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Researach Article

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Platinum derivatives: generic brands vs. original, *in vitro* tests

Derivaţii de platină: medicamente generice vs. originale, teste in vitro

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Abstract

The entry of the generic drugs on the market was an impressive development of the pharmaceutical industry and due to their lower prices also a decrease in the cost price for the treatment of patients. The difference in price (sometimes even 50%) between generics and original and different response to therapy sometimes raised serious questions related to their therapeutic equivalence. The scientific community is increasingly interested in this aspect, with studies (in vitro and on patients) demonstrated statistically significant differences in terms of differences generic / original drug. In this context, the aim of our study was to assess the in vitro cytotoxic activity of oxaliplatin (original and generic drug) on DLD-1 cell lines, HT-29, and carboplatin cytotoxic activity (and the reference molecule from Santa Cruz Biotechnology) on cell line A2780. Cell viability was evaluated using the MTT assay.

Regarding the cell line DLD-1, IC50 values of generics was lower than the original after exposure for 24 hours to oxaliplatin but after 48 hours of exposure were not statistically significant differences. HT-29 line has a higher resistance to chemotherapy compared with oxaliplatin, the IC 50 values after 48 hours of exposure are higher than those for the line DLD-1. IC50 values are confirmed by morphological analysis of cells. Regarding carboplatin were not recorded statistically significant differences between the two generic drugs tested.

Although other studies reported differences between generic and branded drugs in terms of hypersensibility reactions, adverse effects and efficacity, we cannot extrapolate our findings to the patients. Further studies on patients are neeeded for a better evaluation of the efficacity of generic vs. original drugs.

***Keywords**: Oxaliplatin, generic vs original, cancer, carboplatin*

Rezumat

Intrarea pe piaţă a medicamentelor generice a reprezentat o dezvoltare impresionantă a industriei farmaceutice şi de asemenea o scădere a preţului de cost pentru tratamentul bolnavilor datorită preţurilor mai mici a acestora. Diferenţa de preţ (uneori chiar de 50%) între medicamentele generice şi originale precum şi răspunsul uneori diferit la terapie a ridicat serioase semne de întrebare legat de echivalenţa terapeutică a acestora. Comunitatea ştiinţifică este din ce în ce mai interesată de acest aspect, existând studii (in vitro dar şi pe pacienţi) care au

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demonstrat diferenţe statistic semnificative în ceea ce priveşte diferenţele medicament generic/original. În acest context, studiul nostru şi-a propus evaluarea citotoxică in vitro a oxaliplatinului (medicament original şi generice) pe liniile celulare DLD-1, HT-29, şi a carboplatinului (şi molecula de referinţă achiziţionată de la Santa Cruz Biotechnology) pe linia celulară A2780. Viabilitatea celulară a fost testată folosind testul MTT.

În ceea ce priveşte linia DLD-1, valoarea IC50 a medicamentelor generice a fost mai mică comparativ cu originalul după expunerea timp de 24 de ore la oxaliplatin însă după 48 de ore de expunere nu au fost diferenţe statistic semnificative. Linia HT-29 prezintă o rezistenţă mai mare la chimioterapia cu oxaliplatin, valorile IC50 după 48 de ore de expunere sunt mai mari comparativ cu cele pentru linia DLD-1. Valorile IC50 sunt confirmate şi de analiza morfologică a celulelor. În ceea ce priveşte carboplatinul, nu au fost înregistrate diferenţe statistic semnificative între cele două medicamente generice testate.

Deşi unele studii au arătat diferenţe între medicamentele generice şi originale în ceea ce priveşte apariţia reacţiilor adverse, reacţiilor de hipersensibilitate şi a eficacităţii, nu putem extrapola rezultatele noastre la rezultatele din clinică. Este nevoie de investigaţii suplimentare pentru a stabili dacă există o diferenţă între medicamente şi în ce măsură acestea afectează evoluţia bolii.

