ROMANIAN ACADEMY

***ŞTEFAN S. NICOLAU* INSTITUTE OF VIROLOGY**

BUCHAREST

PhD THESIS

Involvement of topoisomerases in tumor cells resistance to treatment with chemotherapeutic agents"

SUMMARY

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BUCHAREST

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INTRODUCTION

Despite all advances made in the fields of surgery, radiotherapy, but especially chemotherapy, advances that have lead to major changes in cancer treatment, the development of tumor cell resistance to chemotherapeutic agents is still an important issue which reduces the efficacy of available therapies.

The large number of non-responsive patients to cytotoxic therapy may be due to the development of both intrinsic resistance of some tumors such as renal cell carcinoma, colon cancer, pancreatic cancer, melanomas, different types of lung cancer, or acquired resistance commonly found in small cell lung cancer, breast and. ovarian cancer (Fodale 2011, Holohan 2013).

Usually, tumor cells exposed to a single chemotherapeutic agent (anthracyclines, vinca alkaloids, taxol) develop cross-resistance to a wide variety of structurally and functionally unrelated drugs, a process called multidrug resistance (MDR) (Ganapathy 2013, Kibria 2014) .

Anticancer drugs' resistance is a multifactorial phenomenon, at the level of tumour cell populations may be expressed at the same time as a series of mechanisms that determine an increased degree of cellular resistance (Jie Qi 2011, Xing Ke 2017).

Mechanisms related to the changes in accumulation and intracellular distribution of chemotherapeutic agents or cellular metabolism are primarily involved in cancer drug resistance. Other mechanisms responsible for the development of resistant phenotype are related to the activation of detoxification systems or the alteration of apoptotic signaling pathways, increased levels of DNA repair as well as alterations in drug targets. (Cox 2016, Holohan 2013). Within these mechanisms the alterations in topoisomerases occupy an important position.

The ability of topoisomerases to cut double stranded DNA and then to release cleaved strands makes these enzymes a primary cellular target for a number of chemotherapeutic agents currently being used to treat many types of cancer (Kellner 2002, Nitiss 2009, Darzynkiewicz 2009).

The cytotoxic potential of topoisomerase inhibitors (camptothecin, irinotecan, topotecan, etoposide, teniposide, doxorubicin) is associated with their ability to stabilize the Topo- DNA covalent complexes (cleavege complexes), temporary intermediates in the normal catalytic cycle of the enzyme. Ternary complexes formed (Topo-ADN- chemotherapeutic

agents) prevent the enzyme to release cleaved strands of DNA, finally leading to DNA damage and cell death (Pommier 2010, Nitiss 2009).

"Atypical" multi-drug resistance (at-MDR) is specific for topoisomerase-targeting agents and is considered to be mediated by quantitative and/or qualitative alterations of topoisomerases

Although the alterations of topoisomerases are probably only one of the many mechanisms of resistance to anticancer therapy, it seems that they can play an important role in the chemotherapy response.There are currently intensive studies to determine which mechanism of resistance predominates in a particular type of cancer.

In recent years, the role of topoisomerases in drug resistance have been investigated in several studies, but the involvement of these enzymes in chemosensitivity or chemoresistance remains to be elucidated because the results were inconclusive, sometimes contradictory and thus their predictive role is still controversial.

This is is due to the fact that the data provided by different studies are difficult to compare because in some it was determined the gene status (amplifications and deletions) in others protein or mRNA expression levels.

In addition, the analysis methods used varied quite a lot (FISH/CISH, IHC, WB, qRT-PCR), because there are no standardized analysis methods for topoisomerases, and the cut-off values were arbitrary chosen.

In addition, most studies have determined drug resistance against a single topoisomerase, simultaneous evaluation of the three types of topoisomerases and their involvement in drug resistance have been too little adressed. Furthermore, in the case of topoisomerase II it is not yet known which of the two isoforms, alpha or beta, are the primary cellular targets for different chemotherapeutic agents

A better knowledge of the molecular mechanisms of chemotherapy resistance could allow the identification of predictive molecular markers which are really relevant for the response to antitumor drugs, as well as to the identification of new therapeutic agents and strategies

SCOPE, OBJECTIVES AND ORIGINALITY OF THE THESIS

The scope of PhD thesis was to determine the involvement of Topoisomerase I (Topo I), alpha and beta isoforms of topoisomerase II (Topo II α and β) in tumor cells resistance to topoisomerases targeting agents, by assessing the changes that occurr in their expressions during the acquisition of the resistance phenotype. It was also determined the cross-resistance between Topoisomerase I (topotecan) and topoisomerase II (adriablastin) inhibitors.

The main objectives of the thesis:

- Obtaining experimental models represented by cellular sublines resistant to representative chemotherapeutic agents that inhibit Topoisomerase I and II
- Determination of sensitivity / resistance or cross – resistance to chemotherapeutic agents, at the level of the cellular sublines with induced resistance (by analysis of cellular cytotoxicity, apoptosis and cell cycle distribution)
- Evaluation of protein and gene expression of Topo I , alpha and beta Topo II isoforms before and during the acquisition of resistance phenotype, using a wide range of modern laboratory techniques (flow cytometry, Western Blot, RT-PCR).
- Determination of possible correlation between topoisomerase expression levels and sensitivity/resistance to chemotherapeutic agents

The originality of the thesis

- Development of experimental in vitro models using five human tumour lines (breast, ovarian or cervical cancer cell lines) for induction of chemotherapy resistance, used in subsequent studies
- Performing complex studies to evaluate concomitant expression of three topoisomerases according to induced resistance to topoisomerase-targeting agents, using all laboratory techniques presented in the thesis
- The use of modern techniques of immunology, molecular biology, biochemistry, flow cytometry allowed to elucidate aspects related to the individual involvement of each topoisomerase in resistance or cross-resistance phenotype

STRUCTURE OF THE THESIS

The PhD thesis **„Involvement of topoisomerases in tumor cells resistance to treatment with chemotherapeutic agents”** comprises two parts:

I. Theorethical part, consisting of scientific substantiation of the subject area, and

II. Experimental part, consisting of original research

Thus, the first chapter, **"Cancer (generalities)"** summarizes information about the epidemiology of human cancers, general aspects of cancer and therapeutic strategies for cancer treatment. The chapter "**Resistance to chemotherapeutic agents targeting topoisomerases”** describes aspects related to the mechanism of resistance to drugs that inhibit DNA topoisomerases: alterations in intracellular transport and metabolism of chemotherapeutic agents, sequestration of chemotherapy drug in different cell compartments, activation of detoxification systems, antioxidant defense systems, proliferative status, mechanisms involving alterations in cellular response to DNA damage or other factors/mechanisms of resistance to chemotherapeutic agents targeting topoisomerases. The third chapter „**ADN topoizomerazele**” presents in detail the classification and characterization of topoisomerases, structure, functions and cellular processes in which type I and II alpha and beta topoisomerases are involved, necessary to understand the studies performed in the thesis. In chapter four **„Chemotherapeutic agents that inhibit DNA topoisomerases”,** some clarifications are made on classification of chemotherapeutic agents targeting topoisomerases as well as the mechanisms of cytotoxicity mediated by topoisomerase I and II. The theoretical part of the thesis ends with chapter five " **Mechanisms of resistance that involved alterations of topoisomerases I and II alpha and beta"** in which alterations of topoisomerases (protein expression, enzymatic activities and mutation) involved in chemotherapy response are described.

The experimental part comprises the **Methodology**, which includes the principles and methods, biological material, consumables, reagents, equipment and working protocols as well as Results, Discussions and partial conclusions that emerged from the studies

At the end of this part are included the **General Conclusions**, the **Abbreviations List**, a vast **Bibliography** consisting of about 300 references, the **List of Tables and Figures**.

EXPERIMENTAL PART

METODOLOGY

Study of the involvement of topoisomerases in tumor cell resistance to representative chemotherapeutic agents for classes targeting the two types of topoisomerases (Topo I and Topo II) was performed on biological material represented by standardized cell lines derived from human adenocarcinomas (mammary, ovarian, cervical) and cell sublines obtained from standardized cell lines by inducing the resistant phenotype in the laboratory.

For this purpose, repeated cell cultures and passages were performed, a number of techniques were used to determine the viability of cells according to the cytotoxicity of chemotherapeutic agents, to evaluate apoptotic events, to analyse the cell cycle dynamics and to evaluate topoisomerases protein and gene expression.

- The studies conducted during this thesis were performed using as biological materials a panel of human cell lines derived from malignant mammary tumors (MCF-7, SK-BR-3, MDA-MB-231), malignant ovary tumors (SK-OV-3) and malignant cervical tumors (HeLa), which according to the molecular classification belong to subclasses representative of the three types of cancer and present a number of characteristics that differentiate them.
- All cell lines studied came from the European Collection of Authenticated Cell Culture (ECACC), with the exception of the SK-BR-3 line that was provided by Dr. Michele Maio (Centro Regionale Oncologico di Aviano, Advanced Immunotherapy Unit).
- As a cellular control line for the studies performed within the thesis was used the line MCF-7/A, adriablastin - resistant, provided by Dr. Liliana Puiu (Oncological Institute "Prof. Dr. Alexandru Trestioreanu", Bucharest).
- **Cell culture:** maintaining in the culture, handling and storage of cell lines taken in the study were performed according to the ECACC recommended protocols for each line.
- **Define experimental models *in vitro:*** adriablastin -resistant cell lines and topotecan - resistant cell lines, respectively were obtained in the laboratory by continuous exposure of parental human breast cancer cell lines (MCF-7, SKBR3, MDA-MB231), human ovarian cancer cell line (SK-OV-3) and human cervical cancer cell line (HeLa) at increasing

concentrations of topotecan (TPT) or adriablastin (ADB) representative drugs for chemotherapeutic agents classes targeting the two types of topoisomerases (Topo I and Topo II, respectively).

