

COMPARATIVE METABOLOMICS OF BREAST CANCER

CHEN YANG[†], ADAM D. RICHARDSON[†],
JEFFREY W. SMITH, and ANDREI OSTERMAN
*The Burnham Institute for Medical Research,
La Jolla, California 92037, USA*

SUPPLEMENTARY MATERIALS

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I. Details of experimental procedures

Cell Lines and Cell Culture

Models representing human mammary epithelium and human breast cancer cells were used in this study. MCF-10A, a spontaneously immortalized nonmalignant cell line derived from normal mammary epithelial cells, was used as a model of normal human mammary epithelium. MCF-10A cells were obtained from American Type Culture Collection (Rockville, MD). MDA-MB-435, a highly metastatic human mammary epithelial cancer cell line, was used as a model of human breast cancer cells. MDA-MB-435 cells were obtained from the National Cancer Institute. This cell line was originally isolated from a 31-year-old Caucasian woman with ductal adenocarcinoma of the breast.

Both MCF-10A and MDA-MB-435 cell lines were cultured in MEM media containing 1 g/L glucose (Cellgrow) supplemented with 1.5 g/L glucose, 10% v/v Fetal Bovine Serum, 1% v/v antibiotic/antimycotic solution (Omega). All cells were maintained in a humidified atmosphere of 5% CO₂ in air, at 37°C. Labeling experiments were started by washing the cells with PBS buffer and replacing the unlabeled media with an identical medium containing 2 g/L unlabeled glucose and 0.5 g/L [U-¹³C] glucose (Cambridge Isotope Laboratories). Media aliquots were taken during the incubation, and glucose and lactate concentrations were determined with enzymatic test kits (Sigma). After

48 h, the cells were rinsed with ice-cold PBS, detached with trypsin and collected by centrifugation.

Analysis of Fatty Acids by GC-MS

The GCMS samples were prepared from about 5×10^7 cells as described previously (7). Briefly, the cell pellet was saponified with 30% KOH and 100% ethanol overnight, and the extraction was performed using petroleum ether. The fatty acids were methylated with 0.5 N methanolic HCl (Supelco, Bellefonte, PA). GC-MS measurements were performed on a Trace GC/Trace MS Plus system (Thermo Electron Corporation, Waltham, MA). The settings are as follows: flow rate of carrier gas (helium), 2 ml/min; source temperature, 200 °C; interface temperature, 250 °C. The column of Rtx-5MS (fused silica, 15m \times 0.25mm \times 0.25 μ m) (Restek, Bellefonte, PA) was used for GC-MS analysis. Electron impact (EI) spectra were obtained at -70 eV. The GC oven temperature was programmed from 180 °C (1 min) to 210 °C at 3 °C/min. Palmitate, palmitoleate, stearate and oleate were monitored at m/z 270, 268, 298 and 296, respectively. Mass isotopomer distribution was determined after correcting the contribution of labeling arising from natural abundances (8). The ^{13}C enrichment of acetyl units and the *de Novo* synthesis of fatty acids were determined from the distribution of mass isotopomers of fatty acids (7).

NMR Analysis

After the experimental incubations, approximately 2.5×10^8 cells were harvested and cell metabolites were extracted using the cold methanol extraction method. It has been reported that the cold methanol extraction is superior to other procedures commonly used for metabolome analysis, which include acid or alkaline treatments, high-temperature extraction in the presence of ethanol or methanol, and extraction with chloroform-methanol (9). Cell pellets were suspended in PBS, and L-methionine in an amount of 5×10^{-6} mol was added for quantification of metabolite concentration and calibration of chemical shift in the NMR spectra. The cells were extracted with an equal volume of cold (-20°C) absolute methanol. After rapid mixing, the tube was transferred into a dry ice bath for 30 min and subsequently thawed in an ice bath for 10 min. Cells were subjected to centrifugation (18,000 \times g, 10 min) and the supernatant was collected. The cell pellet was subjected to extraction another time with 0.5 ml of 50% v/v cold (-20°C) methanol, and the first and second extracts were combined. The cell extract was evaporated to dryness, and then dissolved in 650 μ l of D₂O (99.9% enriched; Cambridge Isotope Laboratories). The sample was filtered through a 0.2- μ m-pore-size filter and used for NMR measurements.

