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Short communication

METABOLOMIC PROFILING IDENTIFIES GLYCOLYSIS PATHWAY ASSOCIATION WITH ASPARAGINASE RESISTANCE IN ACUTE LYMPHOBLASTIC LEUKEMIA CELL LINES

PERFIL METABOLÔMICO IDENTIFICA ASSOCIAÇÃO DA VIA GLICOLÍTICA COM A RESISTÊNCIA À APARAGINASE EM LINHAGENS CELULARES DE LEUCEMIA LINFOBLÁSTICA AGUDA

Carolina Pereira de Souza Melo
Boldrini Children's Hospital, Campinas, SP, Brazil
https://orcid.org/0000-0001-9698-5817
carolinapsmelo@gmail.com

Rafael Renatino Canevarolo
Boldrini Children's Hospital, Campinas, SP, Brazil
https://orcid.org/0000-0002-8722-8512
Rafael.RenatinoCanevarolo@moffitt.org

Silvia Regina Brandalise
Boldrini Children's Hospital, Campinas, SP, Brazil
https://orcid.org/0000-0003-3696-9852
silvia@boldrini.org.br

Ana Carolina de Mattos Zeri*
Brazilian Biosciences National Laboratory (LNBio), Brazilian Center for Research in Energy and Materials (CNPEM), Campinas, SP, Brazil
https://orcid.org/0000-0003-4169-4523
ana.zeri@lnls.br

José Andrés Yunes*
Boldrini Children's Hospital, Campinas, SP, Brazil
Medical Genetics Department, Faculty of Medical Sciences, State University of Campinas, Campinas, SP, Brazil
https://orcid.org/0000-0002-1316-3525
andres@boldrini.org.br

**These Authors Jointly Coordinated the Work.*

ABSTRACT

Early response to therapy has consistently shown independent prognostic significance in pediatric Acute Lymphoblastic Leukemia (ALL) and can be attributed primarily to the intrinsic resistance/sensitivity of leukemic lymphoblasts to chemotherapy. In this study, our objective was to determine the metabolic profile after L-asparaginase (ASNase) treatment of cell lines resistant and sensitive to this drug by NMR metabolomics. ALL cell lines Nalm6, Nalm21, REH and RS4;11 were cultured in RPMI 1640 medium plus 10% FBS, supplemented or not with ASNase (0.8 IU/ml). After 24hs, NMR spectra of the culture medium were acquired and quantified. Unsupervised PCA analysis of the obtained metabolites concentrations showed that resistant cells culture medium are characterized by lower glucose and higher lactate levels. Interestingly, inhibition of the glycolysis pathway synergistically increased sensitivity of the resistant cell lines to ASNase (F_{syn} = 0.19 for Nalm6 and F_{syn} = 0.50 for REH) but not of the sensitive one (F_{syn} = 1.11 for Nalm21 and F_{syn} = 1.85 for RS4;11). In conclusion, this study shows a potential metabolomics approach for the identification not only of patients with possible resistance to treatment, but also of target molecules for the development of future therapeutic interventions.

***Keyword**: Metabolomics; Acute Lymphoblastic Leukemia; Asparaginase resistance.*

RESUMO

A resposta precoce à terapia na leucemia linfoblástica aguda (LLA) pediátrica pode ser atribuída principalmente à resistência intrínseca dos linfoblastos leucêmicos à quimioterapia. Neste estudo, nosso objetivo foi determinar o perfil metabólico de linhagens celulares resistentes e sensíveis a L-asparaginase (ASNase) após o tratamento com a mesma utilizando metabolômica por RMN. As linhagens celulares de LLA Nalm6, Nalm21, REH e RS4;11 foram cultivadas em meio RPMI 1640 com 10% de SFB, suplementado ou não com ASNase (0,8 UI/ml). Após 24hs, os espectros de RMN do meio de cultura foram adquiridos e quantificados. A análise de PCA não supervisionada das concentrações obtidas dos metabólitos mostrou que o meio de cultura de células resistentes é caracterizado por níveis mais baixos de glicose e mais elevados de lactato. Curiosamente, a inibição da via da glicólise aumentou sinergicamente a sensibilidade das linhagens celulares resistentes à ASNase (F_{syn} = 0,19 para Nalm6 e F_{syn} = 0,50 para REH), mas não das sensíveis (F_{syn} = 1,11 para Nalm21 e F_{syn} = 1,85 para RS4;11). Concluindo, este estudo apresenta uma potencial abordagem metabolômica para a identificação não apenas de pacientes com possível resistência ao tratamento, mas também de moléculas-alvo para o desenvolvimento de futuras intervenções terapêuticas.

