REAL-TIME ANALYSIS OF QUERCETIN, RESVERATROL AND /OR DOXORUBICIN EFFECTS IN MCF-7 CELLS

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Abstract

Breast cancer represents a malignancy with high incidence and mortality throughout women, its etiology involving many genetic, immunological and biochemical factors. Adriamycin (ADR) is one of the most effective anti-cancer agents used to treat breast cancer, but chronic cardiotoxicity is a major limiting factor of its use. In order to choose the most effective ADR chemotherapeutic dose, and thereby diminish the side-effects, we used new technical assays to modulate the chemo-preventive or anticancer properties of ADR by adding dietary natural compounds.

Our study focused on the role of natural compounds such as resveratrol (RSV) or quercetin (QCT) in sensitization of MCF-7 human breast cancer cell line to ADR action. Real-time cell analysis (RTCA) by xCELLigence System was used to continuously monitor the cytotoxic effects of treatments on MCF-7 cells. RTCA allowed us to choose the proper concentrations for further end-point assays, as flow-cytometry techniques used for the evaluation of apoptotic events or progression through cell cycle phases of compound-treated MCF-7 cells. Data obtained showed additional effects of QCT or RSV to ADR treatments on the increase of early apoptosis events, and suggested alternative approaches to obtain a stronger anti-tumor response, diminish the side-effects when use lower concentrations of anticancer drugs.

Keywords: RTCA, xCELLigence, breast cancer, cytotoxicity, quercetin, resveratrol, doxorubicin, apoptosis, cell cycle.

Introduction

Cancer is a disease of the cells, so it is essential to identify the stages of development and use of basic information in the prediction, prevention, early detection and development of drugs targeted action [1]. Breast cancer is one of the malignant diseases with high incidence and mortality among women. The etiology of breast cancer involves a multitude of genetic, immunological and biochemical modifications [2]. Therefore, the transformation of normal cells, tumor progression and advanced metastasis involve a complex series of events such as genetic alterations, the aberrant progression of the cell cycle, induction of angiogenesis, inhibition of apoptosis and modification of cell adhesion. Failure to eradicate the entire tumor cell population and consequent development of resistance to chemotherapy, are the main obstacles to successful treatment of many malignant diseases, including breast cancer [3]. Increase of replicative capacity, loss of cell adhesion and angiogenesis process represent aggravating factors of clinical evolution for cancer patients [4,5].

The focus on finding chemotherapeutic agents has recently shifted to natural products. Various plants and their bioactive compounds have been shown to have anti-carcinogenic and anti-proliferative effects towards breast cancer cells [6]. Studies have also reported positive
correlation between antioxidant activities of plants and their anti-proliferative effects, suggesting the potential action of antioxidants in inhibiting cancer cell growth [7]. Phenolic compounds such as epigallocatechin gallate and quercetin, which have high antioxidant activities, demonstrated anti-proliferative effects against breast cancer cell lines [8].

Flavonoids are chemical substances (over 5000) found in vegetables and fruits, wines, seeds, nuts, grains and teas, herbs, and represent a class of plant secondary metabolites, known for their antioxidant properties. This property catalogued flavonoids as “immune system” of plants. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities [6,7,8]. Studies on animal models reveal that flavonoids exert positive preventive effects in carcinogenesis and neurodegenerative disorders, essentially because of their antioxidant activity, their capacity to affect the expression of several detoxifying enzymes and their ability to modulate protein signaling cascades [9,10]. Flavonoids can interfere with specific stages of the carcinogenic process, and can inhibit cell proliferation and induce apoptosis in several types of cancer cells [11].

Apoptosis represents a cellular “suicide” mechanism which allows the control of cell number from tissues and elimination of cells that present DNA mutations or having an aberrant cell cycle, those cells being predisposed to malignant transformation [12]. Thus, elucidating the mechanisms of programmed cell death process seems to be of great importance for malignant transformation, tumour evasion and therefore for anti-cancer therapy. New approaches to cancer therapy involves restored cellular mechanisms responsible for inducing cell death (apoptosis) in tumor cells [13,14,15]. Apoptosis induction is the most potent defense against cancer, therefore chemopreventive approach entails the use of agents that eliminate premalignant/malignant cells by inducing them to undergo apoptosis [6,16].