NMR experiments were performed at 30°C and 500 MHz on a Bruker Avance 500 spectrometer (Bruker, Karlsruhe, Germany). One-dimensional ^1H spectra were acquired using a spectral width of 5000 Hz and 32 K data points. Two-dimensional [$^{13}\text{C}, ^1\text{H}$] HSQC spectra were obtained using a standard gradient-based sequence. The acquisition parameters were $t_{1\text{max}} = 183$ ms, $t_{2\text{max}} = 157$ ms; data size was 3072 points in t_1 and 1024 points in t_2 . Sweep width was 80 ppm and the carrier position was 50 ppm for ^{13}C . ^{13}C decoupling during t_2 was achieved using WALTZ-16, and quadrature detection in ω_1 was accomplished with States-TPPI. Before Fourier transformation, the time domain data were multiplied in t_1 and t_2 with sine-bell windows shifted by $\pi/2$. The digital resolution along ω_1 after linear prediction and zero-filling was 1.23 Hz/point.

The ^{13}C - ^{13}C scalar coupling fine structures were extracted from the cross sections taken along the ^{13}C axis in a HSQC spectrum by using the Bruker XWINNMR software. After manual baseline correction, the individual multiplet components of the scalar coupling fine structures were integrated to quantify the relative contributions of singlet, doublet, and quartet signals. The obtained relative multiplet intensities were used in the following ^{13}C isotopomer model to assess the metabolic fluxes through individual pathways.

The concentrations of metabolites were determined by integrating the cross peaks in the HSQC spectra using the NMRPipe (10) and Sparky (<http://www.cgl.ucsf.edu/home/sparky/>) software packages, comparing with the integral of resonances peaks of the L-methionine that was treated as an internal standard, and normalizing to the amount of total cellular protein. The culture for each cell line was repeated, and for each sample the spectra were recorded twice using the same acquisition parameters. The significance of the various concentration values was analyzed using Student's t -test. Probability values (5%) were taken as significant results ($p < 0.05$)

II. Details of NMR peak assignment

Amino acids alanine, arginine, glycine, isoleucine, leucine, lysine, proline, and valine were identified using ^{13}C - ^1H cross-correlations. Glutamate, one of the most abundant amino acids, showed prominent cross peaks in the HSQC spectra (Fig. 2). Two signals close to the resonances of glutamate were assigned to the C3 (2.18 ppm for ^1H ; 28.91 ppm for ^{13}C) and C4 (2.57 ppm for ^1H ; 34.14 ppm for ^{13}C) of the γ -glutamyl moiety of glutathione based on spiking experiments. The C2 of glutamate and γ -glutamyl of glutathione were unresolved. The other cross peaks of glutathione, *i.e.*, C2 of glycine (3.79 ppm for ^1H ; 46.10 ppm for ^{13}C), C2 (4.58 ppm for ^1H ; 58.40 ppm for ^{13}C) and C3 (2.97 ppm for ^1H ; 28.25 ppm for ^{13}C) of cysteine were also identified. The peak at 2.46 ppm for ^1H and 33.56 ppm for ^{13}C was assigned to C4 of glutamine based on spiking experiments.

A very intense cross peak was observed for the C2 (4.12 ppm for ^1H ; 71.22 ppm for ^{13}C) and C3 (1.33 ppm for ^1H ; 22.78 ppm for ^{13}C) of lactate. The resonance of C2 or C3 of succinate was assigned to the peak at 2.42 ppm for ^1H and 36.90 ppm for ^{13}C .

The resonances of *m*-inositol were assigned by recording the spectrum of this compound. Based on the spiking experiments, the cross peak at 2.01 ppm for ^1H and 24.62 ppm for ^{13}C was assigned to the acetyl group of *N*-acetylglucosamine (GlcNAc) or *N*-acetyl-galactosamine (GalNAc), while the peak at 2.08 ppm for ^1H and 24.83 ppm for ^{13}C to UDP-GlcNAc or UDP-GalNAc.

The resonance from the $\text{N}^+(\text{CH}_3)_3$ group of choline, phosphocholine, and glycerophosphocholine was overlapped. It is difficult to distinguish between phosphocholine and glycerophosphocholine in the ^1H NMR spectrum (18). However, in this work, [^{13}C , ^1H] HSQC spectra was recorded so that the resonances due to choline, phosphocholine, and glycerophosphocholine could be easily distinguished. For example, the cross peak at 4.07 ppm for ^1H and 58.35 ppm for ^{13}C was assigned to the O- CH_2 group of choline, the peak at 4.18 ppm for ^1H and 60.79 ppm for ^{13}C to phosphocholine, and the peak at 4.33 ppm for ^1H and 62.17 ppm for ^{13}C to glycerophosphocholine based on spiking experiments.

Resonances for creatine and phosphocreatine can not be distinguished, hence the corresponding cross peaks represent the total creatine pool. The CH_2 and CH_3 of creatine were assigned using ^{13}C - ^1H cross correlation and by the exact matching of the carbon and proton chemical shifts of these peaks with the values obtained by recording the spectra of authentic compounds. The N- CH_2 and S- CH_2 groups of taurine were assigned to the peak at 3.44 ppm for ^1H and

38.15 ppm for ^{13}C and the peak at 3.28 ppm for ^1H and 50.23 ppm for ^{13}C , respectively.

