Metabolomics by Proton Nuclear Magnetic Resonance Spectroscopy of the Response to Chloroethyl Nitrosourea Reveals Drug Efficacy and Tumor Adaptive Metabolic Pathways

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Abstract

Metabolomics of tumors may allow discovery of tumor biomarkers and metabolic therapeutic targets. Metabolomics by two-dimensional proton high-resolution magic angle spinning nuclear magnetic resonance spectroscopy was applied to investigate metabolite disorders following treatment by chloroethyl nitrosourea of murine B16 melanoma (n = 33) and 3LL pulmonary carcinoma (n = 31) in vivo. Treated tumors of both types resumed growth after a delay. Nitrosoureas provoke DNA damage but the metabolic consequences of genotoxic stress are little known yet. Although some differences were observed in the metabolite profile of untreated tumor types, the prominent metabolic features of the response to nitrosourea were common to both. During the growth inhibition phase, there was an accumulation of glucose (more than \( \times 10; P < 0.05 \)), glutamine (\( \times 3 \) to 4; \( P < 0.01 \)), and aspartate (\( \times 2 \) to 5; \( P < 0.01 \)). This response testified to nucleotide de novo synthesis down-regulation and drug efficacy. However, this phase also involved the increase in alanine (\( P < 0.001 \) in B16 melanoma), the decrease in succinate (\( P < 0.001 \)), and the accumulation of serine-derived metabolites (glycine, phosphoethanolamine, and formate; \( P < 0.01 \)). This response witnessed the activation of pathways implicated in energy production and resumption of nucleotide de novo synthesis, thus metabolic pathways of DNA repair and adaptation to treatment. During the growth recovery phase, it remained polyunsaturated fatty acid accumulation (\( \times 1.5 \) to 2; \( P < 0.05 \)) and reduced utilization of glucose compared with glutamine (\( P < 0.05 \)), a metabolic fingerprint of adaptation. Thus, this study provides the proof of principle that metabolomics of tumor response to an anticancer agent may help discover metabolic pathways of drug efficacy and adaptation to treatment.

Introduction

Although little applied in the cancer field yet, metabolomics, the latest functional genomics technique, is of great promise (1–3). Because it uses tools for mining biochemical information, which operate without preconception and at high throughput, metabolomics is a technique for discovery. First, it can provide metabolic biomarkers or fingerprints for cancer detection (4), staging, and prognosis. Second, it can provide biomarkers or fingerprints of the response to an anticancer agent and of the prediction of the response to a treatment as shown in the toxicology field (5). In these aims, biofluids easily obtainable in vivo (plasma and urine) may be analyzed, given the fact that the biofluid metabolome reflects the response of the whole organism to cancer or to anticancer therapy. Third, metabolomics can be applied to the discovery of tumor metabolic pathways. In this respect, metabolomics is done directly on tumor cells or tumor samples. This approach may be applied to investigate the mechanisms of response to treatment, treatment efficacy, or tumor adaptation to treatment. Practical implications are the identification of tumor biomarkers of the response and the proposal of complementary treatments to conventional anticancer agents.

Metabolomic analysis consists in identifying the largest set of metabolites visible to a technique and, as much as possible, to quantify them. It is presently acknowledged that nuclear magnetic resonance (NMR) spectroscopy is a first-line tool for metabolomics (1). One-dimensional proton NMR spectroscopy cumulates the advantages of being sensitive and quantitative, taking advantage of semiautomatic data processing (6). Two-dimensional NMR spectroscopy entered the field of metabolomics recently. This technique has the advantages of having improved metabolite specificity and solving most one-dimensional spectral superimpositions while keeping good sensitivity, especially in association with high-resolution coils, such as those using magic angle spinning (HRMAS) of samples (7, 8). This makes it possible to detect 20 to 40 metabolites in tissue samples (1).

In this article, we propose a novel exploitation of NMR spectroscopy data to do differential metabolomics of tumors (e.g., the comparison between tumor types and between before and after treatment). The technique was applied to in vivo tumor response to chloroethyl nitrosourea (CENU), an anticancer agent indicated in the treatment of melanoma and glioma, to provide the proof of principle that metabolomics may serve to unravel metabolic pathways associated with treatment efficacy or tumor adaptation to treatment.

In contrast to induced DNA damage, metabolic changes associated with tumor response to CENU have attracted little attention yet. However, DNA damage is followed by a genotoxic stress response involving cell cycle arrest and ATP/NAD+ consumption through activation of DNA repair processes (9, 10). This response may profoundly alter cellular metabolism so as to lead to cell death by apoptosis or necrosis (10). Cells may also survive through DNA repair processes, most of them consuming ATP, and nucleotide base synthesis following the activation of transcription factors (9), resistance, or adaptive mechanisms. In response to nitrosourea, increased phosphocreatine content was reported in primary glioma and attributed to tumor size reduction-related reoxygention (11, 12). Decreased sodium ion content in glioma tumors was attributed to treatment-induced Na-K-ATPase activation (12). Phospholipid metabolism alterations in melanoma...
tumors were described as participating in tumor cell differentiation and/or survival (7, 13). Alteration in fluoro-2-deoxyglucose uptake on positron emission tomography (PET) scans showed the involvement of glycolysis (14).

