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ORIGINAL ARTICLE

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1-Benzyl indazole derivative-based ^{18}F-labeled PET radiotracer: Radiosynthesis and cell uptake study in cancer cells

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ABSTRACT

Hypoxia-inducible factor-1 (HIF-1α) is a transcription factor activated in response to low oxygen level, and is highly expressed in many solid tumors. Moreover, HIF-1α is a representative biomarker of hypoxia and also helps to maintain cell homeostasis under hypoxic condition. Most solid tumors show hypoxia, which induces poor prognosis and resistance to conventional cancer therapies. Thus, early diagnosis of hypoxia with positron emission tomography (PET) radiotracer would be highly beneficial for management of malignant solid tumors with effective cancer therapy. YC-1 is a most promising candidate among several HIF-1α inhibitors. As an effort to develop a hypoxia imaging tool as a PET radiotracer, we designed and synthesized [^{18}F]DFYC based on potent derivative of YC-1 and performed preliminary in vitro cell uptake study. [^{18}F]DFYC showed a significant accumulation in SKBR-3 cells among other cancer cells, proving as a good lead to develop a hypoxic solid tumor such as breast cancer.

Key Word: Hypoxia, HIF-1α inhibitors, YC-1, Positron emission tomography, [^{18}F]DFYC

Introduction

Tumor hypoxia is a distinct feature of rapidly growing solid tumors and characterized as a significantly low level of O_2 due to high demands of nutrients and oxygen for continues growth of tumors (1, 2). Hypoxia is an indicator of malignant progression, altered metabolism, poor prognosis, aggressive nature of cancers. Moreover, hypoxia is associated with the resistance to cancer therapies such as radiotherapy and chemotherapy. Thus, hypoxia can serve as a sign of advanced stage solid tumors, and the detection of hypoxia might help to guide a way for effective diagnosis and therapy against cancer. Activation of cellular processes as hypoxia-inducible factor-1α (HIF-1α) pathway is one of the critical post hypoxic responses (3). HIF-1α is a transcription factor that activates more than 100 downstream genes such as vascular endothelial growth factor (VEGF) involved in angiogenesis, inducible nitric oxide synthase (iNOS) leading nitric oxide production (NO), and insulin-like growth factors (IGF-1) that regulate growth, differentiation, and survival in cells (4-7). Therefore, HIF-1α is not only a promising target for cancer therapy but also a novel biomarker for noninvasive imaging study such as Positron emission tomography (PET), that will be of great importance for

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diagnosis of the advanced stage tumors and developing an effective cancer therapy. Several HIF-1α inhibitors that block the activity of HIF-1α under hypoxic conditions have been discovered and some of them are under clinical study (8, 9).

YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole] is a synthetic molecule known to possess several pharmacological actions such as inhibition of platelet aggregation and vascular contraction via the stimulation of soluble guanylate cyclase (sGC) activity (10-14). It has been previously reported that the effects of YC-1 are very similar to those of nitric oxide (NO) but are independent of NO (15). Nitric oxide (NO) is known to inhibit hypoxic responses through suppression of HIF-1α accumulation and activity (16, 17). In an effort to discover the mechanism behind the similarity of effects of YC-1 and NO, Park and co-workers performed a study and found that YC-1 completely blocks HIF-1α protein expression (18). This finding allowed some research groups to perform the studies for the development of novel potent HIF-1α inhibitors based on YC-1 structure (19, 20). Takeuchi and co-workers showed that the replacement of 1-benzyl group in YC-1 by di- or tetra-substituted benzyl groups improved the inhibitory activity from single digit micromolar to submicromolar IC_{50} (19). Among the synthesized derivatives, YC-1 derivative with a difluorobenzyl group (12l) improved the potency of HIF-1α inhibition by 20 times (2 μM to 0.1 μM).

Based on the study by Takeuchi and co-workers, we herein designed and synthesized ^{18}F-labelled PET radiotracer (1, [^{18}F]DFYC) based on potent YC-1 derivative with a difluorobenzyl group (12l) and performed its initial validation as a marker for early detection and good retention in cancer cells (Figure 1). *In vitro* cell uptake study of [^{18}F]DFYC PET radiotracer (1) were performed in a few cancer cells, including CT-26, MCF-7, MDA-MB-231, SKBR-3 and 4T1 cells, to develop the experimental method for *in vitro* cellular uptake and understand the potential of our novel PET radiotracer for future tumor imaging.

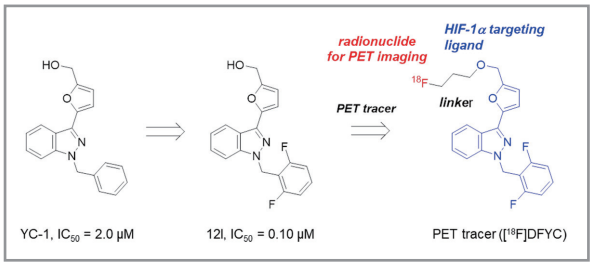


Figure 1. Designing of HIF-1α targeting PET radiotracer ([^{18}F]DFYC).

