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

Roberta M. Maria$^a$, Wanessa F. Altei$^b$, Heloisa S. Selistre-de-Araujo$^b$, Luiz A. Colnago$^{a,*}$

$^a$ Embrapa Instrumentação, Rua XV de Novembro, 1452, São Carlos, SP, 13560-970, Brazil

$^b$ Laboratório de Bioquímica e Biologia Molecular, Departamento de Ciências Fisiológicas, Universidade Federal de São Carlos (UFSCar), Rodovia Washington Luis, km 235, Caixa Postal 676, São Carlos, SP, 13565-905, Brazil

ARTICLE INFO

Article history:
Received 19 May 2017
Received in revised form 21 August 2017
Accepted 28 August 2017

Keywords:
Breast cancer
Anticancer treatment
Metabolomics
Pathways
Nuclear magnetic resonance

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.

© 2017 Elsevier B.V. All rights reserved.

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].

* Corresponding author.
E-mail address: luiz.colnago@embrapa.br (L.A. Colnago).

http://dx.doi.org/10.1016/j.jpba.2017.08.038
0731-7085/© 2017 Elsevier B.V. All rights reserved.
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 1H 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, 1H 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 1H 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. 1H 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 (BCR, 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 × 10^5 cells per 75 cm^2 of cell culture flask and maintained at 37°C in a humidified incubator containing 5% of CO₂ 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 D₂O 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 24h 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 D₂O to NMR analysis. This procedure was carried out in triplicate for each drug. The samples pH were approximately 7.2.

2.3. 1H 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). 1H-HR-MAS spectroscopy was carried out at 14.1 T (600MHz for 1H) 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 (32K 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), 1H-13C-HSQC (proton-carbon heteronuclear single–quantum correlation spectroscopy), and 1H-1H-C-13C-HMBC (proton-carbon heteronuclear multiple bond correlation spectroscopy). 1H HR-MAS spectra of cancer cells were identified using correlated spectroscopy (2D homonuclear and heteronuclear NMR experiments, including COSY, 1H-13C-HSQC, and 1H-13C-HMBC). Online databases – e.g., Human Metabolome Database (HMDB) and Chenomx – were also suitable for such assignments. One-dimensional (1D) 1H 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 1H 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 1H 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 (T₂) filter (T₂ filter) since it attenuates more efficiently broad (shorter T₂) rather than sharp (longer T₂) NMR peaks. Therefore, all signals presented in Fig. 2 were attenuated according to their T₂ 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 1H 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 1H 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 1H HR-MAS NMR spectra of the three cultures for each treatment (triplicates) are shown in Supplementary Information.
3.1. Spectra assignments

All spectra displayed in Fig. 1 showed strong, broad peaks overlapped with sharp, weak ones, the latter being assigned to water-soluble metabolites similar to those observed in MCF-7 cells [11]. The broad, strong peaks at 0.9 [1] and 1.3 ppm [2] have been assigned to methyl and methylene groups of fatty acids and proteins, respectively. These peaks also comprised signals from metabolites that better resolved in Fig. 2. The Con spectrum (Fig. 1A) also exhibited strong peaks at 3.22 [5], 3.26 [6], and 3.78 ppm [7], which have been attributed to phosphocholine, betaine, and guanidinoacetate, respectively. The broad peak at 5.3 ppm [8] has been assigned to olefinic hydrogens of unsaturated fatty acids.

Fig. 2 shows CPMG spectra in which sixteen metabolites have been assigned: lactate/threonine (1/2), alanine [3], acetate [4], glutamate/glutamine (5/6), acetone [7], creatine [8], choline [9], phosphocholine [10], taurine [11], glycine [12], guanidino acetate [13], tyrosine [14], phenylalanine [15], and uridine-triphosphate/uridine-diphosphate/uridine-monophosphate (UTP/UDP/UMP) [16]. These assignments have been published elsewhere [14,11].

3.2. Impact of chemotherapy on MDA-MB-231 cell metabolism

The spectrum (Fig. 1B), wherein virtually only broad peaks are observed, evidences a strong effect of tamoxifen on the metabolic profile. The phosphocholine peak [5], considered as a biomarker of breast cancer malignant transformation, was very small in Tamo spectrum (see also Fig. S1B, Supplementary Information). The strong, sharp peak observed at 1.91 ppm [4] has been assigned to acetate (Fig. 2B).

Cis spectrum (Fig. 1C) also shows the strong effect of chemotherapy on MDA-MB-231 metabolic profile. Phosphocholine [5] and betaine [6] peaks at approximately 3.25 ppm were remarkably suppressed by Cis. The expansion of Cis spectrum from 5.0 to 10.0 ppm (Fig. 1C) revealed peaks at 6.8 and 7.2 ppm [9]; 7.31, 7.36, and 7.41 ppm [10]; and 7.94 ppm [11]. The aforementioned peaks have been assigned to tyrosine, phenylalanine, and UTP/UDP/UMP, respectively. The stronger sharp peaks observed at 1.30/1.32, 1.47, and 3.56 ppm have been attributed to lactate/threonine (1/2), alanine [3], and glycine [12], respectively (Fig. 2C).