Palavras-chave: Metabolômica; Leucemia Linfoblástica Aguda; Resistência à Asparaginase.

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1.INTRODUCTION

Early response to therapy has consistently shown independent prognostic significance in pediatric Acute Lymphoblastic Leukemia (ALL) and can be attributed primarily to the intrinsic resistance/sensitivity of leukemic lymphoblasts to chemotherapy. [1] A variety of cellular mechanisms can give rise to drug resistance, including increased expression of cellular transporters, changes in detoxification mechanisms, increased DNA repair process, reduced production of drug-targeted metabolites and changes in cell cycle regulation and apoptotic pathways. [2-4]

Metabolomics has been an important tool contributing to unravel these mechanisms, combining the use of analytical technologies for the identification and quantification of cellular metabolites with statistical methods of multivariate analysis for information extraction and data interpretation. [5, 6] The biochemical data obtained and interpreted using this approach provide a more comprehensive view of pathological processes than that obtained on the basis of isolated biological markers. In the last

decade, clinical metabolomics has seen a rapid growth in the number of studies aimed at finding diagnostic biomarkers and assess the response to therapy of specific diseases, [7-9] including leukemia. [10]

A correlation between higher glycolytic activity and either glucocorticoid or arabinofuranosyl cytidine (Ara-C) cell resistance has been shown previously for ALL [11] and AML patients, [12] respectively. In this study, we analysed the metabolic profile of the ALL cell lines resistance to L-asparaginase (ASNase), an effective drug utilized in the pediatric ALL remission induction therapy, [13, 14] and found that glucose consumption differs between ASNase sensitive and resistant cell lines. A possible synergistic effect between a glycolysis pathway inhibitor and ASNase was investigated.

2.MATERIAL AND METHODS

2.1 ALL cell lines metabolic profile

Cell lines for this study were selected based on the work developed by Fine and collaborators. [15] Human B-precursor ALL cell lines sensitive (Nalm21, Nalm29 and RS4;11) and resistant (Nalm6, 697 and REH) to ASNase were cultured at 2 x 10^6 cells/ml in the absence (control) or presence of ASNase (0.8 IU/ml; Merck Sharp & Dohme), in RPMI 1640 medium (Cultilab) supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin (PS; Sigma-Aldrich), at 37°C in a 5% CO_2 humidified atmosphere. Nalm21 cell line were kindly provided by Dr. Akira Harashima of Hayashibara Biochemical Labs in Japan. After 24 hours of incubation, 1 ml of the medium was collected, centrifuged, and the supernatant were kept frozen at -80°C for NMR analysis. All experiments were performed in triplicate.

2.2 Nuclear magnetic resonance (NMR) spectroscopy data acquisition

Approximately 0.6 ml of conditioned culture medium was thawed and filtered at room temperature through a Microcon YM-3 column (Millipore, Massachusetts, USA). Of this, 480 µl was mixed with 60 µl of deuterium oxide (D2O, 99.9%; Cambridge Isotope Laboratories Inc., Massachusetts, USA) and 60 ml of a 5 mM solution of the

internal reference sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS; Cambridge Isotope Laboratories Inc., Massachusetts, USA) in 100 mM phosphate buffer, pH 7.0. Next, 550 µl of sample was added to a 5 mm NMR tube. ^{1}H-NMR spectra of samples were acquired immediately using a Varian Inova NMR spectrometer (Agilent Technologies Inc., Santa Clara, USA) operating at a ^{1}H resonance frequency of 500 MHz and constant temperature of 298 K (25^{o}C). A total of 256 free induction decays (FIDs) were collected with 32-k data points over a spectral width of 16 ppm, 1.5-s relaxation delay and continual water presaturation radio frequency (RF) field. Data treatment and quantitative analyses were done using the software Chenomx NMR Suite 4.6 (Chenomx Inc., Edmonton, Canada). For peaks that were difficult to identify, a two-dimensional total correlation spectroscopy (TOCSY) was used with the same parameters applied in 1D acquisition.