Metabolomics by proton NMR spectroscopy of the response of two tumor models in vivo (murine B16 melanoma and 3LL pulmonary carcinoma) to CENU revealed or strongly supported the involvement of still unreported metabolic pathways, accounting for drug efficacy but also adaptation to treatment of the investigated tumor types. Especially, the probable role of aminotransferases other than aspartate aminotransferase in tumor adaptation to nitrosourea is emphasized.

Materials and Methods

Chemicals and Standards

\(\text{N}^\alpha\text{-[2-chloroethyl]-N}[2-(methylsulfonyl)ethyl]-\text{N}^\beta\text{-nitrosourea (cystemusine; Orphachem, Clermont-Ferrand, France)}\) was the used CENU antineoplastic agent. Before administration, it was prepared as a 5 mmol/L solution in 0.9% NaCl. D3O was used as a solvent and locking medium for NMR spectroscopy. Histidine type IIa from calf thymus, \(\text{N}^\beta\text{-fucose (6-deoxy-\text{N}^\alpha\text{-galactose)}, and lysine (from Sigma, St. Louis, MO)}\) were used as standards for two-dimensional NMR spectroscopy correlation assignments.

Cell Cultures

The transplantable B16 (F1) melanoma cells and Lewis lung carcinoma cells (3LL) originating from C57BL6/6J Ico mice were obtained from ICIG (Villejuif, France) and adapted to grow in culture. The cells were maintained as monolayers in culture flasks using culture medium consisting of Eagle's MEM-GlutaMAX medium (Invitrogen, Carlsbad, CA).

Animal Model

Six- to 8-week-old C57BL6/6J male mice were purchased from IFFA CREDO (L'Arbresle, France). All procedures were approved by the Animal Experimental Ethical Committee. Mice were shaved before s.c. injections into their flank of \(5 \times 10^5\) tumor cells (B16 melanoma or 3LL pulmonary carcinoma cells). Tumors became palpable at days 8 to 10 after cell inoculation. Six groups of mice were done: two untreated groups (untreated B16 and untreated 3LL tumors) receiving sham injections of saline solution and four CENU-treated groups that received CENU at a dose of 15 mg/kg body weight, intratumorally, at days 11, 14, and 18 from cell inoculation (15). For each treated tumor type, two subsets of mice served for metabolite profiling of the growth inhibition phase and the growth recovery phase. Mice bearing untreated tumors were followed until day 25 because they died few days later.

Tumors were removed from day 14 to day 25 in the B16 (\(n = 8\) mice) and 3LL (\(n = 12\)) untreated tumor groups, from day 18 to day 28 in the B16 (\(n = 12\) and 3LL (\(n = 6\)) growth inhibition phase groups, from day 42 to day 53 in the B16 (\(n = 13\)) growth recovery phase groups, and from day 46 to day 56 in the 3LL (\(n = 13\)) growth recovery phase groups. Mice of each group were sacrificed. Tumors were dissected and weighed. A piece of the tumor \(<50\) mg or the whole tumor was frozen and stored at \(-80\text{°C}\) in preparation of NMR spectroscopy analysis.

Growth curves of all tumors were established between day 11 and the day of the animal sacrifice using percutaneous tumor diameter measurements by a caliper and the application of the ellipsoid formula to estimate tumor mass (15). The tumor weight measured after dissection correlated excellently with the mass calculation derived from percutaneous measurements (\(r = +0.95\)). The initial rate of the growth curve (\(\alpha\)) of untreated and posttreatment recovering tumors was calculated by fitting their initial growth curve to an exponential.

NMR Spectroscopy

NMR spectroscopy was done on a small bore Bruker DRX-500 magnet (Bruker, Karlsruhe, Germany) equipped with a HRMAS probe. All experiments were done on intact tumor samples that were set into 4-mm-diameter, 50-\(\mu\)L free volume ZrO2 rotor tubes without upper spacer. Rotors were spun at 4 kHz at room temperature. NMR spectroscopy acquisition and processing were run from a workstation.

One-dimensional proton NMR spectroscopy. The one-dimensional sequence was a saturation-recovery sequence with an 8-\(\mu\)s radiofrequency pulse, water signal suppression at low power, a 10 ppm spectral width, 8 K complex data points, a 10-s relaxation delay, and 32 repetitions. This resulted in a 6:05 min acquisition duration. After Fourier transformation, a baseline correction was applied using a spline algorithm in the spectral domain of interest.