Materials and Methods

General

Starting materials were obtained from commercial sources and used without purification (Sigma-Aldrich, Combi-Block, Acros, TCI, Alfa Aesar, Daejung). Anhydrous toluene (Sigma-Aldrich, ≥ 99%), anhydrous ethanol (EtOH, Sigma-Aldrich, ≥ 99%) dichloromethane (MC, Sigma-Aldrich, ≥ 99%) and methanol (MeOH, Sigma-Aldrich, ≥ 99%) were purchased and used without any further purification and used under dry nitrogen atmosphere. All synthesized cold compounds were purified by flash column chromatography using ZEOprep 60 (40-63) μM silica gel from ZEOCHEM. ^{1}H NMR and ^{13}C spectra were recorded on JEOL JNM-ECZ400s/L1 (400 MHz) and used $CDCl_3$ or DMSO-d_6 as the solvent. Chemical shifts were quoted in parts per million (ppm) and coupling constants (J) were reported in hertz unit (Hz). Chemical shifts (in ppm) were referenced to tetramethylsilane (δ = 0 ppm) in $CDCl_3$ as an internal standard. ^{13}C NMR spectra were obtained by using the same NMR spectrometers and chemical shifts were reported in ppm referenced to the center line of a triplet at 77.0 ppm of $CDCl_3$ or 39.5 ppm of DMSO-d_6. The reactions containing ^{18}F radioisotope were monitored by radio-thin-layer chromatography using TLC 60 F_{254} -(Merck), analytical reverse phase-

high pressure liquid chromatography (RP-HPLC) and gamma-counter (Wizard 1470, Perkin-Elmer).

1. Synthesis of precursor 10 for ^{18}F-labeling

1.1) 3-Iodo-1H-indazole (1)

To a solution of indazole (2 g, 17.00 mmol) in N,N-dimethylformamide (DMF, 50 mL) were added iodine (8.30 g, 34.00 mmol) and potassium hydroxide (2.20 g, 34.00 mmol). After stirring for 2 h at RT, 10% $NaHSO_3$ (250 mL) was added to the reaction mixture and extracted product with ethyl acetate. Organic layer was washed with brine, dried over Na_2SO_4, concentrated and purified on column chromatography using Hex:EA (4:1) as eluent to provide 3-iodo-1H-indazole (1) in 98% of yield (4.05 g); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.51 (d, *J* = 8.7 Hz, 1H), 7.45-7.34 (m, 2H), 7.16 (t, *J* = 7.1 Hz, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 140.97, 127.76, 127.34, 121.77, 120.93, 111.07, 94.00; LRMS (ESI$^+$): m/z Calcd for $C_7H_{61}N_2$ $[M+H]^+$: 244.9, Found: 244.9.

1.2) 3-Iodo-1-((2-(trimethylsilyl)ethoxy) methyl)-1H-indazole (2)

To a stirred suspension of 3-iodo-1H-indazole (1) (4 g, 16.39 mmol) and TBABr (0.054 g, 0.164 mmol) in 105 mL of CH_2Cl_2 and 30 mL of 50% of KOH was added 2-(trimethylsilyl)ethoxymethyl chloride (3.35 g, 19.67 mmol) dropwise at 0 °C. After being stirred for 2 h at 0 °C, stirring was stopped and the reaction mixture was transferred to separating funnel and two layers were separated. Organic phase was washed with brine and dried over Na_2SO_4, filtered and concentrated. Resulting residue was purified by column chromatography using Hex:EA (4:1) as eluent to obtained 3-iodo-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-indazole (2) in 98% yield (6 g); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.72 (d, *J* = 8.7 Hz, 1H), 7.50 (td, *J* = 7.5, 0.9 Hz, 1H), 7.43 (d, *J* = 8.2 Hz, 1H), 7.24 (td, *J* = 7.5, 0.9 Hz, 1H), 3.48 (t, *J* = 8.0 Hz, 2H), 3.26 (s, 2H), 0.75 (t, *J* = 7.8 Hz, 2H), -0.15 (s, 9H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 140.80, 128.74, 128.37, 122.62, 121.47, 110.87, 95.26, 77.34, 66.26, 17.65, -0.90; LRMS (ESI+): m/z Calcd for $C1_3H_{20}IN_2OSi$ $[M+H]^+$: 375.0, Found: 375.0.

1.3) 5-(1-((2-(Trimethylsilyl)ethoxy) methyl)-1H-indazol-3-yl) furan-2-carbaldehyde (3)

To solution of 3-iodo-1-((2-(trimethylsilyl)ethoxy) methyl)-1H-indazole (2) (0.96 g, 2.56 mmol), and (5-formylfuran-2-yl)boronic acid (0.431 g, 3.08 mmol) in EtOH (2.5 mL) and toluene (25 mL) were added $Pd(dppf)Cl_2.CH_2Cl_2$ (0.21 g, 0.26 mmol) and saturated aq. Na_2CO_3 (2.5 mL). The reaction mixture was heated to 80 °C for 10 h. After solvent was removed under reduced pressure, the residue was passed through bed of Celite using dichloromethane (DCM) as eluent, concentrated and purified on column chromatography using Hex:EA (10:1 to 5:1) as eluent to provide

5-(1-((2-(trimethylsilyl)ethoxy)methyl)-1H-indazol-3-yl)furan-2-carbaldehyde (3) in 64% yield (0.56 g); ^{1}H-NMR (400 MHz, DMSO-d6) δ 9.66 (s, 1H), 8.16 (d, *J* = 8.2 Hz, 1H), 7.83 (d, *J* = 8.7 Hz, 1H), 7.70 (d, *J* = 3.7 Hz, 1H), 7.53 (td, *J* = 7.5, 0.9 Hz, 1H), 7.36 (td, *J* = 7.5, 0.9 Hz, 1H), 7.32 (d, *J* = 3.7 Hz, 1H), 3.53 (t, *J* = 8.0 Hz, 2H), 3.27 (s, 2H), 0.78 (t, *J* = 8.0 Hz, 2H), -0.16 (s, 9H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 178.54, 153.53, 152.29, 149.73, 141.31, 135.14, 128.09, 125.29, 123.51, 121.55, 121.35, 111.36, 110.41, 77.82, 66.44, 17.66, -0.90; LRMS (ESI$^+$): m/z Calcd for $C_{18}H_{23}N_2O_3Si$ $[M+H]^+$: 343.1, Found: 343.1.