***Cuvinte cheie**: Oxaliplatin, generic vs original, cancer, carboplatin*

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Introduction

Introduced in therapy since the early 80's, platinum derivatives still represent a reference class of anticancer compounds. In 1845, Michele Peyrone discovered cisplatin, lately known as Peyrone's salt, whose antitumor properties have been serendipitously highlighted in 1965 by Barnett Rosenberg et al. (1). The extraordinary results of cisplatin in oncological therapy pushed researchers to continue the synthesis of platinum compounds with anticancer activity.

Until the present date, in oncological therapy there are three compounds that are being used on a large scale: Cisplatin – the first generation, Carboplatin – the second generation, and Oxaliplatin – the third generation. Other platinum derivatives that are being used on a smaller scale are Nedaplatin – approved in Japan, Lobaplatin – approved in China, and Heptaplatin – approved in the Republic of Korea (2). Platinum derivatives play an essential role in the treatment of cancers of various etiologies such as: ovarian cancer, colon cancer, stomach cancer and pulmonary cancer (3).

In terms of mechanism of action, platinum derivatives belong to the alkylating agents category. Forming intra-strand or inter-strand adducts with the cancer cell's DNA is characteristic for this class of drugs, having an affinity for guanine's 7th position nitrogen atom. The forming of these adducts induces modifications in DNA structure, which inhibits cellular replication (4). The *cis* position of the ligands offers a greater stability to the drug than the *trans* position (5).

The efficiency of generic drugs compared to branded drugs is a debatable subject among professional healthcare providers and patients. The European Medicines Agency defines a generic drug as a "medicine that contains the same active substances as the reference medicine, and is used at the same doses to treat the same diseases. However, a generic medicine's inactive ingredients, name, appearance and packaging can be different from the reference medicine's" (6). Usually a branded drug loses about 80% of its market shares after 1 year of generic brand authorization (7). It has been observed that patients respond differently to generic and original drugs treatment and therefore the first question mark appears: "If there is no difference between the generic and original drugs, why do patients respond differently to the treatment?" and "If there are differences between the two types of

drugs, what are they and how serious is their effect on the treatment?" Some studies already revealed a difference regarding the efficiency and the occurrence of adverse effects when using generic drugs vs. the original drug (8-13). Our study is based on the clinical observation regarding the patients overall survival and drug-free interval in which our team observed a significant drop once the generic drugs entered the hospital system. Our aim was to study the *in vitro* activity of oxaliplatin (L-OHP) and carboplatin (original vs. generic drugs) against several human carcinoma cell lines.

Material and methods

Cell lines and cultures

A2780 is an epithelial human ovarian carcinoma cell line and DLD-1 and HT-29 are epithelial colorectal human carcinoma cell lines, which were obtained from ECACC through Sigma Aldrich. DLD-1 and A2780 were cultivated in RPMI-1640 and HT-29 in McCoy`s 5A Modified Medium, all supplemented with Fetal Calf Serum 10%, 2mM L-glutamine and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). Experiments were performed at a 70-80% cell confluence and confirmed in at least two independent experiments unless stated otherwise.

DLD-1 and HT-29 cell lines were treated with oxaliplatin brand and two generic drugs with concentrations ranging from 0.5 to 300 µg/mL. The cells were seeded at a density of $1x10^4$ DLD-1 cells/well and $12x10^3$ HT-29 cells/well using 96-well plates. The treatment was applied after 24 hours and the cells were incubated for an additional 24 or 48 hours.

A2780 cell line was treated with two carboplatin generic drugs and the reference molecule from Santa Cruz Biotechnology, with concentrations ranging from 1 to 1000 µg/mL. The cells were seeded at a density of 15x 103 cells/well using 96-well plates. The treatment was applied after 48 hours and the cells were incubated for an additional 24 hours.

Cytotoxicity evaluation

The evaluation of drugs cytotoxicity was done by MTT assay. This is a colorimetric assay for assessing cell viability. It is based on the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a purple colored formazan product by the viable cells (dead cells cannot do the conversion thus they remain uncolored) (14). Absorbance was recorded with a Biotek Synergy 2 at 570nm wavelength. The half maximal inhibitory concentration (IC50) values were calculated as the concentrations corresponding to a 50% reduction of the cellular growth.