Peak referencing was done on the signal of 3-trimethylsilyl-propionate sulfonate. Metabolites that did not give rise to correlation signals in two-dimensional spectra, namely those identified from their methyl group (total creatine \((\text{tCr} = \text{creatine + phosphocreatine})\) at 3.03 ppm, acetate at 1.92 ppm, dimethylglycine at 2.89 ppm, and phosphatidylcholine at 3.26 ppm), from their uncoupled methylene signal (glycine at 3.56 ppm and succinate at 2.40 ppm), or from other type of uncoupled hydrogen spins (formate at 8.45 ppm), were measured in the one-dimensional spectrum using deconvolution procedures (XWIN-NMR v2.6 software, Bruker; refs. 7, 16). The one-dimensional signal of these metabolites was standardized to the one-dimensional signal of taurine at 3.43 ppm and then multiplied by the ratio of the taurine cross-peak to the protein cross-peaks of the corresponding two-dimensional spectrum (see below). This made possible the standardization of one-dimensional signals to the same protein signal than two-dimensional NMR spectroscopy-resolved metabolites.

Two-dimensional proton NMR spectroscopy. The one-dimensional spectrum was immediately followed by a two-dimensional spectrum, itself by a second one-dimensional spectrum, to ensure global sample preservation. The used sequence was a two-dimensional total correlation spectroscopy (TOCSY) sequence (7, 13). It was done with water signal suppression at low power, with a 6 ppm spectral bandwidth along both frequency axes (256 samples along the first axis and 2 K samples along the second axis), a mixing time of 75 ms during which the spin-lock pulse train (DIPSI-2) was applied, a 1-s relaxation delay, and 16 repetitions. The two-dimensional spectrum duration was \(1:41\) h, a duration in excellent compatibility with the preservation of the sample in the magnet at room temperature (7).

For quantification, TOCSY spectra were phased along both frequency axes, baseline corrected using a low order spline function, and filtered at 1 Hz exponential in both dimensions. Cross-peak volumes (CPV) were integrated in the absolute intensity mode using the XWIN-NMR v2.6 software from a template of regions of interest. Rectangular measurement areas were chosen so as not to superimpose with any other and so as cross-peaks themselves do not superimpose with any other. In addition, CPVs were measured in \(1\)-noise-free areas and in the upper half plane of the spectrum where signals were unaffected by water signal suppression so that the following cross-peaks were used for quantification (the bar indicates that two cross-peaks of the same metabolite were integrated together): polyunsaturated fatty acid (PUF) at 2.79–5.33 ppm, \(\beta\)-glucose and \(\beta\)-glucose-6-phosphate (Glc) together at 3.25/3.49–4.65 ppm, lactate at 1.34–4.11 ppm, alanine at 1.47–3.77 ppm, glutamine at 2.12–2.46 ppm, glutamate at 2.06–3.76 ppm, aspartate at 2.70/2.80–3.89 ppm, asparagine at 2.88/2.95–3.99 ppm, hypotaurine at 2.63–3.35 ppm, taurine at 3.27–3.43 ppm, total glutathione (GSX = reduced glutathione + 2 \times\) oxidized glutathione; ref. 16) at 2.17–2.53 ppm, arginine at 1.68/1.92–3.23 ppm, leucine at 0.96–3.73 ppm, lysine at 1.90–3.77 ppm, methionine at 2.14/2.20–2.63 ppm, phenylalanine at 3.13–3.99 ppm, tyrosine at 3.05–3.94 ppm, threonine at 1.32–3.58 ppm, choline at 3.55–4.07 ppm, phosphocholine at 3.62–4.18 ppm, glycerophosphocholine at 3.66–3.94 ppm, phosphoethanolamine at 3.22–3.99 ppm (corrected for the phenylalanine contribution at 3.20–3.99 ppm using the above-mentioned phenylalanine contribution), and glycerophosphateethanolamine at 3.30–4.12 ppm.

Protein amino acid residue resonances were identified: protein lysyl \(\gamma\)- and alanyl \(\beta\)- at 1.40–4.30 ppm. Both cross-peaks were broad signals, wider than those of fatty acid correlations and those of fucose (17). The lysyl resonance is frequent in one-dimensional NMR.
spectra of plasma or intact tissue samples (18). The alanyl resonance can be seen in two-dimensional spectra of tumor samples (19, 20). In our study, the protein lysyl and alanyl resonances displayed similar high intensity in both tumor model two-dimensional spectra. Other protein resonances with quite strong intensities could be found, such as a seryl α–β broad resonance at 3.86–4.86 ppm and a threonyl β–γ broad resonance at 1.23–4.26 ppm. The protein origin of these resonances was finally verified on protein standards, such as histone type IIA, which spectra showed the four above-mentioned protein amino acid correlations. Fucose spectra showed a CH₂-CHOH correlation at 1.17–1.16 ppm near but separable from the alanyl cross-peak (data not shown). However, fucose was not present in significant amount in B16 melanoma and 3LL pulmonary carcinoma tumor as witnessed by lack of galactose resonances (e.g., correlations involving the C-1H resonance at 4.57 ppm) in two-dimensional spectra.