2.3 Multivariate analysis

Metabolite concentrations were used in multivariate analysis in the form of unsupervised Principal Component Analysis (PCA) using the Pirouette 4.0 software (Infometrix Inc., Washington, USA). Data were auto-scaled, as this method of scaling applies equal weight to all variables regardless of their absolute value, thus ensuring that all variables retain equal importance during the generation of the model.

2.4 Glucose consumption assay

Glucose consumption was determined using the Glucose (HK) Assay Kit (Sigma-Aldrich), as described by the manufacturer. Briefly, 60,000 cells were cultured in a 9-well plate for four days under the following conditions: without drug (control), with ASNase (0.1 IU /ml or 1x10^{-6} IU/ml for ASNase resistant or sensitive ALL cell line, respectively) or with 2-deoxyglucose (2-DG) (1 mM or 0.5 mM for ASNase resistant or sensitive, respectively). After the incubation period, the plates were centrifuged and the medium was collected to measure the amount of remaining glucose.

2.5 In vitro drug resistance assay

ALL cell lines were treated with increasing doses of ASNase (ranging from 10^{-7} to 10^{-2} IU/mL and from 10^{-3} to 10 IU/mL for ASNase sensitive and resistant cell lines,

respectively), 2-DG (ranging from 10^{-3} to 10 mM), or a combination of both, using the methodology described by Hulleman and colleagues [11]. Briefly, 60,000 cells were grown in a 96-well plate for four days, with five different drug concentration. Cell survival was quantified by the in vitro 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) drug resistance assay, as previously described. [16]LC_{50} doses were determined through dose response curves as the drug concentration lethal to 50% of the ALL cell lines. The calculation of the synergy factor (F_{syn}) was performed according to the method described by Berenbaum, [17] where

F_{syn} = [LC_{50} Drug A combined with B]/[LC_{50} Drug A Alone] + [LC_{50} Drug B combined with A]/ [LC_{50} Drug B alone]

An F_{syn} value less than 1 indicates synergy between the two drugs.

3. RESULTS AND DISCUSSION

A total of 32 metabolites were identified in the ALL cell lines culture media using ^{1}H-NMR spectroscopy. Unsupervised PCA analysis of the obtained metabolites concentrations grouped the samples into four clusters (Figure 1A). Cells cultured without ASNase clustered on the left side, while cells cultured in presence of the chemotherapeutic agent located on the right side. Compounds responsible for this stratification are shown in the loading plot. As expected, higher concentrations of asparagine and glutamine in the culture medium segregated control from treated samples, while aspartate and glutamate were enriched in the culture media added with ASNase. ASNase resistant cell lines clustered on the upper right diagonal while the sensitive ones clustered on the opposite side. The culture medium of drug-resistant cells contained higher concentrations of pyroglutamate, lactate, and 5,6-dihydrothymine, while the culture medium of sensitive cells has higher concentrations of glucose, 2-oxoisocaproate and 3-methyl-2- oxovalerate.

