MOLECULAR MECHANISMS OF ACTION AND PREDICTION OF RESPONSE TO OXALIPLATIN IN COLORECTAL CANCER CELLS

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Summary
In our study we investigated the contribution of oxaliplatin to mediated toxicity and transcription profile of genes involves in cancer in order to understand the mechanisms involved in tumor cell apoptosis and cancer disease progression. As colorectal cancer model we used the Colo320 cell line. We evaluated the oxaliplatin induced toxicity of a single dose of different concentrations at 24, 48 and 72 hours, and also multiple doses at different times, in order to establish the IC50 concentration. p53, NF2B and PDGF gene expression levels were evaluated after treatment with a concentration close to the IC50 using the RT-PCR technique. The purpose of the study is to observe the changes in pharmacokinetic parameters after administration of a single dose in order to establish IC50, using the MTT cell proliferation assay on the line Colo320. We compared the parameters of cell proliferation observed after administration of a single dose and multiple doses, and tried to establish an optimal time and frequency of oxaliplatin treatment that induces the minimal cytotoxic effect. After evaluating the modulation of gene expression after treatment with oxaliplatin, we obtained a high gene expression of two major pro-apoptotic genes, p53 and NF2B (nuclear factor-2B) and the inhibition of a pro-angiogenic factor, the platelet-derived growth factor (PDGF). In conclusion, the frequency of drug administration is important, and may establish the minimum dose required and the frequency of administration, with maximum biological results and without adverse effect.

Key Words: oxaliplatin, colorectal cancer, p53, NF2B, PDGF.

Introduction
Colorectal cancer is the second most common cause of cancer (Klijn et al, 2003) and the third cancer related death worldwide (Stewart et al, 2003). Most cases of colorectal cancer occur due to mutations in proto-oncogenes, suppressor genes and genes involved in the maintenance and repair of DNA. But so far, all the aspects regarding the genes and causes involved in the process of carcinogenesis of the gastrointestinal tract are still not well established (Chirila et al, 2011).

Several preclinical in vitro and in vivo tumor models are being used for the screening of potential anticancer agents, including Colo320 cancer cell line for colorectal cancer (Temmink et al, 2007)

The chemotherapeutic regime for colorectal cancer has improved substantially during the past decade with the introduction of new and more effective drugs and advances in the understanding of the disease's molecular biology (Adamsa et al, 2011). In patients who develop metastatic colorectal cancer oxaliplatin is the most extensively used treatment (Midgley et al, 2009). It is an alkylating agent that inhibits DNA synthesis and replication by generating DNA damage. Apoptosis of cancer cells can be caused by formation of DNA lesions, arrest of DNA
synthesis, inhibition of RNA synthesis, and triggering of immunologic reactions (Suh et al, 2005; Alcindor and Beauger, 2011).

The effect of chemotherapeutic drugs on malignant cells is obtained primarily through the induction of apoptosis. Among the numerous signaling pathways that regulate apoptosis, the most important one in the malignization process is the one involving the p53 protein and its activities. The p53 gene is mutated in about 50% of the cancers (Klein et al, 2009; Hollstein et al, 1991), while in the rest of 50% its function is inhibited (Azmi et al, 2011). The p53 protein in cancer cells is involved in cell cycle control, drug resistance and apoptosis. But there is increasing evidence that mutations in p53 play an important role in regulating the survival and antigenic responses of tumors, being able to sustain cell proliferation for a longer period of (Royds et al, 1998).

Genetic mutations of p53 are found in > 60% of gastrointestinal cancers, and they were proven to have a close correlation with the onset and prognosis of malignant tumors, combined with the chemosensitivity and response to treatment (Jones et al, 2008; Baker et al, 1990; Hussain et al, 2000). p53 acts as a central mediator of the cellular response to stressful stimuli which play an important role in chemosensitivity and radiosensitivity of tumor cells. These events take place only in wild-type and mutant expressed p53 proteins (Weekes et al, 2009; de Bruijn et al, 2010).

Oxaliplatin is an anticancer agent that acts by formation of Platinum-DNA (Pt-DNA) adducts resulting in DNA-strand breaks, and is used for the treatment of colorectal cancer. Oxaliplatin has distinct biochemical, pharmacological and cytotoxic properties compared to the related platinum compounds cisplatin and carboplatin, and shows no crossresistance (Temmink et al, 2007).

In this in vitro study we aimed to analyze the effect of single and multiple doses of oxaliplatin on human colorectal cancer cell line. We also evaluated the capacity of oxaliplatin to modulate the expression the genes implicated in different pathways leading to apoptosis.

Material and methods

Cell culture: In our study, we used the human colorectal cancer cell line Colo320. It is colon adenocarcinoma, isolated from a sigmoid colon of a 55 year old Caucasian female, with a karyotype model of 53 chromosomes. The cell line was cultured using RPMI 1640 culture medium, completed with: 10% fetal bovine serum (FBS-Sigma, Germany), 2 mM glutamine (Sigma, Germany), 100 international units (IU/ml penicillin and 100 mg/ml streptomycin (Sigma, Germany). Cells were incubated at 37°C in humidified atmosphere with 5% CO2.

