



¹⁸Oxygen Substituted Nucleosides Combined with Proton Beam Therapy: Therapeutic Transmutation In Vitro

Tyvin Rich, MD¹⁻⁴; Dongfeng Pan, PhD⁵; Mahendra Chordia, PhD⁵; Cynthia Keppel, PhD^{4,6}; David Beylin, MS⁷; Pavel Stepanov, MS⁷; Mira Jung, PhD²; Dalong Pang, PhD²; Scott Grindrod, PhD³; Anatoly Dritschilo, MD^{2,3}

¹The University of Virginia, Radiation Oncology and Diagnostic Radiology, Charlottesville, VA, USA

²Georgetown University, Radiation Medicine, Washington, DC, USA

³Shuttle Pharmaceuticals, Inc, Rockville, MD, USA

⁴Hampton University Proton Therapy Institute, Hampton, VA, USA

⁵The University of Virginia, Radiology and Medical Imaging, Charlottesville, VA, USA

⁶Thomas Jefferson National Accelerator Facility, Newport News, VA, USA

⁷Brain Biosciences, Inc, Rockville, MD, USA

Abstract

Purpose: Proton therapy precisely delivers radiation to cancers to cause damaging strand breaks to cellular DNA, kill malignant cells, and stop tumor growth. Therapeutic protons also generate short-lived activated nuclei of carbon, oxygen, and nitrogen atoms in patients as a result of atomic transmutations that are imaged by positron emission tomography (PET). We hypothesized that the transition of ¹⁸O to ¹⁸F in an ¹⁸O-substituted nucleoside irradiated with therapeutic protons may result in the potential for combined diagnosis and treatment for cancer with proton therapy.

Materials and Methods: Reported here is a feasibility study with a therapeutic proton beam used to irradiate H₂¹⁸O to a dose of 10 Gy produced by an 85 MeV pristine Bragg peak. PET imaging initiated >45 minutes later showed an ¹⁸F decay signal with T_{1/2} of ~111 minutes.

Results: The ¹⁸O to ¹⁸F transmutation effect on cell survival was tested by exposing SQ20B squamous carcinoma cells to physiologic ¹⁸O-thymidine concentrations of 5 μM for 48 hours followed by 1- to 9-Gy graded doses of proton radiation given 24 hours later. Survival analyses show radiation sensitization with a dose modification factor (DMF) of 1.2.

Conclusions: These data support the idea of therapeutic transmutation in vitro as a biochemical consequence of proton activation of ¹⁸O to ¹⁸F in substituted thymidine enabling proton radiation enhancement in a cancer cell. ¹⁸O-substituted molecules that incorporate into cancer targets may hold promise for improving the therapeutic window of protons and can be evaluated further for postproton therapy PET imaging.

Keywords: proton therapy; oxygen-18; nucleoside; positron emission tomography (PET); theranostic

Introduction

Fluorine atoms substituted into biologically active molecules are widely used in drug synthesis because of their powerful clinical effects [1–4]. In nuclear medicine practice, the positron emitter ¹⁸F is commonly activated by proton bombardment of ¹⁸O ex vivo and then

Submitted 18 Jul 2020
Accepted 17 Dec 2020
Published 19 Mar 2021

Corresponding Author:

Tyvin Rich, MD
40 Enterprise Parkway
Hampton, VA 23666, USA
Phone: +1 (434) 996-9145
tyvinr@gmail.com

Original Article

DOI
10.14338/IJPT-D-20-00036.1

© Copyright
2021 The Author(s)

Distributed under
Creative Commons CC-BY

OPEN ACCESS

<http://theijpt.org>

the ¹⁸F is combined chemically with biologic molecules for use in diagnostic PET [5]. Examples include ¹⁸F, which is inserted onto 2-deoxy-glucose (2-deoxy-2[¹⁸F]-fluoroglucose) or onto the ribose of thymidine (3'-deoxy-3'-¹⁸F-fluorothymidine), where they provide valuable anatomic and cell proliferation information in cancer patients [6, 7]. A means of producing *targeted fluorine* in a cancer cell is reported here by using an ¹⁸O-substituted nucleoside [8–12] that is used to treat cells first and then combined with therapeutic protons. This approach is similar to using therapeutic protons to generate positron emitters from ¹²C, ¹⁴N, and ¹⁶O atoms, which are short lived, and form the basis of in-room postproton positron emission tomography (PET) for range analysis that may be used to assess proton beam treatment accuracy [13, 14]. An overlapping goal is to address the concept of using a therapeutic proton beam's nuclear atomic effects to harness an increased and selective cytotoxicity effect in cancer cells [15, 16].

