Drug modulation of soluble biomarkers and oxidative stress in LLC-WRC 256 cell line: role in cell proliferation and angiogenesis

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Abstract
During the last years, studies were focused to understanding cancer mechanisms at the molecular level, identifying new biomarkers that interfere at different stages of tumour initiation and progression, but also in metastasis. Angiogenesis is a complex, multistage process that includes the interaction between endothelial cells, neoplastic cells and extracellular matrix components. The molecular basis of this mechanism may be the increase of the production of angiogenic factors or may be the loss of angiogenesis inhibitors. Angiogenesis is regulated by growth factors, pro-angiogenic cytokines, angiogenesis modulators and neovascularization inhibitors. The regulation of tumour angiogenesis is achieved by the balance between activating factors and angiogenesis inhibitors. Aberrant glycosylation is often considered a "cancer fingerprint", playing an important role in signaling cancer cells, dissociating and invasion of tumor cells, cell-matrix interactions, and angiogenesis, metastasis and immune modulation. Resistance to treatment, escape from host immune system control, tumour invasion and angiogenesis, metastasis are closely related to aberrant sialilation of glycoproteins and glycolipids. Oxidative stress can cause angiogenesis in breast carcinoma because oxygen radicals increase the production of angiogenic factors (IL-8 and VEGF), promote secretion of MMP-1, that aids the growth of vessels in the tumour microenvironment. Therefore, our study focused on modulation of oxidative stress and biomarkers release in cell culture supernatants of LLC-WRC 256 rat carcinosarcoma cell line, molecules with important functions in tumour proliferation and angiogenesis. In order to find alternative therapeutic approaches based on lower concentrations of anti-cancer drugs (EPI, EDX), we investigated the regulatory roles of natural compounds (Rsv) added to cancer drugs, in order to diminish the undesirable side-effects, obtain a stronger anti-tumour responses and decrease the drug-resistance.

Keywords: LLC-WRC 256 cell line, cathepsin D, sialic acid, ROS, angiogenic factors

1. Introduction
Cancer is the second leading cause of death worldwide, following cardiovascular disease. Therefore, in recent years, many studies have been devoted to understanding this disease at the molecular level, identifying new biomarkers that intervene at different stages of tumour initiation and progression, but also in metastasis. The initial stages of tumour development are associated with a fibrinogenic response and the development of a hypoxic environment that promotes the survival and proliferation of cancer stem cells. Part of the survival strategy of these cells can be manifested by changes in cellular metabolism. After the onset of tumours, growth and metastasis can be sustained by overproduction of hormones (in hormone dependent cancers), by promoting angiogenesis, triggering autophagy (WEBER & al. [1]; JEMAL & al. [2]).
Angiogenesis is a complex, multistage process that includes the interaction between endothelial cells, neoplastic cells and extracellular matrix components (CARMELEIT & al. [3]; PETROVIC & al. [4]). The molecular basis of this mechanism may be to increase the production of angiogenic factors or may be the loss of angiogenesis inhibitors. Switching to an angiogenic phenotype is regulated by a change in balance between positive and negative regulators (YADAV & al. [5]). Under normal conditions, the balance leans towards the anti-angiogenic phenotype. Hypoxia is the primary stimulus of the angiogenic "switch" (TAMEEMI & al. [6]; HASHIMOTO & al. [7]). Angiogenesis is regulated by growth factors, proangiogenic cytokines, angiogenesis modulators and neovascularization inhibitors (HUANG & al. [8]). The regulation of tumour angiogenesis is achieved by the balance between activating factors and angiogenesis inhibitors. The main pro-angiogenic factors are VEGF, bFGF, PDGF, EGF, TGF-β, TNF-α. The most well-known endogenous anti-angiogenic factors are interferons, interleukins, tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, TIMP-3), angiostatin and endostatin (YADAV & al. [5]).