The metabolic profile of MDA-MB-231 cells was not significantly changed by Doxo treatment (Fig. 1D). The main difference was the phosphocholine peak at 3.22 ppm [5], which decreased in intensity, while the betaine peak at 3.25 ppm [6] remained practically unchanged when compared with untreated cells (Fig. 1A). Like in
Cis treatment – in smaller intensities, though – the spectrum expanded from 5.0 to 10.0 ppm (Fig. 1D) demonstrated peaks at 6.8 and 7.2 ppm [9]; 7.31, 7.36, and 7.41 ppm [10]; and 7.94 ppm [11], which have been assigned to tyrosine, phenylalanine, and UTP/UDP/UMP, respectively. The stronger sharp peaks observed at 1.30/1.32 and 3.22 ppm were as attributed to lactate/threonine (1/2) and phosphocholine [10], respectively (Fig. 2D). However, other minor sharp peaks were observed, such as that close to 2.5 ppm that was assigned to glutamate/glutamine (5/6) (Fig. 2D). Creatine, taurine, and guanidine acetate (peaks at 3.03, 3.45, and 3.78 ppm, respectively) also presented reduced intensities in the spectrum recorded in Doxo-treated MDA-MB-231 cells.

The areas below single pulse 1H HR-MAS NMR spectra (Fig. 1) showed the following variations: stronger peak 3 in Cis > Doxo > Tamo ~ Con as well as stronger peak 4 in Tamo > Cis ~ Doxo ~ Con. Tamo and Cis spectra showed stronger peaks 1 and 2 than those of Con and Doxo (refer also to Fig. S1B-B' and C-C'). Larger areas corresponding to methyl [1] and methylene [2] indicate that Tamo and Cis either induced the synthesis or reduced the uptake of saturated fatty acids. Therefore, the spectrum of control (Fig. 1A) and Doxo-treated cells (Fig. 1D) showed the highest of phosphocholine [5] contents. This peak significantly decreased in Tamo and Cis spectra.

The betaine peak [6] was similar in Con and Doxo spectra, but substantially decreased in Tamo and Cis spectra (Fig. 1B and C). Betaine is considered to be a major osmolyte in cells and plays an essential role in cellular protection against environmental stress, including high temperature and osmotic imbalance [15]. It also regulates cell volume and stabilizes proteins [16]. Therefore, regardless of the therapeutic intervention, only doxorubicin induced a stress condition in MDA-MB-231 cells.

The peak at 3.78 ppm, which was assigned to guanidine acetate [7], decreased after all treatments (Fig. 1). The area attributed to olefinic hydrogens [8] was larger in Cis > Tamo > Doxo > Con (see also Supplementary Information). The spectra of drug-treated cells revealed higher protein contents (signal from 6.5 to 10 ppm) than those of control cells (Fig. 1A). However, the spectra cisplatin-treated cells (Fig. 1C) and, to a lower extent, of doxorubicin-treated cells (Fig. 1D) indicated the presence of tyrosine, phenylalanine, and UTP/UDP/UMP (peaks 9–11, respectively).

The areas under CPMG 1H HR-MAS NMR signals (Fig. 2) assigned to lactate [1], threonine [2], alanine [3], acetate [4], taurine [11], and glycine [12] were found to be smaller in untreated cells (Con) than in their treated counterparts. Phosphocholine [10] was present at higher concentrations in Con and Doxo-treated cells (Fig. 2A and D), whereas acetate [4] content was higher in cells treated with Tamo (Fig. 2B). Alanine [3] and glycine [12] contents were higher in Cis spectra (Fig. 2C). The area attributed to glutamate/glutamine (5/6) was smaller in untreated cells (Con) than in the treated ones, but acetone [7] was observed in higher contents in untreated cells (Con). The spectra of drug-treated cells evidenced higher protein contents (signal from 6.5 to 10 ppm) than those of control cells (Fig. 2A). Tyrosine, phenylalanine, and UTP/UDP/UMP peaks (14–18, respectively), in particular, were more intense in Cis spectrum (Fig. 2C) than in Tamo/Doxo spectra.

4. Discussion

Identification of specific metabolites displaying altered levels as well as of their associative metabolic pathways can improve the understanding of biological and pathological aspects involved in the process from normal to eventually cancerous state. Altered pathways include changes in ketone bodies (acetone), glycolysis (lactate), and energy and lipid metabolisms.

Un-treated MDA-MB-231 cells showed broad peaks related to fatty acids, as previously observed in MCF-7 cells [11]. The higher concentrations of fatty acids in untreated tumor cells are in line with the high activity of fatty acid synthase in tumor tissue, which in turn is associated with both proliferation and malignant transformation [17]. Essentially, fatty acid synthase activity supports cancer growth by increasing the building blocks for cell membranes and lipid-containing molecules involved in cell signaling [18]. As a result, the de novo synthesis of fatty acids in tumor cells would be possible [19]. The broad peak assigned to olefinic hydrogens of unsaturated fatty acids became stronger in treated cells. Consequently, chemotherapies are believed to significantly induce fatty acid synthesis.

