis buffer. Proteins were released from the beads by boiling in SDS sample buffer and loaded onto a 10% SDS-polyacrylamide gel. The immunoprecipitates were analyzed by Western immunoblot.

**SDS-PAGE** Slab SDS/PAGE was used, according to the method of Laemmli, 24) by using 10% SDS-polyacrylamide gel. The immunoprecipitates were analyzed by Western immunoblot.

**Western Immunoblot Analysis** After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/l Tris–HCl, 0.2 mol/l glycine in 20% (v/v) MeOH. The protein was transferred to 0.2 μm pore-sized nitrocellulose at 100 mA for 1 h by using a LKB 2117 Multiphor II electrophoresis unit. The nitrocellulose was incubated with monoclonal antibody against human caspase-3 at concentration 1:5000 in 5% dried milk in Tris buffered saline with 0.05% Tween 20. The nitrocellulose was washed with 150 mmol/l NaCl and 0.05% Tween 20) for 1 h. In order to analyze caspase-3, second antibody-alkaline phosphatase conjugated anti-mouse IgG (whole molecule) was added at concentration 1:7500 in TBS-T and incubated for 60 min under gentle shaking. Then nitrocellulose was washed with TBS-T (5×5 min) and submitted to Sigma-Fast BCIP/NBT reagent. The intensity of the bands was quantified by densitometric analysis.

**Relaxation Assay of Topoisomerase I and II** Supercoiled pHOT1 DNA (0.5 μg) was incubated with 4 units...
of human topoisomerase I, in relaxation buffer (10 mM Tris–HCl (pH 7.8), 1 mM EDTA, 0.15 mM NaCl, 0.1% BSA, 0.1 spermidine, 5% glycerol), in the presence of varying concentrations of the tested compounds. Supercoiled pRIG DNA (0.5 μg) was incubated with 4 units of human topoisomerase II in cleavage buffer (30 mM Tris–HCl (pH 7.8), 50 mM KCl, 10 mM MgCl₂, 3 mM ATP, 15 mM mercaptoethanol), in the presence of varying concentrations of the tested compounds. Reactions were carried out at 37 °C for 1 h and then terminated by the addition of sodium dodecyl sulphate (SDS) to 0.25% and proteinase K to 250 mg/ml. The reaction mixture was subjected to electrophoresis through a 0.8% agarose gel containing 0.5 mg/ml ethidium bromide in TBE buffer (90 mM Tris–borate and 2 mM EDTA). The gels were stained with ethidium bromide and photographed under UV light.

**Determination of Intracellular Free Calcium Concentration (\[^{45}\text{Ca}^{2+}\])** MCF-7 and MDA-MB-231 breast cancer cells were treated with compounds 1—3 for 24 h. Cell suspension (1 ml containing 3×10⁶ cells) was loaded with 6 μg fura-2 acetoximethyl ester (Fura 2/AM) dissolved in 6 μl dimethyl sulfoxide. They were incubated in the dark for 30 min at 37 °C. After extensive washing, 1×10⁶ cells were resuspended in 2.5 ml loading buffer, consisting of 152 mM NaCl, 1.2 mM MgCl₂, 2.2 mM CaCl₂, 5 mM KCl and 10 mM Hepes. Fluorescence measurements were performed using a dual wavelength spectrometer system (Hitachi spectrophotometer F-2500 FL, Japan). Excitation of Fura-2 was performed at 340 and 380 nm, with excitation band widths set at 5 nm. The ratio of emitted fluorescence signals (510 nm) was used to calculate the cytosolic free Ca²⁺ concentration according to the method of Grynkiewicz et al.²⁵ Dye was considered saturated after lysis with 0.16 mM digitonin (maximum fluorescence). Minimum fluorescence was determined by adding EGTA to a final concentration of 8 mM.

**Statistical Analysis** The results were analysed by Analysis of Variance (ANOVA) and multiple comparison were done to check statistical significance. The data were expressed as mean value for 3 independent assays±S.D. The statistical significance between means was verified by Sheffe’s comparison test accepting p<0.05 as significant.