The protein lysyl signal was corrected for the free lysine signal. The latter was estimated to 2-fold the CPV of the measured correlation at 1.90 to 3.77 ppm from the analysis of free lysine standard. No significant polyamine signal (21) was found to contaminate the lysyl signal but could be subtracted similarly to that of free lysine. The ratio of a metabolite CPV to the sum of CPVs of the most intense protein resonances, alanyl and lysyl, was calculated as: CPVR (metabolite) = CPV (metabolite) / [CPV (alanyl) + CPV (lysyl)].

The obtained CPV ratio, CPVR(metabolite), thus, was proportional to a metabolite quantity per protein tissue (cell) content. Because lysyl and alanyl cross-peaks had similar intensities, these signals were simply added without weighting factors. CPVR(metabolite) was averaged for the untreated groups, growth inhibition phase groups, and growth recovery phase groups. Then, percentage variations of metabolites were calculated based on the data of the growth inhibition phase groups or the growth recovery phase groups and data of the untreated groups. In addition, percentage variations in metabolites were calculated between untreated tumor types (3LL tumors compared with B16 tumors).

Statistical Analysis

Data are presented as mean ± SD. Comparisons between CPVR(metabolite) of the growth inhibition phase or the growth recovery phase groups with the untreated group were done using the Mann-Whitney test. Principal component analysis using the Spearman’s rank correlation matrix was applied to the whole set of data to show the most contributive metabolic trends among groups.

Results

Growth curves of untreated and CENU-treated tumor models. The initial growth rate of tumors was 0.082 ± 0.038 day⁻¹ and 0.087 ± 0.050 day⁻¹ (P = not significant) in untreated B16 melanoma and 3LL pulmonary carcinoma, respectively (Figs. 1A and 2A).

CENU-treated B16 melanoma tumor proliferation curve exhibited two phases: the growth inhibition phase with a growth delay of 20 ± 5 days followed by the growth recovery phase. The growth rate during the growth inhibition phase was 0.006 ± 0.016 day⁻¹ and 0.053 ± 0.038 day⁻¹ during the growth recovery phase (P < 0.05 versus untreated).

CENU-treated 3LL tumors showed three phases: an initial proliferation from day 11 to day 14, excluded from the growth inhibition phase metabolome analysis, followed by the so-called growth inhibition phase with a growth delay of 22 ± 8 days, and a growth recovery phase. The growth rate during the growth inhibition phase was 0.017 ± 0.018 day⁻¹ and 0.063 ± 0.031 day⁻¹ during the growth recovery phase (P < 0.05 versus untreated).

Metabolite profiling of untreated 3LL tumors versus untreated B16 tumors. For the sake of simplicity, metabolites were clustered into five subsets (Table 1), although there may be intersubset partial covering. Glc and glutamine derivative clusters were supported by literature data on the most abundant labeled metabolites recovered after tumor cell exposure to labeled glucose or glutamine (22, 23). 3LL pulmonary carcinoma metabolite profiling was compared with that of B16 melanoma (Figs. 1, 2, and 3A). The glycolysis/tricarboxylic acid (TCA) cycle derivative subset was not altered; the glutamine derivative subset showed increased content in aspartate, asparagine, and proline in 3LL tumors (3LL versus B16, P < 0.01). The essential amino acid subset showed an increase in arginine and threonine in 3LL tumors (3LL versus B16, P < 0.01). The amino acid derivative subset showed a significant decrease in tCr in 3LL tumors (3LL versus B16, P < 0.001). The phospholipid derivative subset showed increases in choline and glycerophosphoethanolamine (3LL versus B16, P < 0.01), a dramatic increase in glycerophosphocholine (P < 0.001), and a decrease in phosphatidylcholine (P < 0.01) in 3LL tumors relative to B16 tumors.

Metabolite profiling of CENU-treated B16 melanoma tumors. During the growth inhibition phase (Figs. 1B, 2B, and 3B), the glycolysis/TCA cycle derivative subset showed an increase in Glc (P < 0.05) despite an unmodified lactate level. There was no significant change in acetate and PUF, but there was a decrease in succinate (P < 0.001), and alanine was strongly increased (P < 0.001). The glutamine derivative subset showed a dramatic increase in glutamine and aspartate (P < 0.001) and an increase in glutamate, asparagine, and proline (P < 0.01). The essential amino acid subset showed an increase in arginine and leucine (P < 0.001) and in lysine, methionine, phenylalanine, threonine, and tyrosine. The amino acid derivative subset showed a moderate but significant increase in dimethylglycine, glycine (P < 0.01), and taurine (P < 0.05) and a strong increase in tCr (P < 0.001) and unmodified hypotaurine and GSx levels. The phospholipid derivative subset showed an increase in glycerophosphocholine and phosphoethanolamine (P < 0.001), phosphatidylcholine (P < 0.01), glycerophosphoethanolamine, and phosphocholine (P < 0.05).

