Hyperpolarized $^{15}$N-pyridine Derivatives as pH-Sensitive MRI Agents

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Highly sensitive MR imaging agents that can accurately and rapidly monitor changes in pH would have diagnostic and prognostic value for many diseases. Here, we report an investigation of hyperpolarized $^{15}$N-pyridine derivatives as ultrasensitive pH-sensitive imaging probes. These molecules are easily polarized to high levels using standard dynamic nuclear polarization (DNP) techniques and their $^{15}$N chemical shifts were found to be highly sensitive to pH. These probes displayed sharp $^{15}$N resonances and large differences in chemical shifts ($\Delta \delta \sim 90$ ppm) between their free base and protonated forms. These favorable features make these agents highly suitable candidates for the detection of small changes in tissue pH near physiological values.
Derivatives were chosen for evaluation because it is known that their imaging agents are highly sensitive to pH changes. Pyridine of pH probes improve the 15N NMR sensitivity of pH-sensitive pyridine derivatives. A shortcoming, we investigated DNP hyperpolarization as a means to very limited mainly due to its poor sensitivity. To overcome such limitations, the role of 15N NMR in molecular imaging is lent sensing capability, the role of 15N NMR in molecular imaging is very limited mainly due to its poor sensitivity. To overcome such shortcomings, we investigated DNP hyperpolarization as a means to improve the 15N NMR sensitivity of pH-sensitive pyridine derivatives. Our results showed that these pyridine derivatives can be well polarized by DNP with high 15N signal enhancements. After dissolution into water, the 15N signal enhancements of 5437 gpd 200, 8702 ± 700, 7877 ± 400, 10065 ± 600, and 3598 ± 700 were observed for 15N-pyridine, 15N-2,6-lutidine, 15N-2-picoline, 15N-2,4,6-collidine and 15N-nicotinamide, respectively. This level of enhancement is comparable to previously reported values for aromatic 15N compounds. The observed higher polarization of methyl-substituted pyridine compounds was likely due to their greater tendency to form frozen glasses when mixed with the DMSO/Sulfolane glassing agent. 15N NMR spectra of hyperpolarized and thermal samples of 15N-pyridine are compared in Fig. 2a. Here, the 15N NMR spectra of the HP sample was collected from a single 10-degree pulse while the thermally polarized spectra represent a total signal from 256 scans using 90-degree pulses. It is important to note that both the HP and thermally polarized spectra were acquired from the same sample. The big difference in signal intensity demonstrates the tremendous signal enhancement achieved by DNP hyperpolarization.

Fig. 2b shows an array of representative 15N NMR spectra of HP 15N-pyridine collected every 5 s using a 5-degree pulse after dissolution with water. By fitting such time-dependent 15N signal intensity decay curves (Fig. 2c), the T1 of HP 15N-pyridine (pH 8.4), 15N-2,6-lutidine (pH 9.5), 15N-2-picoline (pH 8.5), and 15N-nicotinamide (pH 5.9) were found to be 41 ± 3 s, 31 ± 2 s, 38 ± 2 s and 22 ± 0.3 s, respectively (Supplementary Fig. S1 online). Due to poor water solubility, dissolution of 2,4,6-collidine required the use of methanol. Here, the measured T1 was 36 ± 4 s. The presence of methyl substituents on the pyridine ring results in a larger number of protons in close proximity to the nitrogen which results in more efficient dipole-dipole relaxation of the pyridyl 15N. Nevertheless, these T1 values are comparable to many 13C carboxyl groups at this same field currently used for metabolic imaging. The hyperpolarized 15N signal of pyridyl derivatives decayed more rapidly near physiological pH (Fig. 3a), likely reflecting an added relaxation pathway by the exchanging protons as these molecules become partially protonated. As expected, the T1 of 15N-pyridine measured in plasma at 37 °C was shorter (11 s, Fig. 3b). While undesirable, this should not prevent the in vivo application of 15N-labeled pyridine derivatives as pH probes because protonation equilibria are established nearly instantaneously. If we assume that the T1 of the 13C atoms in these molecules were only ~10 s.