The flavonoids possess a remarkable spectrum of biochemical and pharmacological activities suggesting that they significantly effects cell functions such as growth, differentiation and programmed cell death (apoptosis). Quercetin (QCT) and resveratrol (RSV) are active biological compounds that present anti-inflammatory, -oxidant, -tumoral and -angiogenic properties [6,7]. They are considered viable chemopreventives because they are absorbed and metabolized rapidly in vivo [16].

Quercetin, (3,3',4',5,7- pentahydroxyflavone) is an important dietary flavonoid, present in different vegetables, fruits, seeds, nuts, tea and red wine. The study of QCT as potential chemopreventer is assuming increasing importance considering its involvement in the suppression of many tumor-related processes including oxidative stress, apoptosis, proliferation and metastasis [17]. QCT has also received greater attention as pro-apoptotic flavonoid with a specific and almost exclusive activity on tumor cell lines rather than normal, non-transformed cells. Numerous in vitro studies show that quercetin induces apoptosis of cancer cells through different mechanisms [18,19,20].

Resveratrol (RSV) (3,5,4'-trihydroxystilbene) is a phytoestrogen found in many plants and products including peanuts, seeds, grapes and red wine, and has many beneficial effects in different biological systems, that include anti-inflammatory, antioxidant, anti-neoplastic, anti-carcinogenic, anti-tumorigenic, cardioprotective, neuroprotective, anti-aging and antiviral effects [21,22]. The growth-inhibitory effect of RSV is mediated through different mechanisms [16,23, 24].

Many anti-cancer drugs act during physiological pathways of apoptosis, leading to tumour cell distruction [25]. By combining flavonoids with anti-cancer drugs, an increase of the effects might be obtained, specifically in highly invasive cancer cells, while in nontumoral cells the natural compounds could reduce the cytotoxic side effects [26,27,28]. The most effective anticancer agents used to treat breast cancer is Adriamycin (ADR, doxorubicin), an
anthracycline antibiotic that exerts its cytotoxic effect by intercalating between DNA base pairs on the double helix and inhibiting topoisomerase II (TOPO-II), the enzyme responsible for DNA helix conformation and stability. Unfortunately, chronic cardiotoxicity including development of a cardiomyopathy is a major limiting factor of the chemotherapeutic use of doxorubicin. Therefore, it is necessary to develop new therapeutic combinations to improve doxorubicin effects at lower concentration of the drug associated with protective effects for non-tumoral cells [29,30].

Real-Time Cell Analysis (RTCA) is a non-invasive way to continuously monitor the cellular behavior that utilizes the inherent morphological and adhesive characteristics of the cell. It could be performed with xCELLigence System (ACEA Biociences/ trademark of Roche) that consists of a cell-based label-free platform technology with good sensitivity and reproducibility in monitoring an entire cell population. The continuous monitoring of cell viability by the xCELLigence system makes possible to distinguish between different perturbations of cell viability, such as senescence, cell toxicity (cell death), and reduced proliferation (cell cycle arrest) [31].

Therefore, this study aimed to explore by the new technology of Real-Time Cell Analysis whether RSV or QCT could enhance the cytotoxic effect of ADR against the growth of human breast cancer cell lines (MCF-7) and to screen for the proper concentrations of compounds with cytotoxic or cytostatic potential, in order to minimize the undesirable side effects of doxorubicin treatment. We investigated the possible mechanisms of interaction between ADR and RSV or QCT regarding compound-cytotoxicity, apoptosis induction, and cell cycle progression of breast cancer cells.