During the growth resumption phase, Glc still accumulated (P < 0.05) and acetate and PUF increased (P < 0.01). Alterations in the glutamate derivative subset were found with increased levels of glutamine and aspartate (P < 0.05) but with a decrease in glutamate levels (P < 0.05). The essential amino acid subset showed a return to untreated values for most species, except arginine (P < 0.01). The amino acid derivative subset showed a decrease in GSx (P < 0.05). The phospholipid derivative subset showed a decrease in glycerophosphocholine (P < 0.05) and a persistent increase in phosphoethanolamine and phosphatidylcholine (P < 0.01).

The Glc-to-glutamine CPVR ratio was calculated (Fig. 4A). During the growth inhibition and recovery phases, it was significantly increased (P < 0.05), indicating a utilization of glutamine larger than that of Glc.

Metabolite profiling of CENU-treated 3LL pulmonary carcinoma tumors. During the growth inhibition phase of 3LL pulmonary carcinoma tumors (Figs. 2C and 3C), the glycolysis/TCA cycle derivative subset showed an increase in Glc (P < 0.05), an unmodified lactate level, and an increase in acetate and PUF (P < 0.01). The glutamine derivative subset showed an increase in glutamine (P < 0.01), glutamate (P < 0.05), aspartate, and asparagine (P < 0.01) but a decrease in proline (P < 0.05). The essential amino acid subset showed an increase in leucine (P < 0.001), lysine, and methionine. The amino acid derivative subset showed a significant increase in tCr (P < 0.05) and formate (P < 0.01). The phospholipid derivative subset showed increases in choline (P < 0.001), phosphocholine (P < 0.05), phosphoethanolamine, and
phosphatidylcholine (P < 0.01) but a marked decrease in glycerophosphocholine (P < 0.001).

During the growth recovery phase, Glc was increased (P < 0.05) as well as acetate and PUF (P < 0.05). Glutamine derivatives returned to untreated values but proline decreased (P < 0.05). The essential amino acid subset showed a return to untreated values. The amino acid derivative subset showed a persistent increase in tCr (P < 0.05). The phospholipid derivative subset showed increases in choline (P < 0.01), phosphocholine, phosphoethanolamine, and phosphatidylcholine (P < 0.05) but a decrease in glycerophosphocholine (P < 0.001) and glycerophosphoethanolamine (P < 0.01).

In 3LL tumors, the Glc-to-glutamine CPVR ratio was significantly increased during both the growth inhibition and recovery phases (P < 0.05; Fig. 4A), indicating a persistent mismatch between Glc and glutamine utilization with respect to the untreated group.

**Metabolism general trends and metabolite covariations.**

Principal component analysis of the six data sets showed a first axis accounting for 30% of the variance that opposed, in the loads plot (Fig. 4B), glutamine, phosphoethanolamine, glycine, and other amino acid levels to the initial rate of proliferation. This axis was mainly explained by the growth inhibition phase as shown by the scores plot (Fig. 4C). The second axis accounted for 15% of the variance and opposed glycerophosphocholine levels to the set of acetate, PUF, and lactate levels. The third factor accounting for 11% of the variance opposed PUF to succinate.

The initial rate of proliferation of growth curves correlated negatively with glutamate, aspartate, and leucine levels (P < 0.05, Spearman’s rank correlation) and positively with succinate levels (P < 0.05).
Discussion

To our knowledge, this is the first metabolomics approach of the response of tumors to a chemotherapy agent. It provides the proof of principle that metabolomics may serve to unravel metabolic pathways of drug responsiveness and adaptive mechanisms to treatment.

Metabolomics is an integrated biochemistry technique. From the use of enzyme inhibitors, it was shown that upstream and downstream metabolites closely reflected the modulation of these enzymes (24). Tracer studies showing metabolites deriving from a common precursor can be used to put together metabolites into biochemically coherent subsets (22, 23) to assist interpretation.

With large amount of data, pattern recognition tools, such as principal component analysis, reveal precious to show covariations between metabolites and metabolism global trends retaining the largest part of information (1).

The used differential metabolomics technique involving quantitative two-dimensional proton NMR spectroscopy was innovative among the other proposed quantification procedures (6, 7, 13, 17, 25, 26). Because correlation signals in two-dimensional proton NMR spectroscopy are all variably related to concentration (19) and because no standardization of the signal has emerged, it remains a major aim in metabolomics to develop novel quantitative procedures. In this article, we propose to normalize the

![Figure 2](image.png)

**Figure 2.** A to C, growth curves and typical one-dimensional and two-dimensional \(^1\text{H}\) NMR spectra of 3LL pulmonary carcinoma. A, growth curves of untreated (white squares) and CENU-treated tumors during the growth inhibition phase (black squares) and the growth recovery phase (gray squares). CENU was given intratumorally at days 11, 14, and 18. Bars, SD. B, one-dimensional \(^1\text{H}\) NMR spectra. Bottom, untreated tumor; middle, growth inhibition phase; top, growth recovery phase. Inset, spectral reduction for the formate area. 4, succinate; 5, acetate; 19, tCr (CH\(_2\) group); 20, dimethylglycine; 21, formate; 22, glycine; 31, phosphatidylcholine; W, residual water signal. C, two-dimensional \(^1\text{H}\)-\(^1\text{H}\) NMR spectra. Bottom, untreated tumor; top, growth inhibition phase. 1, \(\beta\)Glc; 2, lactate; 3, alanine; 6, PUFA; 7, glutamine; 8, glutamate; 9, aspartate; 10, asparagine; 11, proline; 12, arginine; 13, leucine; 14, lysine; 15, methionine; 16, phenylalanine; 17, threonine; 18, tyrosine; 23, GSx; 24, hypotaurine; 25, taurine; 26, choline; 27, glycerophosphocholine; 28, glycerophosphoethanolamine; 29, phosphocholine; 30, phosphoethanolamine.
Metabolomics of Tumor Response to CENU

