Role of topoisomerases during modulation of drug sensitivity in resistance-induced variants of MDA-MB-231 breast cancer cell line

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Abstract
Resistance to chemotherapy is determined by the development of resistance mechanisms to one or more chemotherapy compounds. Multi-drug resistance (MDR) is the process by which tumor cells exposed to a single chemotherapeutic compound develop cross-resistance to a series of structurally and functionally unrelated compounds, which reduces the effectiveness of therapies. New data that explain the mechanisms that govern the interplay between the topoisomerases TopoI, TopoIIα and TopoIIβ, molecules that are differentially expressed and modulated by drug treatments in tumour cells, might add to knowledge in the domain of oncologic treatment resistance. Since many studies were focused on the resistance to a single topoisomerase, while the simultaneous evaluation of the expression of the three topoisomerases and implication in resistance was less analyzed, the aim of the present study was to evaluate the involvement of TopoI, TopoIIα and TopoIIβ in the induced resistance to Topo-inhibitors chemotherapeutic agents (Adriablastin (ADB) and Topotecan (TPT)). Changes in their expression during the acquisition of the resistance phenotype in human breast cancer MDA-MB-231 cell line were evaluated using MTS cell viability assay, evaluation of nuclear antigen expression during cell cycle phases and apoptotic events by flow-cytometry, or Western blotting. Moreover, we investigated the cross-resistance developed against ADB or TPT. The evaluation of apoptotic events by flow cytometry confirms the acquired phenotype during the resistance acquisition process. Furthermore, we have shown that TopoI (TPT) and Topo II (ADB) inhibitors do not interfere giving cross resistance. Thus, topoisomerases could be taken into account as important factors during setting of more selective treatment approaches for cancer patients.

Keywords: Topoisomerases, topotecan, adriablastin, breast cancer, drug-resistance

1. Introduction
Clinical resistance to chemotherapy is a complex phenomenon, multifactorial, involving simultaneously many factors and mechanisms and generating an increased degree of cellular resistance (GOTTEŠMAN [1]). Either intrinsic or acquired, tumor cell resistance remains a major challenge for both cytotoxic chemotherapy and hormone therapy as well as immunotherapy and targeted therapy (DESOIZE & al. [2]). Resistance to chemotherapy is determined by the development of resistance mechanisms to one or more chemotherapy compounds. Multi-drug resistance (MDR) is the process by which tumor cells exposed to a single chemotherapeutic compound develop cross-resistance to a series of structurally and functionally unrelated compounds, which reduces the effectiveness of therapies...
(CALLAGHAN & al. [3]; HOLOHAN & al. [4]; HOUSMAN & al. [5]). Therefore, it is often necessary to permanently change the chemotherapy strategies for treating cancer patients.

The pharmacokinetic factors, and components of tumor micromedium have a great influence on how a tumor responds to chemotherapy (FODALE & al. [6]; SENTHEBANE & al. [7]; IZAR & al. [8]). In addition, there are many mechanisms that can contribute substantially to the induction of resistance at the cellular level. They reduce the cytotoxic accumulation in cells as a consequence of decreased influx or increase in chemotherapy efflux (overexpression of P-gp, MRP-1, BCRP), disruption of intracellular distribution of the compound, changes in chemotherapy metabolism, activation of detoxification systems such as cytochrome p450, glutathione. In addition, there are mechanisms involving altered apoptosis signaling pathways, increased repair of DNA lesions, but also mechanisms that cause alterations in both cytotoxic and quantitative targets (amplification or deletions, mutations) (MASUI & al. [9]; RODRIGUES & al. [10]). All of these factors and mechanisms greatly influence how the tumor responds to chemotherapy.