Results and Discussion

15N-NMR signal enhancement and spin-lattice relaxation time of pH probes. In this study, we aim to develop HP spectroscopic imaging agents that are highly sensitive to pH changes. Pyridine derivatives were chosen for evaluation because it is known that their 15N resonances have relatively long T1 and T2. The pH sensitivity of HP 15N-enriched pyridine derivatives is optimal for tissue pH measurements and can also be altered considerably by adding substituents. Therefore, it is highly possible that pyridine derivatives having suitable pKa values for any specific pH measurement applications can be designed and synthesized. Despite the excellent sensing capability, the role of 15N NMR in molecular imaging is very limited mainly due to its poor sensitivity. To overcome such shortcomings, we investigated DNP hyperpolarization as a means to improve the 15N NMR sensitivity of pH-sensitive pyridine derivatives. Our results showed that these pyridine derivatives can be well polarized by DNP with high 15N signal enhancements. After dissolution into water, the 15N signal enhancements of 5437 ± 200, 8702 ± 700, 7877 ± 400, 10065 ± 600, and 3598 ± 700 were observed for 15N-pyridine, 15N-2,6-lutidine, 15N-2-picoline, 15N-2,4,6-collidine and 15N-nicotinamide, respectively. This level of enhancement is comparable to previously reported values for aromatic 15N compounds. The observed higher polarization of methyl-substituted pyridine compounds was likely due to their greater tendency to form frozen glasses when mixed with the DMSO/Sulfolane glassing agent. 15N NMR spectra of hyperpolarized and thermal samples of 15N-pyridine are compared in Fig. 2a. Here, the 15N NMR spectra of the HP sample was collected from a single 10-degree pulse while the thermally polarized spectra represent a total signal from 256 scans using 90-degree pulses. It is important to note that both the HP and thermally polarized spectra were acquired from the same sample. The big difference in signal intensity demonstrates the tremendous signal enhancement achieved by DNP hyperpolarization.

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PH sensitivity of HP 15N-pyridine derivatives. 15N NMR has long been known to have a great sensing capability for different chemical environments such as acidity and metal ion concentrations. Protonation or metal chelation of some nitrogen-containing compounds can lead to a significant change in 15N chemical shift, making them suitable MR sensors for pH or metal ions. For pyridine derivatives, the electronic properties of the sp2-hybridized, aromatic nitrogen center are greatly altered upon protonation leading to a significant change in

Figure 1 | 15N NMR spectra of HP-pyridine as the free base (green) and fully protonated (red) forms. The large chemical shift difference demonstrates that pyridine derivatives may serve as ultra-sensitive pH probes. The two spectra were acquired separately from HP-pyridine samples dissolved in either base or acid.
15N chemical shift. 15N NMR spectra of HP15N-pyridine samples adjusted to different pH values are shown in Fig. 4a. All five 15N-pyridine derivatives shift upfield upon protonation (Fig. 4b). The 15N NMR linewidth of each probe was narrow in both the fully protonated or fully deprotonated forms but broaden somewhat at pH values near their respective pKₐ's. This broadening reflects intermediate rates of exchange of protons between the 15Na atom and water. The pKₐ values estimated by fitting the pH titration curves were 5.17 ± 0.07 for pyridine, 6.60 ± 0.02 for 2,6-lutidine, 7.65 ± 0.05 for 2-picoline, and 4.14 ± 0.02 for nicotinamide. These pKₐ values agreed well with values previously reported. The 15N chemical shift differences between the free base and fully protonated forms were all in the range, 88–94 ppm (Table 1). The chemical shifts of all five 15N enriched compounds changed linearly with pH at least over 6 pH units of each ligand pKₐ. The data demonstrate that these 15N probes are quite sensitive to changes in pH and that structures can be modified to fine-tune the probe to readout any desired pH value from 5 to 8.5.