The cytotoxic effect evaluated here used ¹⁸O-thymidine or ¹⁸O-uridine combined with therapeutic protons. ¹⁸O-substituted nucleosides are incorporated into DNA and RNA [17–21]; ¹⁸O is activated efficiently by protons [22], and a substituted nucleoside may leverage kinetic and metabolic differences between proliferative cancers versus normal tissues [23]. We hypothesize that this combination produces therapeutic transmutation in vitro (TTiv) by ¹⁸O + protons → ¹⁸F + neutron, (T/2 = 109.8 minutes). Depending on the precise location of an ¹⁸O substitution inserted into a nucleoside, the in situ ¹⁸F created by the proton irradiation at critical locations may disrupt repair mechanisms, promote single- or double-DNA strand breaks, create cluster lesions, and produce low-dose activation neutrons; all could lead to preferential tumor cell kill by protons. Human cancer cells were treated with ¹⁸O nucleosides substituted at the C2 position of the pyrimidine ring and exposed to protons forming a ¹⁸F-C bond [24], indicating support for the TTiv hypothesis.

Materials and Methods

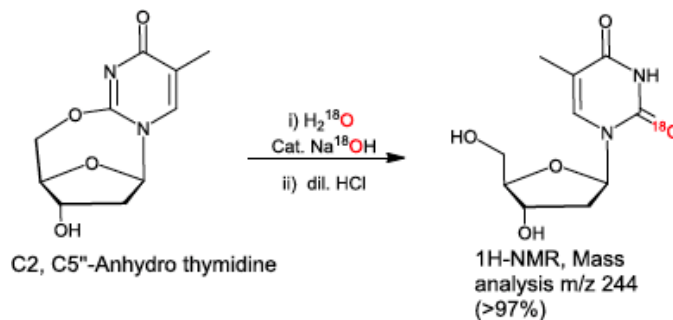
Synthesis of C2-¹⁸O-Thymidine and C2-¹⁸O-Uridine

Solutions of 2,5'-cyclic-anhydro thymidine (224.0 mg, 1 mmol; Berry and Associates, Inc, Dexter, Michigan; Catalog No. PY 7060, CAS No. 15425-09-9), or 2,5'-cyclic-anhydro uridine (210.0 mg, 1 mmol; Asta Tech, Inc, Bristol, Pennsylvania; Catalog No. F13342, CAS No. 20701-12-6) were suspended in 1.0 mL of H₂¹⁸O, to which was added 0.2 mL of Na¹⁸OH solution. The mixture was stirred at room temperature overnight (~12 h). Thin-layer chromatography analysis of the reaction mixture indicated complete loss of starting cyclic thymidine, and a new compound matching the retention time with thymidine was formed. The mixture was then quenched with dilute HCl (0.1 N) until slightly acidic (pH ~3.0) in an ice-water bath (5°C to 10°C). The reaction mixture was frozen by keeping it in a -70°C refrigerator for 30 minutes and then lyophilized to remove all water to yield a white powder (232.0 mg, 95%). This was characterized by ¹H-nuclear magnetic resonance and mass spectroscopy, and purity was checked by high-performance liquid chromatography analysis. Both syntheses were analyzed with nuclear magnetic resonance spectroscopy (Figure 1).

Proton Irradiation of H₂¹⁸O Water

The H₂¹⁸O containing tubes were placed on a 5 cm solid water slab, covered with 2 cm tissue equivalent bolus, and computed tomography scans were acquired at a slice thickness of 1.25 mm on a GE Light speed computed tomography scanner. A treatment plan was created on a Raystation Version 8 planning system (Raysearch Laboratories, Stockholm, Sweden) for a 2 Gy physical dose (the treatment plan defines 1.1 Gy = 1 Gye [Gray equivalent]) and uniform dose coverage of the tubes. The plan was delivered on the Mevion S250i pencil beam scanning proton system (Mevion, Littleton, MA) at Medstar Georgetown University Hospital and repeated 5 times to achieve a total dose of 10 Gy.

Figure 1. Schema for ¹⁸O thymidine production and confirmation by mass spectroscopy. A similar schema was used to synthesize ¹⁸O-uridine.



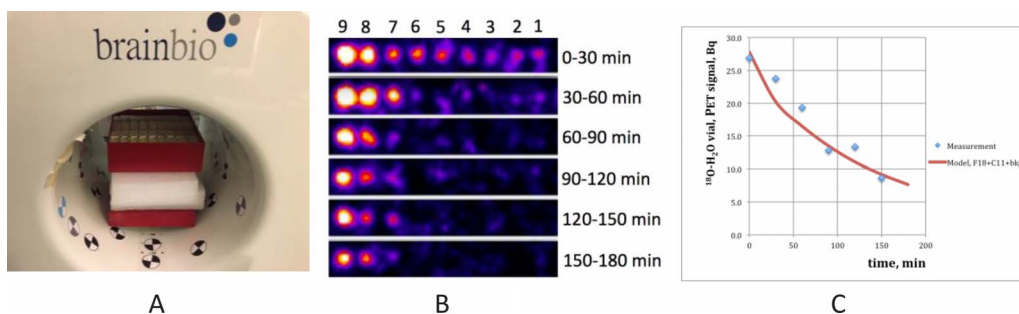


Figure 2. Positron emission tomography (PET) scan of the irradiated vials. (A) Irradiated vials positioned for the PET scan. (B) PET activity of vials containing irradiated ¹⁸O-water mixed with ¹⁶O-water (deionized water) in decreasing concentrations from 100% to 0%. (C) PET coincidence data was acquired from vial 9 for 180 minutes. Acquisition started >45 minutes after the last proton fraction. Bq = Becquerel.