Within these events, topoisomerase alterations seem to be an important factor (GANAPATH & al. [11]; BATES & al. [12]; BAI & al. [13]). The ability of these enzymes to cleave the double-stranded DNA helix through a transient (single/double strand) break and then to release down the broken strands makes them the primary targets for a number of chemotherapeutic agents commonly used to treat many cancers, compounds that at least partially exert cytotoxicity through interactions with these enzymes (BASSI & al. [14]; CHAMPOUX & al. [15]; CHEN & al. [16]). For example, Topo I is the intracellular target for camptothecin and its derivatives (irinotecan, topotecan (TPT)), while Topo II is of major interest in cancer therapy, being the intracellular target for antitumor agents, both intercalants like antraciclines (doxorubicin or Adriablastin (ADB), daunomycin, idarubicin), anthracendiones (mitoxantrone), aminoacridines (amsacrine), and nonintercalants, as epipodophyllotoxins (etoposide and teniposide), quinolones (NITISS & al. [17]; POMMIER & al. [18]; POMMIER & al. [19]). All these compounds are called poisons of topoisomerases in order to distinguish from catalytic inhibitors, such as bis-dioxopiperazone (ICRF-159, 193), aclarubicin, merbarone, fostricin, staurosporine (POMMIER & al. [20]). The cytotoxic potential of topoisomerase poisons is associated with their ability to stabilize the covalent complexes Topo-cleaved DNA (cleavage complexes) (LI & al. [21]; DEWEESE & al. [22]). Camptothecin, irinotecan, topotecan, etoposide, teniposide, doxorubicin bind to DNA, forming ternary complexes Topo-DNA-chemotherapeutic agent and prevent releasing thebreaks, leading to permanent catenary breaks at the DNA level (CALDERWOOD & al [23]; KIBRIA & al. [24]; KLEJEWSKI & al. [25]; LOVITT & al. [26]; LUKYANOVA & al. [27]; XU & al. [28]). In recent years, the role of topoisomerases in chemotherapy resistance has been investigated in a series of in vitro and in vivo studies (CAO & al. [29]; DE CAMPOS-NEBEL & al. [30]; DURBECQ & al. [31]; VILLMAN & al. [32]; JIN & al. [33]), but the involvement of these enzymes in chemotherapy sensitivity/resistance remains to be elucidated as the results were inconclusive, sometimes contradictory, depending on the high variety of the investigation methods (qRT-PCR, IHC, WB, FISH/ CISH), but not many used in the same study. In addition, many studies were focused on the resistance to a single topoisomerase, the simultaneous evaluation of the expression of the three topoisomerases and implication in resistance was less analyzed. Moreover, for Topo II, it is not yet clear which of the two isoforms, alpha or beta, are primary cell targets for various chemotherapeutic agents. We have previously demonstrated the cytotoxic role of ADR on drug treated MCF-7 breast cancer cells (MIHAILA & al. [34]). The aim of the present study was to evaluate the
involvement of topoisomerase I and the alpha and beta isoforms of topoisomerase II in the induced resistance to chemotherapeutic agents, resulting in changes in their expression during the acquisition of the resistance phenotype in human breast cancer MDA-MB-231 cell line. The drugs belong to different classes of inhibitors (ADB and TPT), and cells were subjected to continuous exposure to increased chemotherapeutic concentrations compared to susceptible cells. In addition, we investigated the cross-resistance developed against ADB or TPT. For this purpose various experimental approaches like MTS cell viability assay, evaluation of nuclear antigen expression during cell cycle phases and apoptotic events by flow-cytometry, or SDS-PAGE/ Western blotting were performed.

2. Materials and methods

2.1. Reagents: Adriablastin (ADB), Topotecan (TPT), dimethyl sulfoxide (DMSO), paraformaldehyde (PFA) were purchased from Sigma. High concentrated stock solutions of ADB were prepared as recommended, in ultrapure sterile water. Working drug concentrations were prepared from the stocks in complete culture medium before each experiment. Annexin V-FITC/Propidium Iodide (PI) Apoptosis Detection Kit for flow-cytometry was purchased from BioVision Inc., USA. Specific monoclonal primary antibodies (mAb): anti-Topoisomerase I, anti-Topoisomerase II alpha and anti-Topoisomerase II beta mAbs, and secondary antibody, FITC labeled, were purchased from Abcam (Cambridge, UK). CycleTEST PLUS DNA Reagent kit was provided by BD Biosciences (Becton Dickinson, SUA).