Aberrant glycosylation is often considered a "cancer fingerprint", playing a fundamental role in tumour development and progression. Thus, glycans play an important role in signaling cancer cells, dissociating and invasion of tumour cells, cell-matrix interactions, and angiogenesis, metastasis and immune modulation (LAZESCU & al. [9]); XIAOLOU & al. [10]). Resistance to treatment, escape from host immune system control, tumour invasion and angiogenesis, metastasis are closely related to aberrant sialilation of glycoproteins and glycolipids (TEOH & al. [11]; KOHNZ &al. [12]; CUI & al. [13]). Basal membrane disruption is a feature of malignancy. Matrix metalloproteinases (MMPs) are a family of endopeptidases, enzymes that play an important role in invasion and metastasis by proteolytic degradation of the extracellular matrix in migration and angiogenesis by the destruction of cell-cell adhesions and matrix-cell adhesions. Elevated MMP levels correlate with the invasion, metastasis and prognosis reserved in many cancers, and animal models demonstrate the causal role of MMP activity in cancer progression. Another biomarker studied for its role in stimulating growth and proliferation of cancer cells as well as in the formation of metastases is another proteinase, cathepsin D, a prognostic factor in breast cancer and endometrium (DIAN & al. [14]; PRANIOL & al. [15]; PRANIOL & al. [16]).

Oxidative stress was first described in 1985 and refers to the imbalance between free radicals that form the pro-oxidant system and endogenous antioxidants, either due to the exaggerated action of the oxidants or because of the reduction in endogenous fighting ability. The role of reactive oxygen species (ROS) in cancer has been extensively investigated in recent years, as the accumulation of reactive species in cancer cells plays an important role in the initiation and evolution of this disease (GRUIA & al. [17]; GRIGORESCU & al. [18]). ROS regulates each stage of tumour development, including transformation, survival, proliferation, invasion, metastasis and angiogenesis (REUTER & al. [19]; PANIERI [20]).

Oxidative stress can cause angiogenesis in breast carcinoma because oxygen radicals increase the production of angiogenic factors (IL-8 and VEGF), promote secretion of MMP-1, a collagenase that aids the growth of vessels in the tumour microenvironment and activates MMP-2, possibly by reacting oxygen radicals with thiol groups in MMP-2. The contradictory roles of ROS and oxidative stress in breast cancer, either induction and tumour progression or the prevention of tumourigenesis by other mechanisms, have led various research groups to investigate potential oxidative stress modulators useful in new anticancer strategies (GURER-ORHAN & al. [21]; GRIGORESCU & al. [18]).
Epirubicin (EPI) and Endoxan (EDX) are several therapeutic agents commonly used in breast cancer treatment. The number of drugs that have been shown to induce resistance in cancer cell killing are rapidly increasing, possibly through the modulation of survival cell components, such as proliferative or anti-apoptotic proteins. Thus, recent approaches in breast cancer treatment are focused on finding better combined multi-drug chemotherapy, the reason being the potential additive or synergistic tumour cytotoxicity produced. Resveratrol (trans-3,4',5-trihydroxystilbene), a naturally occurring polyphenol phytoalexin, is found in red wine, chocolate, peanuts, berries, and black grapes, exhibited anticancer properties by inhibiting cell proliferation, inducing apoptosis, scavenging the free radicals, decreasing angiogenesis, and causing cell cycle arrest in several cancer cell lines.

Therefore, our study focused on modulation of oxidative stress and biomarkers release in cell culture supernatants of LLC-WRC 256 rat carcinosarcoma cell line, molecules with important functions in tumour proliferation and angiogenesis. In order to find alternative therapeutic approaches based on using lower concentrations of anti-cancer drugs (EPI, EDX), we investigated the regulatory roles of natural compounds (RSV) added to cancer drugs, in order to diminish the undesirable side-effects, obtain a stronger anti-tumour response and decrease the drug-resistance.

2. Materials and methods

2.1. Reagents: Epirubicin (EPI), endoxan (EDX), dimethyl sulfoxide (DMSO), resveratrol (RSV) were purchased from Sigma Aldrich (St. Louis, Mo, US). High concentrated stock solutions were prepared as recommended, in ultrapure sterile water (EPI) or DMSO. Working drug concentrations were prepared from the stocks in complete culture medium before each experiment.

