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Toward hyperpolarized molecular imaging of HIV: synthesis and longitudinal relaxation properties of ^{15}N -Azidothymidine

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Abstract

Previously unreported ^{15}N labeled Azidothymidine (AZT) was prepared as an equimolar mixture of two isotopomers: 1- ^{15}N -AZT and 3- ^{15}N -AZT. Polarization decay of ^{15}N NMR signal was studied in high (9.4 T) and low (~50 mT) magnetic fields. ^{15}N T_1 values were 45 ± 5 s (1- ^{15}N -AZT) and 37 ± 2 s (3- ^{15}N -AZT) at 9.4 T, and 140 ± 16 s (3- ^{15}N -AZT) at 50 mT. ^{15}N -AZT can be potentially ^{15}N hyperpolarized by several methods. These sufficiently long ^{15}N -AZT T_1 values potentially enable hyperpolarized *in vivo* imaging of ^{15}N -AZT, because of the known favorable efficient (i.e., of the time scale shorter than the longest reported here ^{15}N T_1) kinetics of uptake of injected AZT. Therefore, 3- ^{15}N -AZT can be potentially used for HIV molecular imaging using hyperpolarized magnetic resonance imaging.

Keywords

hyperpolarization; contrast agent; azidothymidine; ^{15}N ; MRI; AZT; HIV; AIDS

Introduction

There are approximately 35 million people with HIV worldwide as of 2012¹ with 1.2 million of them living in the USA. This disease has no cure or vaccine, and HIV/AIDS is managed by administration of one or more antiretroviral drugs. The course of management and the disease progression is monitored by blood tests to measure the viral and CD4 cell counts in blood. However, these tests repeated every 3–6 months do not directly report on the viral load in other tissues such as lymph nodes, spleen, or brain. Furthermore, when patient's viral count is reduced below the detection limit (<50 copies/mL),² there is no

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Conflict of Interest

The authors did not report any conflict of interest.

Supporting Information

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gauge to measure the response to treatment and identify more effective choice of treatment until the disease manifests again as the elevated level of HIV virus in blood. Information about disease progression beyond blood tests is critically important for disease management, progression, response to treatment, and development of new drugs and vaccines.

Molecular imaging significantly improved patients' management in cancer, where the elevated metabolism of cancer cells can be imaged with radioactive and hyperpolarized (HP)³ contrast agents. HIV/AIDS does not have clear metabolic signatures that can be interrogated by F18-fluorodeoxyglucose positron emission tomography for distinguishing the disease progression.⁴ Therefore, other molecular imaging probes are required to report on HIV progression by targeting HIV/AIDS signatures. Multiple approaches of molecular imaging beyond metabolite (e.g., glucose, glutamine, and choline) imaging are feasible including receptor imaging via targeted positron emission tomography⁵ and HP molecular magnetic resonance imaging (MRI)⁶ probes. An alternative approach is to image the drug molecule itself. While this can be challenging if not impossible from the perspective of radioactive isotope incorporation due to sophisticated chemistry and short life time of biomedically useful nuclei, emerging HP MRI offers clear benefit of dividing the challenging task of contrast agent preparation in two parts: (i) chemical synthesis of the molecule with isotopically enriched nuclei biochemically identical to the drug, for example, incorporation of ¹³C or ¹⁵N and (ii) contrast agent preparation step, for example, hyperpolarization that makes the enriched molecule activated and useful for molecular imaging. Despite MRI sensitivity gains by orders of magnitude achieved by hyperpolarization process,^{7,8} the ultimate limitation of HP approaches is still the detection sensitivity,⁹ which requires a human dose of hundreds of milligrams of imaging contrast agent. Therefore, the use of HP molecular imaging using isotopically enriched treatment drugs is limited to those injected in relatively large quantities.

Azidothymidine (AZT) or Zidovudine is a Food and Drug Administration approved nucleoside analog reverse transcriptase inhibitor, a type of antiretroviral medication.¹⁰ It is used for the treatment of HIV/AIDS as well as for prevention of the infection transmission such as mother to child during the period of birth.¹¹ It has been shown that AZT acts by competing with 3'-deoxythymidine-5'-triphosphate, for incorporation into DNA by HIV-reverse transcriptase.¹² Incorporation of AZT monophosphate into DNA by HIV-reverse transcriptase results in chain termination. Because of its potency as well as the fact that AZT presently available as a 'generic drug', it is used in many combined formulations including Combivir and Trizivir.¹³ Furthermore, AZT can be administered in doses of ~1 g and therefore is a suitable candidate for HP imaging probe, which can potentially allow for imaging of injected HP contrast agent that has good affinity to HIV's reverse transcriptase¹² and cell membrane permeability enabled by the azide moiety.