2.2. Cell cultures: MDA-MB-231 cell line, derived from breast adenocarcinoma was purchased from European Collection of Authenticated Cell Cultures (ECACC). Adherent cells were routinely maintained in culture in DMEM:F12 medium added by 2mM L-glutamine and 10% fetal bovine serum (Sigma Aldrich, St. Louis, Mo, USA), and incubated at 37°C/ 5% CO₂ humified atmosphere. Then cells were detached with a nonenzymatic solution of PBS/1mM EDTA, washed twice in PBS and immediately used for the evaluation of apoptosis, antigen expression by flow-cytometry or frozen at -80°C for preparation of lysates.

2.3. Cell treatments: After 24h of culture, when cells achieved 50-70% confluency, cultures were treated with different concentrations of anti-cancer drugs (ADB, TPT), for various periods of time. Induction of drug resistance was achieved by continuously exposing MDA-MB-231 cells to increasing doses of topotecan (TPT) or adriablastin (ADB). Treatments started concurrently under IC50, 3nM for TPT and 10nM for ADB. The increase in concentration from one exposure to the next one was performed based on cell recovery time (for short recovery times of 1-2 weeks, concentrations were 1-1.5 times increased; for 2-4 weeks recoveryperiods, the concentration increase was 10-50%). During the process of achieving drug resistance, the cells were incubated with the same concentration at least for one week; when the destruction of the cells was severe, the drug concentration was decreased for one week. A similar selection was made at the level of parental cell line in the absence of chemotherapy, in order to determine possible changes in cell phenotype as a result of long-term cultivation.

2.3. Cytotoxicity assays: MTS cell viability assay was used to measure the cytotoxicity of reagents and cell viability using a standard colorimetric assay. All assays were performed in triplicate in 96-well microtiter plates with flat bottom (Falcon), using CellTiter 96 Aqueous One Solution Cell proliferation Assay (Promega). The method is based on the ability of metabolically active cells to reduce MTS, a yellow tetrazolium salt to the colored formazan that is soluble in the culture medium. Briefly, 15x10³ cells/well were cultured in 100 uL for 24h, culture supernatants discarded, then cells were treated for 24-72 h with increasing
concentrations of ADB or TPT. After the end of incubation time, 20 μL reagent containing: a) MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt], and b) PES (phenazine ethosulfate) were added in each well. After adding the colouring solution, plates were incubated 4h at 37°C, with mild agitation every 15 min. The colour developed during incubation was spectrophotometrically quantified at λ = 492 nm.

2.4. Apoptosis analysis: The apoptosis assay was carried out using the Annexin V-FITC/PI Apoptosis Detection Kit and the manufacturer’s protocol from BioVision. The percentages of apoptotic cells were evaluated after Annexin V-FITC/PI double staining, followed by sample data acquisition with FACS CantoII flow-cytometer and analysis using Kaluza and WinMDI 2.9 softwares.

2.5. Evaluation of nuclear antigen expression during cell cycle phases by flow-cytometry. Cells were treated for 1h/4°C in 2% PBS-PFA buffer for fixing the surface molecules, and permeabilized by additional incubation for 15 min/ 37°C in PBS-Tween-20 (0.2%) buffer. After two washings with PBS, 10^5 cells were sequentially stained by 0.5 μg of specific mouse anti-human monoclonal antibodies, and 1:50 dilutions of FITC-labelled pAb (Abcam, Cambridge, UK). Then, cells were processed for cell cycle analysis using CycleTEST PLUS DNA Reagent kit. Acquisition of data was performed using a FACS Calibur flow-cytometer, then data were analyzed with Kaluza software.