- The resistance character was induced in a long period of time, 10 months, with concentrations between 20 nM and700nM for ADB or 3 nM and 60nM for TPT, depending on the resistance of each parental line and IC50 determined using cytotoxicity assay (MTS) or real-time cell analysis (RTCA).
- Resistent sublines were named MCF-7/AR and MCF-7/TR; SK-BR-3/AR and SK-BR-3/TR; MDA-MB-231/AR and MDA-MB-231/TR; SK-OV-3/AR and SK-OV-3/TR; HeLa/AR and HeLa/TR.
- **Determination of resistance degree** to anti-topoisomerase treatments was performed by colorimetric techniques, CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega), calculating the IC50 cytotoxicity index
- Results obtained from cytotoxicity tests by MTS technique and spectrophotometric determinations (= 490 nm) were expressed as values of optical density (O.D.) and converted into percentage values of cell viability, representing the average values resulting from three experiments, calculated by the following formula *Viability(%) = OD probe – OD blank/ OD control – OD blank*. The Results were confirmed by the real-time cell analysis (RTCA) with xCELLigence systems (RTCA analyer and RTCA software).
- **Evaluation of apoptotic events** by flow cytometry for the purpose of determining and monitoring drug sensitivity/resistance on the basis of double-labeling for annexin V – FITC and PI, Annexin V-FITC Apoptosis Detection kit (BioVision Inc, USA). By double labeling, were determined cells found in different cell death phases: early apoptosis (annexin V^+/ PI^-), late apoptosis (annexin V^+ /PI^+) or necrosis (annexin V^-/PI^+). The data acquired with FACSCanto II Flow Cytometer were analyzed using softwares such as Diva 7.2., WinMDI 2.9 and Kaluza
- **Analysis of progression through cell cycle phases** in the parental cell lines and **resistant sublines** derived from these parental lines was performed using propidium iodide (PI) staining

after prior pre-incubation **with RNase** followed by the acquisition of data with FACS Calibur flow cytometer and their analysis using softwares such as ModFIT or CellFIT. The Intensity of fluorescent light is directly proportional to the amount of DNA, thus allowing the estimation of cell cycle phases (G0/G1, S and G2/M).

- **Evaluation (Topo I, α and β isoforms of Topo II) nuclear antigens expression** by flow cytometry was performed before and during the acquisition of resistant phenotype in the

panel of human breast cell lines, ovarian cell line and cervical cell line, to which induced resistance to ADB or TPT. Data acquisition were performed with FACS Calibur flow cytometer and fluorescence of the cells was analysed using software WinMdi 2.9. The results were expressed as percentages of positive cells (IIF %) and Mean fluorescence intensity (MFI) values, the samples being defined as positive if more than 5% of the cells bound specific mAb

- **Determination of Topo I, α and β isoforms of Topo II expression function of the cell cycle phases** by flow cytometry, with FACS Calibur flow cytometer in order to evaluate the expression of the antigens function of the cell cycle phases by labeling the cells with both fluorescent specific monoclonal antibodies and PI. Sample analysis was done as in the case of cell cycle analysis. A sample only with secondary antibody and PI was used as control.
- **Determination of Topo I, α and β isoforms of Topo II protein expression by Western blot** was performed in the lysates of untreated or treated cells with ADB or TPT. From the lysates containing protein mixtures, the topoisomerases were separated on the basis of molecular weight, by vertical polyacrylamide gel electrophoresis, and then were transferred to PVDF membranes (Polyvinylidene). Detection of target proteins was performed after binding of the specific primary antibodies and the secondary antibodies peroxidase conjugated by ECL chemiluminescence ("enhanced chemiluminescence"), having luminol as a substrate, which is oxidified by HRP in the presence of H2O2.
- **Evaluation of topoiomerases gene expression by RT-PCR** was performed in total ARNm samples, isolated from cell line suspensions with TRIzol reagent and purified on mini spin columns with DNase using the RNeasy Mini kit (*Qiagen Inc, USA*). After mRNA quantification at an optical density (260, 280 and 230 nm) using Nanodrop 1000 spectrophotometer (*Nanodrop Technologies, USA*), mRNA samples were subjected to reverse transcription using non-specific, random primers with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA amplification was performed using TaqMan technology, using *TaqMan gene expression assay (*Thermo Fisher Scientific). The samples were tested in duplicate and normalized by reporting to the reference gene GAPDH. The data were analyzed using the Comparative Ct Method ($\Delta\Delta$ Ct method).
- **Statistical analysis** of the results obtained and correlation studies were performed using GraphPad Prism 7.0 (GraphPad Software) or Excel (Student's *t*, The Pearson correlation coefficient). The data with $p < 0.05$ were considered statistically significant.

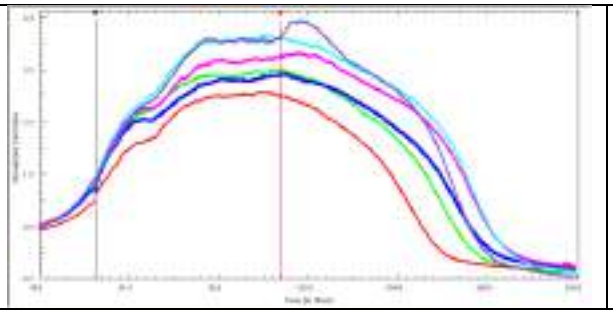
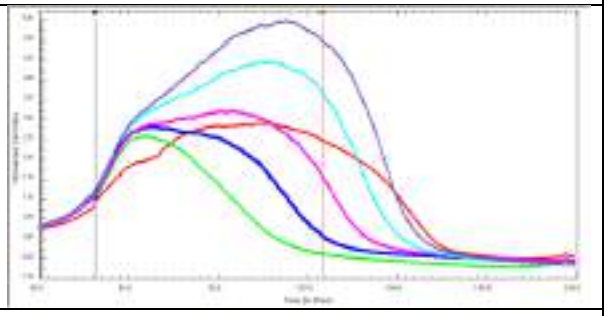
RESULTS AND DISCUSSIONS

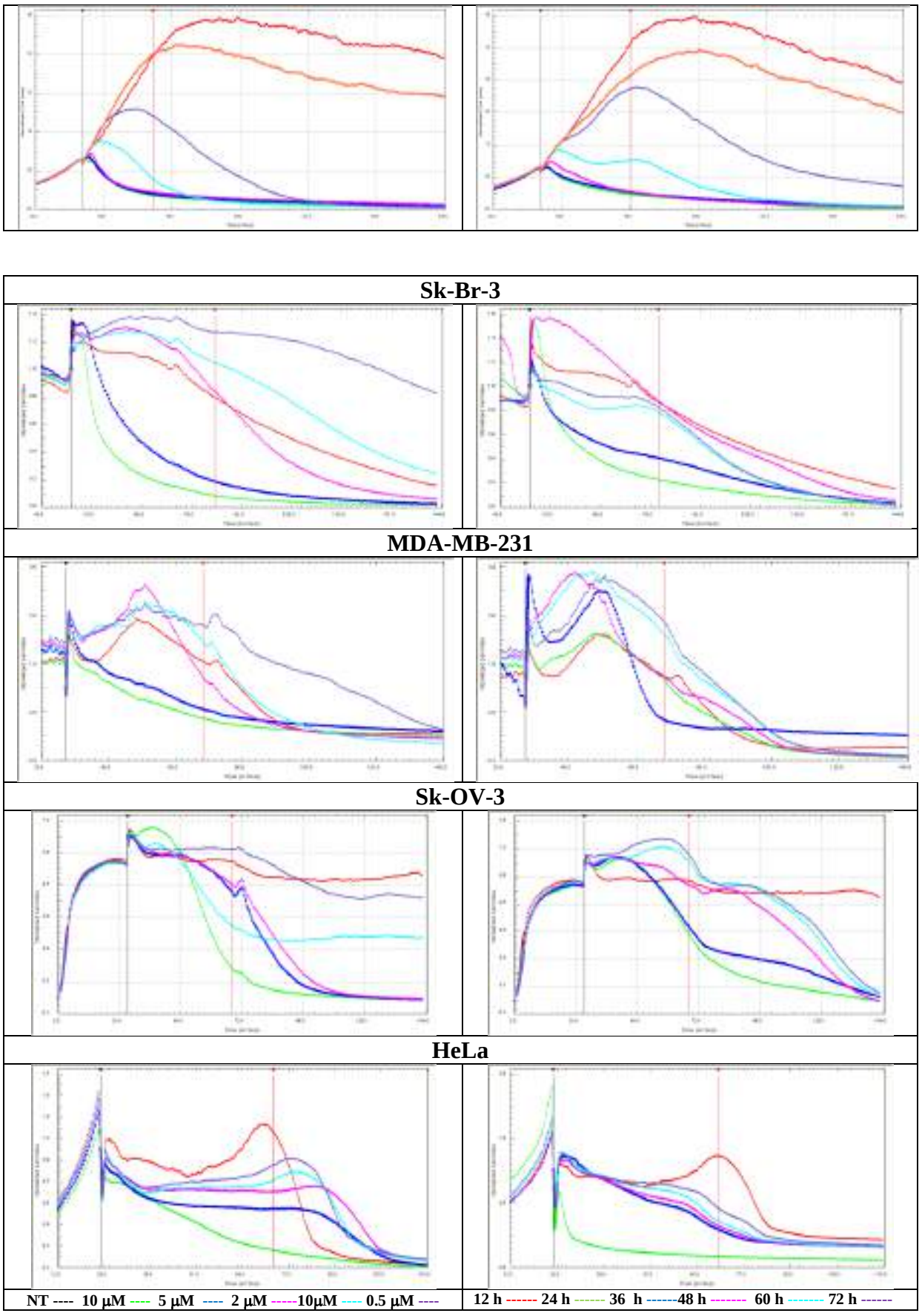
I. Profiles of sensitivity / resistance or cross – resistance to chemotherapeutic agents

1) Evaluation of cytotoxicity of chemotherapeutic agents by the real-time cell analysis (RTCA)

Real-time evaluation of the effects of Adriablastina (ADB) and Topotecan (TPT) treatment performed in all parental lines, was used to determine the doses and time periods of specific treatment for each chemotherapeutic agent and cell line. In order to evaluate the cytotoxicity of modulators by RTCA assay, tumor cells were cultivated for 24h in 75 cm2 culture plates, in complete culture medium, until reaching a 50% density. After removal of the culture medium, the cells were detached, washed and incubated in E-Plate VIEW 16 PET with impedance sensors (Roche). Drug concentrations subsequently used for treating the cells were established by using the RTCA cell proliferation assay system, RTCA-DP (Acea Biosciences/ Roche). To select optimal cell density In order to carry out the cell proliferation inhibition tests, density curves were plotted for each cell line by adding cell suspensions of different densities (5000, 10000, 15000, 20000 cells / well). Cell growth curves were automatically recorded on the xCELLigence System in real time. Raw growth data (expressed by cell index, IC) were normalized by soft ("normalized cell index") at the time of addition of the tested compounds (Fig. 1).