The images were taken using a Carl Zeiss Axiovert D1 microscope, 40X objective, equipped with a MRC CCD camera. After 1 hour of incubation with MTT, images were captured and analyzed with a morphometric software, AxioRel 4.8.

Statistical analysis was performed using Graph Pad Prism software program version 5.0 (San Diego, CA, USA). Statistical comparison between groups was made by one-way ANOVA (One-way analysis of variance) and by unpaired one-tailed t test.

Results

DLD-1 cells treated with L-OHP

After 24 hours of exposure to L-OHP we found a significantly higher IC50 value for the original drug compared to the Oxaliplatin generic I ($p= 0.0086$) and Oxaliplatin generic II ($p= 0.0339$). There were no statistically significant differences between the two generics ($p= 0.182$) (Figure 1). Comparing the exposure after 48 hours of treatment, the differences were not statistically significant: oxaliplatin original vs. oxaliplatin generic I ($p=0.238$) and oxaliplatin

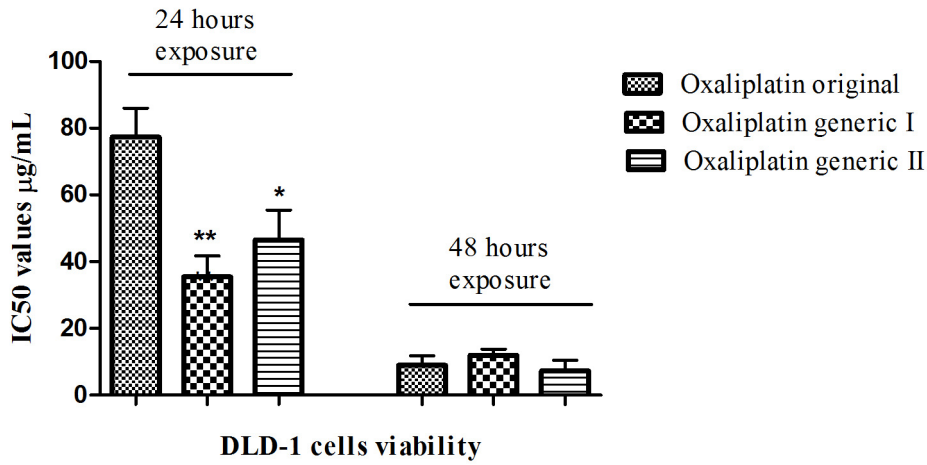


Figure 1. DLD-1 cells viability after 24 and 48 hours exposure at L-OHP brand and generics drug.

original vs. oxaliplatin generic II (p=0.364) but a decrease in IC50 values for the original drug was observed (Table I).

HT-29 cells treated with L-OHP

After 24 and 48 hours of exposure to L-OHP there were no statistical differences between the original and generic drugs. A slight increase in IC50 was observed for L-OHP original drug at 24 hours and a decrease in IC50 after 48 hours of treatment (Table II).

A2780 cells treated with carboplatin

Regarding the A2780 cells' response to carboplatin, no difference in IC 50 values between the reference molecule and the two generic drugs was observed after 24 hours of exposure (Table III).

Table I. DLD-1 cells IC50 values (µg/mL) after exposure at oxaliplatin brand and generic drugs. Values are means ±SEM.

DLD-1	Oxaliplatin original	Oxaliplatin generic I	Oxaliplatin generic II
IC50 values after 24 hours exposure	77.403 ± 8.656	35.417 ± 6.292	46.550 ± 8.896
IC50 values after 48 hours exposure	8.991 ± 2.829	11.940 ± 1.880	7.847 ± 2.623

Table II. HT-29 cells IC50 values (µg/mL) after exposure at oxaliplatin brand and generic drugs. Values are means ±SEM.

HT-29	Oxaliplatin original	Oxaliplatin generic I	Oxaliplatin generic II
IC50 values after 24 hours exposure	42.670 ± 0.140	33.980 ± 9.720	38.450 ± 6.870
IC50 values after 48 hours exposure	19.82 ± 5.227	24.14 ± 1.374	27.77 ± 1.597

Table III. A2780 cells IC50 values (µg/mL) after exposure at carboplatin generic drugs and the reference molecule purchased from Santa Cruz Biotechnology. Values are means ±SEM.