1.4) 5-(1H-Indazol-3-yl)furan 2-carbaldehyde (4)

To the solution of 5-(1-((2-(trimethylsilyl)ethoxy) methyl)-1H-indazol-3-yl)furan-2-carbaldehyde (3) (0.620 g, 1.81 mmol) in THF (60 mL) were added ethylene diamine (1.09 g, 18.10 mmol) and TBAF (2.37 g, 9.05 mmol). The reaction mixture was heated at 80 °C for 10

h. After the completion of reaction was confirmed, the reaction mixture was cooled and poured into the mixture of DCM:MeOH (10:1). The organic layer was washed by saturated aqueous solution of NH_4Cl. The organic layer was dried over Na_2SO_4, filtered, concentrated and purified on column chromatography using DCM:MeOH (30:1) as eluent to obtain 5-(1H-indazol-3-yl)furan-2-carbaldehyde (4) in 52% yield (0.016 g); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.64 (s, 1H), 8.13 (dd, *J* = 8.2, 0.9 Hz, 1H), 7.69 (d, *J* = 3.7 Hz, 1H), 7.61 (d, *J* = 8.7 Hz, 1H), 7.48-7.41 (m, 1H), 7.32-7.26 (m, 1H), 7.25 (d, *J* = 3.7 Hz, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 178.29, 154.65, 152.03, 141.54, 135.03, 127.47, 125.57, 122.71, 120.98, 120.41, 111.48, 109.54; LRMS (ESI+): m/z Calcd for $C_{12}H_9N_2O_2$ $[M+H]^+$: 213.0, Found: 213.0.

1.5) 5-(1-(2,6-Difluorobenzyl)-1H-indazol-3-yl) furan-2-carbaldehyde (5)

To the solution of 5-(1H-indazol-3-yl)furan-2-carbaldehyde (4) (0.25 g, 1.18 mmol) in THF (30 mL) was added t-BuOK (135 g, 1.18 mmol) at 0 °C. After stirring reaction for 1 h at 0°C was added 2-(bromomethyl)-1,3-difluorobenzene (0.199 g, 0.94 mmol) dropwise. The reaction mixture was stirred at RT by for 4 h. After the completion of reaction was confirmed, cooled the reaction mixture and poured EtOAc. The organic layer was washed by water followed by brine. The organic layer was dried over Na_2SO_4, filtered, concentrated and purified on column chromatography using Hex:EA (2:1) as eluent to obtain 5-(1-(2,6-difluorobenzyl)-1H-indazol-3-yl)furan-2-carbaldehyde (5) in 58% yield (0.23 g); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 9.72 (s, 1H), 8.24 (d, *J* = 7.8 Hz, 1H), 7.58 (d, *J* = 8.7 Hz, 1H), 7.49-7.41 (m, 1H), 7.36 (d, *J* = 3.7 Hz, 1H), 7.34-7.26 (m, 2H), 7.08 (d, *J* = 3.7 Hz, 1H), 6.91 (t, *J* = 8.0 Hz, 2H), 5.69 (s, 2H).

1.6) (5-(1-(2,6-Difluorobenzyl)-1H-indazol-3-yl)furan-2-yl)methanol (6)

To the solution of 5-(1-(2,6-difluorobenzyl)-1H-indazol-3-yl)furan-2-carbaldehyde (5) (0.41 g, 1.21 mmol) in EtOH (6 mL) was added $NaBH_4$ (0.228 g, 6.06 mmol) slowly in portion. The reaction mixture was stirred at RT for 3h. After the completion of reaction was confirmed, the mixture was quenched with water and extracted with the mixture of DCM:MeOH (100:1). The organic layer was washed by brine. The organic layer was dried over Na_2SO_4, filtered, concentrated and purified on column chromatography using DCM:EA (9:1) as eluent to obtain (5-(1-(2,6-difluorobenzyl)-1H-indazol-3-yl)furan-2-yl)methanol (6) in 96% yield (0.39 g); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.04 (d, *J* = 8.2 Hz, 1H), 7.74 (d, *J* = 8.7 Hz, 1H), 7.51-7.36 (m, 2H), 7.21 (t, *J* = 7.5 Hz, 1H), 7.09 (t, *J* = 8.0 Hz, 2H), 6.87 (d, *J* = 3.2 Hz, 1H), 6.40 (d, *J* = 3.7 Hz, 1H), 5.68 (s, 2H), 5.30 (t, *J* = 5.7 Hz, ^{1}H), 4.44 (d, *J* = 5.5 Hz, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 162.82, 162.74, 160.34, 160.27, 155.98, 147.52, 140.87, 136.25, 131.51, 127.44, 122.00, 121.58, 120.42, 113.02, 112.40, 110.55, 109.36, 108.60, 56.20; LRMS (ESI^+): m/z Calcd for $C_{19}H_{15}F_2N_2O_2$ $[M+H]^+$: 341.1, Found: 341.1.

1.7) 3-((tert-Butyldimethylsilyl)oxy)propyl 4-methylbenzenesulfonate (7)

At 0 °C, to the solution of 1,3-propanediol (1.99 g, 26.15 mmol) in CH_2Cl_2 (7 mL) were added TEA (0.53 g, 5.23 mmol) and DMAP (0.064 g, 0.53 mmol). A solution of tosyl chloride (1 g, 5.25 mmol) in CH_2Cl_2 (15 mL) was added dropwise to reaction mixture. The reaction mixture was warmed to RT and stirred for 18 h. After the completion of reaction was confirmed, the reaction mixture was poured into saturated aqueous NH_4Cl and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered, concentrated and purified on column chromatography using the mixture

of DCM:EA (9:1) as eluent to obtain 3-hydroxypropyl 4-methylbenzenesulfonate in 69% yield (0.84 g); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 8.2 Hz, 2H), 7.35 (d, *J* = 8.2 Hz, 2H), 4.18 (t, *J* = 5.9 Hz, 2H), 3.71 (t, *J* = 5.9 Hz, 2H), 2.45 (s, 3H), 1.89 (quint, *J* = 6.1 Hz, 2H).