To test the accuracy of these pH probes, a series of samples containing HP 15N-pyridine or 15N-2,6-lutidine with variable amounts of HCl added to the dissolution solution were examined by 15N-NMR (Supplementary Fig. S2 online). The two agents were chosen because of their long T₁ values. For each pH probe, the test was carried out over a pH range near the respective pKₐ values where the 15N chemical shifts are extremely sensitive to pH. The pH of each sample was estimated from the observed 15N chemical shifts (Fig. 4b) and also measured using a pH electrode. The results show an excellent correlation between the two measurements for both probes (R² > 0.99 for 2,6-lutidine and R² > 0.95 for pyridine), demonstrating the high accuracy of pH measurements by 15N NMR of HP-pyridine derivatives.

Magnetic resonance spectroscopic imaging of HP 15N-Pyridine. Chemical shift images of phantoms containing HP 15N-enriched pyridine were collected to test the applicability of applying HP 15N-pyridine derivatives to distinguish pH differences. In this experiment, a solution of HP 15N-pyridine was simultaneously injected into two NMR tubes inserted within a large NMR tube (see Supplementary Fig. S3 online for the phantom setup). One of the small tubes contained a predetermined amount of sulfuric acid while the other contained sodium hydroxide. The imaging plane was positioned axially covering both tubes of HP-pyridine solutions (see Image in Fig. 5). 15N CSI data demonstrate that the 15N signals of both basic and acidic HP-pyridine compartments were detectable by MRI. 15N NMR spectra of selected voxels show 15N resonances at 297.4 and 207.5 ppm reflecting the free base and protonated pyridine samples, respectively. CSI images of both basic and acidic pyridine displayed good localization of these two signals within the tubes (see CSI and merged image). The results show that magnetic resonance spectroscopic imaging (MRSI) of HP 15N-enriched pyridine was able to distinguish different pH environments in adjacent spatial locations in a phantom. It is worth noting that the imaging of HP 15N-pyridine was very rapid and a similar image of thermally polarized 15N-enriched pyridine would have required a much higher 15N spin concentration and a longer imaging time. These results strongly emphasize the sensitivity advantage of HP 15N for molecular imaging applications over thermally polarized imaging practices. To the best of our knowledge, this is the first demonstration that HP 15N probes can be used in a 15N CSI experiment with similar imaging time as traditional thermally polarized imaging.
Potential in vivo imaging applications of these agents include, but not limited to, pH imaging or MR spectroscopic assessment of tissue acidosis that is present in many diseases. However, many considerations must be taken into account before HP pyridine derivatives can be translated for in vivo evaluations. First, the HP pyridine agents should preferably be present as a free base or be partially protonated at the physiological pH. The long $T_1$ of the free base will allow for the HP signal to be retained much longer during the pre-injection quality controls of the agents. Additionally, the pH regions of the targeted tissues should not be very close to the pKa of the agents in order to avoid the rapid signal loss from fast water proton exchanges. Therefore, the ideal HP pyridine derivatives should have a pKa that is slightly below the pH of acidic tissues to be assessed in order to provide a relatively large chemical shift window and relatively long HP signal lifetime. The toxicity of these agents is also an important aspect that needs to be considered. Although the toxicity of pyridine is a concern, there are several naturally occurring pyridine derivatives that can be considered. Some of these compounds are present in the biological systems and play key roles in human physiology. For example, nicotinamide is a building block for nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) while nicotinic acid (vitamin B3) and pyridoxal derivatives (vitamin B6) are essential human nutrients. Another derivative, picolinic acid, is a product of the kynurenine pathway and is a neuroprotective compound. Chemical modifications of these molecules will be required for potential in vivo pH imaging applications to alter their pKa values toward the desirable range while preserving their biocompatibility. Future work will be focused on biocompatible pyridinyl compounds with favorable pKa values for pH imaging of tissue acidosis.

**Conclusion**

We have demonstrated the potential of using HP $^{15}$N-pyridine derivatives as pH-sensitive probes for MRI. These molecules display large changes in $^{15}$N chemical shift with pH and have very sharp chemical shift versus pH titration curves. The combination of hyperpolarization and pH-sensitive $^{15}$N agents offers new opportunities to develop highly sensitive imaging agents with great sensing capability for the characterization of important biomarkers such as tissue acidity. Future work will be focused on in vivo pH imaging of acidic tumors.