Figure 1. Profiles of proliferation curves induced by ADB or TPT in parental cell lines

ADB	TPT
MCF-7/A	
	
MCF-7	



The method allowed the study of cytotoxicity mediated by the studied compounds, drugs that target Topo I or Topo II Alpha and beta. Monitoring in real-time of cytotoxicity

allowed calculation of time-dependent IC50 at any time of cell proliferation. Thus, it is possible to select the moment at which a concentration reaches the maximum response.

Cell growth curves were normalized to control (the untreated cells curve). Using the xCELLigence system for real-time cell proliferation analysis vs. cellular cytotoxicity assay under the action of anti-topoisomerase chemotherapeutic agents has provided important advantages compared to classical, colorimetric methods, " end-point " type;

The whole process of modulation of cell proliferation by the stimuli used (anti-topoisomerase chemotherapeutic agents) could be followed in real time during a long period of time;

The normalization of the data acquired at the time of application of the treatments allowed a better analysis of the effect induced by low concentrations compared with the highest;

The obtained data allowed the selection of concentrations of anti-topoisomerase agents and time periods for the application of treatments, useful in subsequent "end-point" assays (Flux cytometry, RT-PCR, WB);

The method allowed calculation of IC50 values at any time in the proliferation curve. IC50 values obtained by RTCA analysis were comparable to those obtained from conventional, colorimetric cytotoxicity assays (MTS). Thus, following the analysis of the cell proliferation inhibition curves and the IC50 calculation a working concentrations range was chosen function of the constitutive or induced resistance of the cell lines studied.

2) Analysis of parental cell lines and resistant sublines response to adriablastin or topotecan by staining with MTS

The cytotoxic response to different concentrations of adriablastin (ADB) or Topotecan (TPT), chemotherapeutic agents representative of the antitumor drug classes targeting Topo I and Topo II was assessed by evaluating the parental lines chemosensitivity and the resistance of derived sublines based on the cytotoxicity index determination, IC50 (the concentration of drug which determines a 50% decrease in cell viability), calculated for each drug in the survival curves obtained after 72 hours ADB or TPT treatment of both parental lines and resistant sublines.

In the panel of parental cell lines taken in the study (MCF-7, MDA-MB-231, SK-BR-3, HeLa and SK-OV-3) were determined different sensitivity patterns to Adriablastin or Topotecan.

The highest IC50 values for both ADB (IC50 = 103.02 μM) and TPT (IC50 = 7.56 μM) were determined for the SK-BR-3 cell line. The MCF-7 cell line had an IC50 increased at

ADB(IC50 = 58.44 μM), but had a high sensitivity to TPT (IC50 = 1.33 μM); MDA-MB-231 cells were most sensitive to both ADB (IC50 = 8.64 μM) and TPT (IC50 = 1.29 μM); HeLa and SK-OV-3 cell lines had moderate sensitivity with different cytotoxicity indices in the two chemotherapeutic 12.45 μM and 25.92 μM for ADB, 5.27 μM and 2.31 μM for TPT, respecively.

The cells of the resistant sublines presented viability at drug concentrations at which parental cell lines were clearly more affected. Between the parental cell lines and the resistant cell sublines derived from these parental lines, under identical culture conditions, differences in IC50 values were determined for both ADB and TPT (Table 1).

Table 1. Sensitivity and cross resistance of the sensitive and resistant cell lines to the Topo I (TPT) și Topo II α,β (ADB) inhibitors

Cell lines	IC50 (μM)	
	Adriablastin	Topotecan
MCF-7	58,44	1,33
MCF-7/TR	68,95	5,66 (4,25)[b)]
MCF-7/AR	101,15 (1.73)[a)]	3,08
MDA-MB-231	8,64	1,29
MDA-MB-231/TR	9,99	3,54 (2,74)[b)]
MDA-MB-231/AR	18,29 (2,12)[a)]	1,43
SK-BR-3	103,02	7,56
SK-BR-3/TR	108,87	19,02 (2,51)[b)]
SK-BR-3/AR	204,27 (1,98)[a)]	7,57
HeLa	12,45	5,27
HeLa/TR	17,95	10,76 (2,04)[b)]
HeLa/AR	24,68 (1,98)[a)]	6
SK-OV-3	25,92	2,31
SK-OV-3/TR	26,92	6,34 (2,74)[b)]
SK-OV-3/AR	58,94 (2,27)[a)]	2,54
MCF-7/A	129,25	4,84

[a)] Adriablastin resistance factors for adriablastin-resistant lines
[b)] Topotecan resistance factors for topotecan resistant lines

By comparing the resistances between the resistant sublines, we found that with regard to the cross-resistance to ADB, TPT-resistant sublines maintained the sensitivity profile of the parental lines from which they derived; also in the case of ADB-resistant sublines, TPT sensitivity was maintained (Tabel 1).

Comparative analysis of the data showed that the MCF-7/AR and MCF-7/TR, MDA-MB-231/AR and MDA-MB-231/TR, SK-BR3/AR and SK-BR3/TR, HeLa/AR and HeLa/TR, SK-OV-3/AR and SK-OV-3/TR resistant cell sublines are variants with varying degrees of resistance induced to adriablastin or topotecan, through the progressive selection of human

breast cell lines (MCF-7, SK-BR-3, MDA-MB-231), human ovarian cell line (SK-OV-3) and human cervical cell lines (HeLa) grown in medium with increasing concentrations of ADB or TPT.

The results obtained showed that resistance to adriablastin doesn't induce cross resistance to topotecan, and vice versa.

3) Determination of sensitivity or resistance of parental cell lines and ADB or TPT - resistant cell sublines by evaluation of apoptotic events.

Regardless of the mechanism of action, chemotherapeutic agents finally induce cell apoptosis (programmed cell death). Therefore, as an additional method of analysis of the chemosensitivity parental cell lines and ADB or TPT - resistant cell sublines as well as for the determination of cross-resistance, the evaluation of apoptotic events was performed, in parallel with other laboratory tests.

Along with the inhibition of cellular proliferation, it is frequently evaluated in the biological response to treatment with various chemotherapeutic agents

In the present study, the evaluation of apoptosis by double labeling with Annexine V-FITC and Propidium iodide (PI) at flow cytometer was performed along with MTT as an additional method of analyzing the sensitivity of parental cell lines and ADB or TPT - resistant cell sublines obtained in the laboratory.

Therefore, the determination of apoptotic events was used in the monitoring and confirmation of the resistant phenotype, but also to assess the cross resistance to Adriablastin or topotecan.

For this, the parental cells (MCF-7, MDA-MB-231, SK-BR-3, HeLa, SK-OV-3), adriablastin- resistant cells (MCF7/AR, MDA-MB-231/AR, SK-BR-3/AR, HeLa/AR and SK-OV-3/AR) and topotecan-resistant cells (MCF-7/TR, MDA-MB-231/TR, SK-BR-3/TR, HeLa/TR and SK-OV-3/TR), after being cultivated and allowed to adhere for 24 hours at 37^0C, 5% **CO2** in a **humidified** atmosphere, were treated with 50 nM topotecan or 1 μM adriablastine for 24 hours.

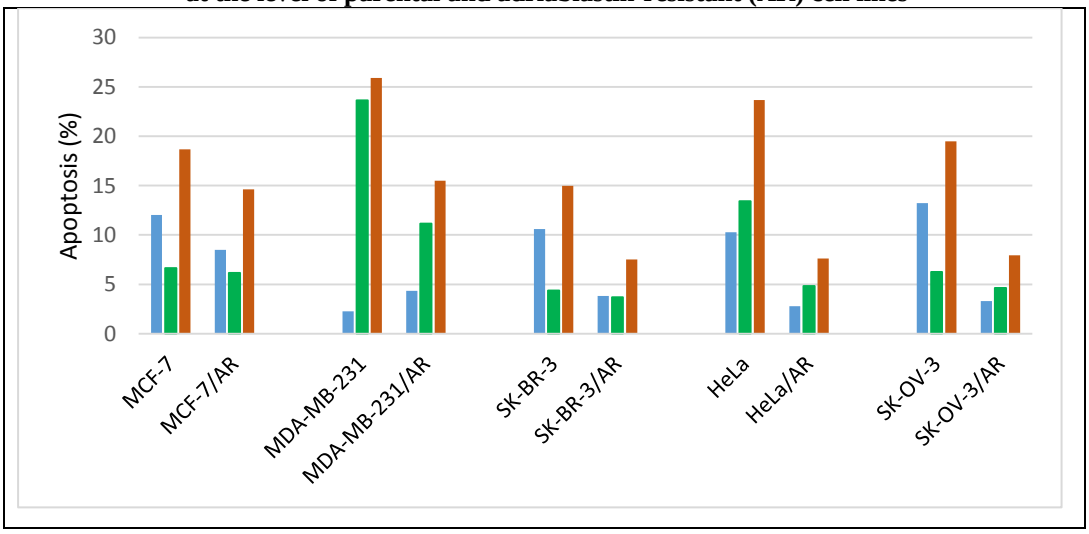
After detachment of the cells, apoptosis analysis of the parental cell line panels and the resistant sublines was performed with Annexin V-FITC Apoptosis Detection Kit, BioVision, with double labeling with Annexine V-FITC and Propidium iodide (PI)

Data acquisition and analysis were performed with FACS Canto II flow cytometer. The percentages of viable cells (negative cells for annexine-V and PI) and cells at different stages of cellular death were determined: early apoptosis (positive cells for annexine V and negative for PI), late apoptosis/necrosis (cells positive for annexine-V and PI).

a) Modulation of apoptosis by ADB in parental cell lines and ADB-induced resistance sublines

Comparative analysis of the parental cell lines sensitivity and adriablastin resistant cell sublines sensitivity, at 24 hours of exposure to ADB (1 μM concentration) showed an increase in the percentage of apoptotic events in all parental cell lines compared to resistant lines (Fig. 2).