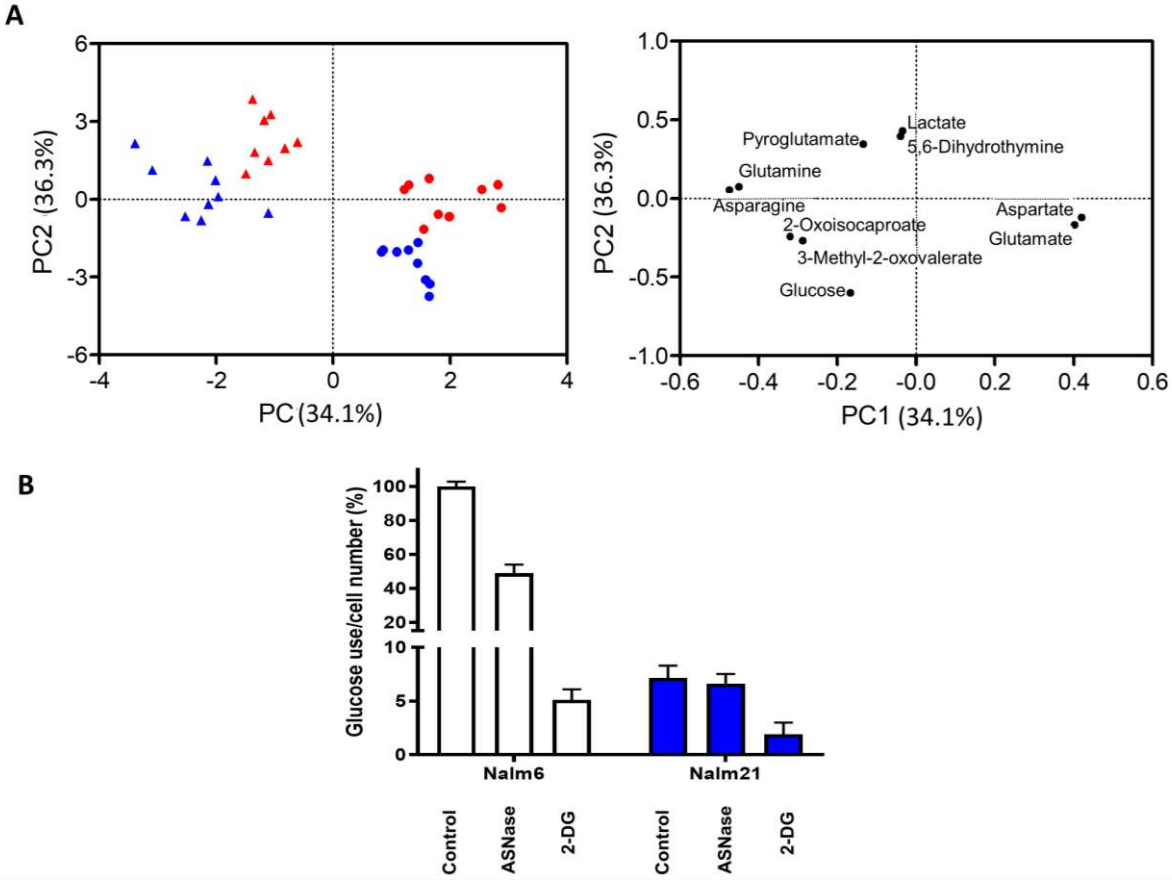


Figure 1. Score and loading plots of PCA analyses and relative glucose consumption of cell lines in different conditions.

(**A**) Score plot (left) shows samples clustered according to treatment (left side = without ASNase; right side = with ASNase) and ASNase sensitivity (blue = sensitive; red = resistant). Metabolites that contributed most to the observed clustering are labeled in the loadings plots (right). (**B**) ASNase resistant cell line (white) consumed more glucose than a sensitive one (blue), irrespectively of the treatment. Nalm6 consumption without treatment (control) was considered 100%. Glucose consumption of each cell line was divided by the respective number of viable cells in the end of the experiment.

Higher lactate and lower glucose concentrations in the resistant's cell culture medium suggested that ASNase resistance could be associated with higher glycolytic activity, as previously observed. [18] To confirm this association, we measured glucose consumption by the different cell lines, under different conditions. As shown in Figure 1B, ASNase resistant cell line Nalm6 showed higher glucose consumption than the sensitive Nalm21 either with or without ASNase (control). Although glucose uptake decreased in Nalm6 cells treated with ASNase, it was still significantly higher than

Nalm21 consumption. This inhibition of glucose uptake in the presence of ASNase has been observed before. [19] We also detected a significant reduction of glucose consumption for both cells when the glycolysis inhibitor 2-DG was added to the culture medium.

If resistance to ASNase depends on the glycolytic pathway, then inhibition of glycolysis is expected to make the cell more sensitive to this drug. To test this hypothesis, ALL cells were treated with increasing doses of ASNase, 2-DG or with a combination of both, and a possible synergistic effect between ASNase and the glycolysis inhibitor was evaluated. Figure 2 shows that inhibition of the glycolysis pathway synergistically increased sensitivity of the resistant cell lines to ASNase (F_{syn} = 0.19 for Nalm6 and F_{syn} = 0.50 for REH) but not that of the sensitive one (F_{syn} = 1.11 for Nalm21 and F_{syn} = 1.85 for RS4;11). In other words, glucose consumption seems to be an important factor in the resistance of cells to ASNase.