**RESULTS**

Ouabain, digoxin, and proscillaridin A were subjected to saponification with LiOH in THF/H₂O (1:1) solution. The sodium salts were acidified to pH 4 with 2 M HCl and diluted with acetone. The structures of the precipitated acids 1—3 (Fig. 1) were confirmed by IR, \(^{1}\text{H}-\) and \(^{13}\text{C}-\)NMR spectral data. The presence of a carboxylic group was indicated at δ 12.08 in the \(^{1}\text{H}-\)NMR spectrum, and δ 172.35 in the \(^{13}\text{C}-\)NMR spectrum, respectively. The primary hydroxy group for compounds 1 and 2 were indicated by methylene resonances at δ 63.61 in the \(^{13}\text{C}-\)NMR spectrum and corresponding resonances at δ 4.18 and 4.21 in the \(^{1}\text{H}-\)NMR spectrum. The presence of a formyl group of compound 3 was indicated at δ 9.8 in \(^{1}\text{H}-\)NMR and δ 203.2 in \(^{13}\text{C}-\)NMR.

Cell viability of breast cancer cells was measured by the method of Carmichael et al.²⁰ using tetrazolium salt (Tables 1, 2). The results of this study show that the acids 1—3 exert significant inhibitory effects on the proliferation of breast cancer cells. In terms of reduction in cell viability, the compounds rank for both 24 h and 48 h of incubation in breast cancer cells in the order 3>2>1. Although growth inhibition was concentration-dependent in either cell line, it was more pronounced at shorter times, in MCF-7 than MDA-MB-231 (Tables 1, 2). Among the derivatives, compounds 1 and 2 in both MDA-MB-231 and MCF-7 proved to be only slightly less potent than ouabain, with IC5₀ values in MCF-7 of 150±2 nm and 143±2 nm, and in MDA-MB-231 of 170±2 and 155±2 nm, respectively, compared to 130±2 nm for ouabain in MCF-7²⁶ and 150±2 nm in MDA-MB-231 cells.²⁷ In contrast, compound 3 is clearly much more active and showed a high level of cytotoxic potency, IC5₀ 65±2 nm and 83±2 nm in MCF-7 and MDA-MB-231, respectively. However, the effect of compound 3 was weaker than proscillaridin A, with IC5₀ respectively 30±2 and 51±2 nm.²⁶,²⁷

To analyze if the inhibition in cell viability was due to decreased cell proliferation, we measured DNA synthesis in presence of compounds 1—3. All of the tested compounds showed concentration-dependent activity, yet with different potency (Tables 3, 4). The concentrations of 1—3 needed to inhibit \(^{3}\text{H}\)-thymidine incorporation into DNA by 50% (IC₅₀) in MDA-MB-231 was found to be 198±2 nm, 160±3 nm and 100±2 nm, respectively, suggesting lower cytotoxic potency compared to proscillaridin (IC₅₀=48±2 nm).²⁷ The concentrations of 1—3 needed to 50% reduction in \(^{3}\text{H}\)-thymidine incorporation into DNA in breast cancer MCF-7 (IC₅₀) was found to be 180±2 nm, 150±2 nm, 95±2 nm, respectively.

Flow cytometric analyses based on the detection of morphological changes, DNA fragmentation, DNA loss, and membrane changes are increasingly used for quantitative

### Table 1. Viability of MCF-7 Cells Treated for 24 or 48 h with Different Concentrations of Compounds 1—3

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>Viability of MCF-7 Cells (% of control)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>91±2</td>
</tr>
<tr>
<td>30</td>
<td>86±3</td>
</tr>
<tr>
<td>60</td>
<td>79±2</td>
</tr>
<tr>
<td>90</td>
<td>68±3</td>
</tr>
<tr>
<td>120</td>
<td>59±2</td>
</tr>
<tr>
<td>150</td>
<td>51±2</td>
</tr>
</tbody>
</table>

a) Mean values±S.D. from three independent experiments (n=3) done in duplicate are presented.
investigations of apoptosis. Apoptosis was determined by assessment of phosphatidylserine exposure by Annexin V-FITC. During the early stage of apoptosis, phosphatidylserine translocates from the interior to the exterior part of the plasma membrane and becomes exposed at the cell surface, facilitating recognition by macrophages.\(^{21,22}\) The incubation of breast cancer cells with compounds 1—3 induced visible phosphatidylserine exposure after 4 and 24 h of treatment (Fig. 2). The percentage of necrotic cells after 4 h of incubation, in MCF-7 and MDA-MB-231 cells was only slightly lower than after 24 h of incubation. Apoptosis induced by 3 was definitely stronger than by 2 or 1. The compounds triggered apoptosis was also estimated by a fluorescent microscopy assay after acridine orange and ethidium bromide staining (Fig. 3). The percentage of early apoptotic cells was similar to the percentage of Annexin V-FITC. We also demonstrated that 3 caused increased necrosis cell death. At the high concentration of 3 (100 nM), cell was attributable to