The treatments, especially those involving cisplatin and tamoxifen, significantly reduced phosphocholine content, compound which is considered as a biomarker of breast cancer malignant transformation [20]. This indicates that doxorubicin and, in particular, tamoxifen and cisplatin reduced its synthesis in MDA-MB-231 cells and, as a consequence, cell invasiveness. Chemotherapeutic intervention also decreased phosphocholine intensities in MCF-7 cells [11], but the decrease was smaller than that in MDA-MB-231 cells.

Ketone bodies – including β-hydroxybutyrate, acetocacete, and acetone – are formed via β-oxidation of fatty acids in mitochondria. Acetoacetate and hydroxybutyrate can originate acetyl-CoA and join the tricarboxylic acid (TCA) cycle, providing the required energy for cellular activity [21]. This probably explains the deprivation of these metabolites in the acquired spectra, since only acetone was observed in untreated MDA-MB-231 cells. Thus, the higher acetone content could be an indication of ketogenesis in untreated cells. Acetone peak was imperceptible in MCF-7 cells [11]. Since MDA-MB-231 cells have an invasive behavior, are poorly differentiated in vivo, and have a worse prognosis than MCF-7 cells, the formers are expected to require more energy for cellular activity than MCF-7 cells.

Similarly to MCF-7 cells [11], lactate levels in untreated MDA-MB-231 cells were lower than in treated cells. This suggests enhanced glycolytic activity after chemotherapy. Because lactate is the end product of glycolysis, its accumulation implies an increased anaerobic glycolysis. However, lactate area increased similarly upon treatments in MCF-7 cells [11]. Conversely, lactate content increased significantly only after treatments with cisplatin and doxorubicin in MDA-MB-231 cells. Tamoxifen treatment did not change such peak significantly. As tamoxifen is a selective estrogen receptor (ER) modulator and MDA-MB-231 cells were demonstrated to be ER-negative, the treatment with tamoxifen probably did not perturb the anaerobic glycolysis in MDA-MB-231 cells as it did in MCF-7 cells, which are ER-positive.

The areas assigned to glutamine and glutamate were slightly larger in MDA-MB-231 treated cells than in untreated cells. Glutamine plays an important role in nucleotide and protein syntheses as well as in mitochondrial energy metabolism [22]. Glutamate and glutamine denote a group of glucogenic amino acids that features many biological functions. These are considered to be very important for cell function maintenance and promotion, being glutamine an important metabolic precursor in rapidly-dividing cells [23]. Cell demands for glutamine and glutamate are enormous during severe illness. Their levels may either decrease or increase, suggesting that these may become conditionally essential amino acids in cancer [24].

The largest creatine area was observed in MDA-MB-231 cells treated with doxorubicin. Provided that creatine is widely recognized as a key intermediate in energy metabolism, increased creatine area upon doxorubicin treatment can be associated with high energy demand.
The area assigned to glycine and alanine was higher in MDA-MB-231 cells treated with cisplatin. High glycine and alanine levels had also been observed previously in breast cancer tissues [22]. The correlation in alanine/glycine levels is because alanine is involved in glycine synthesis from pyruvate and serine [22]. This biochemical pathway is important since glycine is an important amino acid involved in the synthesis of proteins and nucleotides, elements that are decisive in cancer development.

Acetate peak was smaller or absent in all spectra of MCF-7 cells [11]. Similarly, such was not present in untreated MDA-MB-231 cells. However, this peak became stronger in tamoxifen-treated MDA-MB-231 cells. As acetate is the end product of lipid metabolism, these results support the hypothesis that tamoxifen has a higher influence over lipid metabolism disorders in MDA-MB-231 cells than in MCF-7 cells.

In summary, the NMR spectra acquired with CPMG pulse sequence were used only in a qualitative fashion because the areas under the signals were attenuated by the transverse relaxation time (T₂). The CPMG method was used to identify soluble metabolites (e.g., organic acids, amino acids, phosphocholine, taurine, and guanidinoacetate). 1H HR-MAS NMR spectroscopy was remarkably efficient in demonstrating the effects of tamoxifen, cisplatin, and doxorubicin on the metabolic profiles of MDA-MB-231 cells, which presented differences from those observed in treated MCF-7 cells. We demonstrated here that variations in lactate, acetate, and phosphocholine contents, indicated by single pulse HR-MAS NMR spectroscopy, 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. These observations reinforce the theory that alterations in cellular metabolism may contribute to the development of a malignant phenotype as well as cell resistance.

Conflict of interest

The authors declare that there is no conflict of interest.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgements

We would like to acknowledge the financial support of the Brazilian agencies FAPESP (grant # 2013/05128-5and #2013/00798-2) and CNPq (grant # 303837/2013-6).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2017.08.038.

References