Table 1. Metabolites identified and quantified in the present metabolomics study, and significance of their variation in the treated tumor models

<table>
<thead>
<tr>
<th>Subset</th>
<th>Metabolite</th>
<th>Abbreviation</th>
<th>B16 INH</th>
<th>B16 REC</th>
<th>3LL INH</th>
<th>3LL REC</th>
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</thead>
<tbody>
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<td>Glycolysis/TCA cycle derivatives</td>
<td>1. Glucose + glucose-6-phosphate</td>
<td>Glc</td>
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<td></td>
<td>2. Lactate</td>
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<td>3. Alanine</td>
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<td></td>
<td>4. Succinate</td>
<td>Suc</td>
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<td>●●●●●●</td>
<td>●●●●●●</td>
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<td></td>
<td>5. Acetate</td>
<td>Ace</td>
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<td>●●●●●●</td>
<td>●●●●●●</td>
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<td></td>
<td>6. Polyunsaturated fatty acids</td>
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<td>Glutamine derivatives</td>
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<td>8. Glutamate</td>
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<td>9. Aspartate</td>
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<td>10. Asparagine</td>
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<td>11. Proline</td>
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<td>Essential amino acids</td>
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<td>13. Leucine</td>
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<td>14. Lysine</td>
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<td>16. Phenylalanine</td>
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<td>17. Threonine</td>
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<td>18. Tyrosine</td>
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<td>DMG</td>
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<td>21. Formate</td>
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<td>22. Glycine</td>
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<td>23. Total glutathione</td>
<td>GSt</td>
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<td>24. Hypotaurine</td>
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<td>●●●●●●</td>
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<td></td>
<td>25. Taurine</td>
<td>Tau</td>
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<td>●●●●●●</td>
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<td>Phospholipid derivatives</td>
<td>26. Choline</td>
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<tr>
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<td>27. Glycerophosphocholine</td>
<td>GPC</td>
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<td>28. Glycerophosphoethanolamine</td>
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<td></td>
<td>29. Phosphocholine</td>
<td>PC</td>
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<td>30. Phosphoethanolamine</td>
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<tr>
<td></td>
<td>31. Phosphatidylcholine</td>
<td>PC</td>
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NOTE: For the sake of simplicity, metabolites were organized in five subsets as indicated. On the right of the table, the Mann Whitney test result (P) is given for each metabolite together with the sign of the variation during the growth inhibition or the growth recovery phases of the response to CENU treatment in both tumor types (B16 and 3LL tumors). A series of six circles is affected to each metabolite: the first three circles indicate a decrease and the last three circles indicate an increase. ●, P < 0.05; ●●, P < 0.01; ●●●, P < 0.001.

Abbreviations: INH, growth inhibition; REC, growth recovery.

correlation signal of metabolites to the correlation signal of identified protein amino acid residues. It results that calculated CPVRs have an analogy with classic metabolite quantification expressed as metabolite tissue content relative to protein tissue content. The technique was applied for individual comparison of metabolites before and after treatment but also between tumor types.

Metabolic differences between untreated tumor types. There were some differences between untreated tumor types. Aspartate, asparagine, proline, and arginine were larger in 3LL tumors, consistent with increased glutamine utilization. In addition, 3LL tumors had decreased tCr content consistent with decreased transmethylation activity (16) or reduced ATP storage (11). Among phospholipid derivatives, glycerophosphocholine was increased and phosphatidylcholine was reduced, indicating increased hydrolysis of phosphatidylcholine by Ca$^{2+}$-independent phospholipase A2 (27). Extensive phosphatidylcholine biosynthesis from a collateral branch of glycolysis, the glycerol pathway, may explain this pattern. Pyruvate kinase, the key enzyme of net ATP production by glycolysis (28), could be down-regulated in untreated 3LL tumors, thus favoring Glc carbon flux toward phosphatidylcholine synthesis, lower tumor ATP production, and low tCr levels (28, 29).