<table>
<thead>
<tr>
<th>Table 2. Viability of MDA-MB-231 Breast Cancer Cells Treated for 24 or 48 h with Different Concentrations of Compounds 1—3</th>
<th>Concentration (nM)</th>
<th>Viability of MDA-MB-231 cells (% of control)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>96 ± 3</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>30</td>
<td>87 ± 3</td>
<td>80 ± 2</td>
</tr>
<tr>
<td>60</td>
<td>74 ± 2</td>
<td>71 ± 2</td>
</tr>
<tr>
<td>90</td>
<td>67 ± 2</td>
<td>64 ± 2</td>
</tr>
<tr>
<td>120</td>
<td>60 ± 2</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>150</td>
<td>55 ± 2</td>
<td>50 ± 2</td>
</tr>
<tr>
<td>170</td>
<td>51 ± 2</td>
<td>43 ± 2</td>
</tr>
</tbody>
</table>

\(a\) Mean values ± S.D. from three independent experiments \((n=3)\) done in duplicate are presented.

<table>
<thead>
<tr>
<th>Table 3. Antiproliferative Effects of Compounds 1—3 in Cultured Breast Cancer MCF-7 Cells as Measured by Inhibition of [^{3}H]Thymidine Incorporation into DNA</th>
<th>Concentration (nM)</th>
<th>[^{3}H]Thymidine incorporation (% of control)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>95 ± 2</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>30</td>
<td>90 ± 2</td>
<td>80 ± 3</td>
</tr>
<tr>
<td>60</td>
<td>77 ± 3</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>90</td>
<td>63 ± 2</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>120</td>
<td>60 ± 2</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>150</td>
<td>56 ± 2</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>180</td>
<td>52 ± 2</td>
<td>37 ± 2</td>
</tr>
</tbody>
</table>

\(a\) Mean values ± S.D. from three independent experiments \((n=3)\) done in duplicate are presented.

<table>
<thead>
<tr>
<th>Table 4. Antiproliferative Effects of Compounds 1—3 in MDA-MB-231 Breast Cancer Cells as Measured by Inhibition of [^{3}H]Thymidine Incorporation into DNA</th>
<th>Concentration (nM)</th>
<th>[^{3}H]Thymidine incorporation (% of control)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>97 ± 2</td>
<td>88 ± 3</td>
</tr>
<tr>
<td>30</td>
<td>86 ± 3</td>
<td>82 ± 2</td>
</tr>
<tr>
<td>60</td>
<td>71 ± 2</td>
<td>69 ± 2</td>
</tr>
<tr>
<td>90</td>
<td>65 ± 2</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>120</td>
<td>61 ± 2</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>150</td>
<td>58 ± 2</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>180</td>
<td>53 ± 2</td>
<td>48 ± 2</td>
</tr>
<tr>
<td>210</td>
<td>46 ± 2</td>
<td>36 ± 2</td>
</tr>
</tbody>
</table>

\(a\) Mean values ± S.D. from three independent experiments \((n=3)\) done in duplicate are presented.
cells (Fig. 3). It was shown that all of analyzed compounds inhibited the proliferation of cells in the malignant cell lines by increasing the number of apoptotic and necrotic cells. It is important to define the apoptotic action to compounds 1—3 for understanding the apoptotic mechanism in human breast cells. Apoptosis, a morphological distinct form of programmed cell death, requires the participation of endogenous cellular enzymes. Central to the apoptotic program is a family of cysteine proteases termed caspases. In living cells caspases are present as inactive zymogens and become activated after apoptotic stimuli. To date more than 10 distinct human caspase genes have been identified. Of these caspases the activation of caspase-3 is the crucial event in various cells that leads to the execution of apoptosis. Caspase-3 exists as a 32 kDa inactive precursor protein in the cytoplasm of most cell types. Upon proteolytic activation the protein is cleaved at a specific site within the molecule. Immunoblot analyses of cytosolic extracts demonstrated that compounds 1—3 originated cleavage of the 32 kDa procaspase-3 into lower molecular weight fragments (Fig. 4). Differences in the proteolytic process generated by the toxic action of the analyzed compounds were observed—compound 3 induced significantly higher degradation of procaspase-3 into 17 kDa subunit (Fig. 4). Taken together, these results suggest that all the synthesized compounds are able to decrease the viability of MDA-MB-231 cells through induction of caspase-dependent apoptosis. MCF-7, a breast cancer-derived cell line is deficient of caspase 3. It is due to a deletion mutation in exon 3 of the CASP-3 gene.

