NMR-based metabolite studies with $^{15}$N amino acids

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$^{15}$N labeled amino acids are routinely used to label proteins or nucleic acids for study by NMR. However, NMR studies of $^{15}$N labeled amino acids in metabolite studies have not been pursued extensively, presumably due to line broadening present under standard experimental conditions. In this work, we show that lowering the temperature to $-5 \, ^\circ C$ allows facile characterization of $^{15}$N-labeled amino acids. Further, we show that this technique can be exploited to measure $^{15}$NH$_3$ produced in an enzyme catalyzed reaction and the transport and metabolism of individual amino acids in mammalian cell culture. With respect to $^{13}$C-labeled amino acids, $^{15}$N-labeled amino acids are less costly and enable direct characterization of nitrogen metabolism in complex biological systems by NMR. In summary, the present work significantly expands the metabolite pools and their reactions for study by NMR.

The use of $^{15}$N-labeled amino acids for the study of biomolecular structures, interactions and dynamics is very well established. In these studies, $^{15}$N-labeled proteins, RNA or DNA are routinely labeled by preparation in Escherichia coli grown in media containing $^{15}$NH$_4^+$, $^{15}$NH$_3$. In contrast, $^{15}$N-labeled amino acids have not been used extensively for NMR-based metabolite studies. Presumably this stems from the extensive line-broadening of HN signals due to water exchange in aqueous samples at room temperatures (e.g. the exchange rates of NH$_3$ groups with water are $\sim 100/s$), which render the HN-$^{15}$N group undetectable. On the other hand, $^{13}$C-labeled amino acids have been used extensively for NMR-based metabolite studies. For example, $^{13}$C-labeled glutamine is routinely used to characterize TCA cycle intermediates. Recently, Beecher and Larive demonstrated that the HN-$^{15}$N signal of glucosamine is observable at temperatures $< 0 \, ^\circ C$ with the addition of organic solvent to obviate freezing. In another study, Millard et al. observed the HN-$^{15}$N signals of hydrolyzed amino acids prepared from $^{13}$C/$^{15}$N-labeled algal and E. coli extracts. In the present work, we present NMR-based studies of commercially available $^{15}$N-labeled amino acids for biochemical analyses and characterization of amino acid transport and nitrogen metabolism in mammalian cells.

As a first step toward analyzing the potential use of $^{15}$N-labeled amino acids in NMR-based studies of metabolites, we used $^{15}$N-labeled Asn, which contains NH$_3^+$ and NH$_2$ protons that are in fast and slow exchange, respectively. For these experiments we chose acidic conditions, for which HN exchange rate is minimized, and 20% acetone co-solvent, which enables studies below the freezing point of H$_2$O. As shown in Fig. 1, only the NH$_3$ groups are readily observed in the $^{15}$N-filtered NMR spectra at temperatures ranging from 5–25 $^\circ C$. In contrast, the NH$_2^+$ group is observable at $-5 \, ^\circ C$, consistent with previous observations of the exchangeable NH$_2^+$ group in glucosamine under similar experimental conditions. Consequently, subsequent studies of exchangeable HN groups were performed under conditions of low temperature and low pH.

In the next step, we assayed the ability to detect amino acid metabolism in the media from cultured cells. L-Asparaginase (ASNase) is a FDA-approved therapy for the treatment of acute lymphoblastic leukemia. Moreover, preclinical and clinical data have shown that ASNase is a promising therapy for other hematological cancers and select solid tumors. ASNase treatment causes depletion of Asn in the blood by conversion to Asp plus NH$_3$. Accordingly, we assayed the metabolism of $^{15}$N-Asn in cell cultures of LOUCY cells, a model leukemia cell line, as a function of ASNase treatment. As shown by the blue correlations in Fig. 2, the $^{15}$NH$_3^+$ group of Asn is readily observed in media after 60 min in the absence of ASNase (the NH$_2$ groups, which are normally observed at $\sim 115$ ppm are relatively weak and appear at an incorrect $^{15}$N chemical shift in this spectrum due to spectral aliasing). As shown by the red correlations in Fig. 2, treatment with ASNase for 60 min results in quantitative transformation of $^{15}$N-Asn to $^{15}$N-Asp and $^{15}$NH$_3^+$ and thus we are measuring the activity of the enzyme in the media (NH$_3$, the product of ASNase catalysis of Asn, is in equilibrium with NH$_4^+$, which is the predominant species at acidic pH).