A2780	Carboplatin Santa Cruz Biotechnology	Carboplatin generic I	Carboplatin generic II
IC50 values after 24 hours exposure	227.410 ± 16.328	210.100 ± 15.627	234.640 ± 27.150

Morphology analysis

The cells` morphology changed after the drugs` concentration increased as phase contrast images highlighted the stained cells with MTT. We observed a decrease in cells number as drug concentration increased and a decreased mitochondrial capacity to reduce MTT salt to formazan crystals with the appearance of colorless cells (Table IV and V). Signs of characteristic apoptotic changes were observed such as chromatin condensation and membrane blebbing. (Figure 2)(15, 16).

Discussion

IC50 values

Based on the IC50 values, a higher acute toxicity can be observed for the generic drugs, but when it comes to a longer exposure to the cytotoxic (48 hours), the original drug appears to be more efficient *in vitro*.

Also, a difference regarding the DLD-1 and HT-29 response to therapy can be observed. It appears that the HT-29 line is more resistant to oxaliplatin than the DLD-1. The IC50 values are higher in HT-29 after 48 hours of exposure to ox-

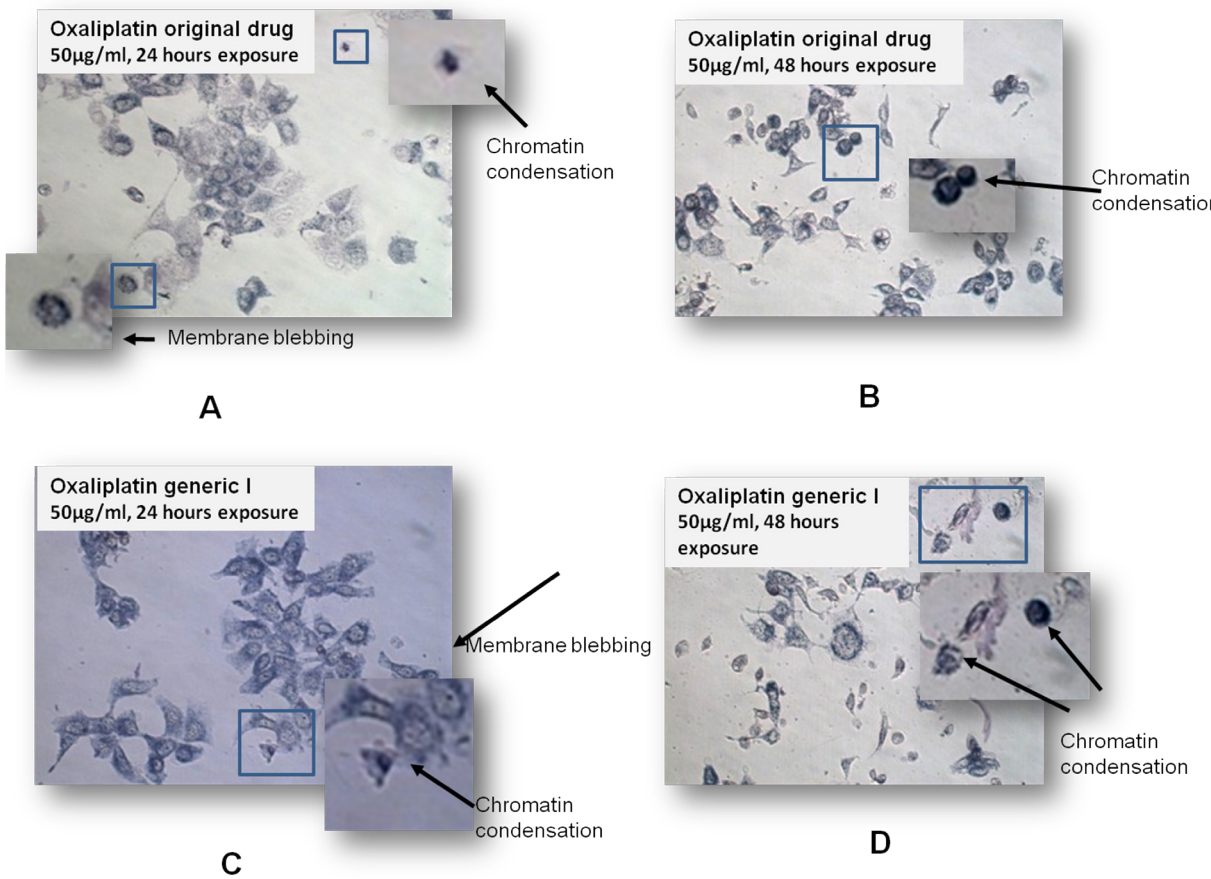


Figure 2. Morphological changes of DLD-1 cells under L-OHP original and generic I drugs

Table IV. DLD-1 cells under L-OHP exposure for 24 or 48 hours.

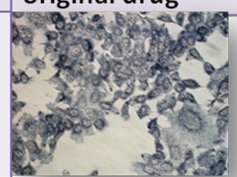
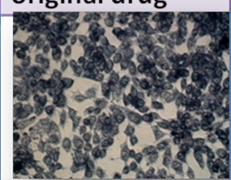
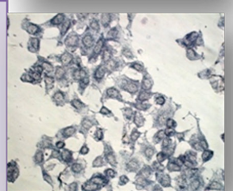
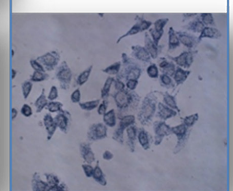
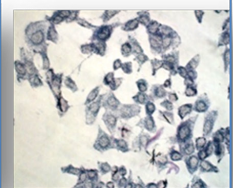
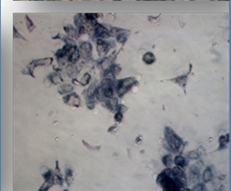