2.6. Evaluation of nuclear antigen expression by Western blotting. Proteins from cell lysates (50 μg/well) were electrophoretically separated on 7.5% SDS-PAA gels. Specific monoclonal primary antibodies (mAb) mouse-anti-human: C-21 anti-TopoI, C-31 anti-Topo II alpha and C-40 anti-TopoII beta mAbs, and secondary antibody were purchased from BD Transduction Laboratories, USA. Western Breeze kit, including the blocking buffer and antibody diluent, was obtained from Invitrogen.

2.7. Statistical Analysis was performed using Student t’ and GraphPad tests; p values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Induction of drug-resistance in MDA-MB-231 cells
Starting from the MDA-MB-231 human breast cancer cell line we generated MDA-MB-231/AR and MDA-MB-231/TR subtypes with different paths of resistance by exposure to increasing doses of chemotherapeutic agents, representative of the chemotherapeutic classes targeting the two topoisomerase types (TPT for Topo I, ADB for Topo II), starting under IC50 value. Chemo-sensibility analysis to various concentrations of ADB (0.25μM - 1μM - 5μM - 10μM - 20μM - 50μM - 100μM) or TPT (0.05μM - 0.1μM - 0.2μM - 0.5μM - 1μM - 2μM - 5μM), using MTS assay, was performed to evaluate the resistance of the subline obtained compared to the parental line from which it derived. The resistance grade for both ADB and TPT were assessed based on the IC50 cytotoxicity index. Its determination was performed for each chemotherapeutic in the survival curves, obtained after treatment with the indicated concentrations for 72h of both the parental cell line and resistance-induced sublines.

3.2. Modulation of cell cytotoxicity by ADB or TPT in breast cancer cells
Analyzing the response to ADB or TPT, for the parental MDA-MB-231 cell line, a cytotoxic dose-dependent effect was observed. IC50 (50% inhibition concentration) values were calculated by dose–response curves using the GraphPad Prism software. Based on the IC50 cytotoxicity index values, we have compared cell susceptibility and found there were
differences in ADB or TPT sensitivity. Resistant cell sublines grew at the drug concentration at which parental cell line was clearly more affected (Table 1, Fig. 1).

Table 1. Sensitivity and cross-resistance of parental and resistant lines to drugs directed to Topo I (TPT) and Topo IIα, β (ADB)

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC50 (µM)</th>
<th>Adriablastin</th>
<th>Topotecan</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>8.64</td>
<td>1.29</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231/TR</td>
<td>9.99</td>
<td>3.54 (2.74)</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231/AR</td>
<td>18.29 (2.12)</td>
<td>1.43</td>
<td></td>
</tr>
</tbody>
</table>

*a* Resistance factor to Adriablastin for ADB-resistant cell subline

*b* Resistance factor to Topotecan for TPT-resistant cell subline

Between MDA-MB-231 parental line and resistant sublines, under identical culture conditions, statistically significant differences were found between IC50 values for ADB or TPT, demonstrating their resistant feature.

Figure 1. Modulation by ADB or TPT of parental and resistance-induced MDA-MB-231 cell sublines

Decreased cell viability was more pronounced in the parental MDA-MB-231 cell line compared to resistant subline. Thus, under conditions of incubation of cells with ADB, viability decreased from 87% for 1 µM to 19% following treatment of cells with 10 µM, and close to 0 after treatment of cells with 50 µM ADB. In the presence of TPT, viability was significantly reduced with increasing concentration, from 58% for 0.5 µM, till 37% for 2 µM, and 18% when 5 µM TPT concentrations were used (Fig. 1). Screening for the proper concentration with cytotoxic or cytostatic potential in inhibiting the growth of adherent tumor cells, made possible the choice of the proper treatment to be further used in end-point assays such as evaluation of apoptosis or evaluation of antigen expression.