Materials and Methods

Cell cultures and treatments: MCF-7 human breast cancer cell line was purchased from American Type Culture Collection (ATCC) and routinely maintained in culture in RPMI-1640 medium added with 2mM L-glutamine and 10% fetal calf serum (Sigma Aldrich, St. Louis, Mo, USA) and incubated at 37°C/5% CO2 humified atmosphere. After 24h cells were treated with different concentrations of doxorubicin (ADR) and/or quercetin (QCT) or resveratrol (RSV) (Sigma) for various periods of time. Then cells were detached with a nonenzymatic solution of PBS/1mM EDTA, washed twice in PBS and immediately used for the evaluation of apoptosis events or fixed in ice-cold ethanol/PBS (70:30) and kept until use at 4°C for cell cycle analysis.

Reagents: Doxorubicin (doxorubicin hydrochloride, adriamycin, ADR), quercetin and resveratrol were purchased from Sigma. The stock solutions were prepared in milliQ sterile water, and preserved at −20°C. Working drug concentrations were prepared from the stocks in culture medium before each experiment. Ribonuclease A (RNase A) from bovine pancreas, propidium iodide (PI), etylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO) were purchased from Sigma. Annexin V-FITC Apoptosis Detection kit was purchased from Becton Dickinson Immunocytometry Systems, Mountain View, Ca, USA (BD).

Real-Time Cell Analysis (RTCA): The xCELLigence System allows label-free and real-time monitoring of cellular processes such as cell proliferation, cytotoxicity, adhesion, viability, invasion, and migration, using electronic cell sensor array technology. RTCA-DP instrument used is composed of three parts: RTCA DP Analyzer for concomitant or independent operation of three E-Plates, RTCA Control Unit and E-Plates with 16 wells. Cellular events are monitored in real time without the incorporation of labels by measuring the electrical impedance across interdigitated micro-electrodes integrated on the bottom of
special tissue culture plates (E-Plates (ACEA Biosciences) [31]. The electrode impedance, which is displayed as cell index (CI) values, was used to provide quantitative information about the biological status of the cells, including cell number, viability, and morphology. Changes in a cell status, such as cell morphology, cell adhesion, or cell viability led to a change in CI, which is a quantitative measure of cell number present in a well. Briefly, MCF-7 breast cancer cells in RPMI-1640 culture medium added by 2mM L-glutamine and 10% FCS were seeded for 24-48h in E-Plates 16 cell and growth curves started to be automatically recorded on the xCELLigence System in real time. Then, scalar concentrations of drugs or dietary natural compounds were added and live cell monitored.

**Cytotoxicity assays**: MTT cell viability assay was used to measure the cytotoxicity of reagents and cell viability using a standard colorimetric assay. All tests were performed in triplicate in 96 flat wells plates, using CellTiter 96 non-radioactive cell proliferation assay, based on metabolic active cells capacity to reduce yellow tetrazolium salt (MTT) to formasan, which has a blue colour and was solubilized in DMSO. The samples were spectrophotometrically detected (λ = 570nm) using Genios/ Tecan spectrophotometer.

**Cell cycle analysis**: Fixed cells were washed twice in PBS and the cell pellet (100 ul) was resuspended in 350 ul of PBS, and added with 50 ul of 10 mg/ml Rnase A (1 mg/ml final concentration). After 10 min incubation at 37°C, 100 ul of 100 ug/ml PI solution were added (20 ug/ml final concentration) and further incubated at 37°C for 10 min. The probes were kept in the dark and at 4°C until acquisition by flow-cytometry using a FACScan cytometer (BD). Then cell cycle analysis was performed and ModFIT LT or CellFIT softwares were used to estimate the DNA index (DI) and cell-cycle phases distribution.

**Apoptosis analysis**: The apoptosis assay was carried out using the Annexin V-FITC kit and the manufacturer’s protocol from BD Pharmingen. The percentages of apoptotic cells were determined by double staining with Annexin V-FITC/ PI, followed by sample analysis by flow-cytometry using WinMDI 2.9 software.

