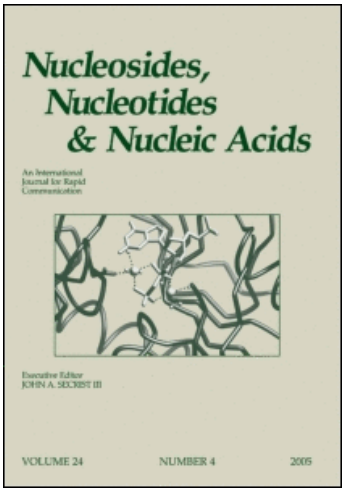

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Synthesis and in Vitro Evaluation of 5-Fluoro-6-[(2-Iminopyrrolidin-1-YL)Methyl]Uracil, TPI(F): An Inhibitor of Human Thymidine Phosphorylase (TP)

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Online publication date: 21 December 2009

To cite this Article Grierson, John R. , Brockenbrough, J. Scott , Rasey, Janet S. , Wiens, Linda and Vesselle, Hubert(2010) 'Synthesis and in Vitro Evaluation of 5-Fluoro-6-[(2-Iminopyrrolidin-1-YL)Methyl]Uracil, TPI(F): An Inhibitor of Human Thymidine Phosphorylase (TP)', Nucleosides, Nucleotides and Nucleic Acids, 29: 1, 49 — 54
To link to this Article: DOI: 10.1080/15257770903451603
URL: http://dx.doi.org/10.1080/15257770903451603

Nucleosides, Nucleotides and Nucleic Acids, 29:49–54, 2010

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770903451603

SYNTHESIS AND IN VITRO EVALUATION OF 5-FLUORO-6-[(2-IMINOPYRROLIDIN-1-YL)METHYL]URACIL, TPI(F): AN INHIBITOR OF HUMAN THYMIDINE PHOSPHORYLASE (TP)

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□ *An investigation was conducted to determine if the 5-fluoro analog of TPI (5-chloro-6-[(2-iminopyrrolidin-1-yl)methyl]uracil), a potent inhibitor of human thymidine phosphorylase (TP), has an IC_{50} in a range that might allow to use it labeled for imaging of TP expression in vivo. The previously unreported fluoro analog, TPI(F), was prepared and tested against TPI and TPI(Br) using an inhibition assay of [H−3]thymidine cleavage. An assay, performed in the presence of 0.4 mg/ml of human TP, yielded IC_{50} values of 2.5 nM, 2.7 nM, and 9.0 nM for TPI, TPI(Br), and TPI(F), respectively. The results indicate that further studies to develop ^{18}F-labeled TPI(F) as a potential radiopharmaceutical for PET imaging of TP expression in vivo are warranted.*

Keywords Human thymidine phosphorylase; TP; transition state inhibitor analog; TPI; TPI(F)

INTRODUCTION

Thymidine phosphorylase (TP) is an essential enzyme involved in endogenous nucleotide salvage.[1,2] It specifically cleaves the glycosidic bond in thymidine to produce thymine and 2-deoxyribose-1α-phosphate (2dR−1P; Scheme 1). The reaction is non-energy dependent, reversible under physiologic conditions, and strongly coupled to thymine degradation. As a result, TP generally functions to clear thymidine from blood, which keeps plasma concentrations low. However, there is growing interest in the potential role TP plays in tumor biology since it was discovered that 2dR-1P

Received 9 February 2009; accepted 29 October 2009.
This work was supported by NIH grant ROI CA 115559.
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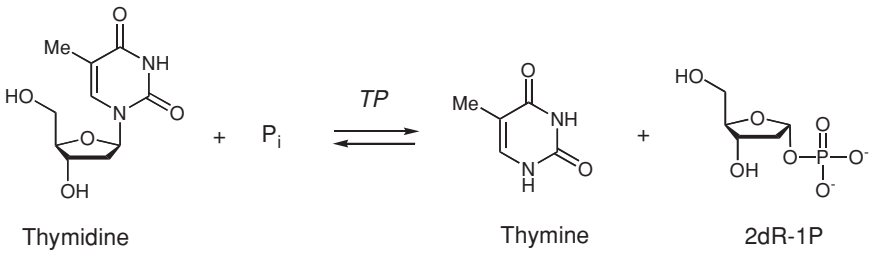

SCHEME 1 Equation of reversible TP catalysis with thymidine as the substrate.