3.3. Modulation of cross-resistance to ADB or TPT by evaluation of apoptosis in parental and anti-topoisomerases drug-induced cell lines

Chemotherapeutic agents irrespective of the mechanism of action ultimately determine cell apoptosis (programmed cell death). Along with the inhibition of cellular proliferation, it is frequently evaluated in the biological response to treatment with various chemotherapeutic agents. In order to evaluate the apoptotic events by flow-cytometry, parental MDA-MB-231
cell line and its variants, TR (resistant to TPT) or AR (resistant to ADB), cancer cells were cultured in complete medium for 24h, culture medium changed, and cells sensitized for 24h with ADB or TPT. Then cells were detached with PBS/1mM EDTA, sequentially washed with PBS, and Wash Buffer, and centrifuged 5 min/300xg. Pellets were suspended in 400 µl Binding Buffer, and 100 µl distributed in flow tubes and stained with 5 µl of Annexin-V/FITC and/or PI for 15 min/RT at 37°C in the dark. The green and red fluorescences were measured by using FACSCantoII flow-cytometer and DIVA and WinMDI2.9 softwares. The live cells were not labelled with Anexin-V/FITC or PI; early apoptotic cells displayed green fluorescence; late apoptotic cells were double stained, while necrotic cells labelled only PI. Total apoptosis was calculated by summing early and late apoptotic events. Figure 2 shows significant experiments of modulated apoptosis by 24h ADB or TPT treatments, performed on all three variants. In the present study, the evaluation of apoptosis by double marking with Anexina V-FITC and propidium iodide (PI) at the flow cytometer was performed along with MTS as an additional method of analyzing the sensitivity of parental lines and doxorubicin or topotecan resistant sublines obtained in laboratory.

<table>
<thead>
<tr>
<th>Drug</th>
<th>MDA-MB-231</th>
<th>MDA-MB-231/TR</th>
<th>MDA-MB-231/AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL</td>
<td><img src="image" alt="CTRL" /></td>
<td><img src="image" alt="CTRL" /></td>
<td><img src="image" alt="CTRL" /></td>
</tr>
<tr>
<td>TPT</td>
<td><img src="image" alt="TPT" /></td>
<td><img src="image" alt="TPT" /></td>
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<tr>
<td>ADB</td>
<td><img src="image" alt="ADB" /></td>
<td><img src="image" alt="ADB" /></td>
<td><img src="image" alt="ADB" /></td>
</tr>
</tbody>
</table>

Figure 2. Modulation of apoptotic events induced by ADB or TPT on parental or resistance-induced MDA-MB-231 cells.
Therefore, the determination of apoptotic events was used to monitor and confirm the resistant phenotype, but also to assess the cross-resistance to ADB or TPT. Following 50 nM TPT treatment for 24 hours, a lower sensitivity was found for TPT-resistant cells, compared to parental cells (Figure 2, Table 2). The results show that ADB- or TPT-resistant sublines have a low apoptosis at exposure to the induced cytostatic in accordance with other studies (Jin Mo Ku, 2015).

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Drug</th>
<th>Early apoptosis (%)</th>
<th>Late apoptosis (%)</th>
<th>Total apoptosis (%)</th>
</tr>
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<tbody>
<tr>
<td>MDA-MB-231</td>
<td>CTRL</td>
<td>1.48</td>
<td>5.16</td>
<td>6.64</td>
</tr>
<tr>
<td></td>
<td>TPT</td>
<td>10.34</td>
<td>12.00</td>
<td>22.34</td>
</tr>
<tr>
<td></td>
<td>ADB</td>
<td>2.27</td>
<td>23.63</td>
<td>25.9</td>
</tr>
<tr>
<td>MDA-MB-231/TR</td>
<td>CTRL</td>
<td>2.67</td>
<td>10.45</td>
<td>13.12</td>
</tr>
<tr>
<td></td>
<td>TPT</td>
<td>8.07</td>
<td>9.56</td>
<td>17.63</td>
</tr>
<tr>
<td></td>
<td>ADB</td>
<td>4.92</td>
<td>21.89</td>
<td>26.81</td>
</tr>
<tr>
<td>MDA-MB-231/AR</td>
<td>CTRL</td>
<td>3.31</td>
<td>8.36</td>
<td>11.67</td>
</tr>
<tr>
<td></td>
<td>TPT</td>
<td>5.78</td>
<td>15.44</td>
<td>21.22</td>
</tr>
<tr>
<td></td>
<td>ADB</td>
<td>4.34</td>
<td>11.14</td>
<td>15.48</td>
</tr>
</tbody>
</table>

3.5. Protein expression associated to parental or drug-resistant MDA-MB-231 cells

Double labeling, first with mAbs specific for TopoI, TopoIIα or TopoIIβ, and then with propidium iodide, allows quantification of the level of these enzymes relative to the cell cycle phases. Information is obtained both on the level and distribution of topoisomerases in the dynamics of DNA progression through the cell cycle phases.