2.2. Cell cultures and treatments: LLC-WRC 256 cell line, derived from Walker 256 rat carcinosarcoma, was purchased from European Collection of Authenticated Cell Cultures (ECACC). Adherent cells were routinely maintained in culture in TC199 medium added by 2mM L-glutamine and 10% fetal bovine serum (Sigma Aldrich) and incubated at 37°C/ 5% CO₂ humidified atmosphere. After 24h of culture, when cells achieved 50-70% confluency, cultures were treated with different concentrations of anti-cancer drugs (EPI, EDX) and/or anti-oxidant bioactive compounds (RSV) for different periods of time. As variants of the experiment, RSV was added before or after the drug treatments. Cell culture supernatants (SN) were harvested, centrifuged 30 min/2000xg, then frozen at -80°C for soluble biomarkers or ROS evaluation.

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

2.4. Viability RTCA assay: To analyze the proliferation profiles of treated murine cells, we continuously monitored cell growth by using real time cells analysis (RTCA) assay and xCELLigence system. Real-time impedance data obtained were used to generate compound-
specific profiles that are dependent on the biological mechanisms of action of each compound. LLC-WRC 256 carcinosarcoma cells were seeded in E-plates 16 in complete culture medium (15x10^3 cells/well/100ul), plates put in xCELLigence DP device and cultured at 37°C/5% CO2 humified atmosphere. After 24h, cells were added by single or combined treatments of EPI, EDX and/or natural compounds (RSV), and growth curves were registered in real time on computer by using RTCA 2.1.2. Software (Figure 1). Acquisition of real-time monitoring of cytotoxicity allowed for calculation of time-dependent IC50 values (KNOP&al. [30]).

2.5 Evaluation of soluble biomarkers: Briefly, cathepsin D concentration used is similar to that described by Capony (CAPONY& al. [22]). To 100 µl of sodium formiate buffer (1M, pH=3,5) was added 100 µl of 4% hemoglobin and 150 µl SN or pure cathepsin D solution. Evaluation of soluble sialic acid: was performed with a colorimetric method described by Kattermann&Kriegel (KATTERMANN&KRIEGEL, [23]). Dosage of free or bound sialic acid is based on periodic oxidation, resulting in β-formylpyruvic acid formation, it reacts with two thiobarbituric acid molecules and forms a pink chromophore with maximum absorbance at 549 nm. VEGF, MMP-2 and TIMP-1 levels in SN of treated cell cultures were evaluated by ELISA, following the manufacturer’s protocols (Rat VEGF ELISA kit; Rat MMP 2 ELISA kit; Rat TIMP 1 ELISA kit, AbFrontier, Seul, Korea).

2.6 ROS evaluation: it was performed in cell culture supernatants. The lipid peroxidation index (PX) was determined by the malondialdehyde assay (KANDAR & al. [24]; GRUIA & al. [20]), while total thiol groups (TSH) were assayed as described by Schosinski, using Ellman reagent (ELMANN & al. [25]; JANERO & al. [26]; GRUIA & al. [20]). Total antioxidants (AO) were evaluated using the method of Benzie & Strain (BENZIE& STRAIN, [27]).

3. Results and discussion
3.1 Cytotoxicity of drugs by MTS
Treatment of Walker 256 carcinosarcoma cell line was performed with increasing concentrations of chemotherapeutic agents or biocompounds.

![Figure 1. Modulation of LLC-WRC 256 cell proliferation by drugs](image_url)

Thus, for EPI, concentrations ranging from 0.25 to 100 µM were used, while for EDX concentrations were used in the range of 10-1000 µM. The same concentration range used for
EPI was used for doxorubicin (DOX) and 5-fluorouracil (5-FU) (positive controls) (HOTNOG & al. [28]; MIHAILA & al. [29]). Treatments for 72h with cytostatics resulted in increased cell lysis rates, and implicitly decreased cell viability. EPI treatments induced a decrease of cell viability from 98.46% for the concentration of 1 μM to 66.22% for 100 μM treatments. Endoxan efficacy has been shown to be reduced at low concentrations, only the concentration of 100 μM being capable of inducing a cell lysis higher than 15%.