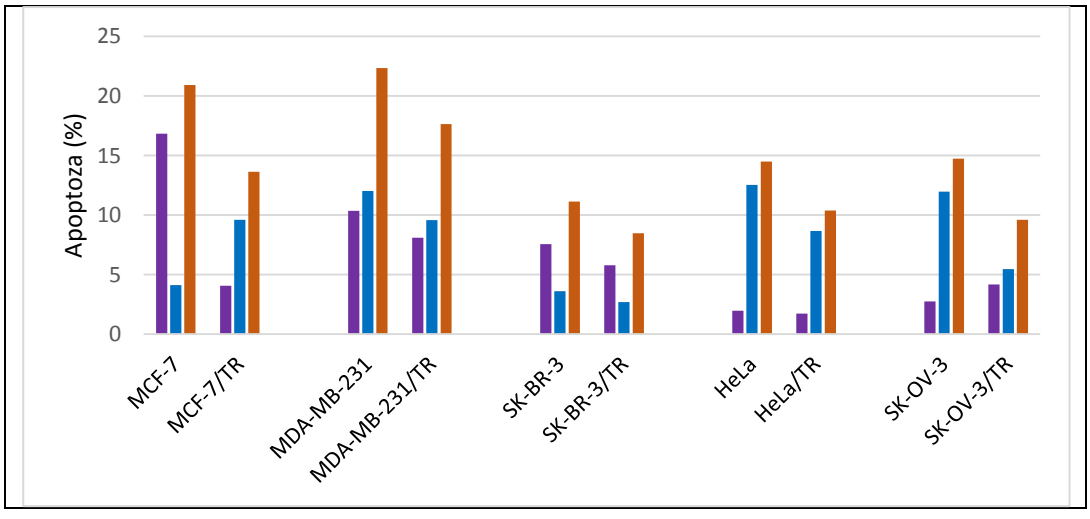
Figure 2. Early apoptotic events □, late □ and total □ induced by ADB at the level of parental and adriablastin-resistant (AR) cell lines



b) **Modulation of apoptosis by TPT in parental cell lines and TPT-induced resistance sublines**

Comparative analysis of parental cell sensitivity and topotecan induced resistance at 24 hours TPT exposure (50nM concentration) showed an increase in the percentage of apoptotic events in all parental cell lines compared to resistant lines, demonstrating a lower sensitivity of topotecan induced resistance cells to parental cells (Fig. 3).

Figure 3. Early apoptotic events □, late □ and total □ induced by TPT at the level of parental and topotecan-resistant (TR) cell lines



The results obtained showed the chemoresistant phenotype of the sublines with ADB or TPT - induced resistance. These findings correspond with the available data of other authors (Potter 2002, Giovanetti 2015, Jin Mo Ku 2015). Thus, studies performed on ADB-resistant MCF-7 cell line showed that exposure to 1μM ADB (24 hour) did not induce apoptosis (Jin Mo Ku 2015).

The involvement of topoisomerases in the apoptotic process is supported by a number of studies. It is known that adriablastin has several mechanisms of action, but both in its case and topotecan has been shown that cytotoxicity is mainly due to interaction with topoisomerases, Topo II and Topo I, respectively and the ternary complexes formed (Topo-DNA-chemotherapeutic agents) (Lee 2017, Giovanetti 2015); subsequent, the cell cycle is blocked and occurs apoptosis of the cells unable to repair efficiently DNA(Lee 2017; Cox 2016; Darzynkiewicz 2009; Giovanetti 2015). Potter's study has shown that DNA destruction occurs predominantly in the phase G2 / M of cell cycle when Topo II is overexpressed (Potter, 2002).

4) Modulation of cross-resistance at ADB and TPT, assessed by determining apoptotic events in parental cell lines and induced resistance sublines

In this study, we also evaluated cross-resistance by quantification of apoptotic events after parallel treatment of the resistant lines to the second cytostatic. The results obtained by flow cytometry analysis showed that irrespective of the type of tumor from which the lines were derived in case of adriablastin-resistant sublines TPT treated, the apoptotic cell fractions generally had values close to those of the parental lines from which they derived (aso parental lines were treated with the same TPT concentration)

The results of apoptosis modulation experiments by anti-topoisomerase agents in parental cell lines or ADB,TPT- induced resistance cell lines are summarized in Table 2.

The evaluation of apoptotic events by flow cytometry confirms the resistant phenotype acquired during the induction process of drug resistance. The TPT sensitivity of ADB-resistant cells and vice versa, the ADB sensitivity of TPT-resistant cells, previously observed in the determination of cytotoxicity by MTS assay, in a concentration-dependent manner are also confirmed in the evaluation of apoptotic events by flow cytometry.

Tabel 2. Percentage distribution of apoptotic events in parental and TPT (TR) or ADB (AR) resistant cell sublines)

Cell lines	Chemotherapeutic agents	Early apoptosis (%)	Late apoptosis (%)	Tota apoptosis (%)
MCF-7	Control	1.18	3.51	4.69

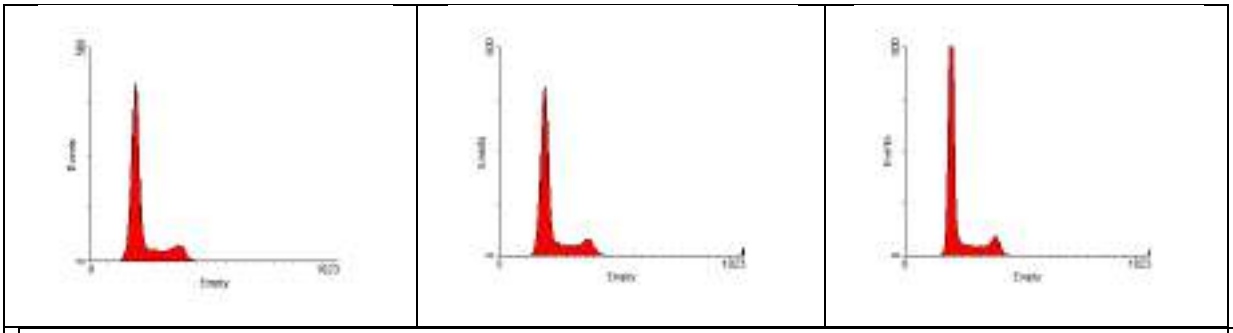
	Topotecan	16.81	4.10	20.91
	Adriablastina	12.01	6.65	18.66
MCF-7/TR	Control	2.61	3.59	6.20
	Topotecan	4.04	9.58	13.62
	Adriablastina	9.75	7.24	16.99
MCF-7/AR	Control	4.21	3.72	7.93
	Topotecan	4.03	11.35	15.38
	Adriablastina	8.49	6.14	14.63
MDA-MB-231	Control	1.48	5.16	6.64
	Topotecan	10.34	12.00	22.34
	Adriablastina	2.27	23.63	25.9
MDA-MB-231/TR	Control	2.67	10.45	13.12
	Topotecan	8.07	9.56	17.63
	Adriablastina	4.92	21.89	26.81
MDA-MB-231/AR	Control	3.31	8.36	11.67
	Topotecan	5.78	15.44	21.22
	Adriablastina	4.34	11.14	15.48
SK-BR-3	Control	2.27	2.06	4.33
	Topotecan	7.54	3.59	11.13
	Adriablastina	10.60	4.38	14.98
SK-BR-3/TR	Control	3.37	4.35	7.72
	Topotecan	5.78	2.67	8.45
	Adriablastina	9.90	4.46	14.36
SK-BR-3/AR	Control	2.11	2.29	4.40
	Topotecan	7.00	3.98	10.98
	Adriablastina	3.81	3.70	7.51
HeLa	Control	2.62	2.62	5.24
	Topotecan	1.96	12.52	14.47
	Adriablastina	10.26	13.42	23.68
HeLa/TR	Control	4.42	4.65	9.07
	Topotecan	1.71	8.65	10.36
	Adriablastina	3.25	18.10	21.35
HeLa/AR	Control	3.74	2.44	6.18
	Topotecan	2.22	12.10	14.32
	Adriablastina	2.78	4.84	7.62
SK-OV-3	Control	0.50	2.64	3.14
	Topotecan	2.73	11.94	14.72
	Adriablastina	13.22	6.26	19.48
SK-OV-3/TR	Control	6.05	2.02	8.07
	Topotecan	4.15	5.45	9.6
	Adriablastina	15.02	2.41	17.43
SK-OV-3/AR	Control	1.87	3.09	4.96
	Topotecan	5.48	8.21	13.69
	Adriablastina	3.31	4.63	7.94
MCF-7/A	Control	1.10	1.76	2.86
	Topotecan	2.67	11.18	13.85
	Adriablastina	3.34	1.77	5.11

5) Evaluation of the proliferation status of parental cell lines and adriablastin or topotecan -resistant sublines by analysis of cell cycle dynamics

Flow cytometry is a superior technique to classical methods of cellular analysis, and is most suitable for monitoring the distribution of DNA in cell cycle phases. In order to determine the proliferative activity of parental cell lines and adriablastin or topotecan resistant sublines by measuring nuclei distribution in the cell cycle phases (G0 / G1, S and G2 / M), the staining with PI of the nuclei of parental lines and and adriablastin or topotecan -resistant sublines were realized followed by the acquisition of 20000 events and data analysis using dedicated software.