(or 2dR) promotes neovacularization, in vitro and in experimental tumor models.[3–8]

Our research group's goal is to investigate tumor biology, particularly lung cancer, using PET imaging and to actively develop novel agents for that purpose. Recently, we reported a radiosynthesis and in vitro characterization of 5′-deoxy-5′-[^{18}F]fluorothymidine (DFT; Figure 1) as a targeted radiopharmaceutical for imaging TP in vivo.[9] This fluorinated thymidine analog is a TP substrate and was actively metabolized to 2,5-dideoxy-5-[^{18}F]fluororibose-1α-phosphate (ddFR-1P) in cells. We hypothesized that labeled ddFR-1P would be trapped in cells due to its phosphate charge and, consequently, accumulate in proportion to intracellular TP activity. Unfortunately, ddFR-1P proved too unstable to be useful as a PET imaging agent.

The instability of ddFR-1P prompted us to consider an alternative strategy that directly targeted TP with a potent ^{18}F-labeled enzyme inhibitor. That goal required a ^{18}F-labeled compound with exceptional binding potency, since the imaging agent and target would have a 1:1 stoichiometry and there would be no metabolic turnover to feed a labeled metabolite pool. Fortunately, a TP inhibitor, TPI (5-chloro-6-[(2-iminopyrrolidin-1-yl)methyl]uracil; Figure 1), has been previously shown to have an IC_{50} of 35 nM.[10] Moreover, TPI is a weak inhibitor of the related enzyme uridine phosphorylase (IC_{50} > 1000 nM).[10]

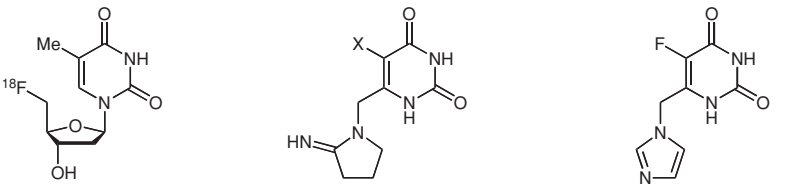

FIGURE 1 Structures of 5′-deoxy-5′-[^{18}F]fluorothymidine (DFT) and TP inhibitors designated as TPI, TPI(Br), TPI(F), and TPI(F)-(IM).

The high TPI inhibitory binding with TP is similar to that found in other receptor-binding ^{18}F-labeled molecules that have been successfully imaged in vivo,[11,12] so a fluorinated analog was of interest. Replacement of the chlorine atom in TPI with a fluorine atom was obvious, but the critical question was whether that analog retained the high inhibitory potency of TPI. Reported herein is a preliminary investigation that focused on the synthesis of 5-fluoro-6-[(2-iminopyrrolidin-1-yl)methyl]uracil (TFI(F)) and determination of its IC_{50} value with TP. The goal was to determine if TPI(F) had adequate potency to justify its fluorine-18 labeling and evaluation as an imaging agent of TP expression in vivo.

RESULTS AND DISCUSSION

TPI(F) was prepared by alkylating 2−iminopyrrolidine with 6-chloromethyl-5-fluorouracil[13,14] in methanol, promoted by 1,8-diazabicyclo[5.4.0]undec-7-ene.[15] TPI and TPI(Br) were also prepared to directly compare the relative 50% inhibitory values (IC_{50}) with human TP. TPI(F) was an obvious choice for a fluorinated TPI analog, given that a fluorine atom represents minimal structural modification of the parent drug. A recent synthesis and evaluation of 5-fluoro-6-[(1H-imidazol-1-yl)methyl]uracil (TPI(F)-(IM) (Figure 1) supports that view.[13,14] However, in that case, the additional impact of the imidazole ring, as the pendent amine moiety, was unclear.

The relative potencies (IC_{50} values) for TPI and TPI(Br) and TPI(F) were determined to be 2.5 nM and 2.7 nM, and 9.0 nM, respectively. The inhibition curves are shown in Figure 2. However, our values for TPI and TPI(Br) are lower than previously reported.[10] This may be attributed to the specific enzyme concentrations used in the respective enzyme assays. Regardless, the trend for TPI, TPI(Br), and TPI(F) is clear. Fluorination, alone, does not improve potency. Nevertheless, the potency of TPI(F) still falls within the range that is typical of many PET radiotracer ligands. Thus, radiolabeling of TPI(F) with high specific activity [^{18}F]fluoride ion (1–2 Ci/μmol) and evaluation of its potential for imaging regional TP expression in vivo is warranted.

