Synthesis and Biodistribution studies of ^{99m}Tc labeled fatty acid derivatives prepared via 'Click approach' for potential use in cardiac imaging

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Short title: Evaluation of ^{99m}Tc-fatty acid derivatives for cardiac imaging

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Abstract

^{123}I-Iodophenylpentadecanoic acid (IPPA) is a metabolic agent used in nuclear medicine for diagnosis of myocardial defects. Efforts are underway worldwide to develop a ^{99m}Tc substitute of the above radiopharmaceutical for the aforementioned application. Herein, we report synthesis and biodistribution studies of ^{99m}Tc labeled fatty acids (8, 11 and 15 carbons) obtained via 'click chemistry' for its potential use in myocardial imaging.

ω-Bromo fatty acids (8C/11C/15C) were synthetically modified at bromo terminal to introduce a heterocyclic triazole with glycine sidearm in a five step procedure. Modified fatty acids were subsequently radiolabeled with preformed $[^{99m}Tc(CO)_3]^+$ synthon to yield the desired fatty acid complexes which were evaluated in Swiss mice.

All the radiolabeled complexes were obtained with radiochemical purities >80%, as characterized by HPLC. Biodistribution studies of all three complexes in Swiss mice showed myocardial uptake of ~6-9% ID/g at 2 min post-injection, close to *I-IPPA (~9% ID/g). Complexes exhibited significant retention in the myocardium up to 30 min (~1% ID/g) but were lower to the standard agent (~7% ID/g).

Similar uptake of activity in myocardium for the newly synthesized complexes in comparison to ^{125}I-IPPA along with favorable *in vivo* pharmacokinetics merits potential for the present 'click' design of complexes for myocardial imaging.

Key words: Fatty acid, click chemistry, ^{99m}Tc carbonyl core, myocardial imaging

Introduction

Mechanistic shift of myocardial metabolism from fats to glucose during ischemic episodes is a sensitive measure for detecting coronary artery diseases. Radiolabeled fatty acids serve as useful substrates for delineating such abnormalities in a functional myocardium.[1-3] In this preview, a number of PET and SPECT labeled fatty acids have been extensively reviewed in past. PET radiotracers offer advantages in terms of high image resolution and quantitative image analyses,[4-7] but complex radiosynthesis protocols employing short lived radionuclides limits their widespread availability for clinical use. ^{123}I-15-(*p*-iodophenyl)pentadecanoic acid (IPPA) and ^{123}I-15-(*p*-iodophenyl)-3-methylpentadecanoic acid (BMIPP) are the only SPECT radiotracers that have shown promise for imaging heart metabolism.[8-10] The relatively short half-life of ^{123}I (~13 h) and its production via high energy beam cyclotron restricts radiopharmaceutical use around cyclotron facility. To circumvent the above limitations, development of ^{99m}Tc labeled fatty acids will continue to attract attention of scientists involved in radiopharmaceutical research. Development of ^{99m}Tc substitute will not only ensure continued radioisotope availability via renewable ^{99}Mo-^{99m}Tc generator but assure round the clock availability of the radiotracer via convenient kit labeling strategy.

Several ^{99m}Tc labeled fatty acids following different ^{99m}Tc chemistries *viz.* oxotechnetium (V) core ($[^{99m}Tc{=}O]^{3+}$),[11] nitrido core ($[^{99m}TcN(PNP)]^{2+}$),[12,13] ^{99m}Tc-4+1 mixed ligand approach,[14,15] tricarbonyl core ($[^{99m}Tc(CO)_3(H_2O)_3]^{+}$),[16,17] cyclopentadienyl tricarbonyl technetium (^{99m}Tc-CpTT) core [18-20] and ^{99m}Tc-HYNIC ,[21] were developed and evaluated in past but all lacked favorable characteristics for *in vivo* imaging. ^{99m}Tc cores/BFCAs alter *in vivo* pharmacokinetic behavior of fatty acids of identical chain lengths. Hence, present work is an evaluation of newly designed ^{99m}Tc fatty acids of variable carbon chain lengths synthesized via 'click chemistry' for its potential use in myocardial imaging.[22]

Copper (I) catalyzed Huisgen 1,3-dipolar cycloaddition reaction (click reaction) between an azide and an alkyne to give a stable 1,4-disubstituted 1,2,3-triazole has been used extensively for preparing ^{18}F-labeled derivatives and for incorporating metallic radionuclides via $[^{99m}Tc(CO)_3]^+$ core.[23-25] Fatty acids of variable chain lengths 8, 11 and 15 carbons were synthetically modified at ω terminal following the same click chemistry approach and labeled via $^{99m}Tc(CO)_3$ synthon. The labeled complexes were subsequently evaluated for potential towards myocardial imaging in comparison with standard *I-IPPA.