Both Ca2+ influx and K+ efflux have been proposed to mediate cardiac glycosides cytotoxicity. We hypothesized...
that the caspase-independent cell death might be associated with the cardiac glycosides induced Ca\(^{2+}\) increase. Previous study reports that digoxin-induced toxicity of prostate cancer cells is accompanied by the increase of intracellular Ca\(^{2+}\),\(^{14,31}\) which is believed to be a key factor in apoptosis. To verify that this mechanism was involved in the proapoptotic effects of compounds 1—3, we measured intracellular Ca\(^{2+}\) concentration in single, fura-2 loaded breast cancer cells by dual-wavelength spectrofluorophotometer (Fig. 5). After an extended treatment period of 24 h compounds 1—3 increased intracellular Ca\(^{2+}\) concentration in breast cancer cells. Figure 5 shows that addition of 100 nM compounds 1—3 induced Ca\(^{2+}\) influx of 127±2, 130±2, and 161±2 nM in MCF-7, and 120±2, 122±2 and 136±2 in MDA-MB-231 cells respectively. These values are significantly greater than control (108±2 nM in MCF-7 and 97±2 in MDA-MB-231 cells). Our results indicate that compounds 1—3 may inhibit proliferation of breast cancer cells, possibly resulting from a process involving elevation of intracellular Ca\(^{2+}\) level and of the induced apoptosis.

Since literature evidence indicated that some glycosides also had inhibitory activity against the mammalian DNA topoisomerases, we decided to study our compounds against both enzymes. To test whether cytotoxic properties were related to topoisomerase action, these compounds were evaluated in a cell-free system. Purified topoisomerase I and II were incubated with increasing concentrations of 1—3 in the presence of supercoiled plasmid DNA, and the products were subjected to electrophoresis in the presence of ethidium bromide to separate closed and open circular DNA. The DNA samples were treated with sodium dodecylsulfate (SDS) and proteinase K to remove any covalently bound protein and were then resolved in a 1% agarose gel.

To examine whether the synthesized compounds stimulate the stabilization of the cleavable complex, supercoiled RYG DNA was incubated with 4 units of topoisomerase II in the presence of compounds 1—3 and etoposide as a control compound (Fig. 6). In this case, the relaxation of DNA was inhibited with all three drugs. The most potent was 3, which

![Fig. 5. Intracellular Ca\(^{2+}\) Concentration in MCF-7 (A) and MDA-MB-231 (B) Cells Treated for 24 h with 100 nM Compounds 1—3. Mean values±S.D. from three independent experiments (n=3) done in duplicate are presented.](image)

![Fig. 6. Inhibition of Topoisomerase II-Mediated DNA Supercoiling in the Presence of Etoposide (ET) and Various Concentration of Compound 1 (A), Compound 2 (B) and Compound 3 (C).](image)
inhibited the topoisomerase II activity almost completely at 30 nm concentration, proving more potent than etoposide, under the same experimental conditions. Enzymatic activity (relaxation of pRYG plasmid) was inhibited for 1 and 2 at concentrations of 100 nm (Fig. 6).

To determine if the synthesized compounds are selective inhibitors of topoisomerase II, we tested their effect on the catalytic activity of topoisomerase I (Fig. 7). The control compound antitumor drug camptothecin specifically targets topoisomerase I, stabilising cleavable complexes through inhibition of the religation step. It can be seen unambiguously that 3 at concentrations of 100 nm (and the reference topoisomerase I inhibitor camptothecin) strongly promote DNA cleavage by human topoisomerase I. As shown in Fig. 7, no inhibitory effect was observed for compounds 1 and 2, even up to the concentration of 100 μM. Results from this work indicate that 3 functions as a poison of topoisomerase I.