**Methods**

**Acquisition of $^{15}$N-NMR spectra of hyperpolarized $^{15}$N-agents.**

Unless otherwise noted, all chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. A mixture of pyridine (6.2 M), 2,6-lutidine (4.3 M), 2-picoline (5.0 M), nicotinamide (2.7 M) or 2,4,6-collidine (3.78 M) and BDPA radical (40 mM) dissolved in 50 mL of DMSO-sulfolane (1:1 v/v) was polarized in a HyperSense polarizer (3.35 T, Oxford Instruments Molecular Biotools, UK) at 1.05 K with 94.055 GHz microwave irradiation for 2 h. The dissolution liquid of HP agents in distilled water (4 mL) from the polarizer was rapidly mixed with a pre-determined volume of hydrochloric acid or sodium hydroxide in a 10-mm NMR tube to achieve the desired pH. $^{15}$N-NMR spectra were acquired at room temperature (~23°C) on a 400-MHz spectrometer using a 5 degree flip angle with a repetition time (TR) of 5 s. All $^{15}$N peaks were externally referenced with $^{15}$N-nitrobenzene (372 ppm). $^{15}$N MRI were acquired on a 9.4 T Agilent vertical bore microimager (Agilent, USA). MR images and spectra were processed using ImageJ (NIH, USA) and ACD/SpecManager (ACD Labs, Canada). The $T_1$, signal enhancement and pH titration experiments were investigated with the natural abundant $^{15}$N compounds. $^{15}$N-labeled pyridine was used in $T_1$ measurement in plasma and CSI experiments.

$T_1$ relaxation times and signal enhancements of HP pyridine derivatives. In a typical protocol, a dissolution liquid (4 mL) of HP pyridine derivative was rapidly transferred into a 10-mm NMR tube. $^{15}$N NMR acquisition was initiated once the transfer was complete. An array of $^{15}$N spectra with one spectra recorded every 5 s was obtained ($TR = 5$ s, flip angle = 5 degree). $^{15}$N signal intensity of the HP nuclei was normalized to the signal intensity of the first time point ($t = 0$ s). By fitting the NMR signal intensity as a function of time, $T_1$ values were calculated using Equation (1)

$$T_1 = \frac{-1}{\log C(t)/C(0)}$$

where $M_0$ is the original magnetization, $TR$ is the repetition time, and $C(t)$ is the signal intensity as a function of time.

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Figure 5 | $^{15}$N MR images of HP-pyridine in basic (top left) and protonated (top right) forms. Localization of the $^{15}$N images to the $^1$H reference image is shown in the merged image. $^1$H MRI of a phantom showing CSI grids is shown in the middle right panel and the $^{15}$N NMR spectra of highlighted voxels are shown in the bottom panel.

### Table 1 | $T_1$ relaxation time, signal enhancement, pKa, and chemical shift changes of HP $^{15}$N-pyridine, $^{15}$N-2,6-lutidine, $^{15}$N-2-picoline $^{15}$N-2,4,6-collidine, and $^{15}$N-nicotinamide. $T_1$, enhancement, and pKa are reported as mean values ± SD

| Compound          | $T_1$ (s)$^{[a]}$ | Enhancement$^{[b]}$ | pKa$^{[c]}$ | $^{15}$N $\Delta$ppm$^{[d]}$ |
|-------------------|-------------------|--------------------|-----------|-----------------|
| pyridine          | 41 ± 3            | 5437 ± 200         | 5.17 ± 0.07| 94              |
| 2,6-lutidine      | 31 ± 2            | 8702 ± 700         | 6.60 ± 0.02| 90              |
| 2-picoline        | 38 ± 2(d)         | 7787 ± 400         | 6.02 ± 0.05| 94              |
| 2,4,6-collidine   | 36 ± 2(d)         | 10065 ± 600        | 7.65 ± 0.05| 88              |
| nicotinamide      | 22 ± 0.3          | 3598 ± 700         | 4.14 ± 0.02| 94              |

$^{[a]}$ $T_1$ was calculated using Equation 1; $^{[b]}$ enhancements were measured after dissolution; $^{[c]}$ pKa was estimated by fitting pH titration curves; $^{[d]}$ pKa values were obtained by 2,6-lutidine was 2 mM. The plasma used in centrifugation of whole rat blood to remove red blood cells. In this experiment, the final concentration of HP $^{15}$N-pyridine was 13 mM. $^{15}$N-pyridine or NaOH in an NMR tube before acquiring $^{15}$N NMR spectra. pH of the solution was measured by pH electrode.