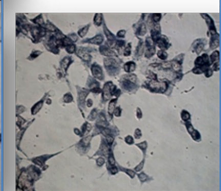
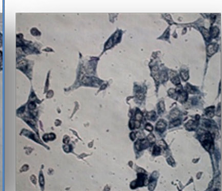
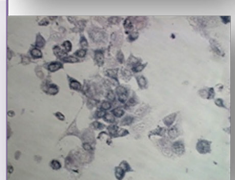
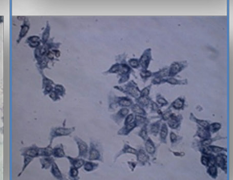
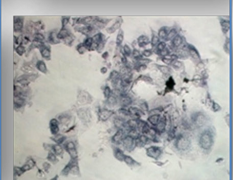
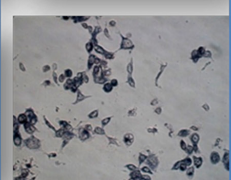
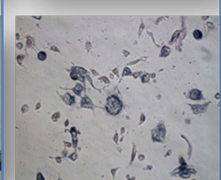
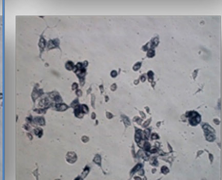
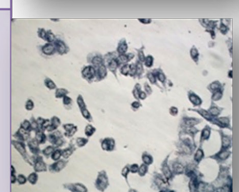
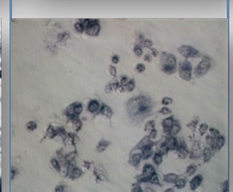
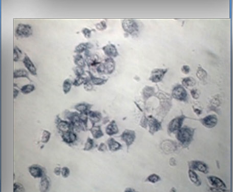
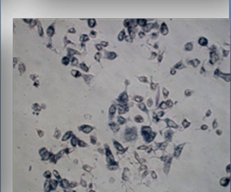
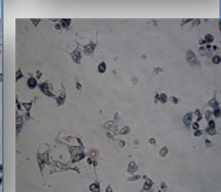
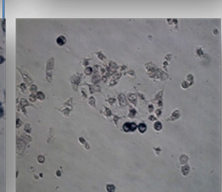
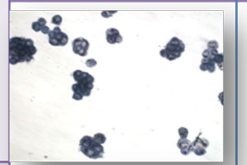
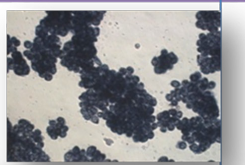
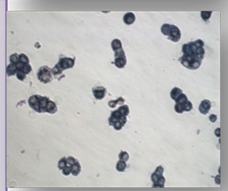
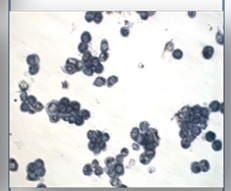
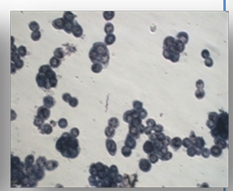
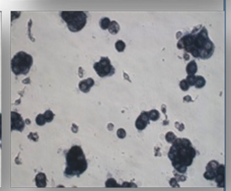
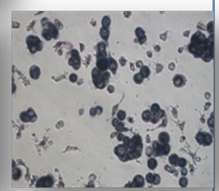
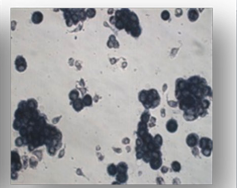
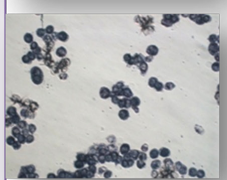
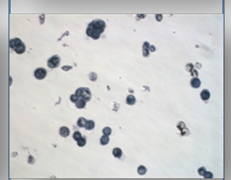
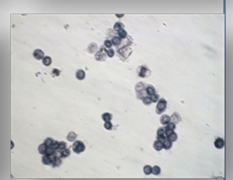
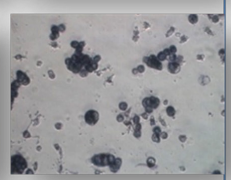
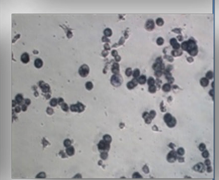
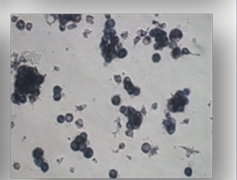
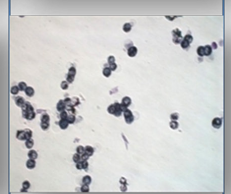
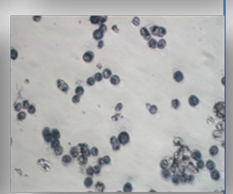
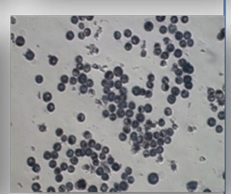
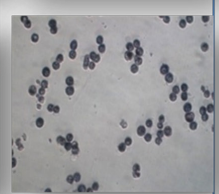
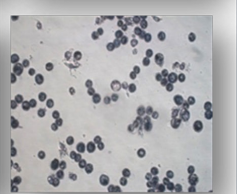
	24 hours exposure			48 hours exposure		
Concentration (µg/mL)	Oxaliplatin original drug	Oxaliplatin generic I	Oxaliplatin generic II	Oxaliplatin original drug	Oxaliplatin generic I	Oxaliplatin generic II
0						
25						
50						
100						