To the solution of 3-hydroxypropyl 4-methylbenzenesulfonate (0.50 g, 2.17 mmol) in CH_2Cl_2 (20 mL) were added imidazole (0.37 g, 5.43 mmol) and DMAP (0.027 g, 0.22 mmol). The reaction mixture was stirred at RT for a while. A solution of t-butyldimethylsilyl chloride (1 g, 5.25 mmol) in CH_2Cl_2 (10 mL) was added slowly to the reaction mixture. The reaction mixture was stirred at RT for 18 h. After the completion of reaction was confirmed, the reaction mixture was poured into saturated aqueous NH_4Cl and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered, concentrated and purified on column chromatography using Hex:EA (4:1) as eluent to obtain 3-((tert-butyldimethylsilyl) oxy)propyl 4-methylbenzenesulfonate (7) in 66% yield (0.49g); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 8.2 Hz, 2H), 7.34 (d, *J* = 8.2 Hz, 2H), 4.14 (t, *J* = 6.2 Hz, 2H), 3.62 (t, *J* = 5.9 Hz, 2H), 2.44 (s, 3H), 1.83 (quint, *J* = 6.1 Hz, 2H), 1.57 (s, 2H), 0.82 (s, 9H), 0.015 (s, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 144.73, 133.17, 129.90, 128.01, 67.64, 58.49, 32.06, 25.88, 21.71, 18.25, 5.44.

1.8) 3-(5-((3-((tert-Butyldimethylsilyl)oxy) propoxy)methyl)furan-2-yl)-1-(2,6-difluorobenzyl)-1H-indazole (8)

To the solution of (5-(1-(2,6-difluorobenzyl)-1H-indazol-3-yl)furan-2-yl)methanol(6)(0.20g,0.59mmol) in DMF (2 mL) was added NaH (0.056 g, 2.36 mmol) slowly in portion. The reaction mixture was stirred at RT for 15 min. A solution of 3-((tert-butyldimethylsilyl) oxy)propyl 4-methylbenzenesulfonate (7) (0.61 g, 1.77 mmol) in DMF (1 mL) was added to the reaction mixture. The reaction mixture was stirred at RT for 6 h. After the completion of reaction was confirmed, the reaction was quenched with water and extracted with ethyl acetate. The organic layer was washed by brine. The organic layer was dried over Na_2SO_4, filtered, concentrated and purified on column chromatography using Hex:EA (9:1) as eluent to obtain 3-(5-((3-((tert-butyldimethylsilyl)oxy)propoxy)methyl)furan-2-yl)-1-(2,6-difluorobenzyl)-1H-indazole (8) in 49% yield (0.15 g); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.09 (d, *J* = 8.2 Hz, 1H), 7.53 (d, *J* = 8.7 Hz, 1H), 7.41 (t, *J* = 7.8 Hz, 1H), 7.31-7.24 (m, 1H), 7.21 (t, *J* = 7.5 Hz, 1H), 6.90 (t, *J* = 7.8 Hz, 2H), 6.86 (d, *J* = 3.2 Hz, 1H), 6.46 (d, *J* = 3.2 Hz, 1H), 5.68 (s, 2H), 4.56 (s, 2H), 3.71 (t, *J* = 6.2 Hz, 2H), 3.62 (t, *J* = 6.2 Hz, 2H), 1.82 (quint, *J* = 6.1 Hz, 2H), 0.87 (s, 9H), 0.03 (s, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 163.02, 162.94, 160.53, 160.45, 151.99, 149.02, 140.43, 136.65, 130.37, 126.84, 121.89, 121.36, 115.26, 112.40, 111.68, 110.91, 109.37, 107.75, 67.04, 65.15, 61.13, 59.98, 40.91, 33.00, 26.02, 18.40, 5.29.

1.9) 3-((5-(1-(2,6-Difluorobenzyl)-1H-indazol-3-yl)furan-2-yl)methoxy)propan-1-ol (9)

To the solution of 3-(5-((3-((tert-butyldimethylsilyl) oxy)propoxy)methyl)furan-2-yl)-1-(2,6-difluorobenzyl)-^{1}H-indazole (8) (0.13 g, 0.25 mmol) in THF (10 mL) was added TBAF (0.099 g, 0.38 mmol, 1M solution (0.38 mL)). The reaction mixture was refluxed for 1 h. The solvent was evaporated and the residue was purified on column chromatography using DCM:EA (9:1) as eluent to obtain 3-((5-(1-(2,6-difluorobenzyl)-1H-indazol-3-yl)furan-2-yl)methoxy)propan-1-ol (9) in 92% yield (0.093 g); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.06 (d, *J* = 8.2 Hz, 1H), 7.80 (d, *J* = 8.7 Hz, 1H), 7.56-7.40 (m, 2H), 7.27 (t, *J* = 7.3 Hz, 1H), 7.14 (t, *J* = 8.0 Hz, 2H), 6.95 (d, *J* = 3.7 Hz, 1H), 6.59 (d, *J* = 3.2 Hz, 1H), 5.74 (s, 2H), 4.49 (s, 2H), 4.41 (t, *J* = 4.8 Hz, 1H), 3.52 (t, *J* = 6.6 Hz, 2H), 3.44 (q, *J* = 5.6 Hz, 2H), 1.67 (quint, *J* = 6.1 Hz, 2H); ^{13}C-NMR (100 MHz,

DMSO-d_6) δ 162.81, 162.73, 160.33, 160.26, 152.40, 148.20, 140.88, 136.06, 131.54, 127.48, 122.15, 121.44, 120.43, 112.99, 112.41, 111.72, 110.62, 108.61, 67.27, 64.47, 58.24, 33.14; LRMS (ESI^+): m/z Calcd for $C_{22}H_{21}F_2N_2O_3$ $[M+H]^+$: 399.1, Found: 399.1.