Table 1. ^1H and ^{13}C chemical shifts of resonances from different metabolites present in the breast cancer cells^a

Seri al no.	Metabolites	Abbreviation in Fig.1	Carbon position	^1H multiplet ^b	^{13}C multiplet ^c	^1H chemical shift (ppm)	^{13}C chemical shift (ppm)
<i>Amino acids</i>							
1	Alanine	Ala	C2	q	m	3.79	53.24
			C3	d	m	1.49	18.89
2	Arginine	Arg	C5	t	s	3.26	43.26
3	Glu (GSH)	Glu (GSH)	C3	m	m	2.18	28.91
			C4	t	m	2.57	34.14
4	Cys (GSH)	Cys (GSH)	C2	t	s	4.58	58.40
			C3	m	s	2.97	28.25
			C2	s	s / m	3.79	46.10
			C2	t	m	3.79	56.90
4	Glutamate	Glu	C3	m	m	2.07, 2.13	29.58
			C4	t	m	2.36	36.22
			C4	m	m	2.46	33.56
5	Glutamine	Gln	C4	m	m	2.46	33.56
6	Glycine	Gly	C2	s	s / m	3.57	44.20
7	Isoleucine	Ile	C4-H ₃	d	s	1.02	17.39
			C5-H ₃	t	s	0.95	13.83
8	Leucine	Leu	C5	d	s	0.98	24.75
			C5'	d	s	0.97	23.67
9	Lysine	Lys	C5	m	s	1.74	29.12
			C6	t	s	3.02	41.95
10	Proline	Pro	C4	m	s / m	2.02	26.49
			C5	m	s / m	3.37, 3.44	48.85
11	Valine	Val	C4	d	s	1.05	20.68
			C4'	d	s	1.00	19.39
<i>Organic acids</i>							
12	Lactate	Lac	CH	d	m	4.12	71.22
			CH ₃	q	m	1.33	22.78
13	Succinate	Suc	CH ₂ -CH ₂	s	m	2.42	36.90
<i>Sugars</i>							
14	<i>m</i> -Inositol	mI	C1H, C3H	q	s	3.55	73.87
			C2H	t	s	4.07	74.92
			C4H, C6H	q	s	3.63	75.15
			C5H	t	s	3.29	77.09
			CH ₃	d	m	2.01	24.62
15	GlcNAc / GalNAc	GlcNAc / GalNAc	CH ₃	d	m	2.01	24.62
16	UDP- GlcNAc /UDP- GalNAc	UDP- GlcNAc /UDP- GalNAc	CH ₃	d	m	2.08	24.83

Table 1. (continued)

Serial no.	Metabolites	Abbreviation in Fig.1	Carbon position	¹ H multiplet ^b	¹³ C multiplet ^c	¹ H chemical shift (ppm)	¹³ C chemical shift (ppm)
<i>Membrane components</i>							
17	Choline	FC	OCH ₂	m	s	4.07	58.35
			NCH ₂	m	s	3.52	70.17
18	Phosphocholine	PC	OCH ₂	m	s	4.18	60.79
			NCH ₂	m	s	3.60	69.23
			N(CH ₃) ₃	s	s	3.23	56.75
19	Glycerophosphocholine	GPC	OCH ₂	m	s	4.33	62.17
			NCH ₂	m	s	3.68	68.74
			CH ₂ -O-P	m	m	3.89, 3.95	69.25
			CHOH	m	m	3.91	73.38
			CH ₂ OH	m	m	3.62, 3.68	64.76
<i>Nucleotides</i>							
20	UTP / UDP	UTP / UDP	C1H	d	m	6.00	91.14
			C2H	t	m	4.39	76.49
			C3H	t	m	4.38	72.39
			C4H	t	m	4.30	85.96
			C5H ₂	m	m	4.24	67.89
<i>Other Compounds</i>							
21	Creatine	Cr	CH ₂	s	s	3.94	56.63
			CH ₃	s	s	3.05	39.69
22	Taurine	Tau	N-CH ₂	t	s	3.44	38.15
			S-CH ₂	t	s	3.28	50.23

^a Abbreviations: GSH, glutathione; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

^b ¹H multiplets were obtained from ¹H-NMR spectra. These data have been reported by Sitter et al (Sitter et al. 2002).

^c ¹³C multiplets were obtained based on [U-¹³C]glucose labeling and 2D [¹³C, ¹H] HSQC spectroscopy.

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