Azide group (N₃) in the AZT molecular structure can be labeled with ¹⁵N isotope, which can be used for hyperpolarization and hyperpolarization storage during agent administration and uptake, Figure 1. Several hyperpolarization approaches can be used for ¹⁵N-AZT hyperpolarization. For instance, dynamic nuclear polarization,¹⁴ presently the most widespread hyperpolarization technique, can be potentially employed similarly to hyperpolarization of other ¹⁵N compounds including choline.¹⁵ Additionally, Signal

Amplification by Reversible Exchange^{16,17} method can provide a good opportunity for ¹⁵N-AZT hyperpolarization, because the unsaturated nitrogens in azide moiety can be potentially employed for reversible exchange. Furthermore, parahydrogen induced polarization (PHIP)^{18,19} can be also potentially applied, because AZT structure allows for the incorporation of an unsaturated C=C bond in the PHIP molecular hyperpolarization precursor. Similar in complexity, ¹⁵N compounds have been developed²⁰ and ¹⁵N HP²¹ by PHIP. Regardless of hyperpolarization approach to be applied, ¹⁵N isotopic enrichment of AZT is required, which was successfully demonstrated here. Moreover, the delivery of HP contrast agent, Figure 1, requires sufficiently long lifetime of produced contrast agent. The ¹⁵N *T*₁ relaxation properties of the produced ¹⁵N-AZT that were investigated here provide critical information for future hyperpolarization and imaging efforts, Figure 1.

Experimental methods

General

All solvents were purchased from common vendors and were used as received. New AZT isotopomer was characterized by ¹H, ¹³C, ¹⁵N NMR (Bruker 400 MHz), and high-resolution mass spectrometry (HR-MS). HR mass spectra were recorded in negative mode using a Synapt hybrid quadrupole/oa-TOF mass spectrometer (Waters Corp., Milford, MA) equipped with a dual chemical ionization/electrospray (ESCI) source. A post-acquisition gain correction was applied using a solution of 3-[(3-holamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, FW 614.88) as the lock mass spray.

¹⁵N-AZT preparation

¹⁵N-AZT was prepared similar to Czernecki procedure,²² where sodium azide-1-¹⁵N (Sigma-Aldrich, 609374) was used in place of natural isotopic abundance lithium azide. No recrystallization of final product was performed because of the small synthetic scale.

2,3'-Anhydro-5'-O-(4-methoxybenzoyl)thymidine²²—Thymidine (3.63 g, 15 mmol) and Ph₃P (5.9 g, 22.5 mmol, 1.5 equiv.) are dissolved in dimethylformamide (DMF) (30 mL), and the solution cooled to 15 °C on a water bath. To this stirred mixture, a solution of diisopropyl azodicarboxylate (4.4 mL, 22.5 mmol, 1.5 equiv.) in DMF (7 mL) and 4-methoxybenzoic acid (3.42 g, 22.5 mmol, 1.5 equiv.) is added drop-by-drop, and stirring is continued at room temperature (r. t.) for 15 min. The same quantity of Ph₃P and diisopropyl azodicarboxylate is then added. After 30 min. at r. t., the mixture is poured into Et₂O (370 mL), and the resulting suspension is chilled for 2 h. The white crystalline precipitate is isolated by suction and washed with Et₂O; yield: 4 g (74% yield).

3'-Azido-3'-deoxy-5'-O-(4-methoxybenzoyl)thymidine—2,3'-Anhydro-5'-O-(4-methoxybenzoyl)thymidine (1.00 g, 2.8 mmol) and sodium azide-1-¹⁵N (273 mg, 4.2 mmol, 1.5 equiv.) are suspended in DMF (10 mL), and the mixture is heated on an oil bath at 125 °C for 5 h. The orange homogeneous mixture is poured in H₂O (25 mL) containing 5% aq HCl (2.0 mL, 1 equiv.), EtOAc (15 mL) is added. The aqueous layer is extracted with EtOAc (2 × 10 mL), and the organic extract is washed with H₂O (7 mL), then with brine (7 mL). After drying (Na₂SO₄), the solvent is evaporated *in vacuo* to give 3'-azido-3'-deoxy-5'-