DISCUSSION

In breast cancer studies, estrogen responsive and -nonresponsive breast cancer cell lines have been extensively used for elucidating the factors responsible for cell growth and for developing new strategies to inhibit cell growth. There are several lines of evidence indicating that cardiac glycosides possess potent anti-breast cancer activity. While it is not completely clear how the suggested anticancer activity of these drugs is achieved, several observations point to cardiac glycosides additionally to inhibition of the plasma membrane Na\(^+/K\(^+\)-ATPase behaving as potential oestrogen receptor antagonists. Chen et al. further to suggesting the utility of the sodium pump as an oncology target have also suggested that it could be useful to develop cardiac glycosides as anti-breast cancer drugs; both as Na\(^+/K\(^+\)-ATPase inhibitors and oestrogen receptor (ER) antagonists. In fact, while anti-oestrogen therapy (used to treat ER-positive breast cancer) is initially successful, a major problem is that most tumours develop resistance and the disease ultimately progresses. In addition, a hypothesis was postulated that alterations in the metabolism of endogenous digitales-like compounds and changes in their interactions with Na\(^+/K\(^+\)-ATPase might be associated with the development of cancer itself. Besides this tissue-specific expression pattern under normal physiological conditions, sodium pump isoform expression is specifically altered in cancer cells and tissues. Also, not only does the activity of Na\(^+/K\(^+\)-ATPase differ between normal and malignant cells, but also their sensitivity towards cardiac glycosides. This may be due to an altered density of Na\(^+/K\(^+\)-ATPase at the plasma cell membrane of tumour cells, as well as differences in isozyme expression.

The well known and accepted mode of action of cardiac glycosides is inhibition of the plasma membrane Na\(^+/K\(^+\)-ATPase. More recently it was found out that the Na\(^+\), K\(^+\)-ATPase might also act as a cell signaling receptor and play a role in the regulation of cell growth and expression of various genes. Interestingly, cardiac glycosides affect various cells at the concentrations lower than that required for the inhibition of the sodium pump. To block the Na\(^+\), K\(^+\)-ATPase relatively high, lethal concentrations (≥10\(^{-5}\)M) of the cardiotoxic steroids are needed. The concentrations used in our experiments were definitely lower—in nanomolar range. Muntau et al.\(^{33}\) have shown that analogs of cardiac steroids without a lactone ring exhibited significantly lower potencies for inhibiting the dog kidney Na\(^+\), K\(^+\)-ATPase (for ouabain EC\(_{50}\) value was 4.8 nmol/l and for analogue of ouabain without a lactone ring 812 nmol/l). Although Na\(^+\), K\(^+\)-ATPase is obviously the major pharmacological target of cardiac steroids, it should be noted that inhibition of Na\(^+\), K\(^+\)-ATPase is not essential in cytotoxic action of this group of compounds. The degree to which cardiac glycosides and their derivatives inhibit cancer cell growth is correlated to DNA topoisomerases-inhibiting activity and caspase-dependent induction of apoptosis.\(^{1,34}\)\(^{3}\)

Our previous studies have demonstrated that ouabain, digoxin and proscillaridin A treatment prevented the exponential growth and decreased the number of viable cells in both estrogen receptor-positive and estrogen receptor-negative breast cancer cells.\(^{26,27}\) We demonstrated that analyzed cardiac glycosides induce apoptosis in MDA-MB-231 cells by increasing free calcium concentration and by activating caspase-3.\(^{27}\) However, it is worth noting that the apoptotic stimulus by relatively high concentrations of cardiac glycosides is hardly attainable since a cardiotoxicity of arrhythmia could be happened.\(^{35}\) Therefore, we were interested to synthesize compounds with cytotoxic activity but devoid of cardiotoxic side effects. We have synthesized three derivatives of ouabain, digoxin and proscillaridin A containing the carboxylic group instead of the lactone moiety (Fig. 1). Our experimental studies have demonstrated that compounds 1—3 treatment prevented the growth and decreased the number of viable cells in both MCF-7 and MDA-MB-231 breast cancer cells. Because the antiproliferative effect of compounds 1—3 is independent of the estrogen receptor status of the breast cancer cells, these potent inhibitors are a potential pharmacological agents for the treatment of both hormone responsive and nonresponsive breast cancer cells. Interestingly, our results show that removal of the chemical groups that are responsible for inhibition of the Na\(^+/K\(^+\)-ATPase weakens but does not abrogate the apoptotic effect of these compounds. Further studies are required to determine the different inhibition potencies of derivatives of cardiac glycosides 1—3.
against different sodium pump isozymes.