Lastly, we assayed the ability to characterize amino acid transport in cell culture by $^{15}$N NMR. For these experiments a mixture of 4 $^{15}$N-labeled amino acids (Asp, G1y, Leu and Ser) was added to the media and the presence
Figure 1. $^{15}$N-filtered 1D spectrum of $^{15}$N-Asn as a function of temperature. Experimental conditions were 10 mM $^{15}$N-Asn in 100 mM $^2$H-acetate/pH 4.0, 10% $^2$H$_2$O, 20% $^2$H-acetone.

Figure 2. $^{15}$N-edited HSQC of $^{15}$N-Asn in the presence (red) and absence (blue) of ASNase. Amino acid labels correspond to the one letter code. Buffer conditions were 100 mM $^2$H-acetate/pH 4.0, 10% $^2$H$_2$O, 20% $^2$H-acetone.
of 15N-labeled groups was assessed in the media and LOUCY cell extracts. Consequently, the media samples assay the amino acid stability and the cell extract samples assay the amino acid transport and stability. As shown in Fig. 3a, the 15NH3+ of the 4 amino acids are readily observed in the media at time = 0. As shown in Fig. 3b, the 4 amino acids are still observed after 60 min and there are no new correlations observed suggesting that they are not metabolized in the media. Interestingly, as shown in Fig. 3c, the 15N-metabolite profile in the cell extracts taken at time = 60 min was very different from that of the media. First note Gly and Ser are readily observed suggesting that they are being transported into the cells. In contrast Asp and Leu are observed at much lower concentrations, suggesting that they are transported at relatively low efficiency and/or being metabolized into other species. Surprisingly, the cell extracts contain a new 15N correlation corresponding to Glu, which is clearly absent from the media, suggesting an intracellular transaminase reaction. We note that aspartate transaminase, which catalyzes the interconversion of Asp ⇔ a-ketoglutarate ⇔ oxaloacetate + Glu and is present in a wide variety of tissues13, is one potential explanation. Alternatively, the branched amino acid transaminase, which catalyzes the interconversion of Leu ⇔ 2-oxoglutarate ⇔ 4-methyl-2-oxopentanoate + Glu and is upregulated in numerous cancers14,15, is an alternative explanation.

In summary, we have demonstrated the ability to observe 15N-labeled amino acids and 15NH4+, a surrogate for NH3, in cell culture by NMR. Based of the results presented herein (Figs 2, 3 and the chemical shifts summarized in Table 1), at least 6 amino acids and NH4+ are sufficiently resolved in the HSQC spectra to be used in future NMR-based metabolite experiments. We note that signal intensity and linewidth may be expected to be further improved through the use of the HSQC sequence, which removes N2 relaxation during nitrogen evolution, and the use of an external D2O standard for the NMR lock signal, which removes cross-peak asymmetry4. Nonetheless, we have exploited the HSQC technique at low pH and low temperature to characterize nitrogen metabolism and amino acid transport. Importantly, the observation of a novel transaminase activity in leukemia cells may suggest a potential metabolic vulnerability to be exploited. Previously 13C-labeled amino acids have been extensively used to study amino acid metabolism5; however, 15N-labeled amino acids are ~4X less expensive and enable direct studies of nitrogen metabolism. With respect to Mass Spectrometry (MS) studies, NMR offers a number of advantages including easy quantitation, the ability to re-use samples for other analyses, the ability

![Figure 3](https://www.nature.com/scientificreports/)

**Figure 3.** Metabolite profiles of 15N-labeled Asp, Gly, Leu and Ser added to LOUCY cells, as assayed by 15N-edited HSQC of the media at time = 0 (a), the media at time = 60 min (b) and the cell extract at time = 60 min (c). Amino acid labels correspond to the one letter code. In all experiments, the buffer conditions were 100 mM 2H-acetate/pH 4.0, 10% 2H2O, 20% 2H-acetone.

| Compound | 1H (ppm) | 15N (ppm) |
|----------|----------|-----------|
| Asn      | 7.98     | 39.81     |
| Asp      | 7.94     | 39.09     |
| Glu      | 8.08     | 40.65     |
| Gly      | 7.81     | 30.33     |
| Leu      | 7.82     | 40.02     |
| Ser      | 7.95     | 35.96     |
| NH4+     | 7.25     | 20.46     |