EXPERIMENTAL

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. NMR spectra: ^{1}H (300 MHz, δ, TMS); ^{13}C (75 MHz, δ, TMS); ^{19}F (282 MHz, δ, CCl_3F), were recorded using a Bruker AV301 multinuclear instrument. Mass spectrometry (MS) was performed using a Micromass Quattro Premier XE instrument and electrospray ionization

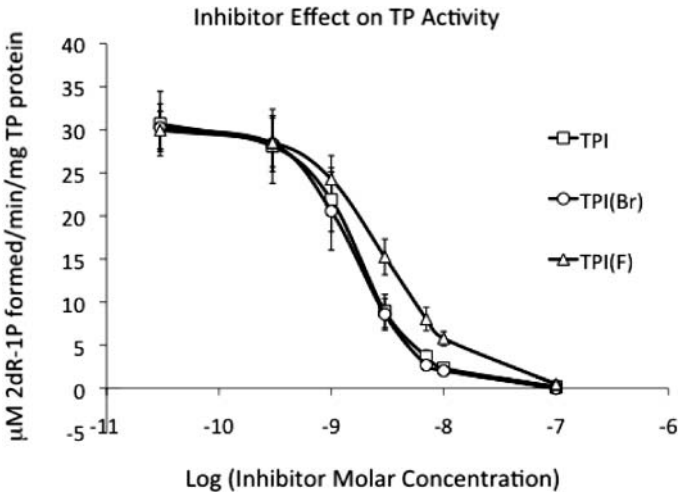

FIGURE 2 Inhibition of TP activity in the presence of various concentrations of TPI(F), TPI(Br), and TPI. The IC_{50} value for each compound was extrapolated from its corresponding concentration curve yielding values of 2.5 nM, 2.7 nM, and 9.0 nM for TPI, TPI(Br), and TPI(F), respectively. Values represent the means with standard deviations of three separate experiments.

(ES). Characteristic data is given for selected synthetic intermediates and products, which were not available.

(A) Chemical Syntheses

i. 5-Fluoro-6-chloromethyluracil was prepared according to methods reported by Lai and Kalman et al.[13,14] and used without purification (MS-ES$^-$ (M-H) m/z 177, 179) for the synthesis of TPI(F). Its synthesis involves the preparation of several key intermediates: (a) 2,4-Difluoro-3-oxo-butyric acid ethyl ester was prepared according to McBee et al.[16] ^{1}H NMR ($CDCl_3$) 1.35 (t, J = 7.5 Hz, 3H), 4.34 (q, J = 7.05 Hz, 2H), 5.23 (d, J_{HF} = 44 Hz, 2H), 5.49 (d, J_{HF} = 47 Hz, 1H); ^{13}C NMR ($CDCl_3$) 13.9, 63.2, 83.2 (dd, J_{CF} = 184 Hz, J_{CF} = 3 Hz), 89.6 (dd, J_{CF} = 196 Hz, J'_{CF} = 1.5 Hz), 162.2 (d, $J_{CF'}$ = 23 Hz), 195.3 (dd, J_{CF} = 18 Hz, J'_{CF} = 22 Hz); ^{19}F NMR ($CDCl_3$) −204.4 (J_{FH} = 47.4 Hz), -236.7 (J_{FH} = 44.3 Hz); (b) 5-Fluoro-6-(fluoromethyl)-2-(methylthio)pyrimidin-4(1H)-one was prepared according to Duschinsky et al.[17]: m.p. 222–223°C; MS-ES$^-$ (M-H) m/z 191; ^{1}H NMR (d^6-DMSO) 2.50 (s, 3H), 5.31 (dd, J_{CF} = 48 Hz, J'_{CF} = 3 Hz, 1H), <10.0 (amide not observed); ^{19}F NMR (D_2O) −157.4, −222.4 (td, J_{FH} = 48 Hz, J_{FF} = 8.5 Hz).

ii. 2-Iminopyrrolidine hydrochloride was prepared by reported methods[18,19] and recrystallized from anhydrous EtOH to afford a hygroscopic, granular, white solid: m.p. 172.4–172.8°C; mass spectrometry (ESI$^+$ (M+H), m/z 85).