1.10) 3-((5-(1-(2,6-Difluorobenzyl)-1H-indazol-3-yl)furan-2-yl)methoxy) propyl 4-methylbenzenesulfonate (10)

To the solution of 3-((5-(1-(2,6-difluorobenzyl)-1H-indazol-3-yl)furan-2-yl)methoxy)propan-1-ol (9) (0.13 g, 0.33 mmol) in CH_2Cl_2 (10 mL) was added TEA (0.099 g, 0.98 mmol) and DMAP (0.019 g, 0.16 mmol). The reaction mixture was stirred at RT for 15 min. p-Toluenesulfonic anhydride (0.27 g, 0.82 mmol) was added to the reaction mixture. The reaction mixture was stirred at RT for 5h. The solvent was evaporated and the residue was purified on column chromatography using DCM:EA (9:1) as eluent to obtain 3-((5-(1-(2,6-difluorobenzyl)-1H-indazol-3-yl) furan-2-yl)methoxy)propyl 4-methylbenzenesulfonate (10) in 59% yield (0.11 g); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.05 (d, *J* = 7.8 Hz, 1H), 7.81 (d, *J* = 8.2 Hz, 1H), 7.73 (d, *J* = 8.2 Hz, 2H), 7.56-7.42 (m, 2H), 7.40 (d, *J* = 8.2 Hz, 2H), 7.27 (t, *J* = 7.3 Hz, 1H), 7.13 (t, *J* = 8.0 Hz, 2H), 6.95 (d, *J* = 3.2 Hz, 1H), 6.54 (d, *J* = 3.7 Hz, 1H), 5.74 (s, 2H), 4.40 (s, 2H), 4.05 (t, *J* = 6.2 Hz, 2H), 3.43 (t, *J* = 6.2 Hz, 2H), 2.33 (s, 3H), 1.83 (quint, *J* = 6.1 Hz, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 162.81, 162.73, 160.33, 160.26, 151.99, 148.32, 145.37, 140.89, 136.03, 132.78, 131.53, 130.66, 128.05, 127.49, 122.17, 121.45, 120.44, 112.98, 112.40, 111.87, 110.63, 108.57, 68.62, 65.59, 64.42, 29.07, 21.53; LRMS (ESI^+): m/z Calcd for $C_{29}H_{27}F_2N_2O_5S$ $[M+H]^+$: 553.2, Found: 553.2.

2. Synthesis of cold DFYC and HPLC analysis.

The cold form of DFYC was synthesized from the tosylated precursor (10) by fluorination reaction in the presence of TBAF as shown in Scheme 2A. The cold DFYC was isolated and characterized by NMR and mass spectra, and was used as a standard for ^{18}F-labeling reaction. The analytical HPLC method was developed using Azura® analytical HPLC system on reverse phase C18 column (Luna® C18(2) 5μm, 4.6*150 mm column, 100Å) by elution with 70 to 90% MeOH in H_2O for 20 min and then 90% MeOH in H_2O till 24 min with final equilibration with 70% MeOH in H_2O for 5 min with the flow rate of 1mL/min and detection by PDA detector at 254 nm. The reaction protocol for ^{18}F-labeling was also developed by performing the reaction of precursor (10) with KF in the presence of using $K_{2.2.2}/K_2CO_3$ which give the cold DFYC (Scheme 2B). The reaction time was monitored and fixed for 20 min for ease of ^{18}F-labeling within half-life of ^{18}F ($t_{1/2}$= 109.8 min).

2.1) 1-(2,6-Difluorobenzyl)-3-(5-((3-fluoropropoxy)methyl)furan-2-yl)-1H-indazole(11, DFYC)

To a solution of tosylated precursor 10 (0.02 g, 0.036 mmol) in THF (5 mL) was added TBAF (0.065 mL of 1 M solution in THF, 0.065 mmol). The reaction mixture was heated to 90 ˚C for 20 min. The volatile was evaporated and the residue was purified on column chromatography using Hex:EA (4:1) as eluent to obtain cold DFYC (11) in 89% yield (0.013 g); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.06 (d, *J* = 8.2 Hz, 1H), 7.80 (d, *J* = 8.7 Hz, 1H), 7.55-7.41 (m, 2H), 7.27 (t, *J* = 7.5 Hz, 1H), 7.14 (t, *J* = 8.0 Hz, 2H), 6.96 (d, *J* = 3.2 Hz, 1H), 6.61 (d, *J* = 3.2 Hz, 1H), 5.74 (s, 2H), 4.55 (t, *J* = 5.9 Hz, 1H), 4.52 (s, 2H), 4.43 (t, *J* = 5.9 Hz, 1H), 3.56 (t, *J* = 6.4 Hz, 2H), 1.93 (quint, *J* = 5.7 Hz, 1H), 1.87 (quint, *J* = 6.2 Hz, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 162.81, 162.73, 160.26, 152.15, 148.32, 140.88,

136.03, 131.54, 127.48, 122.16, 121.42, 120.44, 112.99, 112.405, 111.90, 110.63, 108.60, 82.43, 80.83, 65.82, 65.76, 64.48, 30.77, 30.58, 29.58; LRMS (ESI^+): m/z Calcd for $C_{22}H_{20}F_3N_2O_2$ $[M+H]^+$: 401.1, Found: 401.1

3. ^{18}F-Labeling

3.1) Production of ^{18}F

^{18}F was produced by $^{18}O(p, n)^{18}F$ reaction (10 μA, 0.3 h irradiation) at RFT-30 an indigenous 30 MeV prototype cyclotron (Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute). The produced ^{18}F was trapped on an anion exchange QMA resin (Sep-Pak light, Waters) and eluted with $K_{2.2.2.}$ (25 mg/mL in H_2O) and K_2CO_3 (100 mg/9 mL in CH_3CN) by 3 times repeated azeotropic distillation with 1 mL of CH_3CN.