**Table 1.** Summary of chemical shifts. Experimental conditions were 20% d-acetone, 100 mM d-acetate/pH 4.0 and 10% 2H2O at −5°C.
to identify novel metabolites using other NMR experiments (e.g. COSY, ROESY), and the facile interpretation of data (e.g. a typical LC-MS study of cell extracts yields >10,000 signals versus the few signals observed in the 15N-edited HSQC spectrum). Nonetheless, MS is clearly much more sensitive and thus more desirable in sample limited situations (e.g. patient biopsy or the study of metabolites at very low concentrations). Finally, we note that the NMR-based technique could be easily extended to in vivo studies of amino acids and nitrogen metabolism. For example, non-toxic 15N-labeled compounds, which could include amino acids, nucleotides or ammonium, are readily introduced into animals either in the diet or by IV delivery, as previously shown by MS studies.

Methods

Isotope-labeled compounds. 15N-labeled amino acids were purchased from CIL; 2H-acetate and 2H-acetone were purchased from Sigma.

Preparation of media and cells. For the analysis of media and cell extracts, the T-cell acute lymphoblastic leukemia suspension cell line LOUCY12 was used. Cells were harvested and re-suspended to 1×10⁶ cells/mL of fresh RPMI 1640 media supplemented with 10% FBS. Cells were allowed to acclimate to new media for 1 hour in a humidified 37°C incubator. In the enzyme treated flasks, 1 IU/mL of Era-TM ASNase12 was added. After 1 hour in the incubator, either 15N-Asn or a mixture of 15N-Asp, Gly, Ser and Leu was added to a final concentration of 500µM of each. For media samples, 800µL of media was spun for 5 minutes at 1k RCF at 4°C. 500µL of supernatant was transferred to a clean 1.5 mL Eppendorf tubes and stored at −20°C. 1 hour after adding the 15N-amino acids mixture, each flask was harvested, chilled on ice and centrifuged for 5 minutes at 1k RCF. The cells were re-suspended in 1mL of cold DPBS, centrifuged for 5 minutes at 1k RCF. The supernatant was discarded and the cell pellet and conditioned media were stored at −20°C.

Preparation of NMR samples. Samples of media +−ASNase were prepared directly by diluting media into buffer with final concentrations: 25% media, 20% d-acetone, 100mM d-acetate/phosphate buffer (pH 4.0 and 10% 2H2O). Media with individual 15N amino acids was prepared by adding 1mL of 100% ice cold methanol to 0.2mL media, incubating on ice for 30min, centrifugation (15min at 10k), 4 hours of lyophilization, and suspension in buffer (20% d-acetone, 100mM d-acetate/phosphate buffer (pH 4.0 and 10% 2H2O)). Cell extracts were prepared by resuspension of cells in 80% ice cold methanol, incubating on ice for 30min, centrifugation (15min at 10k), 4 hours of lyophilization, and suspension in buffer (20% d-acetone, 100mM d-acetate/phosphate buffer (pH 4.0 and 10% 2H2O)).

NMR experiments. NMR experiments were performed on a Bruker AVANCE 800 MHz spectrometer with a liquid nitrogen cooling unit to enable studies below 5°C and using a TXI room temperature probe. For detection of HN−15N correlations, a standard HSQC sequence with water suppression by flip back pulses was employed on samples in 3 mm NMR tubes with a final volume of 200µL. Total experimental times ranged from 0.5 to 2 hours. Chemical shifts were calibrated using DSS and indirect referencing of 15N17. The effects of low temperature and acetone on the chemical shifts were observed to be minimal (<0.04 ppm). Assignments for the 1H−15N correlations were obtained from the analysis of individual reference compounds (15N-Asn, 15N-Asp, 15N-Glu, 15N-Gly, 15N-Ser, 15N-Leu and 15NH4Cl).

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Author Contributions
B.R., A.L. and M.C. designed the research. M.D. prepared the enzyme and cells. B.R. and M.C. performed the NMR experiments. B.R. and M.C. wrote the manuscript and B.R., M.D., A.L. and M.C. discussed and improved the manuscript.

Additional Information
Competing Interests: The authors declare no competing interests.

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