O-(4-methoxybenzoyl)thymidine-1-¹⁵N as a foamy solid; yield: 1 g (90% nominal yield). Crude 3'-azido-3'-deoxythymidine: 3'-azido-3'-deoxy-5'-*O*-(4-methoxybenzoyl)thymidine-1-¹⁵N (1.0 g, 2.5 mmol) is suspended in dry MeOH (10 mL), 1 M NaOMe in MeOH (2.9 mL, 1.15 equiv.) is added, and the mixture is stirred at r.t. overnight (12 h). H₂O (15 mL) is added, and MeOH is evaporated. The aqueous solution is extracted with Et₂O (2 × 10 mL), then Amberlite IRN-77 (H⁺ form, 1 g) is added, and the mixture is stirred slowly at r.t. for 15 min. The resin is filtered, washed well with H₂O, and the filtrate is concentrated *in vacuo* to give a white glassy solid: 3'-azido-3'-deoxythymidine-1-¹⁵N (¹⁵N-AZT) yield: 0.270 g (40%).

¹H (D₂O, 400 MHz) δ: 7.50 (d, 1H, *J*(HH) = 1.2 Hz), 6.05 (pseudo-t, 1H, *J*(HH) = 6.5 Hz), 4.22 (dd, 1H, *J*(HH) = 6.4 Hz, and *J*(HH) = 12.0 Hz), 3.87 (m, 1H), 3.73 and 3.63 (dAB, 2 H, *J*(HH)_{gem} = 12.6 Hz, *J*(HH)_{vic} = 4.6 Hz), 2.35 (superposition of normal triplet (3-¹⁵N-AZT), 1H, *J*(HH) = 6.5 Hz and doublet of triplets (1-¹⁵N-AZT), 1H, *J*(HH) = 6.5 Hz and *J*(H-¹⁵N) = 2.6 Hz) 1.74 (d, 3H, *J*(HH) = 1.1); ¹³C{¹H} (D₂O) The spectrum was referenced externally to chloroform-*d* (77 ppm), 100 MHz δ: 166.7, 151.7, 137.2, 111.1, 84.7, 83.9, 60.7, 59.6, 35.8, 11.4; ¹⁵N{¹H} (D₂O, The spectrum was referenced externally to ¹⁵N-urea, 76 ppm) 214.9, 75.9 (*J*(H-¹⁵N) = 2.6 Hz). HR-MS calculated for (M-1): 267.0865; found: 267.0864 (0.4 ppm).

¹⁵N T₁ NMR Measurements at 9.4 T: Nuclear magnetic resonance sample was prepared by dissolving ¹⁵N-AZT in 0.50 mL of D₂O at the maximum concentration (~20 mg/mL at 25 °C). T₁ inversion recovery pulse sequence (as supplied by Bruker Biospin) was used to measure ¹⁵N T₁ at 9.4 T. ¹⁵N NMR signals of the two ¹⁵N sites were integrated (using the same scale for all the spectra) in resulting set of 1D spectra with varied inversion recovery delay, Figure 2b and 2c. Mono-exponential decay fitting model ($y = C \cdot \exp(-x/T_1) + y_0$) was used for each ¹⁵N resonance time series data.

¹⁵N T₁ NMR Measurements at 50 mT: The aforementioned NMR sample was used in the field-cycling ¹⁵N T₁ experiment, where the NMR sample was first polarized inside 400 MHz NMR spectrometer for ~5 min, which significantly exceeds T₁ at 9.4 T. Then, the sample was lifted from 9.4 T magnetic field, and it was exposed to the low fringe field ($B_0 = 50 \pm 5$ mT) of the main NMR magnet. Finally, the sample was quickly placed back inside the 400 MHz NMR spectrometer, and a single scan ¹⁵N NMR spectrum was collected without any additional delay. This cycling operation was repeated with variable low-field sample exposure. Integral values of 3-¹⁵N signal were plotted versus time spent in the low magnetic field. Mono-exponential decay fitting model ($y = C \cdot \exp(-x/T_1) + y_0$) was used for 3-¹⁵N resonance time series data. All fitting and error analyses were performed using Microcal Origin software.

Results and discussion

In Czernecki procedure²² (Figure 2a), thymidine is protected with *p*-methoxybenzyl ether with inversion of 3'-OH center. Lithium azide with natural isotopic abundance was replaced by sodium 1-¹⁵N-azide substitution step (Figure 2a, II). Because of the double inversion overall stereochemistry of AZT is the same as that of thymidine. Because symmetric azide

anion was labeled only on one of two ends, the reaction sequence produced equimolar mixture of two isotopomers **1-¹⁵N-AZT** and **3-¹⁵N-AZT** (Figure 2a) in excellent isotopic purity and satisfactory overall purity (Figures S1–S4).