Experimental

Materials and Methods

8-Bromooctanoic acid, 15-bromopentadecanoic acid, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 4-dimethylaminopyridine (DMAP), sodium azide and palladium on activated carbon (10% wt) were purchased from Aldrich, USA. 11-bromoundecanoic acid was purchased from TCI Chemicals, Japan. Sodium ascorbate was obtained from Alfa Aesar (Germany). Benzyl alcohol, copper (II) sulfate, *tert*-butanol and trifluoroacetic acid were obtained from SD Fine Chemicals (India). All reagents were of analytical grade and were used as received without further purification. Sodium pertechnetate ($Na^{99m}TcO4$) was eluted with normal saline prior to use from a ^{99}Mo-^{99m}Tc column generator, supplied by Board of Radiation and Isotope Technology (BRIT), India. ^{125}I-IPPA was prepared according to the procedure described previously.[13] Silica gel plates (Silica Gel 60 F_{254}) and 60-120 mesh silica gel used for column chromatography was procured from Merck, India. The HPLC of the prepared complexes was carried out on a JASCO PU 2080 Plus dual pump HPLC system, Japan, with a JASCO 2075 Plus tunable absorption detector and Gina Star radiometric detector system, using a C18 reversed phase HiQ Sil (5 µm, 4 × 250 mm) column. FT-IR spectra were recorded on JASCO FT/IR-460 Spectrophotometer, Japan. The ^{1}H-NMR spectra were recorded on a 60 MHz Nanalysis NMR spectrometer, Canada. Mass

spectra were recorded on expression^{L} CMS Advion Mass Spectrometer, USA using electrospray ionization (ESI) in both positive and negative modes.

Synthesis

ω-bromo fatty acid benzyl ester **(1a-3a)**

ω-bromo fatty acid (2.24 mmol) and benzyl alcohol (0.25 mL, 2.5 mmol) in DCM (10 mL) were stirred overnight at room temperature in presence of EDC (471 mg, 2.46 mmol) and catalytic amount of DMAP (27 mg, 0.22 mmol). Upon completion of reaction (Cf. TLC), the reaction mixture was washed with water and DCM layer dried over anhydrous Na_2SO_4. Thereafter, DCM was removed under vacuum and purified using silica gel column chromatography to yield the desired compound.

Benzyl 8-bromooctanoate (**1a**)

Yield: 74% (520 mg) R_f =0.9 ($CHCl_3$)

IR (neat, $\bar{\upsilon}$ cm^{-1}) 1735.6 (C=O ester)

^{1}H NMR ($CDCl_3$, 60 MHz): δ 7.30 (s, 5H, PhCH_2COO), 5.10 (s, 2H, PhCH_2COOCH_2), 3.30 (t, 2H, *J* = 8 Hz, BrCH_2), 2.31 (t, 2H, *J* = 7.2 Hz, BzCOOCH_2), 1.60–2.1 (m, 4H, BrCH_2CH_2- and BzCOOCH_2CH_2-), 1.30 (s, 6H, (CH_2)$_3$).

Benzyl 11-bromoundecanoate **(2a)**

Yield: 80% (635 mg)

IR (neat, $\bar{\upsilon}$ cm^{-1}) 1735.2 (C=O, ester)

^{1}H NMR ($CDCl_3$, 60 MHz): δ 7.33 (s, 4H, PhCH_2COO), 5.10 (s, 2H, PhCH_2COOCH_2), 3.36 (t, 2H, *J* = 8 Hz, BrCH_2), 2.34 (t, 2H, *J* = 7.2 Hz, BzCOOCH_2), 1.60–2.12 (m, 4H, BrCH_2CH_2- and BzCOOCH_2CH_2-), 1.34 (s, 12H, (CH_2)$_6$).