![MDA-MB-231 cells](image)
The results highlighted elevated expressions for all of the three topoisomerases in all phases of the cell cycle, including phase G0 / G1. The distribution of nuclei in TopoI, TopoIIα or TopoIIβ cells is also similar (Figure 3).

By analyzing the correlation between Topo I, Topo IIα and β levels and IC50 values (or IC50 based resistance factor) for the two anti-Topo drugs and parental cell line, we found that Topo I levels correlated with TPT sensitivity/ resistance, while Topo IIα level correlated with ADB sensitivity/ resistance.

3.5. Protein expression associated to WT or drug-resistant MDA-MB-231 cells

Western Blot technique is the most commonly used technique for evaluating the expression of protein topoisomerases. Proteins were separated from cell lysates obtained from both the parental and ADB or TPT resistant cell lines in a polyacrylamide gel with a degree of retention of 7.5%, and then transferred to a PVDF membrane. Blots were sequentially stained by anti-TopoI, anti-TopoIIα and anti-TopoIIβ mAbs, and the peroxidase-coupled secondary antibody. As control, we used beta actin (MW = 42 kDa) and for detection we used the chemiluminescent amplification (ECL). Western Blot analysis of Topo I, Topo IIα and IIβ expressions was performed at the parental line level and during the acquisition of the TPT or ADB resistant phenotype.

From the data obtained, it was found that the intensity of the Topo I bands is lower in TPT-induced resistance subline compared to parental line, but also with ADB-resistant line (Figure 3). Regarding the protein expression of Topo IIα and Topo IIβ, small variations were found. In ADB-resistant subline, by comparing the intensity of the Topo IIα bands, there is also a decrease in the intensity.
MDA-MB-231 cells

<table>
<thead>
<tr>
<th></th>
<th>Protein</th>
<th>MW</th>
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<tr>
<td>WT</td>
<td>Topo IIβ</td>
<td>180 kDa</td>
</tr>
<tr>
<td>AR</td>
<td>Topo IIα</td>
<td>170 kDa</td>
</tr>
<tr>
<td>TR</td>
<td>Topo I</td>
<td>100 kDa</td>
</tr>
<tr>
<td></td>
<td>β-actina</td>
<td>42 kDa</td>
</tr>
</tbody>
</table>

Figure 4. Protein expression of topoisomerases detected by SDS-PAGE/WB in parental and resistance-induced MDA-MB-231 cells

Conclusions
The study brings additional information on the diversity of chemotherapeutic resistance phenotypes. The evaluation of apoptotic events by flow cytometry confirms the acquired phenotype acquired during the resistance acquisition process. The results previously obtained in the determination of cytotoxicity in a concentration-dependent manner (by MTS technique), TPT sensitivity of ADB-resistant cells (AR subline) and vice versa, ADB sensitivity of TPT-resistant cells (TR subline) were also confirmed in the evaluation of apoptotic events by flow cytometry. The results presented showed that the decrease of TopoI or Topo IIα levels is sufficient to determine ADB or TPT resistance in MDA-MB-231 breast cancer cell line. Furthermore, we have shown that TopoI (TPT) and Topo II inhibitors (ADB) do not interfere giving cross resistance. Better knowledge of molecular mechanisms of resistance to chemotherapy might lead to identification of predictive molecular biomarkers, relevant to the response to chemotherapy. Thus, topoisomerases could be taken into account as important factors during setting of more selective treatment approaches for cancer patients.

Acknowledgments
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References