20-mM solution of a pyridine compound was used. 256 scans of $^{15}$N NMR spectra were acquired using 90-degree pulses with a 500-second delay (>5 times $T_1$). Enhancement levels were calculated as ratios of the $^{15}$N signal from the two polarized states, taking into account for the different $^{15}$N concentrations and number of scans (1 vs 256). PBS buffer solution (pH = 7.4) was used as a dissolution solvent for $T_1$ measurements at the physiological pH. The final concentration of HP $^{15}$N-pyridine or 2,6-lutidine was 2 mM. The plasma used in $T_1$ of HP $^{15}$N-pyridine was obtained by centrifugation of whole rat blood to remove red blood cells. In this experiment, the dissolution liquid of HP $^{15}$N-pyridine (2 mL) was well mixed with rat plasma (2 mL) in a 10-mm NMR tube. The final concentration of HP $^{15}$N-pyridine was 13 mM. $^{15}$N-NMR spectrum was acquired at ~37 °C.

$$M_2(t) = M_0 (\sin \theta) (\cos \theta)^{\frac{t}{T_1}} e^{-\frac{t}{T_1}}$$

(1)

**pH titration curves.** Titration curves were created by plotting $^{15}$N chemical shift versus pH as measured from a pH meter. For the determination of pH by HP $^{15}$N-NMR, HP-pyridine or HP-2,6-lutidine was mixed with an unknown amount of HCl or NaOH in an NMR tube before acquiring $^{15}$N NMR spectra. pH of the solution was calculated from the apparent $^{15}$N chemical shift using the Henderson–Hasselbalch equation and the pKa value estimated by HP-NMR titration. The pH values obtained from HP $^{15}$N-NMR were plotted against the values measured by a pH electrode.

**Chemical shift imaging (CSI) of HP $^{15}$N-pyridine.** The phantom for this experiment was a 25-mm NMR tube encasing two 10-mm NMR tubes, with one pre-added sulfuric acid (200 μL, 7 M) and another had sodium hydroxide (200 μL, 5 M). The phantom was inserted into a 25-mm NMR tube containing DI water (~10 mL). The imaging plane was positioned axially across the phantom (Supplementary Fig. S3a online). $^{15}$N CSI was acquired after the transfer was completed with a 10-s delay to allow for complete mixing. pH values of the HP-pyridine solutions measured by pH electrode in the small tubes were 2 and 11. $^{15}$N CSI parameters: CSI2d sequence (Agilent VnmrI 4 Imaging, Agilent, USA), field of view (FOV) = 40 × 40 mm²; TR = 200 ms; TE = 1.30 ms; flip angle = 20°; number of average (NA) = 1. $^1$H reference image was acquired at the same slice position using a GEMS sequence. $^1$H imaging parameters: FOV = 40 × 40 mm²; TR = 200 ms; TE = 4 ms; flip angle = 20°; NA = 1. Matrix = 8 × 8; voxel size = 5 × 5 × 15 mm³. The CSI data were processed to 128 × 128 matrix.

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Acknowledgments
This research was supported in part by the Department of Defense Prostate Cancer Research Program (W81XWH-12-1-0289 and W81XWH-12-1-0134), the National Institutes of Health (P41EB015908 and R37 HL34557), and the Robert A. Welch Foundation (AT-584).

Author contributions
C.K. initiated and directed the project, analyzed data, and wrote the manuscript. W.J. performed the experiments, analyzed data, and wrote the manuscript. L.L., W.C., S.Z., Z.K. and A.D.S. contributed to data analyses and manuscript preparation.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Jiang, W. et al. Hyperpolarized 15N-pyridine Derivatives as pH-Sensitive MRI Agents. Sci. Rep. 5, 9104; DOI:10.1038/srep09104 (2015).

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