	Vial No.									
	9	8	7	6	5	4	3	2	1	0
H ₂ ¹⁸ O, %*	100	50	25	5	2.5	0.5	0.5	0.05		

*Percentage dilutions of 200 μL H₂¹⁸O water with deionized water.

PET Imaging with ¹⁸O-Substituted Water

An evaluation of the time course of the decay of ¹⁸F generated from proton activated ¹⁸O water was first performed using a clinical, spread out Bragg peak. This showed activation with an ¹⁸F signal of decay using a sensitive NaI detector. For the experiments reported here, H₂¹⁸O water solutions irradiated with protons were transferred to nonirradiated plastic vials for PET scanning. The vials were placed in the field of view of a portable, high-resolution, dedicated brain research PET scanner (CerePET, Brain Biosciences, Inc, Rockville, Maryland) to image the ¹⁸F signal as shown in **Figure 2A**. PET imaging began >45 minutes later. The energy gate was set from 430 to 650 keV. PET data were split into 6 × 30 minute frames, and PET images were reconstructed using an iterative, quantitative maximum-likelihood expectation maximization algorithm with 1.875 mm isotropic voxel on a 128 × 128 × 122 grid.

Cell Irradiation

SQ20B cells were irradiated on the Mevion S250i hyperscan pencil beam system at MedStar Georgetown University Hospital. The clinical proton unit is a compact, gantry-mounted synchrocyclotron capable of delivering protons of 1 to 227 MeV energies. The energy modulator design of this proton system results in a constant Bragg peak width (80% to 80%) of 8.4 mm regardless of the energy produced. Radiation dose was determined using a National Institute of Standards and Technology traceable calibrated parallel plate chamber (Model PPC05, IBA dosimetry; Herndon, VA).

We placed the cell layer (<2 mm thick) in flasks in the 90% to 90% dose region of the peak. To determine the required thickness of the solid water plates, we placed varying thicknesses of the plates in front of a calibrated parallel plate chamber to measure dose variation. The dose varied less than 5% within a 6 mm region around the Bragg peak. We used a 20 × 20 cm² spot map of 85 MeV energy consisting of 1681 spots at 5 mm spot spacing to deliver a uniform dose of 1 Gy to a set of 6 flasks each time. The delivery was repeated multiple times to achieve doses of 1, 3, 5, 7, and 9 Gy. The cell samples were placed on top of the solid water plates whose thickness was determined as described previously. A posterior beam angle was used.

Photon Irradiation

Cells were treated with gamma rays on a ¹³⁷Cs-irradiator (Model 30, Mark I; J. L. Shepherd, San Fernando, CA) at room temperature in ambient air.

Proton Dose Responses of SQ20B Cells

Radiation-resistant SQ20B squamous carcinoma cells were used in a radiation dose-response experiment to examine the combined effect of an ¹⁸O nucleoside plus irradiation with γ-rays or protons. These cells were maintained in exponential

Downloaded from http://meridian.allenpress.com/ijparticle/pdf/7/4/1/2798888/2331-5180-7-4-11.pdf by guest on 16 August 2022

