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Impact of chemotherapy on metabolic reprogramming: Characterization of the metabolic profile of breast cancer MDA-MB-231 cells using ^{1}H HR-MAS NMR spectroscopy

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ABSTRACT

Doxorubicin, cisplatin, and tamoxifen are part of many chemotherapeutic regimens. However, studies investigating the effect of chemotherapy on the metabolism of breast cancer cells are still limited. We used ^{1}H high-resolution magic angle spinning (HR-MAS) NMR spectroscopy to study the metabolic profile of human breast cancer MDA-MB-231 cells either untreated (control) or treated with tamoxifen, cisplatin, and doxorubicin. ^{1}H HR-MAS NMR single pulse spectra evidenced signals from all mobile cell compounds, including fatty acids (membranes), water-soluble proteins, and metabolites. NMR spectra showed that phosphocholine (*i.e.*, a biomarker of breast cancer malignant transformation) signals were stronger in control than in treated cells, but significantly decreased upon treatment with tamoxifen/cisplatin. NMR spectra acquired with Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence were interpreted only qualitatively because signal areas were attenuated according to their transverse relaxation times (T_2). The CPMG method was used to identify soluble metabolites such as organic acids, amino acids, choline and derivatives, taurine, guanidine acetate, tyrosine, and phenylalanine. The fatty acid variations observed by single pulse as well as the lactate, acetate, glycine, and phosphocholine variations observed through CPMG ^{1}H HR-MAS NMR have potential to characterize both responder and non-responder tumors in a molecular level. Additionally, we emphasized that comparable tumors (*i.e.*, with the same origin, in this case breast cancer) may respond totally differently to chemotherapy. Our observations reinforce the theory that alterations in cellular metabolism may contribute to the development of a malignant phenotype and cell resistance.

1. Introduction

Chemotherapy is the fundamental treatment for most of the disseminated cancers[1]. Nevertheless, some tumor cells can survive and become resistant to the therapy [2]. Drug resistance has been a considerable limitation for treatments to succeed [1]. Fortunately, extensive efforts have been made towards the understanding of resistance mechanisms, such as oncogenic signaling pathways that result from genomic instability of cancer cells [2].

There are thousands of alterations in point mutations, translocation, amplifications, and deletions that may contribute to the resistance of cancer cells to drugs, being the mutational range able to diverge even among histopathologically similar tumors [3]. Moreover, these alterations are varied and appear to change between distinct cell types and different time courses, increasing the complexity of the tumor process [4]. It is becoming perceptible, though, that many key oncogenic signaling pathways converge to readjust tumor cell metabolism in order to support tumor growth [3]. These observations support the theory that alterations in cellular metabolism may contribute to the development of a malignant phenotype [5]. Due to its high sensitivity and rapid response to changes in the environment, metabolism analysis is often claimed to best reflect the tumor phenotype [6]. The rapid metabolite changes caused by diseases or therapeutic agents may be considered as advantages for understanding the mechanisms involved in cell response [6]. Should anticancer chemotherapies be the case, rapid metabolic changes can be often detected prior to any clinical symptoms, allowing the prevention of side effects or even to switch therapies [6].

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Nowadays, studies investigating how metabolism in breast cancer is affected by chemotherapy are still limited [7–10]. We have previously studied the influence of chemotherapy on metabolic profile of the estrogen receptor-positive human breast cancer cell line MCF-7 using ^{1}H high-resolution magic angle spinning (HR-MAS) NMR spectroscopy [11]. This technique confirmed that doxorubicin, cisplatin, and tamoxifen had strong effects on the metabolic profile of MCF-7 cells [11].

In this paper, ^{1}H HR-MAS NMR spectroscopy was also used to investigate the effect of the anticancer drugs – particularly doxorubicin, cisplatin, and tamoxifen – on intact estrogen-negative human breast cancer MDA-MB-231 cells. These cells lack the expressions of estrogen, progesterone, and human epidermal growth factor receptor-2/neu (TNBC), which implies in totally hormone-independent, poorly-differentiated tumors [4]. The ^{1}H HR-MAS NMR results presented here demonstrate that the metabolic profile of intact MDA-MB-231 cells is more affected by these drugs than that of MCF-7 cells. ^{1}H HR-MAS NMR has been shown to be a highly reproducible tool in the field of cancer cell metabolomics. Additionally, we emphasized that comparable tumors (*i.e.*, with the same origin, in this case breast cancer) may respond remarkably differently to chemotherapy. These observations strengthen the theory that alterations in cellular metabolism may contribute to the development a malignant phenotype and cell resistance to therapy.

2. Materials and methods

2.1. Breast cancer cell culture

MDA-MB-231–estrogen receptor (ER)-negative human breast adenocarcinoma cell line – cells were obtained from the Rio de Janeiro Cell Bank (BCRJ, Rio de Janeiro, RJ, Brazil). MDA-MB-231 tumor cells were chosen because these are poorly differentiated *in vivo* and lead to the worst prognosis. MDA-MB-231 cells were grown in Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific Inc.). Cells were seeded at a density of 1.0×10^6 cells per 75 cm^2 of cell culture flask and maintained at 37 °C in a humidified incubator containing with 5% of CO_2 for 72 h. This allowed cells to attach to the flask and become confluent. The medium was removed and subcultures were obtained by treating cells with trypsin in phosphate buffered saline (PBS, Sigma Aldrich) for 2 min. Subsequently, 4 mL of culture medium was added and cells were centrifuged at 1.200 rpm for 5 min to form pellets. After centrifuging, 10 μL of D_2O was added to approximately 40 μL of MDA-MB-231 pellet in PBS. This procedure was accomplished in triplicate. The cell viability was tested by Trypan Blue exclusion prior to the measurements.