iii. TPI(F)-hydrochloride was prepared by adaptation of the TPI synthesis of Yano et al.[15] to obtain a white crystalline hydrochloride salt that exhibited: m.p. 240°C–243°C (dec); MS-ES$^-$ (M-H), m/z 225; ^{1}H-NMR (D_2O) 2.11 (pentuplet, J = 7.7 Hz, 2H), 2.93 (t, J = 8.0 Hz, 2H), 3.68 (t, J = 7.3 Hz, 2H), 4.55 (s, 2H), 4.7 (HOD); ^{19}F-NMR (D_2O) -169.42 (t, J_{FH} = 2.5 Hz);

iv. TPI-hydrochloride and TPI(Br)-hydrochloride were prepared, as described by Yano[15] and were characterized by mass spectrometry and ^{1}H-NMR. The ^{1}H NMR spectra of TPI, TPI(Br) and TPI(F) were essentially identical.

(B) Determination of IC_{50} for TPI(F) with [H-3]Thymidine and Recombinant Human TP

Thymidine phosphorylase activity was assayed in vitro using a modification of previously described methods.[20] An amount of 100 μl aliquots of 0.4 mg/ml human recombinant thymidine phosphorylase (Sigma-Aldrich Catalog No. T9319) in 0.5% bovine serum albumin were placed on ice. At time zero minutes the reaction was initiated by addition of 300 μl of assay buffer (4°C) containing 76 mM sodium phosphate, pH 7.4 and 1.6 mM [^{3}H-5′]-thymidine (Moravek Radiochemicals Catalog No. MT-846W; Moravek, Brea, CA, USA) plus or minus the appropriate concentrations of TPI compounds, serially diluted in double distilled water. The mixture was vortexed and placed in a 37°C gently shaking water bath. Time zero minute controls were terminated immediately without being removed from ice. After 30 minutes at 37°C, reactions were terminated by the addition of 400 μl of a 4°C slurry of 5% charcoal (Sigma-Aldrich Catalog No. 242276, "Darco G-60") in 10% trichloroacetic acid. The terminated mixture was centrifuged (10,000 × g/10 minutes/room temperature) and 0.4 ml of the supernate was counted in 5 ml of EcoScint A (National Diagnostics, Atlanta, GA, USA) in a Beckman LS5000C liquid scintillation counter. For an individual experiment, values for each condition were determined from the mean of two separate determinations after subtracting the time zero control values. IC_{50} values were extrapolated from the averaged data from three separate experiments and represent the concentration of inhibitor that inhibited TP activity by 50%.

REFERENCES

1. Cleaver J.E. *Thymidine metabolism and cell kinetics*. North-Holland Publishing Co., Amsterdam, 1967.
2. Rabinowitz, Y.; Wilhite, B.A. Thymidine salvage pathway in normal and leukemic leukocytes with effects of ATP on enzyme control. *Blood* **1969**, 33, 759–771.
3. Toi, M.; Atiqur Rahman, M.; Bando, H.; Chow, L.W.C. Thymidine phosphorylase (platelet-derived endothelial-cell growth factor) in cancer biology and treatment. *Lancet Oncol.* **2005**, 6, 158–166.