Benzyl 15-bromopentadecanoate **(3a)**

Yield: 70% (644 mg)

IR (neat, $\bar{\upsilon}$ cm^{-1}) 1735.4 (C=O, ester)

^{1}H NMR ($CDCl_3$, 60 MHz): δ 7.37 (s, 4H, PhCH_2COO), 5.14 (s, 2H, PhCH_2COOCH_2), 3.41 (t, 2H, *J* = 8 Hz, BrCH_2), 2.37 (t, 2H, *J* = 7.2 Hz, BzCOOCH_2), 1.60–2.2 (m, 4H, BrCH_2CH_2- and BzCOOCH_2CH_2-), 1.29 (s, 20H, (CH_2)$_{10}$).

ω-azido fatty acid benzyl ester **(1b-3b)**

Sodium azide (130 mg, 2 mmol) was added to a solution of ω-bromo benzyl ester (**1a-3a**; 0.5 mmol) in DMF (5 mL), and mixture stirred overnight at room temperature. Thereafter DMF was removed under vacuum and reaction mixture re-dissolved in water (10 mL). The aqueous mixture was then extracted with $CHCl_3$ (3 × 10 mL) and pooled extracts were dried over anhydrous Na_2SO_4. The chloroform was then removed to give azide derivative which was used as such without further purification.

Benzyl 8-azidooctanoate (**1b**)

Yield: 85% (117 mg)

IR (neat, $\bar{\upsilon}$ cm^{-1}) 1735.6 (C=O, ester), 2095.3 (N=N=N azide), 2854.1 (C-H sym), 2926.4 (C-H asymm)

^{1}H NMR ($CDCl_3$, 60 MHz): δ 7.30 (s, 4H, PhCH_2COO), 5.10 (s, 2H, PhCH_2COOCH_2), 3.30 (t, 2H, *J* = 8 Hz, N_3CH_2), 2.31 (t, 2H, *J* = 7.2 Hz, BzCOOCH_2), 1.60–2.1 (m, 4H, N_3CH_2CH_2- and BzCOOCH_2CH_2-), 1.30 (s, 6H, (CH_2)$_3$).

Benzyl 11-azidoundecanoate **(2b)**

Yield: 90% (142 mg)

IR (neat, $\bar{\upsilon}$ cm^{-1}) 1735.6 (C=O, ester), 2094.3 (N=N=N, azide), 2855.1 (C-H sym), 2927.4 (C-H asymm).

^{1}H NMR ($CDCl_3$, 60 MHz): δ 7.33 (s, 4H, PhCH_2COO), 5.10 (s, 2H, PhCH_2COOCH_2), 3.36 (t, 2H, *J* = 8 Hz, N_3CH_2), 2.34 (t, 2H, *J* = 7.2 Hz, BzCOOCH_2), 1.60–2.12 (m, 4H, N_3CH_2CH_2- and BzCOOCH_2CH_2-), 1.34 (s, 12H, (CH_2)$_6$).

Benzyl 15-azidopentadecanoate **(3b)**

Yield: 89% (166 mg)

IR (neat, $\bar{\upsilon}$ cm^{-1}) 1735.6 (C=O, ester), 2095.3 (N=N=N, azide), 2854.1 (C-H sym), 2926.4 (C-H asymm)

^{1}H NMR ($CDCl_3$, 60 MHz): δ 7.37 (s, 4H, PhCH_2COO), 5.14 (s, 2H, PhCH_2COOCH_2), 3.41 (t, 2H, *J* = 8 Hz, N_3CH_2), 2.37 (t, 2H, *J* = 7.2 Hz, BzCOOCH_2), 1.60–2.2 (m, 4H, N_3CH_2CH_2- and BzCOOCH_2CH_2-), 1.29 (s, 20H, (CH_2)$_{10}$).

ω-(N-Boc-amino propanoic acid) triazole fatty benzyl ester **(1c-3c)**

N-Boc-propargyl glycine (82 mg, 0.38 mmol) and azido compound (**1b-3b**, 0.45 mmol) was refluxed overnight in presence of $CuSO_4.5H_2O$ (21 mg, 0.11 mmol) and sodium ascorbate (42 mg, 0.21 mmol) in *tert*-butanol: water mixture (1:1 v/v). Reaction mixture was then dried under vacuum and crude product purified using silica gel column chromatography to yield compound **1c-3c**.