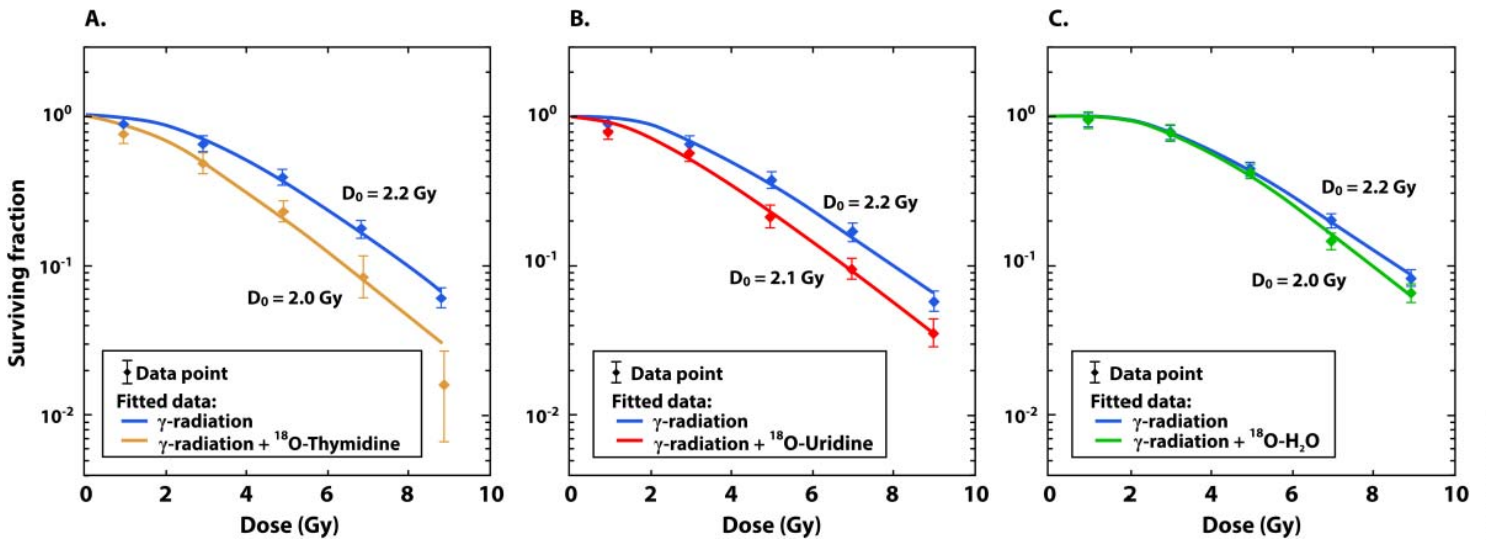


Figure 3. SQ20B cells were grown in log phase with (A) 5 μM ¹⁸O-thymidine or 10 μM ¹⁸O-uridine for (B) 48 hours or (C) 5 μM H₂¹⁸O water as described in the Materials and Methods section. No change was seen in the D₀ (radiation sensitization) with γ-rays under these conditions.

growth in 5% CO₂ at 37°C. Complete Media (Dulbecco's modified Eagle's medium; ThermoFisher, Waltham, MA) was supplemented with 20% fetal bovine serum, 100 mg/mL of streptomycin, 100 mg/mL of penicillin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 1 mg/mL of hydrocortisone. Cells were split 1:10, plated into T75 flasks, and exposed to 5 μM ¹⁸O-thymidine or 10 μM ¹⁸O-uridine. After 48 hours of drug treatment, cells were plated in triplicate in T25 flasks in fresh Complete Media for 24 hours. Cells were then exposed to graded doses of γ-rays or proton radiation (1, 3, 5, 7, and 9 Gy). The flasks were incubated in 5% CO₂ and 37°C for 14 days for colony formation. Flasks were stained, and colonies containing more than 50 cells were scored to determine survival data. Data were fit to the single hit and multi-target models [25].

Results

Protons Activate H₂¹⁸O Indicating ¹⁸F decay

PET signal was observed from all 9 vials at different time points (Figure 2B). Noise events from self-radioactivity of the PET scanner material (LYSO) contributed to background. Decay of the PET signal was consistent with the presence of ¹⁸F (109.8 min half-life, 89% at the start of measurement) and ¹¹C (20 min half-life, 11% at the start of measurement). A time-activity curve of a PET signal was detected from a proton-irradiated vial (100% ¹⁸O-water, measured over 3 hours) (Figure 2C). Contributions of ¹⁸F and ¹¹C were derived from the time-activity curves using least squares fit. Potential contributions of ¹³N and ¹⁵O to the PET signal were neglected due to the length of time (45 min) between the irradiation and the start of data acquisition.