3.2. Modulation of cell proliferation by drugs and natural compounds in drug-treated LLC-WRC 256 cell line

We have previously demonstrated the cytotoxic role of DOX on drug treated MCF-7 cancer cells (MIHAILA & al. [29]). In the present study we have further investigated the effect of adding bioactive compounds like Rsv to cancer drugs, using a murine in vitro model, based on LLC-WRC 256 cell line. To analyze the proliferation profiles of treated murine cells, we continuously monitored cell growth by using real time cells analysis (RTCA) assay and xCELLigence system.

<table>
<thead>
<tr>
<th>LLC-WRC 256 cells</th>
<th>EPI</th>
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<tbody>
<tr>
<td>NT ---- 100 μM --- 25 μM --- 10 μM --- 5 μM --- 1 μM ---</td>
<td></td>
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<tr>
<td>12 h ---- 24 h ---- 36 h ---- 48 h ---- 60 h ---- 84 h ----</td>
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<tr>
<th>EDX</th>
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<tr>
<td>NT ---- 200 μM --- 100 μM --- 10 μM --- 5 μM --- 1 μM ---</td>
</tr>
<tr>
<td>12 h ---- 24 h ---- 36 h ---- 48 h ---- 60 h ---- 72 h ----</td>
</tr>
</tbody>
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<tr>
<th>RSV</th>
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<tr>
<td>NT ---- 200 μM --- 100 μM --- 25 μM --- 12.5 μM ---</td>
</tr>
<tr>
<td>24 h ---- 36 h ---- 48 h ---- 60 h ---- 72 h ---- 84 h ----</td>
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</tbody>
</table>

Figure 2. Real time cell analysis of the modulation of LLC-WRC 256 cell line proliferation induced by drugs or RSV

Real-time impedance data obtained were used to generate compound-specific profiles that are dependent on the biological mechanisms of action of each compound. Using xCELLigence System and LoVo cancer cell line we obtained continuous compound-dependent cell
impedance profiles as our \textit{in vitro} models (Figure 2). RTCA analysis of LLC-WRC 256 cell line proliferation curves treated with concentrations between 1-100 μM of EPI demonstrated a pronounced cytotoxicity of the cells. All cell proliferation curves, normalized vs. control (the untreated cell curve), indicate that prolongation of cell culture time with EPI causes the decrease of viability for almost all concentrations used in the assay: 5, 10, 25 or 100, excepting the 1 μM concentration, whose profile is above the untreated cell curve. RTCA analysis confirms the results obtained by colorimetric method with MTS. Thus, in subsequent biomarker modulation experiments, LLC-WRC 256 cells will be treated with 10 μM EPI and 200 μM EDX. Cellular growth curve analysis indicates concentrations of the bioactive compounds between 60-100 μM, in order to reach an IC50. Thus, in subsequent experiments, fixed concentrations of RSV 50 μM will be used. Screening for the proper combination of compounds with cytotoxic or cytostatic potential in inhibiting the growth of adherent tumour cells made possible the choice of proper combinations to be further used in end-point assays such as evaluation of apoptosis or evaluation of expression.

3.3. Modulation of the release of cathepsin D and sialic acid in cell culture supernatants

Supernatants, prepared according to the established protocol, were tested for the assessment of biomarker levels by colorimetric techniques. After spectrophotometric reading and interpretation of the results, the analysis of the data obtained in cathepsin D modulation in the LLC-WRC 256 line demonstrated that the combined cytotoxic treatment (EPI + EDX), prolonged at 72h, results in decreased proteinase levels.

Analysis of the data obtained when LLC-WRC 256 line was treated with cancer drugs and/or bioactive compounds demonstrated that levels of sialic acid decreased in cell culture supernatants after the combined cytotoxic treatment (EPI + EDX), extended up to 48h, similar to , cathepsin D. Sequential cytostatic treatment with drugs, followed by RSV, determines for both 48 and 72h a decrease in cathepsin D, as well as AS, comparing with RSV treatment added prior to cytostatics.
3.4. Modulation of the release of VEGF, MMP-2 and TIMP-1 in cell culture supernatants