Figure 4. Cell cycle phase distribution in MCF-7 parental and resistant lines

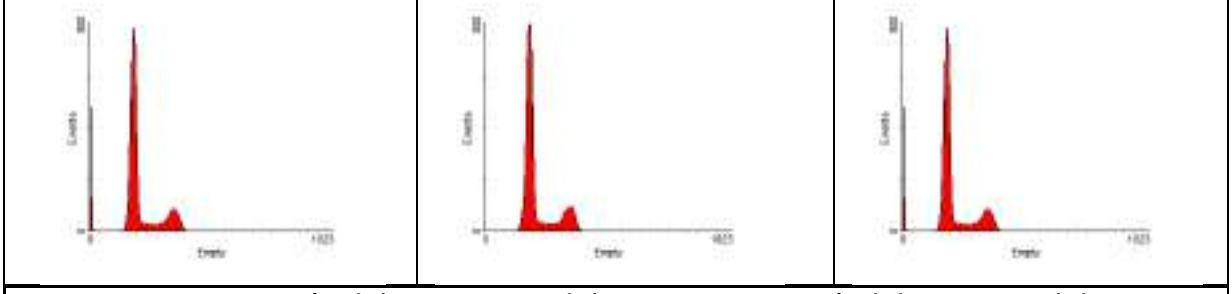
MCF-7	MCF-7/AR	MCF-7/TR



Cell lines	G0/G1 (%)	S (%)	G2/M (%)	IP (%)
MCF-7	72.22	16.86	10.92	27.78
MCF-7/AR	70.17	19.17	10.66	29.83
MCF-7 /TR	71.11	19.01	9.88	28.89

Figure 5. Cell cycle phase distribution in SK-OV-3 parental and resistant lines

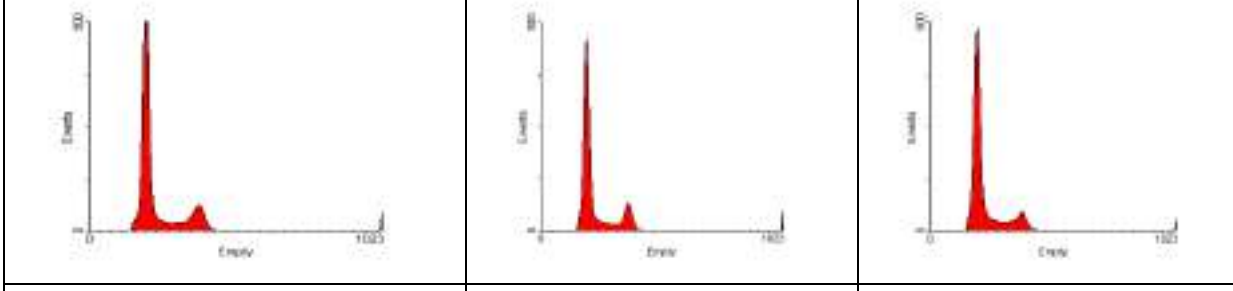
SK-OV-3	SK-OV-3/AR	SK-OV-3 /TR



Cell lines	G0/G1 (%)	S (%)	G2/M (%)	IP (%)
SK-OV-3	73.08	10.38	15.82	26.2
SK-OV-3/AR	71.70	10.09	18.21	28.3
SK-OV-3/TR	74.85	9.12	16.03	25.15

Figure 6. Cell cycle phase distribution in SK-BR-3 parental and resistant lines

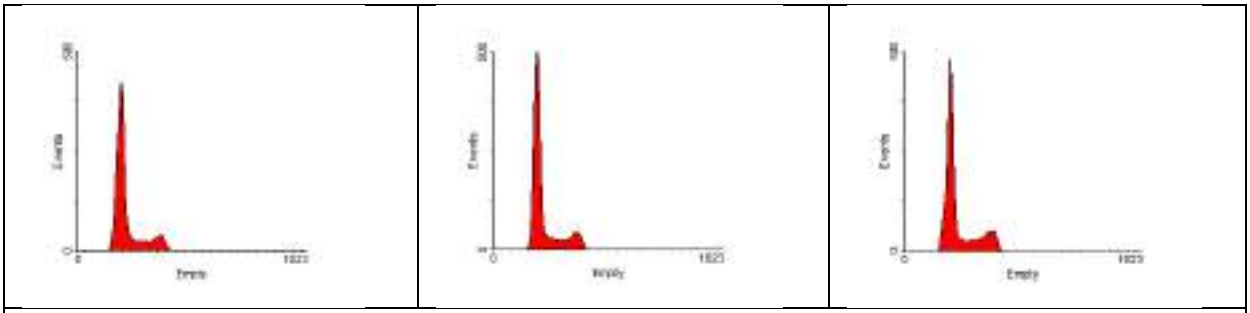
SK-BR-3	SK-BR-3/AR	SK-BR-3/TR



Cell li	G0/G1 (%)	S (%)	G2/M (%)	PI (%)
SK-BR-3	62.36	29.59	8.05	37.64
SK-BR-3/AR	66.15	20.37	13.47	33,84
SK-BR-3/TR	64.20	29.78	6.02	35,8

Figure 7. Cell cycle phase distribution in HeLa parental and resistant lines

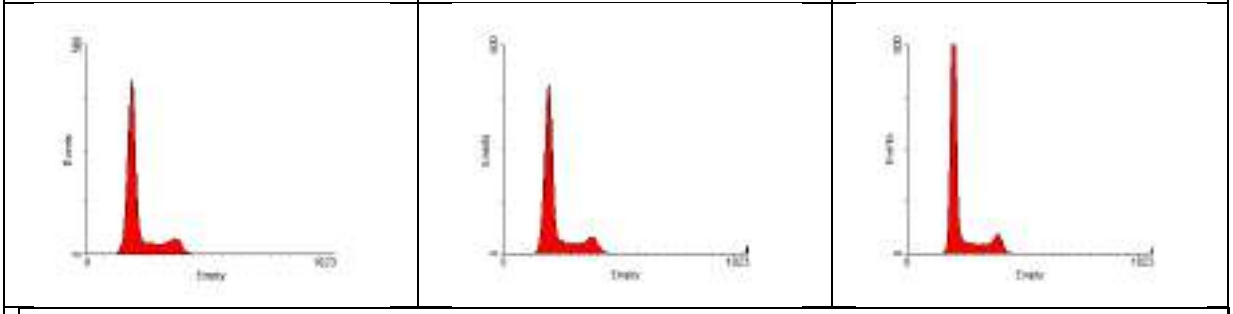
HeLa	HeLa /AR	HeLa /TR



Cell lines	G0/G1 (%)	S (%)	G2/M (%)	IP (%)
HeLa	74.57	13.25	12.18	25.43
HeLa/AR	77.62	10.35	11.57	21.92
HeLa/TR	74.03	7.94	13.83	21.77

Figure 8. Cell cycle phase distribution in MCF-7/AR parental and resistant lines

MCF-7	MCF-7/AR	MCF-7/TR



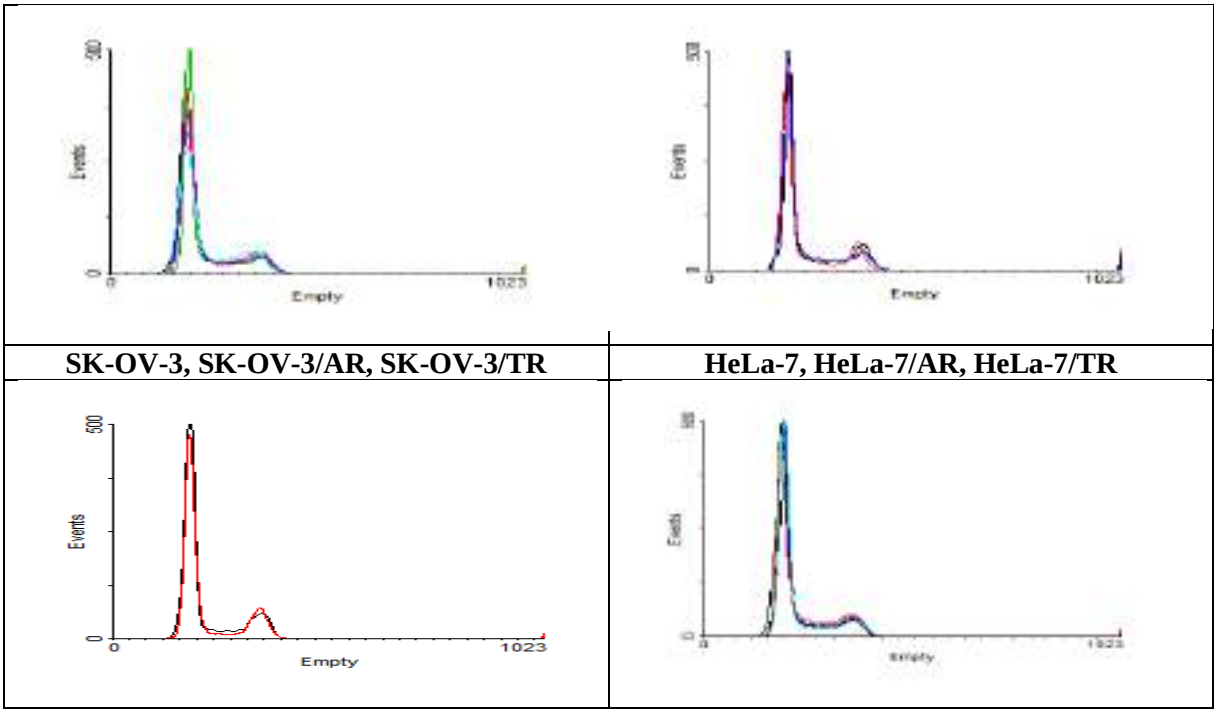
Cell lines	G0/G1 (%)	S (%)	G2/M (%)	IP (%)
MCF-7	72.22	16.86	10.92	27.78
MCF-7/AR	70.17	19.17	10.66	29.83
MCF-7 /TR	71.11	19.01	9.88	28.89

Results from the analysis of the cellular cycle dynamics showed that between the distribution patterns in the cell cycles of the resistant cell sublines and the corresponding parental lines for MCF-7, SK-OV-3 do not appear notable differences in the case of ADB or TPT resistance. A slight decrease in proliferative activity was recorded in some resistant sublines (SK-BR-3/Ar, HeLa/AR, HeLa/TR).