3.2) Preparation of [^{18}F]DFYC

3.7 GBq of ^{18}F containing $K_{2.2.2.}/K_2CO_3$ (200 μL of CH_3CN) was reacted with 5 mg of precursor 10 dispersed in 300 μL of CH_3CN. The mixture was reacted at 95 ℃ for 20 min and was cooled at RT for 10 min as shown in Scheme 3. The labeling efficiency of [^{18}F] DFYC was measured by radio-TLC (Stationary phase: silica gel, mobile phase: Hex/EA=4/1). The crude [^{18}F] DFYC was directly purified by RP-HPLC (Gradient: MeOH in H_2O system developed during the synthesis of cold form, flow rate: 1 mL/min, UV wavelength: 254 nm). The peaks on chromatogram were separated and collected manually, observing the gamma peak. Radiochemical purity (RCP) of the purified [^{18}F]DFYC was calculated by Radio-TLC.

3.2) Preparation of [^{18}F]DFYC

Lipophilicity (Log P) was calculated using the ratio of radioactivity of [^{18}F]DFYC in n-octanol phase and aqueous phase. 3.7 MBq/100 μL of [^{18}F]DFYC was added in the mixture of n-octanol (1 mL) and water (1 mL). This solution was thoroughly mixed for 10 min at RT using a vortex shaker, and centrifuged at 10,000 rpm for 5 min. Each phase aliquot (100 μL) was taken and counted using gamma-counter (Wizard 1470, Perkin-Elmer). The lipophilicity was calculated as following;

$$\text{Log P} = \frac{\text{(activity (cpm)in n-Octanol)}}{\text{(activity (cpm)in water)}}$$

3.4) *In vitro* stability study

The stability of [^{18}F]DFYC in human serum was monitored using a radio-TLC (Stationary phase: silica gel, mobile phase: Hex/EA=4/1). Purified [^{18}F]DFYC was dried under a stream of N_2 gas and dissolved in 200 μL of 0.9% NaCl. The 1 mL of human serum in a 15 mL conical tube equilibrated to 37 ℃ in water bath was added to 3.7 MBq (100 μCi) of [^{18}F]DFYC in 0.9% NaCl. After incubation, 1 μL (<5 μCi) of [^{18}F] DFYC was spotted onto silica gel TLC plate and the radiochemical purity (%) was determined by radio-TLC.

3.5) Cell culturing for *In vitro* cellular uptake study

Breast cancer cells (MCF-7, MDA-MB-231 and SKBR-3) and mouse colon cancer cell (CT-26) were purchased from Korea Cell Lines Bank (KCLB), and breast cancer cell (4T1) was purchased from American Type Culture Collection (ATCC). Cancer cell lines were cultured in Dulbecco's modified medium (DMEM) with 10% fetal bovine serum, 4 mM L-glutamine, 4500 mg/L glucose and sodium pyruvate. SKBR-3, MCF-7 and 4T1 breast cancer cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and L-glutamine. All cancer cell lines were cultivated and maintained at 37 °C in 5% CO_2 atmosphere. Cancer cell lines were plated to evaluate the binding affinity on a 24 well plate and incubated in the cell culture media.

3.6) *In vitro* cellular uptake study

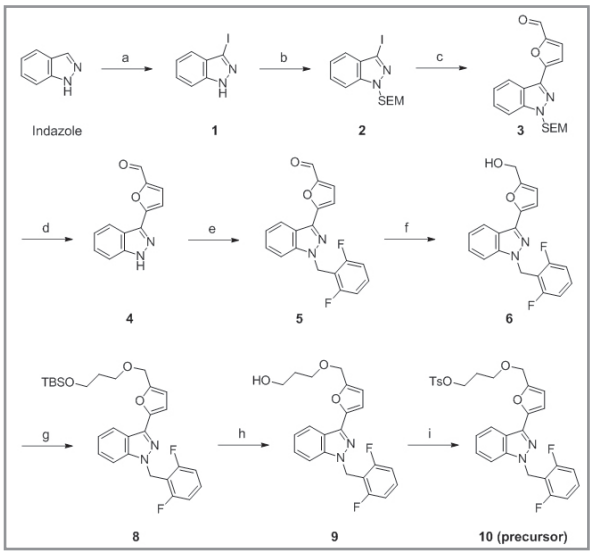
The cellular uptake of [^{18}F]DFYC was measured in CT-26, MCF-7, MDA-MB-231, SKBR-3 and 4T1 cells. The cells were sub-cultured in 12-well plates (1x106 of cells) and incubated with 185 kBq/well of [^{18}F]DFYC at 37 ℃ for 15, 60, and 120 min. After incubation, 20 μL of supernatant was collected and the remaining media was discarded, after which cells were washed with 500 μL of cold PBS to remove the unbounded [^{18}F]DFYC. 400 μL of 0.05% trypsin/EDTA solution was added to each well containing harvesting cancer cell lines. After collecting cell pellets, the number of cells was counted by cell counter (Mini Automated Cell Counter, ORFLO technologies) for accurate measurement of cell uptake. The radio-activity of supernatant and cell suspension was determined by gamma-counter.

Results and Discussion

Synthesis of cold DFYC

The precursor 10 for ^{18}F-labeling, containing a linker with a good leaving group (-OTs) for nucleophilic substitution with 18F, was synthesized as shown in scheme 1. The synthesis was started with iodination at C-3 position of commercially available indazole to obtained compound 1. The protection of N-1 of compound 1 with SEM chloride under basic conditions followed by Pd-catalyst coupling at C-3 position with (5-formylfuran-2-yl)boronic acid by Suzuki coupling reation under conventional condition provided compound 3. Deprotection of SEM from compound 3 with TBAF provided compound 4, which was sub*J*ected to benzylation with o-difluorobenzyl bromide to provide compound 5. Furfuryl alcohol 6 was obtained by reduction of 5-formyl group on carboxaldehyde 5, which was subjected to the coupling reaction with compound 7 in basic condition to give TBS-protected alcohol 8. Finally, precursor 10 with a tosyl group was prepared by deprotection of TBS group from 8 followed by tosylation.