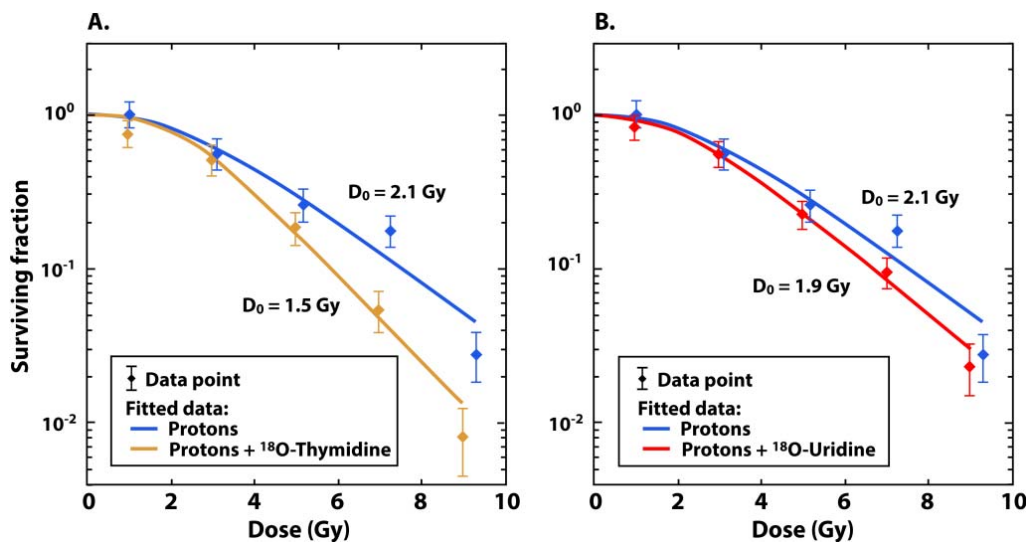
Cell Studies

Evaluation with thymidine or ¹⁸O-thymidine alone used at 10 μM for 1 week indicated no delay of growth. An ~10% radiation sensitization was seen with protons plus thymidine compared with x-rays alone, consistent with an accepted relative biological effectiveness difference between protons and γ-rays (data not shown). Increased cytotoxicity was found with 0.5 μM ¹⁸O-thymidine combined with 8 Gy protons compared with a thymidine plus 8 Gy proton control. These preliminary evaluations provided a rationale for study of full γ-ray and proton dose response relationships described in the following sections.

Photon and Proton Dose Responses with ¹⁸O Nucleosides

Radiation sensitization was assessed with 2 different ¹⁸O nucleosides treated with 5 or 10 μM concentrations for 48 hours followed by irradiation with γ-rays (Figures 3 and 4).

Figure 4. Radiation-resistant SQ20B squamous carcinoma cells were grown in log phase with 5 μM ¹⁸O-thymidine or 10 μM ¹⁸O-uridine for 48 hours as described in the Materials and Methods section. (A) Radiation sensitization was observed with ¹⁸O-thymidine and protons indicated by a change in D₀ with a dose modification factor ~1.2 calculated at 1 log survival. (B) Radiation sensitization was not seen with ¹⁸O-uridine and protons.

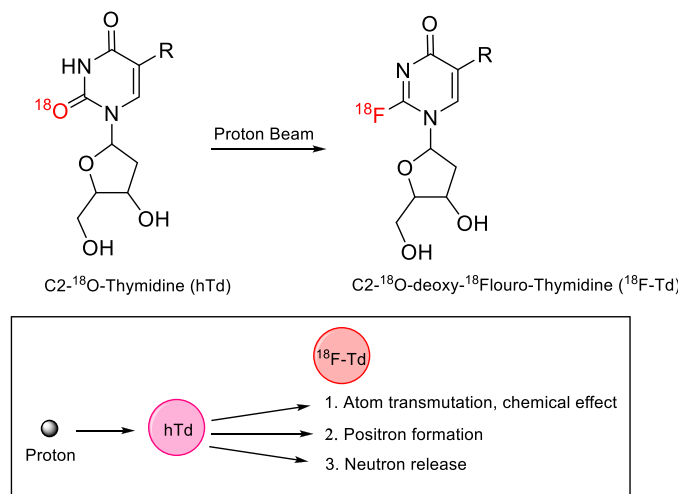


Discussion

Well-known properties of thymidine led us to construct a heavy nucleoside because it substitutes well with ¹⁸O label (up to 97%), is nonradioactive, and is safe to handle [9–12]. Thymidine incorporates into DNA, or uridine into RNA; the heavy substituted versions with ¹⁸O have been used for nucleic acid research, while ¹⁸O water alone has been used in tracing and protein metabolism studies in vivo with analysis by mass spectroscopy [26–34]. Thymidine is critical for cell growth, although in high doses it can perturb nucleotide pools and the cell cycle [35]. This observation led to clinical investigations showing a concentration of thymidine in plasma and extracellular fluids that averages in the range of 0.4 to 6 μM, with higher levels present within the cell [36]. Thymidine doses given in kg/m² have been used for its cytostatic effects (as a chemo sensitizer) in cancer patients, and these large doses were tolerated [37]. In contrast, we evaluated *physiologic* concentrations of ¹⁸O nucleosides with 0.5 to 10 μM × 48 to 96 hours and did not find evidence of inhibition of cell growth in vitro. Further, when human radiation resistant SQ208 log phase cells were exposed to graded doses of therapeutic protons plus 5 μM ¹⁸O-thymidine, there was a dose-modifying factor of ~1.2 compared with protons alone or compared with γ-rays [38].

The mechanism of the TTiv effect is hypothesized to relate to the presence of an ¹⁸F containing nucleoside created in DNA by therapeutic proton irradiation (**Figure 5**). Fluorine atoms are electronegative, and their introduction into biologic molecules has been successful in using fluorine substitutions for improving a drug’s action through mechanisms that are understood [4]. A proposed mechanism for TTiv with proton treatment may be the formation of ¹⁸F, which acts as a strong dipole in the C-F bond [24] that alters base pairing caused by disrupted hydrogen bonding in DNA (**Figure 6**).

Figure 5. Proton transmutation of ¹⁸O- to ¹⁸F-thymidine.