We demonstrated that analyzed compounds 1—3 induce apoptosis in breast cancer cells by increasing free calcium concentration and by activating caspase-3. Toxin-induced cell killing or cell apoptosis are processes provoked by sustained elevation of cytosolic Ca\textsuperscript{2+}. Therefore, manipulation aimed at increasing the concentration of intracellular Ca\textsuperscript{2+} may induce necrosis or apoptosis or enhance the concentration of intracellular Ca\textsuperscript{2+}. Disturbance of intracellular Ca\textsuperscript{2+} homeostasis causes the induction of apoptosis in many types of cells. It has been suggested that a stimulation of Ca\textsuperscript{2+} mobilization from intracellular stores and an elevation of cytosolic Ca\textsuperscript{2+} are able to induce apoptotic responses via Bcl-xL and caspase-3 involved mechanism in human prostate cancer LNCaP cells. More recent works have shown that caspase activation and DNA fragmentation are preceded by a drop in intracellular K\textsuperscript{+} levels. Inhibition of this drop blocks caspase activation and cell death. Importantly, cardiac glycosides induce both an increase in Ca\textsuperscript{2+} and a decrease in K\textsuperscript{+}. In addition, previously studies have shown that oleandrin suppresses NF-κB (nuclear factor-κB) activation, which could also contribute to cell death induction. However, the cell death-promoting activity of cardiac glycosides appears cell type specific, because other work has shown that they inhibit multiple pathways of apoptosis in vascular smooth muscle cells.

The signalling pathways that are rapidly activated by the interaction of cardiac glycosides and their derivatives with the sodium pump and which are independent of changes in intra-cellular Na\textsuperscript{+} and K\textsuperscript{+} concentrations, include activation of Src kinase, transactivation of the epidermal growth factor receptor (EGFR) by Src, modulation of the NF-κB activity, activation of Ras and P42/P44 mitogen-activated protein kinases and increased generation of reactive oxygen species by mitochondria. Several cardiac glycosides provoke an increase in [Ca\textsuperscript{2+}], as the result of the activation of these downstream signalling pathways. More studies are required to evaluate whether compounds 1—3 affect directly these processes, including modulation of the NF-κB activity and inhibition of the signalling cascade. There are several facts that suggest that other mechanisms than just the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase inhibition might be involved in the anticancer activity of cardiac glycosides. Steroid substances such as corticosteroids have been extensively used for a long time in medical oncology in the treatment of lymphoproliferative cancers or prostate and breast cancer. Cardiac glycosides have also shown a radiosensitising effect on malignant cancer cell lines but not on normal ones, and interestingly, malfunction of topoisomerases has been proposed to be involved in the radiosensitisation processes. Our previous study indicates also that ouabain, digoxin and proscillaridin A are able in a cell-free system to inhibit topoisomerase II. It was found that other cardiac glycosides—bufalin selectively inhibited the growth of various lines of human cancer cells and induced apoptosis, due at least in part to its specific effect on topoisomerase II. Hashimoto \textit{et al.} have demonstrated that bufalin caused a marked decrease in the steady-state level of topoisomerase II in human leukemia cells, which led to the fragmentation of DNA, a typical feature of apoptosis. Poisoning of topoisomerases is frequently associated with apoptosis and the level of topoisomerase in cancer cells are generally higher than in normal ones. Therefore, drugs able to interact with topoisomerases may show selectivity for cancer cells. In our previous experiments we have showed that while both digoxin and ouabain inhibited topoisomerase II catalytic activity at nanomolar concentrations (100 nM), neither agent inhibited topoisomerase I catalytic activity even at concentrations as high as 100 μM. On the other hand, proscillaridin A was a potent poison of topoisomerase I and II activity at nanomolar drug concentrations (30 nM, 100 nM, respectively), suggesting that this agent may produce its cytotoxic activity by targeting both enzymes simultaneously. Interestingly, in this study we have proved that analogues of ouabain, digoxin and proscillaridin A without the lactone ring are able to inhibit topoisomerase II and compound 3 is even capable to inhibit topoisomerase I.

In summary, this study presents evidence that cardiac steroids devoid of unsaturated lactone, which has been seemed to be crucial in cardiotoxic action of cardiac glycosides, induce anticancer effect in estrogen-dependent MCF-7 and estrogen-independent MDA-MB-231 breast cancer cell lines. Compound 3 is the most cytotoxic analogue of cardiac glycosides among a series of derivatives we have synthesized to date. The increase in potency for this compound may be related, in part, to an activation of caspase-3 and topoisomerase II inhibition. All these data emphasize the potential usefulness of derivatives of cardiac glycosides as anticancer agents. It should be noted that other factors such as low penetration into cell, cellular distribution and metabolic deactivation may also influence the cytotoxicity results, but they are not assessed in the present study. Further biological evaluation is underway and these results will be described in due course.

**REFERENCES**