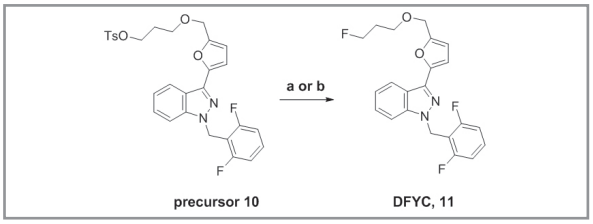
Scheme 1. Synthesis of ^{18}F-Labeling Precursor 10.[a]



[a] Reagents: (a) I2, KOH, DMF, RT; (b) KOH, TBABr, SEMCl, H_2O, CH_2Cl_2, 0 ℃; (c) (5-formylfuran-2-yl) boronic acid, Pd(dppf).CH_2 Cl_2 , Aq. Na_2CO_3, EtOH, PhMe, 80 ℃; (d) TBAF, CH_2NH_2, THF, Reflux; (e) 2-(bromomethyl)-1,3-difluorobenzene, t-BuOK, THF, 0 oC to RT; (f) $NaBH_4$, THF, RT; (g) 7, NaH, DMF, RT; (h) TBAF, THF, 90 ℃; (i) Ts_2O. Et_3N, DMAP, DCM, RT.

The cold DFYC was finally prepared by fluorination of tosylated precursor 10 using TBAF in 89% of yield (Scheme 2). The analytical LCMS analysis showed the molecular ion peak of DFYC at m/z = 401.1 (M+H, Figure 2C). The model study for ^{18}F-labeling by the reaction of precursor 10 with KF in the presence of $K_{2.2.2}$/K_2CO_3 resulted in 85% of yield (Figure 2) as per HPLC analysis.

Scheme 2. Synthesis of cold DFYC.[a)]



[a)] Reagents: (a) TBAF, THF, 90 °C; (b) KF/$K_{2.2.2}$, K_2CO_3, CH_3CN, 95 °C.

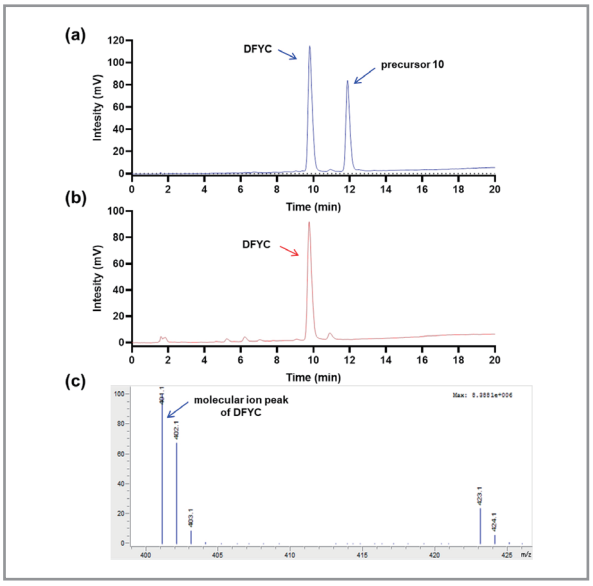
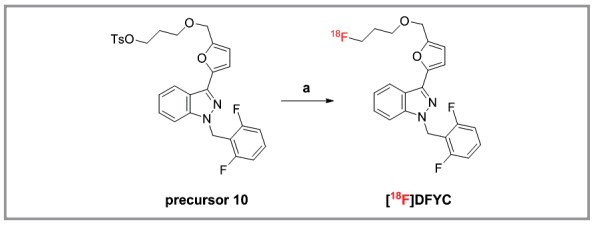


Figure 2. Characterization of cold DFYC. (a) HPLC chromatograms of the mixture of precursor 10 and DFYC (11), (b) HPLC chromatogram of the crude reaction mixture of model study for ^{18}F-labeling, (c) Mass spectrum of DFYC.

^{18}F-Labeling and purification of [^{18}F]DFYC

Radiosynthesis of [^{18}F]DFYC is carried out using $K_{2.2.2}$/K_2CO_3-mediated nucleophilic radiofluorination of the precursor 10 with the generated ^{18}F (Scheme 3). Substitution reaction with ^{18}F and subsequent purification by RP-HPLC (MeOH/water, gradient elution, Figure 3) was performed for 80 ± 5 min, which resulted in decay-corrected yields of 17.71 ± 1.83% (n=2). Radiofluorination was performed on 5 mg of the precursor 10 with ^{18}F-fluoride in 500 µL of CH_3CN at 95 °C for 20 min. The radiochemical purity of [^{18}F]DFYC was determined to be in 95.05 ± 0.25% by radio-TLC (Figure 3). The specific activity (SA= radioactivity/mol) of purified [^{18}F]DFYC was determined with 2.20 GBq/µmol.

Scheme 3. ^{18}F-Labeling for Synthesis of [^{18}F]DFYC.[a)]



[a)] Reagents: (a) ^{18}F/$K_{2.2.2}$, K_2CO_3, CH_3CN, 95 °C.