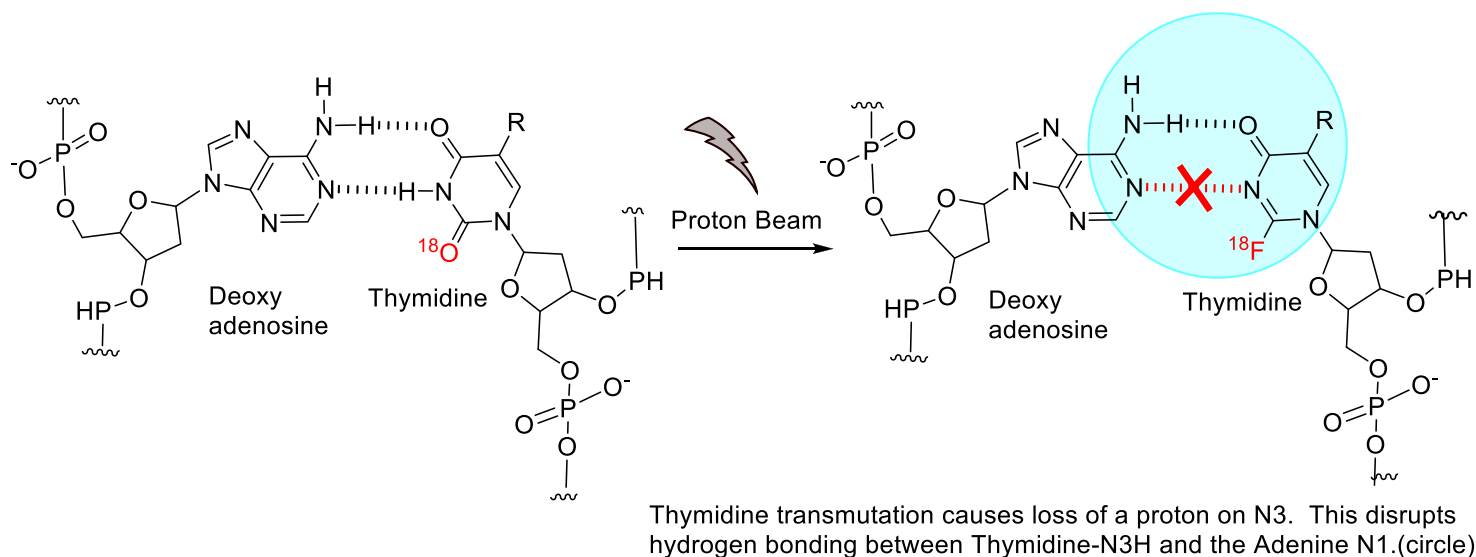


Figure 6. A hypothesized mechanism of therapeutic transformation: the ¹⁸F-C2 bond (a strong dipole) causes chemical change in DNA.

The ¹⁸O insertion at the C2 position of the pyrimidine ring appears to cause a deleterious transmutation when irradiated with protons. ¹⁸O substituted thymidine, but not ¹⁸O-labeled uridine, suggest a DNA-targeted mechanism for cytotoxicity, but the precise molecular mechanism is not understood and is beyond the scope of this investigation.

A clinical application of proton-induced TTiv could be considered using ¹⁸O substitutions on thymidine analogues such as BUdR or IUdR, which are known radiation sensitizers [39, 40]. Tumor biopsies after repeated IUdR infusions resulted in ~3% to 26% DNA incorporation of this analogue in gliomas, head and neck squamous cancers, sarcomas, and other cancers when used during a course of treatment [41]. A multiple dose schedule of an ¹⁸O-substituted thymidine analogue could be attractive when combined with fractionated-proton spread out Bragg peak irradiation. Animal studies will be needed to determine the degree of proton enhancement in tumors in vivo and to assess normal tissue sensitization. The entrance-dose sensitization of the proton beam could be ameliorated by the use of multiple entrance beams to reduce transit activation in normal tissues while treating a sensitized cancer volume. These chemical, physical, and treatment-planning aspects could eventually result in the use of lower overall radiation doses to sensitive normal tissues to reduce acute and late morbidity. Furthermore, an ¹⁸O-substituted nucleoside or thymidine analogue, such as IUdR, which has been shown to incorporate into DNA, could be evaluated for out-of-room, postproton PET.

A limitation of this study is that we did not make direct measurements of the heavy nucleoside incorporation into DNA or show direct damage to this molecule as the cause of radiation sensitization. However, the published incorporation models based on DNA/RNA, protein, and intracellular water studies with substituted ¹⁸O precursors support a DNA targeted mechanism. The detection of ¹⁸F created in the water study showed feasibility, but higher signal will be required for useful postproton imaging PET studies and could be addressed with understanding ¹⁸O-substituted nucleoside incorporation into DNA.

ADDITIONAL INFORMATION AND DECLARATIONS

Conflicts of Interest: Tyvin Rich, MD, Dongfeng Pan, PhD, and Mahendra Chordia, PhD, report inventorship/patents owned by the University of Virginia. Anatoly Dritschilo, MD, and Mira Jung, PhD, report industry/equity ownership in Shuttle Pharmaceuticals, Inc. David Beylin, MS, reports industry/equity ownership in BrainBIO, Inc. The authors have no additional conflicts of interest to disclose.