4. Brown, N.S.; Bicknell, R. Thymidine phosphorylase, 2-deoxy-D-ribose and angiogenesis. *Biochem. J.* **1998**, 334, 1–8.
5. Hotchkiss, K.A.; Ashton, A.W.; Klein, R.S.; Lenzi, M.L.; Zhu, G.H.; Schwartz, E.L. Mechanisms by which tumor cells and monocytes expressing the angiogenic factor thymidine phosphorylase mediate human endothelial cell migration. *Cancer Res.* **2003**, 63, 527–533.
6. Koukourakis, M.I.; Giatromanolaki, A.; O'Byrne, K.J.; Comley, M.; Whitehouse, R.M.; Talbot, D.C.; Gatter, K.C.; Harris, A.L. Platelet-derived endothelial cell growth factor expression correlates with tumour angiogenesis and prognosis in non-small-cell lung cancer. *Br. J. Cancer* **1997**, 75, 477–481.
7. de Bruin, M.; Smid, K.; Laan, A.C.; Noordhuis, P.; Fukushima, M.; Hoekman, K.; Pinedo, H.M.; Peters, G.J. Rapid disappearance of deoxyribose-1-phosphate in platelet derived endothelial cell growth factor/thymidine phosphorylase overexpressing cells. *Biochem. Biophys. Res. Comm.* **2003**, 301, 675–679.
8. Pula, G.; Mayr, U.; Evans, C.; Prokopi, M.; Vara, D.S.; Yin, X.; Astroulakis, Z.; Xiao, Q.; Hill, J.; Xu, Q.; Mayr, M. Proteomics identifies thymidine phosphorylase as a key regulator of the angiogenic potential of colony-forming units of endothelial progenitor cell cultures. *Circ. Res.* **2009**, 104, 32–40.
9. Grierson, J.R.; Brokenbrough, J.S.; Rasey, J.S.; Wiens, L.W.; Schwartz, J.L.; Jordan, R.; Vesselle, H. Evaluation of 5′-deoxy-5′-[F-18]fluorothymidine ([F-18]DFT) as a tracer of intracellular thymidine phosphorylase activity. *Nucl. Med. Biol.* **2007**, 34, 471–478.
10. Fukushima, M.; Suzuki, N.; Emura, T.; Yano, S.; Kazuno, H.; Tada, Y.; Yamada, Y.; Asao, T. Structure and activity of specific inhibitors of thymidine phosphorylase to potentiate the function of antitumor 2-deoxyribonucleosides. *Biochem. Pharmacol.* **2000**, 59, 1227–1236.
11. Zhou, D.; Chu, W.; Rothfuss, J.; Zeng, C.; Xu, J.; Jones, L.; Welch, M.J.; Mach, R.H. Synthesis, radiolabeling, and in vivo evaluation of an 18F-labeled isatin analog for imaging caspase-3 activation in apoptosis. *Bioorg. Med. Chem. Lett.* **2006**, 16, 5041–5046.
12. Zhou D.; Chu, W.; Chen, D.L.; Wang, Q.; Reichert, E.E.; Rothfuss, J.; D'Avignon, A.; Welch, M.J.; Mach, R.H. [F-18]- and [C-11]-Labeled N-benzyl-isatin sulfonamide analogues as PET tracers for Apoptosis: synthesis, radiolabeling mechanism, and in vivo imaging study of apoptosis in Fas-treated mice using [C-11]WC-98. *Org. Biomol. Chem.* **2009**, 7, 1337–1348.
13. Kalman, T.I.; Lai, L. 6-Substituted 5-fluorouracil derivatives as transition state analogue inhibitors of thymidine phosphorylase. *Nucleosides Nucleotides Nucleic Acids* **2005**, 24, 367–373.
14. Lai, L. *Design, synthesis and study of transition state analogue inhibitors of thymidine metabolism. Ph.D. thesis*, Department of Chemistry, SUNY, Buffalo, NY, 2001 (UMI 3010841).
15. Yano, S.; Kazuno, H.; Sato, T.; Suzuki, N.; Emura, T.; Wierzba, K.; Yamashita, J.-I.; Tada, Y.; Yamada, Y.; Fukushima, M.; Asao, T. Synthesis and evaluation of 6-methylene-bridged uracil derivatives. Part 2: Optimization of inhibitors of human thymidine phosphorylase and their selectivity with uridine phosphorylase. *Bioorg. Med. Chem.* **2004**, 12, 3443–3450.
16. McBee, E.T.; Pierce, O.R.; Kilbourne, H.W.; Wilson, E.R. The preparation and reactions of fluorine-containing acetoacetic esters. *J. Am. Chem. Soc.* **1953**, 75, 3152–3153.
17. Duschinsky, R.; Pleven, E.; Heidelberger, C. Synthesis of 5-fluoropyrimidines. *J. Am. Chem. Soc.* **1957**, 79, 4559–4560.
18. Moriconi, E.J.; Cevasco, A.A. Synthesis and reactions of cyclic amidines. *J. Org. Chem.* **1968**, 33, 2109–2111.
19. Moore, W.M.; Webber, R.K.; Fok, K.F.; Jerome, G.M.; Connor, J.R.; Manning, P.T.; Wyatt, P.S.; Misko, P.T.; Tjoeng, F.S.; Currie, M.G. 2-Iminopiperidine and other 2-iminoazaheterocycles as potent inhibitors of human nitric oxide synthase isoforms. *J. Med. Chem.* **1996**, 39, 669–672.
20. Zhu, G.H.; Lenzi, M.; Schwartz, E.L. The Sp1 transcription factor contributes to the tumor necrosis factor-induced expression of the angiogenic factor thymidine phosphorylase in human colon carcinoma cells. *Oncogene* **2002**, 21, 8477–85.