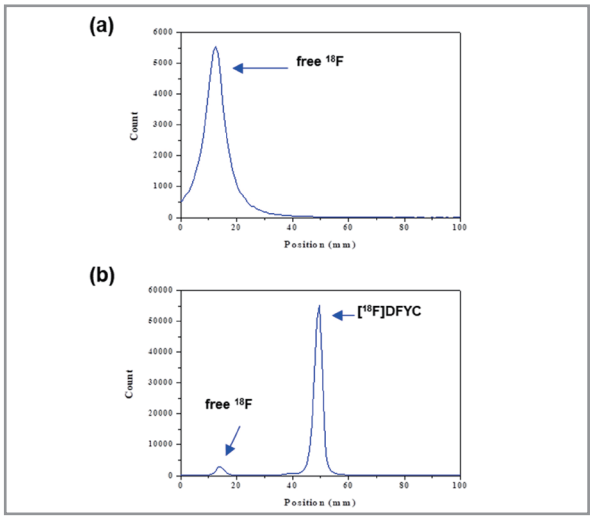


Figure 3. Radio-TLC chromatograms of ^{18}F-labeling reaction.

Lipophilicity of [^{18}F]DFYC and in vitro stability

The lipophilicity is one of the most important pharmacokinetic properties for drugs, which is decides permeability of drug molecule into cells and distribution during circulation. The log p value of 0.74 ± 0.01 suggested the hydrophobic nature of [^{18}F]DFYC would be suitable for permeating the cells to reach the target HIF-1α protein. In vitro stability evaluation of [^{18}F]DFYC was conducted in human serum and showed that 54.22 ± 0.95% radiotracer was intact till 120 min at 37 °C (Figure 4). The results suggested that the [^{18}F]DFYC would be stable for significant time span for

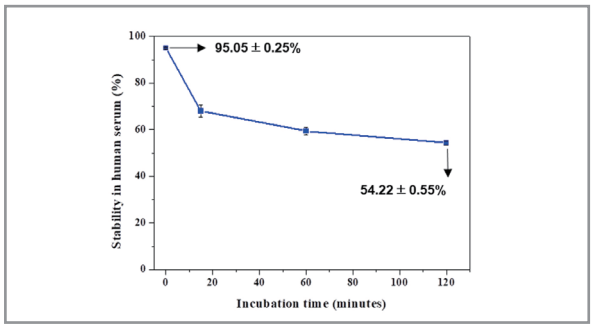


Figure 4. *In vitro* stability of [^{18}F]DFYC in human serum.

imaging study.

In vitro cellular uptake

In vitro uptake of [^{18}F]DFYC was assessed in CT-26, MCF-7, MDA-MB-231, SKBR-3 and 4T1 at three incubation times points with 185 kBq/well of radiotracer and the results are summarized in Figure 5 and Table 1. The [^{18}F]DFYC uptake study was demonstrated a time-dependent cellular accumulation in all cancer cell lines. The linearity of uptake was decreased from 60 min to 120 min for all cells except SKBR-3. However, accumulation of [^{18}F]DFYC in SKBR-3 cells increased further to 2.01 ± 0.02%ID at final interval of 120 min. Human epidermal growth factor receptor-2 (HER2 or Neu) overexpression activates HIF-1α pathways via the activation of Akt independently of hypoxia, which allows angiogenesis and metabolic adaptation in HER2/Akt-activated tumors, even in the absence of hypoxia.(21,22) Additionally, HIF-1α is known as a

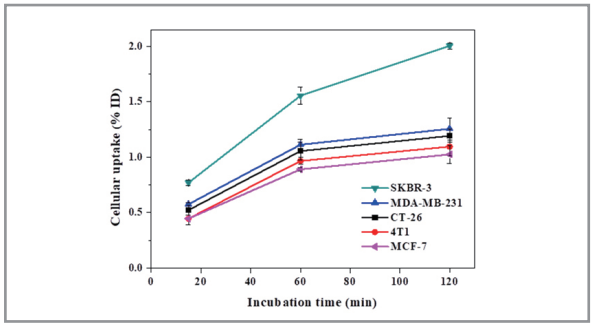


Figure 5. Time courses of in vitro cellular uptake of [^{18}F]DFYC. Cancer cell lines were incubated with [^{18}F]DFYC for 15, 60 and 120 min. Data are expressed as mean ± standard deviation of the percentage of the injected dose (%ID) of [^{18}F]DFYC.

Table 1. Cellular Uptake of [^{18}F]DFYC.

	15 min	60 min	120 min
CT-26	0.52 ± 0.02	1.06 ± 0.5	1.19 ± 0.03
MCF-7	0.45 ± 0.03	0.96 ± 0.02	1.10 ± 0.05
MDA-MB-231	0.57 ± 0.01	1.11 ± 0.03	1.26 ± 0.06
SKBR-3	0.77 ± 0.01	1.56 ± 0.04	2.01 ± 0.02
4T1	0.45 ± 0.03	0.89 ± 0.01	1.03 ± 0.05

regulator of HER2 mediated oncogenesis and anoikis resistance in breast cancer.(23) Thus, the detection of HIF-1α can help for imaging of HER2 overexpressed breast cancers. SKBR-3 cells are breast cancer cell lines with overexpression of HER2 biomarkers, and the cell uptake study suggests that YC-based PET radiotracer can be developed as a nuclear medicine for diagnosis of HER2-positive breast cancer patients.

Conclusion

In conclusion, we have successfully designed a PET radiotracer based on potent derivatives of YC-1, which is a HIF-1α inhibitor. The tosylated precursor 10 was prepared from indazole by 10 step reactions containing iodination, Suzuki cross coupling, N-alkylation, reduction and tosylation with 4.7% of total yield, and the subsequent fluorination with ^{18}F successfully produced [^{18}F]DFYC with 17.71 ± 1.83% of radiochemical yield and 95.05 ± 0.25% of radiochemical. The synthesized [^{18}F]DFYC was found to possess good stability in human serum, adequate hydrophobicity for cell permeation, and significant in vitro cellular uptake in SKBR-3 cells which are breast cancer cells expressing HER2 receptor. Thus, this preliminary cell uptake study showed that [^{18}F]DFYC can be a good candidate for the development of successful imaging agent for solid tumors such as breast cancer.

Acknowledgments

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