Acknowledgments: The authors wish to thank Alfredo Valena for providing technical support, Bill Thomas for administrative support, and Dr. William Dritschilo for editorial support.

Ethical approval: This study was reviewed by the authors' institutional research infrastructure and was determined to be exempt from institutional review board approval.

Funding: This work was supported in part by the Virginia Department of Health, Office of Naval Research, Hampton University, Shuttle Pharmaceuticals Inc, and BrainBio, Inc.

References

- Bohm H, Banner D, Bendels S, Kansy M, Kuhn B, Muller K, Obst-Sander U, Sander M. Fluorine in medicinal chemistry. *ChemBioChem*. 2004;5:637–43.
- Chambers R. Chapter title. In: *Fluorine in Organic Chemistry*. Oxford, UK: Blackwell Publishing Ltd;2004:16–145.
- Gillis E, Eastman K, Matthew H, Donnelly D, Meanwell N. Applications of fluorine in medicinal chemistry. *J Med Chem*. 2015;58:8315–39.
- Bonjani J, Yue-Zhong S, Xialiang Z, Meanwell N. Metabolic and pharmacological aspects of fluorinated compounds. *J Med Chem*. 2020;63:6315–86.
- Almuhaideb A, Papathanasiou N, Bomanji J. ¹⁸F-FDG PET/CT imaging in oncology. *Ann Saudi Med*. 2011;31:3–13.
- Alauddin M. Positron emission tomography (PET) imaging with ¹⁸F-based radiotracers. *Am J Nucl Med Mol Imaging*. 2012; 2:55–76.
- Tehrani O, Shields A. PET imaging of proliferation with pyrimidines. *J Nucl Med*. 2013;54:903–12.
- Wiebe L. Applications of nucleoside-based molecular probes for the in vivo assessment of tumor biochemistry using positron emission tomography (PET). *Braz Arch Biol Technol*. 2007;50:445–59.
- Puzo G, Schram K, McCloskey J. Incorporation of oxygen-18 into nucleosides and bases. *Nucleic Acid Res*. 1977;4:2075–61.
- Schram K, Ratcliff S, Neenan J. Oxygen-18-labeled nucleosides. 1. A general method for the synthesis of specifically labeled pyrimidine deoxyribosides. *J Labelled Comp Radiopharm*. 1982;19:399–404.
- Solsten RT, McCloskey JA, Schram KH. Oxygen-18-labeled nucleosides. 2. A general method for the synthesis of specifically labeled pyrimidine ribonucleosides. *Nucleosides Nucleotides*. 1982;1:57.
- Richards O, Boyer P. ¹⁸O labeling of deoxyribonucleic acid during synthesis and stability of the label during replication. *J Mol Biol*. 1966;19:109–19.
- Parodi K. PET monitoring of hadrotherapy. *Nucl Med Rev*. 2012;15(suppl C):37–42.
- Zhu X, Fakhri G. Proton therapy verification with PET scanning. *Theranostics*. 2013;3:731–40.
- Kavanagh JN, Currell FJ, Timson DJ, Savage KI, Richard DJ, McMahon SJ, Hartley O, Cirrone GA, Romano F, Prise KM, Bassler N, Holzscheiter MH, Schettino G. Antiproton induced DNA damage: proton like in flight, carbon-ion like near rest. *Sci Rep*. 2013;3:1770.
- Cirrone GAP, Manti L, Margarone D, Petringa G, Giuffrida L, Minopoli A, Picciotto A, Russo G, Cammarata F, Pisciotto P, Perozziello F, Romano F, Marchese V, Milluzzo G, Scuderi V, Cuttone G, Korn G. First experimental proof of proton boron capture therapy (PBCT) to enhance proton therapy effectiveness. *Sci Rep*. 2018;8:1141.
- Asadi-Atoi P, Barraud P, Tisne C, Kellner S. Benefits of stable isotope labeling in RNA analysis. *Biol Chem*, 2019;400: 847–65.
- Qian WJ, Petritis B, Kaushal A, Finnerty C, Jeschke M, Monroe M, Moore R, Schepmoes A, Xiao W, Moldawer L, Davis R, Tompkins R, Herndon D, Camp D II, Smith R. Inflammation and the host response to injury large scale collaborative research program. Plasma proteome response to severe burn injury revealed by ¹⁸O-labeled “universal” reference-based quantitative proteomics. *J Proteome Res*. 2010;9:4779–89.
- Wilkinson D. Historic and contemporary stable isotope tracer approaches to studying mammalian protein metabolism. *Mass Spectrom Rev*. 2018;37:57–80.
- Hamasaki T, Matsumoto T, Sakamoto N, Shimahara A, Kato S, Yoshitake A, Utsunomiya A, Yurimoto H, Gabazza EC, Ohgi T. Synthesis of ¹⁸O-labeled RNA for application to kinetic studies and imaging. *Nucleic Acids Res*. 2013;41:e126.
- Follmann H, Hogenkamp H. The synthesis of ribose and of adenine nucleotides containing oxygen-18. *J Am Chem Soc*. 1970;92:3.
- Solberg D, Wethington J, Arkinson T. Quantitative determination of oxygen-18 by activation analysis. *Nature*. 1963;197: 611–2.