**Results and Discussions**

**Real time analysis of compound-mediated cytotoxicity of MCF-7 cells.**

The xCELLigence System of Real-Time Cell Analyzers (RTCA) allows cell-based in vitro assays for the assessment of cell viability and cytotoxicity and are disruptive endpoint assays [31]. In a first line of experiments, MCF-7 breast cancer cells were seeded at scalar densities from 10^3 to 10^5/well in E-Plates for a dynamic monitoring of adherent cell proliferation and to establish the optimal cell density to be used in further assays. The cell growth curves were automatically recorded on the xCELLigence System in real time (data not shown).

RTCA growth curves and CI values prompted us to choose the 2x10^4 cells/well for the next compound-mediated cytotoxicity assays (Fig. 1). Therefore, we investigated by RTCA the cytotoxicity of 5 to 500 μM of QCT, 1 to 200 μM of RSV and 1 to 200 μM of ADR in order to screen for the proper concentrations of compounds with cytotoxic or cytostatic potential and continuously monitor the cell growth, proliferation and viability of MCF-7 cells. Results are expressed as normalized cell index (CI) after automatic comparison between the curves of viability for treated and non-treated cells (Fig. 1).
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Real-time monitoring of cellular processes by the xCELLigence Systems offered distinct and important advantages over traditional end-point assays. A comprehensive representation of entire length of the assay was possible allowing us to make informed decisions regarding the timing of certain manipulations or treatments. Acquisition of real-time monitoring of cytotoxicity allowed for calculation of time-dependent IC50 values. Regarding timing of calculating IC50, internally, it was selected the time point where the highest concentration reached maximum response and before the controls started to crash. Therefore, we monitored compound-induced cytotoxicity to produce continuous compound- and concentration-dependent cell impedance profiles using xCELLigence System and MCF-7 cancer cell line as our in vitro models. We screened for the proper concentrations of compounds with cytotoxic or cytostatic potential (e.g. drugs as ADR, or natural compounds) in killing adherent tumor cells, in order to be used in further end-point assays such as apoptosis or cell cycle analyses. The IC50 values obtained by the xCELLigence System were comparable to the IC50 value obtained by colorimetric cytotoxicity assays as MTT or XTT (data not shown).

Modulation of apoptotic events

After automatic data acquisition from the homogenous assays of compound-mediated cytotoxicity, we determined the compound-specific profiling, the optimal point of compound treatment and calculated the real-time IC50 values. In order to evaluate in an end-point assay the apoptosis events induced by anti-cancer drug treatment, alone or in combination with natural compounds, MCF-7 cells were cultured for 24h, medium was changed, and cells were sensitized by 6h with fixed concentrations of 50, 100 or 200 µM QCT or 25, 50 or 100 µM RSV. Then, 1 or 2.5 µM ADR were added for additional 42 h with ADR. After 48h of treatment cells were detached, washed twice with PBS and apoptotic cells detected by using Annexin V-FITC/ PI double staining, followed by flow-cytometry analysis. Apoptotic events

Figure 1. Real-time, label-free monitoring of compound-mediated cytolysis of MCF-7 human breast tumor cells by xCELLigence Real Time Cell Analyzer (RTCA).
are expressed as percentages of cells. In each quadrant of Figure 2 there are represented the live (left down), early apoptotic (right down), late apoptotic (right up), and necrotic (left up) cells.

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**Figure 2.** Effects of treatments with QCT or RSV on ADR induction of apoptosis in MCF-7 breast tumor cells, evaluated by Annexin-FITC/PI double staining and flow-cytometry analysis.

Figure 2 shows a significant experiment of modulated apoptosis by QCT or RSV and/or ADR. QCT treatment induced higher levels of total apoptosis (44%, the sum of early and late apoptosis events) compared to control cells (9%). Moreover, 100μM of QCT induced higher levels of early apoptosis (21%) than control cells (3%) (Fig. 2). Results are similar for 50 μM QCT treatment. In addition, 200 μM of QCT induced higher levels of late apoptosis (data not shown). The 100 μM QCT/1 μM ADR combined treatment enhanced the levels of early apoptosis (Fig. 2). Effect of RSV seemed to be much stronger for a lower concentration than QCT: 50 μM RSV induced 29% of early apoptotic events, while combination with 1 μM ADR enhanced early apoptosis to 47% (Fig. 2). Since 2.5 μM of ADR induced higher necrosis than 1 μM concentration, addition of flavonoids might be an alternative approach in order to obtain the same or a stronger anti-tumor response, enhance the chemo-sensitivity of tumors to ADR and diminish the side-effects by using lower concentrations of anti-cancer drugs. Our results are in accordance to similar studies concerning the additive effect of RSV (30,32) or QCT (29,33) to anti-cancer drug treatment, and in reversing the multi-drug resistance [4,5].