2.2. Breast cancer cell treatment

MDA-MB-231 cells were cultured as described above. After confluence, cells were treated with the drugs (*i.e.*, doxorubicin, cisplatin, and tamoxifen), individually, for 24 h each. The culture medium was supplemented with 3, 1, and 25 μM of doxorubicin, cisplatin, and tamoxifen, respectively. We relied upon previous cytotoxicity assays with doxorubicin [12], cisplatin [13], and tamoxifen [12] to choose such drug concentrations. Cisplatin-, tamoxifen-, and doxorubicin-treated cells were detached from the culture flask using trypsin. Once centrifuged, the pellets were resuspended in D_2O to NMR analysis. This procedure was carried out in triplicate for each drug. The samples pH were approximately 7.2.

2.3. ^{1}H HR-MAS NMR analyses

Each pellet was packed into a zirconium HR-MAS rotor containing 10 μL of deuterium oxide and sodium trimethylsilyl-[2,2,3,3-2H4]-1-propionate (TMSP). The system was adjusted to 0.00 ppm whereas the spin was set to the magic angle (*i.e.*, 54.7° relative to the magnetic field's z direction). ^{1}H-HR-MAS spectroscopy was carried out at 14.1 T (600 MHz for ^{1}H) and 5 kHz of spinning rate using an AVANCE 600 BRUKER NMR spectrometer. The spectra were acquired with a 1.5 s presaturation pulse, an acquisition time of 4.63 s (32 K points), a 4 s recycle delay, and an accumulation of 256 transients. Additionally, a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo train was employed before data acquisition by means of applying 120 cycles separated by 1.2 ms echoes. The free induction decay (FID) signal was multiplied by a 1.0 Hz (0.0025 ppm) line broadening as well as a two-fold zero filling for Fourier transform. The Advanced Chemistry Development (ACD) Labs software was used for automatic phase adjustment and baseline correction. Samples were also analyzed using two-dimensional (2D) NMR spectroscopy methods, such as COSY (*Correlation spectroscopy*), ^{1}H–^{13}C-HSQC (proton-carbon *heteronuclear single-quantum correlation spectroscopy*), and ^{1}H–^{13}C-HMBC (proton-carbon *heteronuclear multiple bond correlation spectroscopy*). ^{1}H HR-MAS spectra of cancer cells were identified using correlated spectroscopy (2D homonuclear and heteronuclear NMR experiments, including COSY, ^{1}H–^{13}C-HSQC, and ^{1}H–^{13}C-HMBC). Online databases – *e.g.*, Human Metabolome Database (HMDB) and Chenomx – were also suitable for such assignments. One-dimensional (1D) ^{1}H HR-MAS spectra were normalized by correcting baseline offset to zero as well as dividing each data point by the sum of all data points, being the resulting values multiplied by 1000.

3. Results

Figs. 1 and 2 show ^{1}H HR-MAS NMR spectra of human breast cancer MDA-MB-231 control cells (Con) and MDA-MB-231 cells treated with tamoxifen (Tamo), cisplatin (cis), and doxorubicin (Doxo). Both single pulse and CPMG ^{1}H HR-MAS NMR spectra of the three cultures for each treatment (triplicates) are shown in Supplementary information.

The spectra presented in these figures were acquired with a single pulse (Fig. 1) and the CPMG sequence (Fig. 2). Single pulse spectra show the signals from all mobile compounds present in the cells, including fatty acids (membranes) as well as some water-soluble proteins and metabolites. Spectra acquired with the CPMG sequence (Fig. 2) show only the sharp signal of the highly mobile water-soluble metabolites.

The CPMG sequence has been used as a transverse relaxation time (T_2) filter (T_2 filter) since it attenuates more efficiently broad (shorter T_2) rather than sharp (longer T_2) NMR peaks. Therefore, all signals presented in Fig. 2 were attenuated according to their T_2 values as well as the total echo time used in the CPMG sequence. Consequently, the CPMG spectra (Fig. 2) cannot be used in quantitative analyses, but only in their qualitative and semi-quantitative counterparts (*i.e.*, only large differences in peak areas can be attributed to variations in metabolite contents).

Figs. 1A–D and 2A–D present ^{1}H HR-MAS NMR spectra, from 0.5 to 4.5 ppm, of Con (A), Tamo (B), Cis (C), and Doxo (D). Figs. 1A'–D' and 2A'–D' show ^{1}H HR-MAS NMR spectra of the same samples – *i.e.*, Con (A'), Tamo (B'), Cis (C'), and Doxo (D') –, but from 5.0 to 10.0 ppm. The vertical axes of spectra A' to D' were four-fold magnified. Both single pulse and CPMG ^{1}H HR-MAS NMR spectra of the three cultures for each treatment (triplicates) are shown in Supplementary Information.