Preparation of ¹⁵N-AZT enabled measurement of the polarization T_1 decay of two ¹⁵N sites, which is a critical parameter determining the feasibility of HP imaging and *in vivo* applications, Figure 1. ¹⁵N T_1 values were 45 ± 5 s for 1-¹⁵N-AZT (¹⁵N peak at 75.9 ppm) and 37 ± 2 s for 3-¹⁵N-AZT (¹⁵N peak at 214.9 ppm), respectively, at 9.4 T, Figure 2. ¹⁵N T_1 values were 140 ± 16 s for 3-¹⁵N-AZT at 50 mT. ¹⁵N T_1 values measured at 9.4 T (35–50 s) were significantly shorter than that at 50 mT, Figure 2. This difference is likely explained by differential contribution of ¹⁵N chemical shift anisotropy (CSA) at high and low magnetic fields, because ¹⁵N CSA over 700 ppm (corresponding to ~30 kHz at this magnetic field) is not uncommon.²³ When the magnetic field is reduced to 50 mT, the CSA contribution is negligible, because CSA scales linearly with magnetic field, it is reduced by ~200-fold in units of Hertz.

¹⁵N T_1 decay of 1-¹⁵N-AZT in the low magnetic field was very rapid, and T_1 was very challenging to quantify, which is related to the relatively slow speed of NMR sample lifting from the NMR spectrometer. A possible explanation of significantly shorter low-field 1-¹⁵N-AZT T_1 is an accelerated relaxation due to the neighboring protons of the five-membered ring.

While the high-field ¹⁵N T_1 values may be sufficient for the preparation of HP AZT and its ¹⁵N *in vivo* imaging²⁴, low-field detection would be clearly advantageous, because of the nearly threefold increase in T_1 . It should be noted that low-field HP imaging can be more sensitive than high-field HP imaging in general,^{25,26} and low-field MRI of HP AZT uptake would be clearly attractive. Furthermore, the detection sensitivity of HP ¹⁵N sites can be further amplified by indirect proton detection of HP contrast agents.^{27,28} While the latter is challenging (but certainly feasible) in high-field MRI applications, because water protons generate large background signal,^{29,30} the water proton background can be attenuated by orders of magnitude in low-field MRI <0.1 T.²⁶

The previous work in cellular model³¹ demonstrated rapid uptake of AZT by human cell with nearly linear intracellular AZT uptake during first 20 s with peak plateau achieved in less than 100 s, which is certainly within $1 \times T_1$ of ¹⁵N spin label that is potentially amenable to hyperpolarization. We point out that HP compounds are useful for imaging within $(1 - 3) \times T_1$ period after their *in vivo* administration.⁹ Moreover, this uptake kinetics holds true even at high extracellular AZT concentration up to 50 mM.³¹ The latter is well below plasma AZT concentration of treated patients with subgram dose. While it remains to be seen (i) if sufficient *in vivo* signal can be detected with HP AZT and (ii) if the *in vivo* viral uptake will be sufficient to generate imaging contrast, the kinetics of hyperpolarization decay (this work) and cellular uptake³¹ are certainly promising to support such future studies.

Conclusions

Synthetic preparation of ^{15}N analog of antiviral drug AZT has been demonstrated. While both ^{15}N -AZT isotopomers have sufficiently long high-field ^{15}N T_1 (~35–50 s) for potential use in future pre-clinical and clinical studies, 3- ^{15}N -AZT is particularly promising, because it has an ultra-long T_1 in excess of 2 min at low magnetic field. This makes 3- ^{15}N -AZT a promising candidate for future HP imaging efforts for molecular imaging of HIV/AIDS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Overall approach of development of hyperpolarized contrast agents for molecular imaging.

Figure 2.

(a) ¹⁵N-AZT preparation: (I) DIAD (1.5 eq)/Ph₃P (1.5 eq), 4-MeOC₆H₄CO₂H (1.5 eq) DMF, DIAD (1.5 eq)/Ph₃P (1.5 eq); (II) Sodium 1-¹⁵N-azide, DMF; (III) MeONa / MeOH. (b) Polarization recovery of ¹⁵N resonance at 75.9 ppm (1-¹⁵N-AZT) at 9.4 T. (c) Polarization recovery of ¹⁵N resonance at 214.9 ppm (3-¹⁵NAZT) at 9.4 T. (d) Polarization decay of ¹⁵N resonance at 214.9 ppm measured by sample exposure to 50 ± 5 mT magnetic field.