23. Cooper G. *The Cell: A Molecular Approach*. 2nd ed. Sunderland, MA: Sinauer Associates; 2000. <https://www.ncbi.nlm.nih.gov/books/NBK9963/>. Accessed April. 16, 2021.
24. O'Hagan D. Understanding organofluorine chemistry. An introduction to the C–F bond. *Chem Soc Rev*. 2008;37:308–19.
25. Albright N. Computer programs for the analysis of cellular data. *Radiat Res*. 1987;112:331–40.
26. Wang M, Shen Y, Turko IV, Nelson DC, Li S. Determining carbapenemase activity with ¹⁸O labeling and targeted mass spectrometry. *Analyt Chem*. 2013;85:11014–9.
27. Bergmann D, Hübner F, Humpf H. Stable isotope dilution analysis of small molecules with carboxylic acid functions using ¹⁸O labeling for HPLC-ESI-MS/MS: analysis of fumonisin B1. *J Agric Food Chem*. 2013;61:7904–8.
28. Mori M, Abe K, Yamaguchi H, Goto J, Shimada M, Mano N. Production of ¹⁸O-single labeled peptide fragments during trypsin digestion of proteins for quantitative proteomics using nanoLC-ESI-MS/MS. *J Proteome Res*. 2010;9:3741–9.
29. Kolodziejska M, Kaminski Z, Paneth P. Preparation of ¹⁸O-labeled nicotinamide. *J Labelled Comp Radiopharm*. 2002;45:1005–10.
30. Xiao B, Li Y, Zhou J, Jiang Y. Method and apparatus for preparation of oxygen 18 labeled water as nuclear medicine diagnostic reagent. Faming Zhuanli Shenqing patent CN 101575086 A 20091111, Jan 1, 2009.
31. Theodorou V, Skobridi K, Alivertis D, Gerathanassis I. Synthetic methodologies in organic chemistry involving incorporation of [O-17] and [O-18] isotopes. *J Labelled Comp Radiopharm*. 2014;57:481–508.
32. Kreuzer H, Quaroni L, Podlesak D, Zlateva T, Bollinger N, McAllister A, Lott M, Hegg E. Detection of metabolic fluxes of O and H atoms into intracellular water in mammalian cells. *PLoS One*. 2012;7:e39685.
33. Amanchy R, Kalume D, Pandey A. Stable isotope labeling with amino acids in cell culture (SILAC) for studying dynamics of protein abundance and posttranslational modifications. *Science's STKE*. 2005;2005:12.
34. Ye X, Luke B, Andresson T, Blonder J. ¹⁸O Stable isotope labeling in MS-based proteomics. *Brief Funct Genomic Proteomic*. 2009;8:136–44.
35. Bjursell G, Reichard P. Effects of thymidine on deoxyribonucleoside triphosphate pools and deoxyribonucleic acid synthesis in Chinese hamster ovary cells. *J Biol Chem*. 1973;248:3901–9.
36. Traut T. Physiological concentrations of purines and pyrimidines. *Mol Cell Biochem*. 1994;140:1–22.
37. Leyva A, Schornagel J, Kraal I, Wadman S, Pinedo H. Clinical and biochemical studies of high-dose thymidine treatment in patients with solid tumors. *J Cancer Res Clin Oncol*. 1984;107:211–6.
38. Paganetti H. The physics of proton biology. 2019, In: Paganetti H, ed. *Proton Therapy Physics*. 2nd ed. Boca Raton, FL: CRC Press; 2019: Chap 22.
39. Urtasun R, Kinsella T, Farnan N, DelRowe J, Lester S, Fulton D. Survival improvement in anaplastic astrocytoma, combining external radiation with halogenated pyrimidines: final report of RTOG 86-12, Phase I-II study. *Int J Radiat Oncol Biol Phys*. 1996;36:1163–7.
40. Prados M, Seiferheld S, Sandler H, Buckner J, Phillips T, Shultz C, Urtasun R, Davis R, Gutin P, Cascino T, Greenberg H, Curran W. Phase III randomized study of radiotherapy plus procarbazine, lomustine, and vincristine with or without BUdR for treatment of anaplastic astrocytoma: final report of RTOG 9404. *Int J Radiat Oncol Biol Phys*. 2004;58:1147–52.
41. Cook J, Glass J, Lebovics R, Bobo H, Pass H, DeLaney T, Oldfield E, Mitchell J, Glatstein E, Goffman T. Measurement of thymidine replacement in patients with high grade gliomas, head and neck tumors, and high-grade sarcomas after continuous intravenous infusions of 5-iododeoxyuridine. *Cancer Res*. 1992;52:719–72.