Cell treatment: Cells were treated with different concentrations of oxaliplatin (0.1, 1, 10, 10, 50, 100 µg/ml) and evaluated after 24, 48 and 72 h for cell viability. We also evaluated the effect of multiple doses of oxaliplatin; cells were first plated and then treated at T = 24 hours with: a) 0.5 µg/ml and 5 µg/ml followed by assessment of cell viability at 48 and 72 hours, and b) 0.33 µg/ml and 3.33 µg/ml followed by assessment of cell viability at 48 hours. Evaluation of gene expression was done at 24 hours after treatment with 10 µg/ml oxaliplatin.

Cell viability assay was performed in 96-well plates using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterrazolium bromide (MTT) assay. The MTT assay is a colorimetric method based on the reduction of tetrazolium salt, yellow, to purple formazan crystals. After the treatment of the cells as mentioned above at 24, 48 and 72 h, the results were evaluated. The medium was first removed and the cells were washed once with 200 µl PBS; then 100 µl of MTT were added to each well. After 2 h of incubation in cell culture conditions, the MTT was removed and 100 µl of dimethyl sulfoxide (DMSO) was added to each well. Absorbance was
measured at 490 nm using a Biotek Synergy HT Microplate Plate Reader.

**Quantitative RT-PCR analysis:** Total RNA was isolated using TriReagent (Sigma, Bucharest, Romania) according to the manufacturer’s protocol. Quantitative and qualitative assessment of the RNA was assessed using NanoDrop ND-1000 and Agilent 2100 Bioanalyzer technology. The reverse transcription was performed by Gradient Palm-Cycler™ RT-PCR device with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Germany). For quantitative mRNA expression analysis, real-time PCR was carried out with 150 ng of cDNA using TaqMan® Master kit (Roche Diagnostics, Germany) on LightCycler®2.0 Real-Time PCR System. PCR analysis was performed according to the manufacturer’s recommended protocol. The primers sequences (TIB MOLBIOL, Germany) used are mentioned in the Table 1.

**Data Analysis:** The fold change of the genes expression was calculated using $\Delta \Delta C_t$ method.

<table>
<thead>
<tr>
<th>Genes studied</th>
<th>Primers left</th>
<th>Primers right</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-actine</td>
<td>CCAACCgCgAAgATgA</td>
<td>CCgAggCgTACAgggATAg</td>
</tr>
<tr>
<td>PDGF</td>
<td>TgATCTCCACgCCTgCT</td>
<td>TgCATGTTCAggTCCAACCTCG</td>
</tr>
<tr>
<td>NFkB</td>
<td>TCCAggTCATAAggCTCA</td>
<td>ggCTggCAgCTCTTCTCA</td>
</tr>
<tr>
<td>p53</td>
<td>AggCCTTggAACTCAAggAT</td>
<td>CCCTTTTTggACTTCAggTg</td>
</tr>
</tbody>
</table>

**Results**

The effects of oxaliplatin on Colo320 tumor cells involve changes in cellular bioactivity. We determined cell viability by quantifying the formazan produced by the mitochondria of the bioactive cells. MTT studies show that cellular activity depends on time and concentration of oxaliplatin. Cell viability results are expressed as log concentration of oxaliplatin (µg/ml) compared to control (as means (n = 4) ± standard error) as shown in Figure 1. At low concentration of the cytostatic, cells exhibit minimal cytotoxicity at 24, 48 and 72 h. The reduction of cellular viability is associated with the increase in oxaliplatin concentration in a time dependent manner.

**Figure 1.** MTT results of cell proliferation after treatment with different doses of oxaliplatin at 24, 48, 72 hours.
Based on the data presented in Figure 1, we also determined IC50 (inhibitory concentration at 50%) for the oxaliplatin activity on the colorectal tumor cells at 24, 48 and 72 hours. The evaluation is based on the statistical parameters obtained with the free trial treatment program GraphPadPrism5. Results are presented in Table 2.

**Table 2. IC50 values after 24, 48 and 72h of treatment with oxaliplatin**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Time (hours)</th>
<th>IC50 (µg/ml)</th>
<th>Slope</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colo320</td>
<td>24</td>
<td>14.45</td>
<td>3.101</td>
<td>0.9511</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>7.858</td>
<td>0.6428</td>
<td>0.9698</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>8.356</td>
<td>2.624</td>
<td>0.9328</td>
</tr>
</tbody>
</table>

Evaluation of the antiproliferative effect of multiple dose administration at 24, 48 and 72 hours is shown in Figure 2. After administering a double dose of 5µg/ml at T = 0 and after 8 hours, we observed no significant change in the antiproliferative effect of the drug, in comparison with the administration of a single dose of 5 µg/ml at T = 0. In the figure we represented a double dose of 5 µg/ml administrated at T = 0 and at 24 hours followed by assessment of cell viability at 48 hours. The administration of a triple dose of 3.33 µg/ml oxaliplatin at T= 0, 24 and 48 hours followed by assessment of cell viability at 72 hours revealed a reduction in cell antiproliferative effect in a more effective way compared to the first treatment arrangement and the administration of a single dose of 10µg/ml oxaliplatin.