Overlapping of DNA histograms (Fig. 9) clearly shows the similar distribution of cell cycle phases in parental lines and resistant sublines. The data are consistent with those in the literature on the decrease of proliferative activity in resistance to chemotherapeutic agents.

Figure 9. DNA histograms of parental and resistant lines

MCF-7, MCF-7/AR, MCF-7/TR	SK-BR-3, SK-BR/AR, SK-BR-3/TR



SK-OV-3, SK-OV-3/AR, SK-OV-3/TR	HeLa-7, HeLa-7/AR, HeLa-7/TR

II. Tooisomerase I, Topoisomerase II alpha and beta status

Although the mechanisms involved in both de novo and acquired resistance have been intensively studied and topoisomerases are also investigated in a series of studies, their involvement in sensitivity / resistance is not fully elucidated. Thus, an important objective of this study was to evaluate the expression of Topoisomerase I and Topoisomerases IIa and IIb in order to determine their involvement in resistance to chemotherapy.

The determination was made at the beginning of the studies and during the acquisition of resistance to ADB (Topo II inhibitor) or TPT (Topo II inhibitor) for the entire panel of parental cell lines selected for study (MCF-7, MDA-MB-231, SK-BR-3, HeLa, SK-OV-3) and for resistant cell lines variants (MCF7/AR, MCF-7/TR, MDA-MB-231/AR, MDA-MB-231/TR, SK-BR-3/AR, SK-BR-3/TR, HeLa/AR, HeLa/TR, SK-OV-3/AR și SK-OV-3/TR).

1) Analysis of constitutive protein expression of topoisomerases by flow cytometry and immunocytochemistry

A) Flow cytometry quantification of the constitutive protein expression levels of Topo I, Topo IIα and IIβ

Determination of Topo I and Topo II (α and β) antigens expressions by flow cytometry was performed, after permeabilization of the cell membranes, and labelling with specific monoclonal antibodies of rabbit anti-human, Anti-topo I, anti-Topo II α or anti-Topo II β, and fluorescent polyclonal secondary antibodies goat anti - rabbit (IgG-FITC) (Abcam, Cambridge, UK).

Table 3. Expression levels of Topo I, Topo IIα și IIβ

Antigens/ cell lines	Positive cells (%)				
	MCF-7	**MDA-MB-231**	**SK-BR-3**	**HeLa**	**SK-OV-3**
Topo I	95,31	94,35	86,79	87,74	91,35
Topo IIα	78,80	90,89	73,18	89,12	86,93
Topo IIβ	83,05	65,03	84,65	77,55	86,74

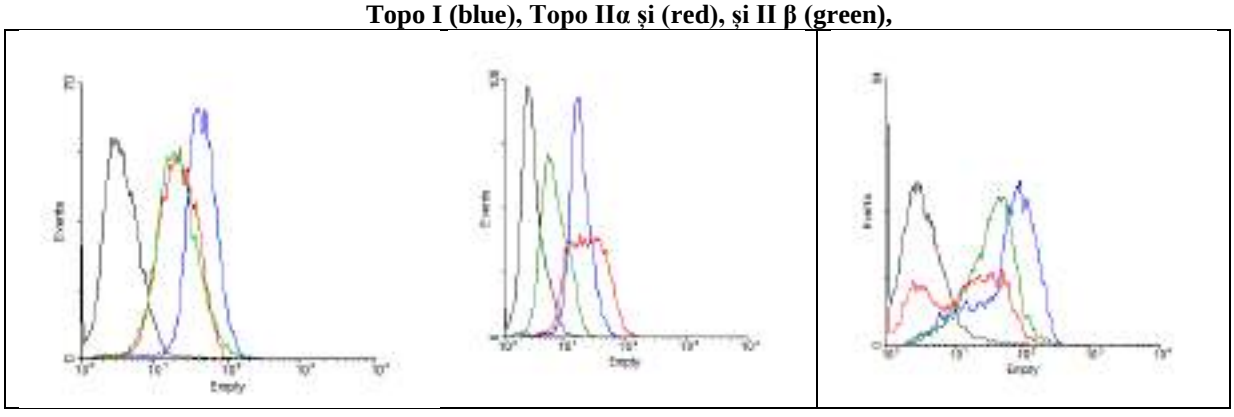
Flow cytometry analysis, represented by histograms of cell fluorescence distribution, showed in the parental lines of the same histologic type MCF-7, MDA-MB-231, SK-BR-3 (breast adenocarcinoma), but also to those belonging to other histologic types HeLa (cervical adenocarcinoma) or SK-OV-3 (ovarian adenocarcinoma) increased protein expression of topoisomerases prior to induction of resistance, expressed both as percentages of positive cells as well as mean fluorescence intensity (MFI) values (Table 3 and 4).

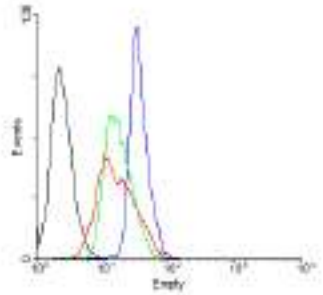
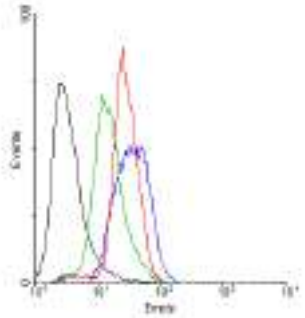
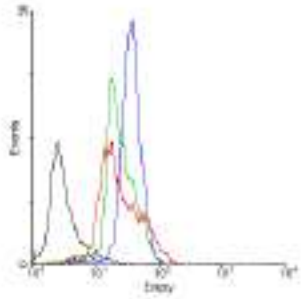
Table 4. Mean fluorescence intensity values of Topo I, Topo IIα și IIβ

Antigens/ cell lines	(MFI)				
	MCF-7	**MDA-MB-231**	**SK-BR-3**	**HeLa**	**SK-OV-3**
Topo I	36,87	20,66	32,12	30	24,4
Topo IIα	21,98	25,3	32,14	27,92	25,14
Topo IIβ	21,79	14,66	37,94	25,69	17,83

The antigen expression levels of Topo I, Topo IIα and IIβ determined in parental cell lines (MCF-7, MDA-MB-231, SK-BR-3, HeLa and SK-OV-3), expressed either as percentages of antigen-positive cells (IIF%), or as mean fluorescence intensity (MFI), are represented by overlapping histograms in Fig. 10.

Figure 10. Protein expression levels of Topo I, Topo IIα and IIβ,
Topo I (blue), Topo IIα și (red), și II β (green),



MCF-7	MDA-MB-231	SK-BR-3
		
MCF-7/A	HeLa	SK-OV-3

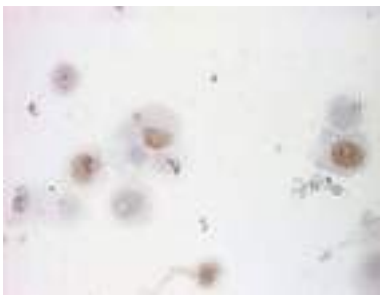
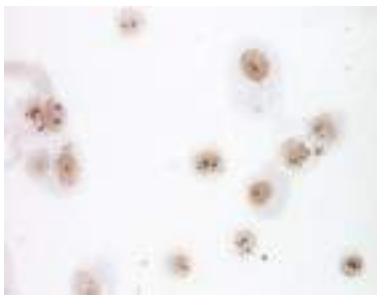
B) Flow cytometry quantification of the Topo I, Topo IIα and IIβ protein expression levels function of cell cycle phases

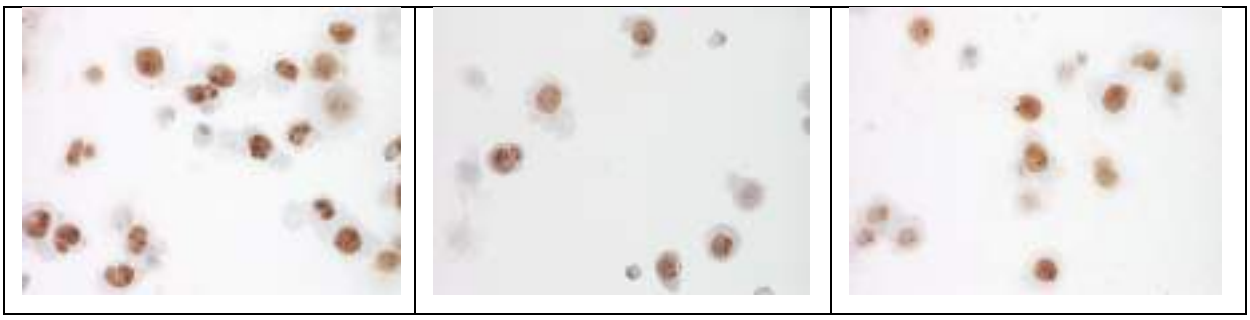
Double labeling with monoclonal antibodies specific to Topo I, Topo IIa, Topo IIb / fluorescent secondary antibodies and propidium iodide allowed the quantification of these enzymes in the different phases of the cell cycle. Analysis of the results revealed at all parental cell lines studied high antigen expressions of the three topoisomerases in all phases of the cell cycle, including phase G0 / G1.

C) Immunocitochemistry quantification of protein expression levels of Topo I, Topo IIα and IIβ

Determination of Topo I and TopoII isoforms α and β isoforms expression of the parental cell lines by immunocytochemistry was performed in cytospine samples labeled with specific primary antibodies after epitope reactivation, endogenous peroxidase blocking and amplification of the reaction by incubation with a HRP-conjugated secondary antibody (Fig.11).