**Modulation of progression through cell cycle phases.**

Perturbations in cell cycle progression may account for the anti-carcinogenic effects of flavonoids. Phytochemicals that may have inhibitory and/or chemopreventive potential like QCT and resveratrol RSV were used in combination with cytotoxic drugs (ADR). In order to evaluate the influence of anti-cancer drug treatment, alone or in combination with natural compounds, upon the end-point assay of progression of MCF-7 cells through cell cycle phases, MCF-7 cells were cultured as in the experiment described above.
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Figure 3. Flow cytometry analysis of the additional effects induced by QCT or RSV on proliferation through cell cycle phases of doxorubicin-treated MCF-7 breast tumor cells.

Adriamycin treatment induced a decrease of cells in S cell cycle phase and an increase of the percentages of cells in G2+M phases on MCF-7 cells: 1μM ADR blocked 32% cells in G2+M compared to 5% of the non-treated cells (Fig. 3 and 4). The same treatment decreases percentages of cells in S phase from 28% to 12%.

One hundred or 200 μM QCT use alone or in combination with 1 μM ADR blocked cells in G0/G1 cell cycle phase, and diminish percentages of cells in S phase, and the effect was QCT dose-dependent. In contrast, 25 μM RSV blocked cells in G2+M (32%), while 50 μM strongly diminish percentages of cells in S phase, from 28 to 16%, and blocked cells in G0/G1 phase (76%) (Fig. 4). ADR has an additive effect, and higher percentages of cells were blocked in G0/G1 phase: 69% when 25 μM RSV were combined with 1 μM ADR, comparing to 25 μM RSV used alone (42% blocked cells); 84% when 50 μM RSV were combined with 1 μM ADR, comparing to 50 μM RSV used alone (76% blocked cells) (Fig. 4).

Figure 4. Modulation of the progression through cell cycle phases of MCF-7 human breast tumor cells treated with flavonoids and/or ADR.

The results suggested that cell cycle arrest in G0/G1 phase might represent an important cause for anti-proliferative effect of QCT. The natural compounds QCT and RSV, used at the concentrations tested in the present study, seemed to have a synergic effect with ADR on apoptosis of tumor cells MCF-7, therefore combined treatments might be used to improve the traditional therapeutic approaches.

Conclusions

Natural compounds have drawn great attention in chemoprevention and for their potential clinical application when used in combination with anti-cancer drugs. Contrast data are available on the anti-cancer effects of flavonoids in breast cancer. The current interest in the potential effects of antioxidants from natural compounds in breast cancer treatment prompted us to investigate using a state-of-the art technology, Real Time Cell Analysis, the cytotoxic vs proliferative capacity of quercetin and resveratrol to modulate the chemo-sensitivity of adriamycin treated MCF-7 breast cancer cells. Real-time impedance data obtained by the xCELLigence System were used to generate compound-specific profiles which were dependent on the biological mechanism of action of the compound used. The actual kinetic response of the cells within an assay prior or subsequent to certain manipulations provided important information regarding the biological status of the cell such as cell growth, arrest, morphological changes and apoptosis.

Stimuli treatment of breast cancer cells differentially induced higher levels of apoptosis as compared to untreated tumour cells, while cell cycle distribution of DNA changed. The effect of modulatory agents on proliferation and apoptosis might be used in clinical departments in order to elaborate new therapeutic approaches and act as useful instruments in elaboration of individualized treatment schemes.

Acknowledgements

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