Table V. HT-29 cells under L-OHP exposure for 24 or 48 hours.

	HT-29 24 hours exposure			HT-29 48hours exposure		
Concentration (μg/mL)	Oxaliplatin original drug	Oxaliplatin generic I	Oxaliplatin generic II	Oxaliplatin original drug	Oxaliplatin generic I	Oxaliplatin generic II
0						
25						
50						
100						

aliplatin and a slower disorganization of the cell structure can be observed, compared to DLD-1 cells (Table V). This difference was noted by other researchers. A study by Chang et al. showed that HT-29 cells were more resistant to curcumin treatment than DLD-1 cells and a study by Liou et al. showed that HT-29 cells were more resistant to arachidonic acid and H2O2-induced apoptosis than DLD-1 cells (17, 18). Another explanation regarding the resistance of HT-29 cell line to L-OHP could be the number of CD133 positive cells, which is higher in the HT-29 cell line comparative with the DLD-1 cell line. CD133 is a marker for cancer stem cells, which are known to be responsible for the resistance to therapy, metastasis and relapse of the disease. A study by Sahlberg et al. showed that HT-29 cells had a higher expression of CD133 positive cells than the DLD-1 cells (19).This fact could influence dramatically the outcome of therapy in patients, since the treatment of colorectal cancer is not yet targeted on the tumor characteristics. Due to the fact that cancer is a heterogeneous group of cells, differences in patients' response could be the result of different chemosensitivity to therapy.