Metabolic profiling of CENU-treated tumors during the growth inhibition phase. Tumor response to nitrosourea may be expected to involve profound metabolic disorders. After genotoxic stress, induced cellular responses are apoptosis, cell cycle retardation, and DNA repair. Cell cycle retardation interferes with phospholipid metabolism (27). Most mechanisms for the repair of CENU-induced DNA damage require ATP. Poly(ADP-ribose) polymerase may be hyperactivated by genotoxic stress and deplete the cell with NAD$^{+}$, its substrate, and ATP, a precursor of NAD$^{+}$...
ATP is produced at the last step of glycolysis by pyruvate kinase or by the burning of acetyl-CoA or α-ketoglutarate in the TCA cycle coupled to oxidative phosphorylation. NAD⁺ is required for oxidative phosphorylation and glycolysis. At present, the metabolic response to nitrosourea treatment has attracted little attention. However, reports of increased phosphocreatine content (11, 12) and increased activation of Na-K-ATPase (12) provide further evidence of alteration of ATP biosynthesis or utilization in targeted cells.

Our integrated interpretation of the metabolic response to CENU of the two tumor types is given in Fig. 5. It was limited to pathways about which we could provide the most convincing evidence of involvement. Taken together, our data suggested the following: (a) the depression of the nucleotide triphosphate/deoxynucleotide triphosphate (NTP/dNTP) de novo synthesis as shown by the accumulation of Glc, glutamine, glutamate, and aspartate, which followed the arrest of cell cycle and DNA replication (this response witnessed drug efficacy) and (b) the activation of aminotransferases other than aspartate aminotransferase to allow ATP production from the burning of α-ketoglutarate in the TCA cycle and to provide the cell with substrates (one-carbon units, glycine) for resumption of NTP/dNTP de novo synthesis (these activated

Figure 3. A to C, metabolite profiling of the differences between tumor types and of primary and growth recovery phases. A, metabolite profiling of the differences between 3LL and B16 tumors. Inset, log scale for metabolites with the largest variations. Lac, lactate; Ala, alanine; Suc, succinate; Ace, acetate; Glu, glutamine; Glu, glutamate; Asp, aspartate; Asn, asparagine; Pro, proline; Arg, arginine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Thr, threonine; Tyr, tyrosine; DMG, dimethylglycine; For, formate; Gly, glycine; hTa, hypotaurine; Tau, taurine; Cho, choline; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; PE, phosphoethanolamine; PC, phosphatidylcholine. Bars, SD. Vertical scale, percentage variation with respect to the untreated group. **, P < 0.01; ***, P < 0.001. B, metabolite profiling of the primary (black columns) and posttreatment (gray columns) responses of B16 melanoma tumors to CENU treatment. Inset, log scale for metabolites with the largest variations. Bars, SD. Vertical scale, percentage variation with respect to the untreated group. *, P < 0.05; **, P < 0.01; ***, P < 0.001. C, metabolite profiling of the primary (black columns) and posttreatment (gray columns) responses of 3LL pulmonary carcinoma tumors to CENU treatment. Inset, log scale for metabolites with the largest variations. Bars, SD. Vertical scale, percentage variation with respect to the untreated group. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
metabolic pathways witnessed DNA repair and adaptation to treatment).

The first prominent metabolic alteration during the growth inhibition phase was retention of Glc, glutamine, glutamate, and aspartate. Glc accumulation indicated reduced Glc carbon flux through glycolysis and/or biosynthetic pathways. This provides a rationale for decreased fluoro-2-deoxyglucose uptake on PET scans of nitrosourea-treated tumors (14) because glucose uptake depends on transmembrane glucose gradient. Glutamine, glutamate, and aspartate accumulation further testified to the down-regulation of pyrroate biosynthesis, coupled to ATP production, from either pyruvate kinase activity or malate efflux from the mitochondria and the activity of the malic enzyme. Pyruvate kinase is activated by the accumulation of phosphorylated glucose derivatives (28). This was found in our study, thus suggesting increased activity of pyruvate kinase under nitrosourea treatment, a hypothesis that we confirmed by enzyme activity measurements (data not shown). Another metabolite, tCr, was closely implicated in the energetic metabolism. tCr was increased in both tumor types under treatment and may indicate either increased substrate availability like methionine or increased ATP storage (11, 12). Because phosphocreatine is an emergency energy regulator, tCr level might increase to compete for ATP availability, thus preventing metabolic consequences of poly(ADP-ribose) polymerase activation and promoting cell survival (10). In addition, glutamine itself behaved as an apoptosis suppressor (30) and its accumulation during growth inhibition was protective for tumor cells.