Figure 11. Topo I (A), Topo IIα (B) și IIβ (C) protein expression in MCF-7 and HeLa cell lines

A	B	C
		
A	B	C



Thus, the levels of expression of TopoI and β-isoform of TopoII are not dependent on the phases of the cell cycle, being expressed independent of the proliferative status, including G0/G1 phase.Protein expressions of Topo I, Topo IIa and IIb were found to be elevated in parental tumor cells (MCF-7, MDA-MB-231, SK-BR-3, HeLa and SK-OV-3). In each cell line, some differences were observed between the expression levels of the three topoisomerases, but there was no significant correlation between them, resulting from from the statistical analysis carried out for the determinations by flow cytometry assays, following both the percentage of positive cells and MFI values.

1) Analysis of Topo I, Topo IIα and IIβ protein expression in adriablastin or topotecan - resistant sublines by flow cytometry

Expression levels of Topo I, Topo IIa and Topo IIb were monitored during the acquisition of the resistance phenotype, by treating cells with increasing concentrations of TPT (between 3 to 60 nM depending on the cell line) for 10 months and analyzing periodically by flow cytometry the antigenic expression of the three topoisomerases in the TPT-resistant sublines (MCF-7 / TR, MDA-MB-231 / TR, SK-BR-3 / TR, HeLa / TR and SK-OV-3 / TR). After 5 months of initiation TPT- resistant phenotype, a marked transient decrease of Topo I expression was observed at at the level of SK-BR-3 / TR and HeLa / TR cell sublines, and more diminished in MCF-7/TR and MDA-MB-231 / TR cell lines. Topo IIa presented small variations of antigenic expression, while Topo IIb expression fluctuated.

Induction of ADB resistance in the cell lines taken in the study was achieved through treatments with concentrations ranging from 20 to 700 nM, depending on the line, over a 10-month period. At the level of ADB-resistant sublines (MCF7 / AR, MDA-MB-231 / AR, SK-BR-3 / AR, HeLa / AR and SK-OV-3 / AR) a decrease in the Topo IIα expression was rapidly recorded after initiation of resistance induction followed by a tendency to return to a higher

level, followed again by a decrease in expression. The most significant decrease of the protein expression was recorded in the MCF-7/AR and SK-BR-3/AR sublines, and the highest level of expression was in HeLa/AR. Topo I expression was generally unchanged, while Topo IIβ expression fluctuated.

In conclusion, the results obtained by flow cytometry analysis of the of Topo I, Topo IIα and Topo IIβ protein expression during induction of TPT-resistance and ADB-resistance showed a decrease of the Topo IIa level during the acquisition of the ADB resistance phenotype without lowering the Topo I level.

3) Analysis of Topo I, Topo IIα and IIβ protein expression in adriablastin or topotecan - resistant sublines by Western Blot

Western Blot is the most commonly used technique for evaluating the protein expression of topoisomerases. The Intensity of the bands corresponding to the molecular masses of the three topoisomerases (MW = 100 kDa for Topo I; MW = 170 kDa for Topo IIα, MW = 180 kDa for Topo IIβ) was different between parental lines, even if they came from the same histological type. The highest expression for Topo I was observed at the level of the MDA-MB-231 cells, and the lowest in SK-BR-3 and SK-OV-3 cells. Topo IIα had the most intense bands in MDA-MB-231 and HeLa cells , the lowest level being recorded in MCF-7/SK-BR-3/SK-OV-3.

From the data obtained, it was found that the intensity of the Topo I bands is lower in TPT- resistant sublines compared to parental lines. Comparative analysis of Topo I levels in the mentioned lines showed that the lowest expression was recorded in SK-BR-3 / TR cells, and the highest in MCF-7 / TR cells. Regarding the protein expression of Topo IIα and Topo II β, small variations were found.

In cell sublines with resistance induced to ADB, analyzing the intensity of the Topo IIα corresponding bands, it is also observed a decrease in the intensity compared to the parental lines.The lowest intensity of the bands was detected in SK-BR-3/AR and MCF-7/AR, the higher intensity being detected at HeLa/AR and MDA-MB-231/AR. Topo I generally had patterns of expression similar to the parental lines from which they derived, while Topo IIβ expression varied.

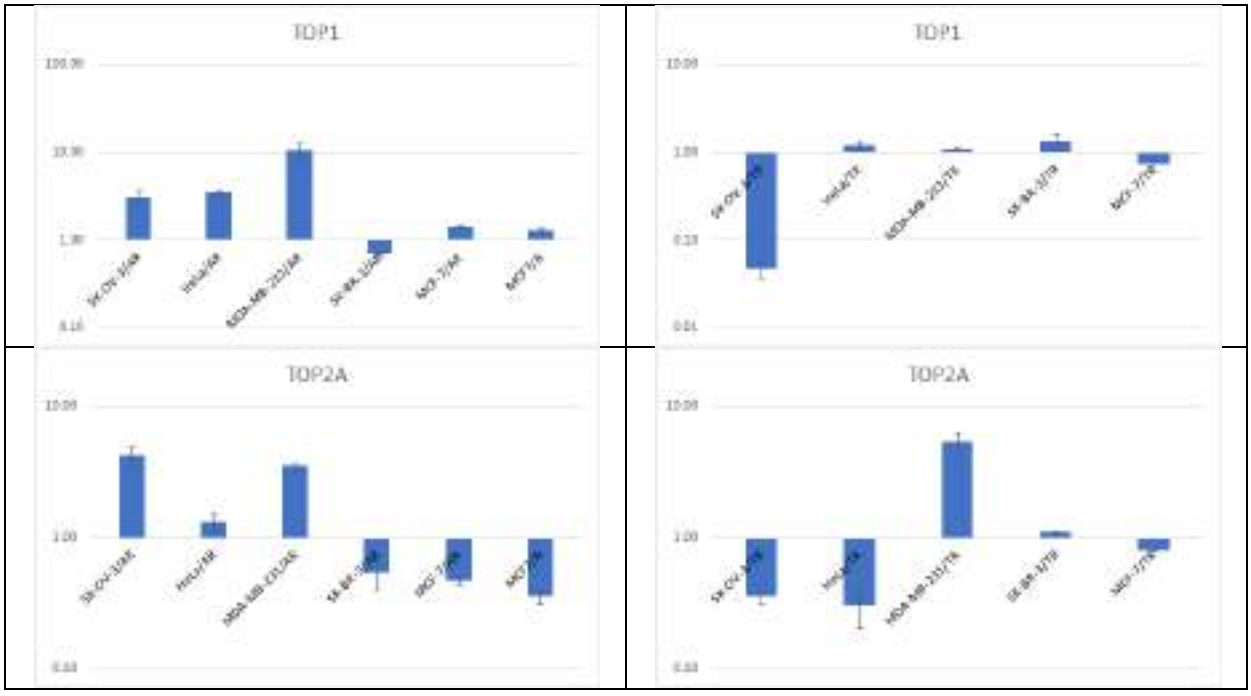
The results obtained by Western Blot are generally consistent with the data obtained from the evaluation of the protein expression by flow cytometry, and support what we found in determining the expression by this technique: by acquiring the resistant phenotype to TPT, Topo I expression decreases, without the expression of Topo IIα to be affected, and in cells

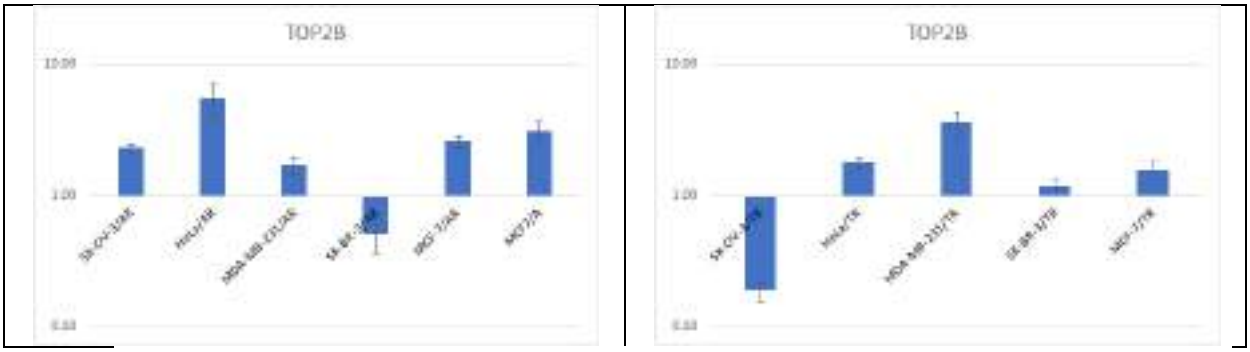
resistant to ADB there is a decrease in Topo IIα expression without affecting the expression of Topo I.

4) Analysis of Topo I, Topo IIα and IIβ gene expression in adriablastin or topotecan - resistant sublines

In order to identify the presence or absence of the topoisomerases gene expression in parental cell lines, TPT- resistant cell sublines and ADB- resistant cell sublines, the cDNA of the cells were subjected to gene expression analysis by RT-PCR, using the Taqman method and specific primers (Fig. 12).

Figura 12. Topoisomerases gene expression levels in resistent cell sublines, related to parental lines (*n-fold*)





The results obtained, represented on the logarithmic scale, demonstrate a differentiated gene expression in the cell lines with induced resistance, reported to the parental lines for all three topoisomerases, Topo I, Topo IIα or Topo IIβ studied.