In our study, the efficiency of carboplatin generic drugs and the reference molecule from Santa Cruz Biotechnology seems to be alike, with no statistically significant differences. Regarding the mechanism of action, carboplatin induces apoptosis due to the formation of DNA adducts and other mechanisms (4). Our findings showed a higher toxicity for the L-OHP generic drugs, which could lead to the presumption that these drugs could have a higher toxicity *in vivo* towards the healthy cells, thus leading to an increased number of side effects. A research by Rasul et al. has shown that patients treated with generic oxaliplatin showed more side effects than the original drug-treated group (20). The differences between the original and generic drugs could also come, as a study of Gallelli et al. has shown, from their excipients - a change in the excipients list may be a cause for the differences in response to therapy and also the occurrence of allergic reactions (7).

Morphology discussion

Apoptosis is a genetically regulated form of cell death. It has a very important role, including the recognition of fetal abnormalities during intrauterine life, aging and many diseases (21). Platinum derivatives manifest their antitumor activity by inducing the apoptosis via different pathways (22). Once the apoptosis is installed, the morphology of the cell changes with the appearance of a blebbing membrane, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies (15, 23).

DLD-1 cells are epithelial-like carcinoma cells that have an irregular shape; they grow side by side, connected as observed on the control images (cells that have not been treated). As the concentration of L-OHP increases, membrane blebbing and decreased number of viable cells (decoloration of the cells) can be observed (Table IV).

HT-29 cells have a round shape, growing in clusters, forming large spheres as can be observed in the control images (cells that have not been treated). We can observe a disorganization of the spheres as the concentration of L-OHP increases, and at high concentrations the cells are shown separately. Also, a decrease in the number of viable cells (decoloration of the cells) was observed. This highlights the increased degree of damage produced by L-OHP (Table V).

Authorization procedure in Romania for generic drugs.

In Romania, the law that regulates the status of drugs (Law 95/2006, Chapter XVII) defines a generic drug as "a drug that has the same qualitative and quantitative composition in terms of active substances and the same pharmaceutical

form as the reference medicinal product, and whose bioequivalence with the reference medicinal product has been demonstrated through appropriate bioavailability studies". It is stipulated that if the manufacturer can prove that the drug is a generic of an original drug which is or was authorized in Romania for at least 8 years, in another state of the European Union or by a centralized procedure in the European Union, the manufacturer of the generic drug is not obliged to attach the results of the pre-clinic and clinical tests. The tests can be replaced by a review of the literature if it's considered that there are enough studies regarding the original drug. Also, the bioequivalence is tested on healthy volunteers and the absorption, distribution, metabolizing and excretion of the drug are investigated, not the therapeutic effect. All these aspects could lead to a poor verification of the efficacy of generic drugs. Therefore a generic drug may be a bioequivalent but not the therapeutic equivalent.

Final remarks

Our study`s findings showed a lower toxicity for acute exposure for the original drug, but a better activity when the time of exposure increased. Although other studies reported differences between generic and branded drugs in terms of hypersensitivity reactions, adverse effects and efficacy, we cannot extrapolate our findings to the patients. Further studies on patients are needed for a better evaluation of the efficacy of original vs. generic drugs. The question regarding the use of generic drugs remains unclear. If the manufacturers prove before the authorization that their products are equivalent to the original drugs, why are there differences? Aren't they the therapeutic equivalent of the branded drugs?

Regarding our study limitations, we can identify the lack of clinical data available before the use of generic drugs, the limited number of cell lines for testing and lack of the original carboplatin drug.

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Abreviations

IC50 - the half maximal inhibitory concentration
L-OHP – oxaliplatin
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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