The second prominent metabolic response accounted for decreased succinate and increased alanine, acetate, glycine, formate, dimethylglycine, phosphoethanolamine, choline, and phosphocholine and witnessed the activation of aminotransferases other than aspartate aminotransferase. Under treatment, oxaloacetate can be expected to be increased because it was no longer consumed to form aspartate. As a consequence, in B16 melanoma tumors, the malate flux was probably redirected outside mitochondria and participated in the formation of pyruvate, so-called glutaminolysis pathway (22). Alanine aminotransferase consumed pyruvate to form alanine that accumulated in treated B16 melanoma. This pathway participated in ATP production because it allowed partial burning of α-ketoglutarate into the TCA cycle. In contrast, in 3LL tumors, excessive oxaloacetate could be driven to citrate, acetate, and PUF production. The accumulation of glycine, formate, dimethylglycine, phosphoethanolamine, choline, and phosphocholine strongly suggested the activation of serine synthesis and metabolism. This hypothesis was further confirmed by the finding that derivatives of [13C-C2]Ser included large amounts of glycine, phosphoethanolamine, and phosphatidylethanolamine in CENU-treated B16 melanoma (data not shown). Phosphoserine aminotransferase consumed 3-phosphohydroxypyruvate to form phosphoserine, itself converted into serine. Serine was mostly metabolized by serine hydroxymethyltransferase into glycine and one-carbon units, and phospholipid base exchange into phosphatidylserine from phosphatidylcholine or phosphatidylethanolamine (31). This reaction released choline and ethanolamine, which were phosphorylated into phosphocholine and phosphoethanolamine by the strong activity of choline and ethanolamine kinases in tumor cells (32). Phosphatidylserine biosynthetic flux was continuously absorbed by decarboxylation into phosphatidylethanolamine.

**Figure 4.** A to C, Glc-to-glutamine ratio and principal component analysis. A, plot of the percentage variation in the Glc-to-glutamine CPRV ratio with respect to the untreated group. Data are given for B16 and 3LL tumor types. Bar, SD. *, P < 0.05. B and C, results of the principal component analysis applied to the whole set of data with variables, all measured metabolites plus the initial rate of proliferation for each tumor. Alp, α, initial rate of the growth curve. B, principal components loads plot (variables) and scores plot (individuals). Void circles, untreated B16 tumors; void squares, untreated 3LL tumors; full circles, growth inhibition phase CENU-treated B16 tumors; full squares, growth inhibition phase CENU-treated 3LL tumors; gray circles, growth recovery phase B16 tumors; gray squares, growth recovery phase 3LL tumors.
Pathways activated during growth inhibition may be considered as metabolic pathways of DNA repair, adaptation to treatment, and growth recovery. Here, our data strongly support the role of alanine or phosphoserine aminotransferase in tumor adaptation to nitrosourea, thus pointing out these enzymes as candidate therapeutic targets complementary to conventional treatment. This agrees with the report that phosphoserine aminotransferase expression was a marker of poor response to tamoxifen of breast cancers (33).

Increased PUF in 3LL tumors may reflect decreased consumption of fatty acids for phosphatidylcholine synthesis or increased fatty acid synthesis to cope with increased acetyl-CoA levels, consistent with increased acetate levels. Increased glycerophosphocholine (and glycerophosphoethanolamine) in B16 melanoma tumors indicated phospholipase A2 activation (27), most likely in relation with cell cycle arrest and excessive involvement of the cytidine diphosphate choline (cytidine diphosphate ethanolamine) pathway. The latter could result from phospholipid base exchange. Decreased glycerophosphocholine in 3LL tumors may be explained by the rerouting of phosphatidylcholine through phospholipid base exchange instead of hydrolysis by phospholipase A2. The accumulation of amino acids (arginine, leucine, lysine, methionine, phenylalanine, threonine, and tyrosine) showed additional metabolic disorders involving either transamination, glutaminolysis, glycolysis, pentose phosphate pathway, serinogenesis, malate; glutathione redox state (36). In addition, we previously showed from histopathology data that CENU-treated tumors during growth recovery exhibited decreased aggressiveness and redifferentiation features (15). Cell differentiation was reported to be associated with reduced GSx levels (37), decreased glycolysis, and increased fatty acid synthesis (38), in agreement with our findings.

An important finding of this study was that the balance between glucose and glutamine utilization was altered as evaluated by the Glc-to-glutamine ratio. This balance could be regulated by phosphoglycerate mutase (28). A down-regulation of this enzyme would account for Glc retention, serine synthesis, and glutamine consumption during growth recovery. Glc retention during this phase would also explain decreased fluoro-2-deoxyglucose uptake on PET scans. Metabolite variations during growth recovery provide a fingerprint of tumor adaptation to nitrosourea.

In conclusion, this article reports the first metabolomics approach of tumor response to a chemotherapy agent. It provides the proof of principle that metabolomics may allow identification of drug responsiveness and tumor adaptive metabolic pathways. Especially, our data point out the role of alanine and phosphoserine...
aminotransferases as candidate therapeutic targets to potentiate the cytotoxicity of nitrosoureas. Confirmation of the prominent metabolic pathways deduced from metabolomics may be achieved by enzyme tests or reverse transcriptomics or genomics. Applied to clinical material, tumor metabolomics offers the overall perspective of early prediction of responsiveness to anticancer drug and personalization of treatments, thus improvement in clinical and therapeutic decisions.

References

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Metabolomics by Proton Nuclear Magnetic Resonance Spectroscopy of the Response to Chloroethylnitrosourea Reveals Drug Efficacy and Tumor Adaptive Metabolic Pathways

Daniel Morvan and Aicha Demidem


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