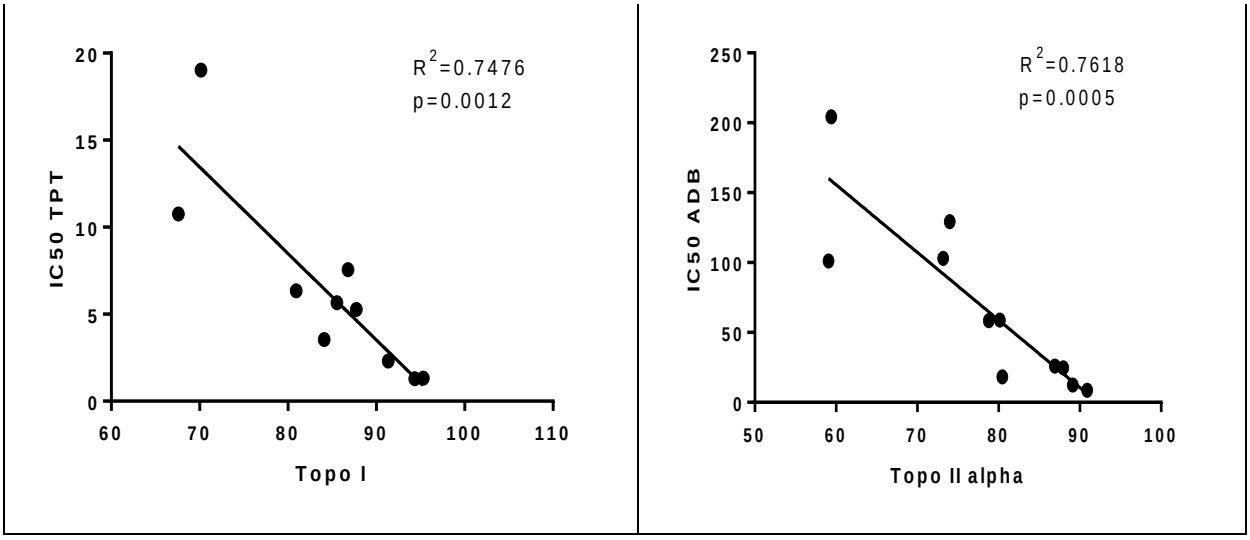
III. Determination of existing correlates between topoisomerase expression levels and sensitivity/resistance to ADB or TPT

In order to determine the involvement of topoisomerases in ADB or TPT resistance, we analyzed possible correlations between the protein expression levels of each topoisomerase (Topo I, Topo IIa and β), assessed by flow cytometry and expressed as percentage of positive cells (IIF%) and cell response to treatment with ADB or TPT, expressed by IC 50 values.

Analysis of correlations between Topo I, Topo IIα or Topo IIβ protein levels and IC50 values for the two drugs demonstrated inverse statistically significant correlations between IC50 to TPT and Topo I levels in TPT-induced resistance lines, while in ADB-induced resistance lines, IC50 to ADB correlated with the Topo IIα level (Figure 13). Parental lines have demonstrated the same type of correlation, both for IC50/TPT and Topo I level, as well as for IC50/ADB and Topo IIα.The existence of inverse correlations between drug concentrations and the expression of topoisomerases actually denotes direct correlations between the degree of sensitivity to treatment and the level of protein expression of these nuclear enzymes

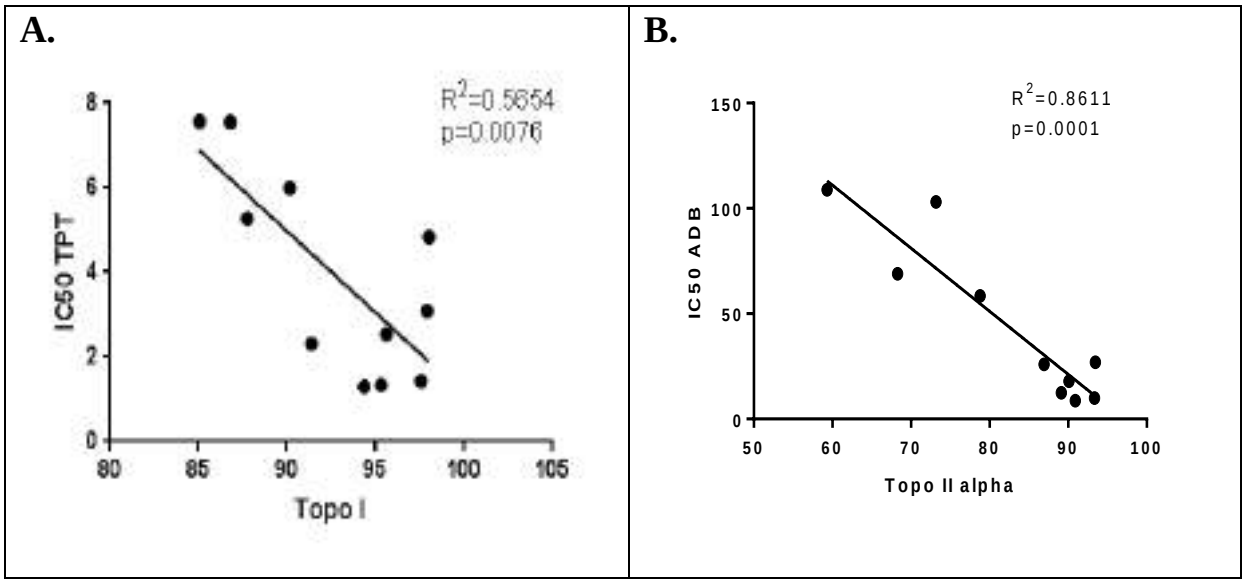
Figura 13. Analysis of correlations between topoisomerase expression (IIF%) and IC50 values in parental cell line and TPT or ADB resistant sublines

A.	**B.**



Based on the statistical analysis, we also found a correlation of TPT response in the parental and ADB-resistant line and the ADB response in parental and TPT-resistant lines (Fig. 14).

Figura 14. Analysis of correlations for cross resistance between topoisomerase expression (IIF%) and IC50 values in parental cell line and TPT or ADB resistant sublines



The existence of a correlation between Topo I protein levels and TPT sensitivity or between Topo IIα protein levels and ADB sensitivity, plus the fact that during the acquisition of resistance phenotype to ADB the decrease in Topo IIa level occurs without a decrease of Topo I level and the decrease Topo I levels in TPT-resistant cells occurs without the reduction of Topo IIa, suggesting that these enzymes are involved in the sensitivity / resistance to chemotherapeutic agents that have primary targets these proteins.

GENERAL CONCLUSION

Using the xCELLigence system for real-time cell proliferation analysis vs. cellular cytotoxicity assay under the action of anti-topoisomerase chemotherapeutic agents has provided important advantages compared to classical, colorimetric methods, " end-point " type.

The obtained data allowed the selection of concentrations of anti-topoisomerase agents and time periods for the application of treatments, useful in subsequent "end-point" assays (Flux cytometry, RT-PCR, WB).

Comparative analysis of the data showed that the MCF-7/AR and MCF-7/TR, MDA-MB-231/AR and MDA-MB-231/TR, SK-BR3/AR and SK-BR3/TR, HeLa/AR and HeLa/TR, SK-OV-3/AR and SK-OV-3/TR resistant cell sublines are variants with varying degrees of resistance induced to adriablastin or topotecan, through the progressive selection of human breast cell lines (MCF-7, SK-BR-3, MDA-MB-231), human ovarian cell line (SK-OV-3) and human cervical cell lines (HeLa) grown in medium with increasing concentrations of ADB or TPT. The results obtained showed that resistance to adriablastin does not induce cross resistance to topotecan, and vice versa.

The method allowed calculation of IC50 values at any time in the proliferation curve. IC50 values obtained by RTCA analysis were comparable to those obtained from conventional, colorimetric cytotoxicity assays (MTS). Thus, following the analysis of the cell proliferation inhibition curves and the IC50 calculation a working concentrations range was chosen function of the constitutive or induced resistance of the cell lines studied.

The evaluation of apoptotic events by flow cytometry confirms the resistant phenotype acquired during the induction process of drug resistance. The TPT sensitivity of ADB-resistant cells and vice versa, the ADB sensitivity of TPT-resistant cells, previously observed in the determination of cytotoxicity by MTS assay, in a concentration-dependent manner are also confirmed in the evaluation of apoptotic events by flow cytometry.

Results from the analysis of the cellular cycle dynamics showed that between the distribution patterns in the cell cycles of the resistant cell sublines and the corresponding parental lines for MCF-7, SK-OV-3 do not appear notable differences in the case of ADB or TPT resistance. A slight decrease in proliferative activity was recorded in some resistant sublines (SK-BR-3/Ar, HeLa/AR, HeLa/TR).The levels of expression of TopoI and β-isoform

of TopoII are not dependent on the phases of the cell cycle, being expressed independent of the proliferative status, including G0/G1 phase.

Protein expressions of Topo I, Topo IIa and IIb were found to be elevated in parental tumor cells (MCF-7, MDA-MB-231, SK-BR-3, HeLa and SK-OV-3). In each cell line, some differences were observed between the expression levels of the three topoisomerases, but there was no significant correlation between them, resulting from the statistical analysis carried out for the determinations by flow cytometry assays, following both the percentage of positive cells and MFI values.

The results obtained by flow cytometry analysis of the of Topo I, Topo IIα and Topo IIβ protein expression during induction of TPT-resistance and ADB-resistance showed a decrease of the Topo IIa level during the acquisition of the ADB resistance phenotype without lowering the Topo I level. The results obtained by Western Blot are generally consistent with the data obtained from the evaluation of the protein expression by flow cytometry, and support what we found in determining the expression by this technique: by acquiring the resistant phenotype to TPT, Topo I expression decreases, without the expression of Topo IIα to be affected, and in cells resistant to ADB there is a decrease in Topo IIα expression without affecting the expression of Topo I.

The results obtained by RT-PCR demonstrate a differentiated gene expression in the cell lines with induced resistance, reported to the parental lines for all three topoisomerases, Topo I, Topo IIα or Topo IIβ studied.

The existence of a correlation between Topo I protein levels and TPT sensitivity or between Topo IIα protein levels and ADB sensitivity, plus the fact that during the acquisition of resistance phenotype to ADB the decrease in Topo IIa level occurs without a decrease of Topo I level and the decrease Topo I levels in TPT-resistant cells occurs without the reduction of Topo IIa, suggesting that these enzymes are involved in the sensitivity / resistance to chemotherapeutic agents that have primary targets these proteins.

Better knowledge of molecular mechanisms of resistance to chemotherapy could lead to the identification of really relevant predictive molecular markers for the response to chemotherapy, and topoisomerases could play an important role in establishing innovative, personalized strategies for patients with neoplasia.

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