**Figure 2. MTT results of cell proliferation after treatment with multiple doses of oxaliplatin at 24, 48, 72 hours.**

**Evaluation of gene expression with the technique of real-time PCR.** The relative mRNA expression of the p53, NFκB and PDGF genes in the Cole320 cell line treated with 10 µg/ml oxaliplatin after 24 hours are presented in Table 2. We used as a reporter the housekeeping gene β-Actine. As shown in Figure 3, the relevant gene transcript expression of the pro-apoptotic genes has significantly higher levels compared to the expression of the PDGF gene that in involved in cell survival and in angiogenic pathways.
Table 3. Relative gene expression of PDGF, NFκB, p53 in cells treated with 10 µg/ml oxaliplatin.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Oxaliplatin (10 µg/ml)</th>
<th>Error (+), (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actine</td>
<td>1.000</td>
<td>0.433</td>
</tr>
<tr>
<td>PDGF</td>
<td>0.368</td>
<td>0.217</td>
</tr>
<tr>
<td>NFκB</td>
<td>1.662</td>
<td>0.552</td>
</tr>
<tr>
<td>p53</td>
<td>1.840</td>
<td>0.658</td>
</tr>
</tbody>
</table>

Figure 3. Levels of gene expression after treatment with oxaliplatin in Colo320 cells.

Discussion
Loss of viability and induction of apoptotic cell death are two major mechanisms by which chemotherapeutic agents kill cancer cells (Banerjee et al, 2009). Apoptosis is a cell death process that is triggered by different factors and is regulated by polymolecular signals. In the extracellular death receptor pathway, external cytotoxic factors stimulate several genes, inducing remarkable increases in the expression of p53 (Nemoto et al, 2009) and NFκB, that are involved in programmed cell death, suggesting that oxaliplatin induces apoptosis (Yang et al, 2011; Kim et al, 2009; Nemoto et al, 2009). These data support our RT-PCR results where the expression of p53 and NFκB were statistically upregulated (p<0.05) after treatment with oxaliplatin.

In this study, we provide evidence that p53 is involved and facilitates chemotherapy-induced apoptosis of Colo 320 tumor cells by cooperating with NFκB and inhibiting genes implicated in cell survival and angiogenesis like PDGF.

Taking in account its side effects that increase with time and dose leaving deep marks on the patients’ health, novel therapeutic strategies of existing cytostatics have nowadays a vast interest in the experimental and clinical medicine. Advances in understanding the action of oxaliplatin on colorectal cancer cells are crucial for improving the design of administering the treatment in order to reduce toxic side effects and to aid in the understanding of their mechanisms of action.

NFκB is a transcription factor often activated in cancer cells in response to
chemotherapeutic agents (du Plessis-Stomana et al., 2011). In 1994, Wu and Lozano reported for the first time the implication of NFκB in regulating the p53 gene expression (Wu and Lozano, 1994). The p53 expression in the oxaliplatine treated cells results in antitumor effects that include inhibition of cell cycle progression and induction of apoptosis through the modulation of the expression of apoptosis- and cell-cycle-related genes, and the sensitization of tumor cells to chemotherapy. Chen et al., in a clinical application study, demonstrated that increase in p53 gene expression strengthens tumor cell sensitivity to chemotherapy and reduces the side effects of chemotherapy (Chen et al., 2011; Peng et al., 2005; Guan et al., 2005).

Pharmacogenomic studies have enabled us to establish the diagnosis and therapeutic approach, as well as new prognostic scores to see the effectiveness of colorectal cancer treatment. The frequency of drug administration is important; it is based on half-life, and may establish the minimum dose required and the frequency of administration, with maximum biological effect and without adverse effect. Colorectal cell line Colo320 was an appropriate model for evaluating anti-tumour agent of oxaliplatin, by measuring cell proliferation and modulation of gene expression.

**Conclusion**

In the present study, we demonstrate that oxaliplatin inhibits colorectal cancer cell growth in a dose and time dependent manner, sensitizing these cells to the chemotherapeutic agent in the case of multiple dose administration strategy, leading to a significant decrease in the number of viable cells. The underlying mechanisms of treatment effects involve the induction of apoptosis, which is achieved via upregulation of p53 and NFκB, as well as the downregulation of PDGF. Our results suggest that a new strategy of dosing oxaliplatin in correlation with a defined multiple administration scheme per interval may increase its effects and also increase patients’ response to treatment with a high impact on survival and the quality of life of cancer patients.

**References**


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