Analysis of the data obtained in the VEGF release modulation by anti-cancer drugs and biocompounds in LLC-WRC 256 cell culture supernatants demonstrated that combined cytotoxic therapy (EPI + EDX), prolonged to 48-72h, results in decreases in VEGF levels. In the case of RSV, the marked decrease in VEGF level occurs when the biocompound is administered prior to cytostatics with 24h. The increase in VEGF expression has been reported to correlate with the prognosis reserved for cancer patients. It has also been suggested that VEGF is a prognostic marker that causes a high risk of recurrence of the disease, for patients that have invaded nodules. Patients with high levels of VEGF in tumours are unlikely to benefit from conventional treatments. This may indicate that VEGF has a predictive value in the treatment of breast carcinoma. VEGF expression correlates with microvascular density, indicating direct involvement of VEGF in angiogenesis. (JOO & al. [31]). Matrix metalloproteinase-2 (MMP-2) is an enzyme with important functions in breast cancer invasion and metastasis, but it is not yet clear whether circulating MMP-2 levels can predict the risk of breast cancer. (ARONER & al. [32]). Other researchers have concluded that MMP-2 and MMP-9 are overexpressed in breast cancer and are closely related to lymph node metastasis and tumour staging. These proteinases could be used as reference indicators for the orientation of breast cancer treatment and prognostic estimation. (LI & al. [33]). Analysis of the data obtained in the case of MMP-2 release modulation in the LLC-WRC 256 supernatants treated with cytostatics and biochemicals has shown that 24h treatment with all three biochemicals studied leads to decreased levels of this proteinase, the same effect being significant in the treatment with cytostatics at 48h and 72h.

![Figure 4](image)

**Figure 4.** VEGF, MMP-2 and TIMP-1 release in culture supernatants of drug and/or RSV treated LLC-WRC 256 cell line

Modulation of TIMP-1 release in cytostatic and biochemical treated LLC-WRC 256 supernatants demonstrated that 48-72h RSV treatments led to decreased levels of this molecule, the same effect being generally observed in combined treatments of drugs and biocompounds, especially for 72h. Liu Z. et al. found that the RSV inhibitory effect on cell proliferation increases with increasing concentration and time of incubation. Resveratrol had an inhibitory effect on VEGF expression and significantly inhibited the proliferation of tumour cells (Li Y.&al. [33], Liu Z.&al [34]).
3.5. Modulation of ROS release by drug and bioactive compounds treatment

Based on the results obtained by treating cells cultured in the cytostatic specific environment dedicated to breast cancer treatment, an increase in EPI-induced lipid peroxidation was observed as a function of time.

Figure 5. Modulation of oxidative stress profile in culture supernatants of drug and/or RSV treated LLC-WRC 256 cell line

Endoxan does not induce specific oxidation, but the combination of the two compounds increases cytotoxicity to untreated normal cells. In contrast, treatment with anti-oxidant biocompounds causes a decrease of PX levels for all incubation periods of time (24, 48, 72h). Administration of antioxidants in culture after cytostatics does not significantly affect the destruction of lipids by peroxidation, but certainly these results are of real use for in vivo models and possibly for patients, since antioxidants administered after chemotherapy could diminish the associated side effects.

The data obtained in the same experimental model that aimed at the oxidative degradation of proteins are similar to PX. In the dynamics of the drug treatment, an oxidative degradation of proteins results in an increase in serum thiol levels depending on time, which determines the cytotoxic effects. Resveratrol has antioxidant effects, the most pronounced effect being observed for single treatment with biochemicals administered for 24h. Measurement of the total antioxidant level demonstrates the ability of RSV to diminish the constitutive levels of cellular AO.

Conclusions

Many anti-cancer drugs act during physiological pathways of tumour proliferation and angiogenesis, leading to tumour cell destruction. Numerous studies to date have shown that the efficacy of various oncostatic therapies is due to the stimulation of oxidative metabolism in tumour cells/ tissues by generating a cytotoxic pro-oxidant status responsible for inducing apoptosis or tumour necrosis, blocking cell proliferation. Using anti-cancer drugs combined with natural compounds might increase their effects, specifically in highly invasive cancer cells, while in nontumoural cells the natural compounds could reduce the cytotoxic side effects. Flavonoid antioxidants have a strong impact on the oxidative stress associated with the tumour, depending on the dose used.
Acknowledgments
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