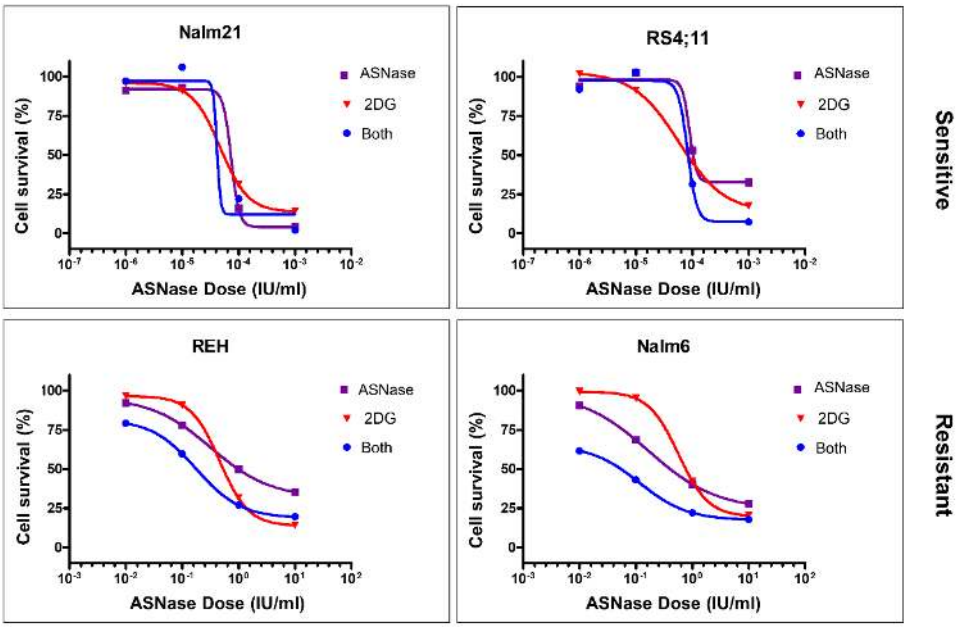


Figure 2. Dose-response curves for ASNase and 2-DG treatments.

A synergistic effect between both drugs was observed for ASNase resistant (F_{syn} = 0.19 for Nalm6 and F_{syn} = 0.50 for REH) but not for the ASNase sensitive cell lines (F_{syn} = 1.11 for Nalm21 and F_{syn} = 1.85 for RS4;11).

Besides causing depletion of asparagine, an amino acid poorly synthesized by leukemic cells, [20] ASNase has also a glutaminase activity that causes a sharp drop in

the glutamine concentration [21] leading to a cell metabolic reprogramming. [19] Indeed, unsupervised PCA data analysis of ALL cell lines conditioned culture media showed that, after ASNase treatment, the concentrations of these amino acids (asparagine and glutamine) decreased resulting in increased glutamate and aspartate concentrations, compared to cells without treatment. Anaerobic glycolytic metabolism even under normoxia conditions is a hallmark of cancer [22] and a recent study has shown that lymphoid leukemia cell lines and primary cells sensitive to ASNase are characterized by low glycolytic activity. [18] We found higher glucose and lower lactate levels in the culture medium of sensitive compared to the resistant cells, either with or without ASNase treatment. As one of the consequences of anaerobic glycolysis is the acidification of the extracellular environment by excess lactic acid, our results corroborate the association of ASNase cell resistance and higher glycolytic activity. In addition, tests with the glycolysis pathway inhibitor 2-DG, functionally confirmed that a difference in the glycolytic metabolism of cells would be involved in their resistance to ASNase. The idea of using glycolytic inhibitors as anticancer agents is not new.[23] In fact, in vitro studies obtained promising results using combinatory treatment with traditional metabolic inhibitors, [24, 25] while new drugs developed with this same objective are in preclinical phase.[26]

4. FINAL CONSIDERATIONS

In conclusion, our results indicated that it is possible to distinguish resistant and ASNase sensitive cell lines using NMR spectra of the culture medium of these cells, even without any treatment. ^{1}H NMR analysis of biofluids such as urine and blood plasma is quite sensitive (micromolar), is non-invasive and requires minimal sample pretreatment, in addition to requiring small sample volume (500-20µL) and short data acquisition time (6 minutes per sample). [27] Although detection of low-abundance metabolites and overlapping resonances, which sometimes turns metabolite identification and quantification into a challenging task, are still limitations for today's NMR analyses, recent improvements resulted in new NMR technologies and techniques that might compensate, at least in part, these drawbacks. [28, 29]

Furthermore, this study shows a potential metabolomics approach for the identification not only of patients with possible resistance to treatment, but also of target molecules for the development of future therapeutic interventions.

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