3-(1-(7-((benzyloxy)carbonyl)heptyl)-1H-1,2,3-triazol-4-yl)-2-N-Boc-aminopropanoic acid **(1c)**

Yield: 80% (148 mg); R_f =0.1 ($CHCl_3$/MeOH 9:1 v/v)

^{1}H NMR ($CDCl_3$, 60 MHz): δ 7.25-7.5 (m, 6H, PhCH_2COO and Trz-CH_2), 5.09 (s, 2 H, PhCH_2COOCH_2), 4.0-4.6 (m, 3H, CH_2-Trz-CH_2-CH(NHBoc)COOH), 3.1-3.4 (m, 2H, Trz-CH_2-CH(NHBoc)COOH), 2.1-2.4 (m, 2H, BzCOOCH_2), 1.60–1.96 (m, 4H, Trz-CH_2CH_2- and BzCOOCH_2CH_2-), 1.1-1.6 (m, 15H, C(CH_3)$_3$ and (CH_2)$_3$).

MS (ESI$^+$): Mass (calculated) $C_{25}H_{37}N_4O_6$ 488.26; *m/z* (observed) 489.6.

3-(1-(10-((benzyloxy)carbonyl)decyl)-1H-1,2,3-triazol-4-yl)-2-N-Boc-aminopropanoic acid **(2c)**

Yield: 84% (170 mg).

^{1}H NMR ($CDCl_3$, 60 MHz): δ 7.25-7.44 (m, 6H, $\underline{Ph}CH_2COO$ and $\underline{Trz}$-CH_2), 5.10 (s, 2H, $PhC\underline{H}_2COOCH_2$), 4.85-5.0 (m, 1H, CH_2-Trz-CH_2-$C\underline{H}$(NHBoc)COOH), 4.2-4.6 (m, 2H, $C\underline{H}_2$-Trz-CH_2-CH(NHBoc)COOH), 3.1-3.4 (m, 2H, Trz-$C\underline{H}_2$-CH(NHBoc)COOH), 2.34 (t, 2H, *J* = 2.8 Hz, $BzCOOC\underline{H}_2$), 1.80–2.10 (m, 2H, Trz-$CH_2C\underline{H}_2$- and $BzCOOCH_2C\underline{H}_2$-), 1.60–1.75 (m, 2H, $BzCOOCH_2C\underline{H}_2$-), 1.1-1.55 (m, 21H, $C(C\underline{H}_3)_3$ and $(C\underline{H}_2)_6$).

MS (ESI^+): Mass (calculated) $C_{28}H_{43}N_4O_6$ 530.66; *m/z* (observed) 531.8; 441.53 [M-Benzyl].

3-(1-(14-((benzyloxy)carbonyl)tetradecyl)-1H-1,2,3-triazol-4-yl)-2-N-Boc-aminopropanoicacid (**3c**)

Yield: 82% (182 mg).

^{1}H NMR ($CDCl_3$, 60 MHz): δ 7.25-7.42 (m, 6H, $\underline{Ph}CH_2COO$ and $\underline{Trz}$-CH_2), 5.10 (s, 2 H, $PhC\underline{H}_2COOCH_2$), 4.6-5.0 (m, 1H, CH_2-Trz-CH_2-$C\underline{H}$(NHBoc)COOH), 4.15-4.35 (m, 2H, $C\underline{H}_2$-Trz-CH_2-CH(NHBoc)COOH), 2.9-3.4 (m, 2H, Trz-$C\underline{H}_2$-CH(NHBoc)COOH), 2.34 (t, 2H, *J* = 2.8 Hz, $BzCOOC\underline{H}_2$), 1.75–2.10 (m, 2H, Trz-$CH_2C\underline{H}_2$- and $BzCOOCH_2C\underline{H}_2$-), 1.55–1.70 (m, 2H, $BzCOOCH_2C\underline{H}_2$-), 1.1-1.55 (m, 29H, $C(C\underline{H}_3)_3$ and $(C\underline{H}_2)_{10}$).

MS (ESI^+): Mass (calculated) $C_{32}H_{51}N_4O_6$ 586.76; *m/z* (observed) 587.8; 497 [M-Benzyl].

ω-(amino propanoic acid) triazole fatty acid benzyl ester **(1d-3d)**

Compound **1c-3c** (0.18 mmol) was treated with trifluoroacetic acid (TFA; 2 mL) at room temperature for 2 h. Thereafter TFA was removed under vacuum to yield the desired compound and was used as such for next step without further purification.

3-(1-(7-((benzyloxy)carbonyl)heptyl)-1H-1,2,3-triazol-4-yl)-2-aminopropanoic acid **(1d)**

Yield: Quantitative (70 mg)

MS (ESI^+): Mass (calculated) $C_{20}H_{29}N_4O_4$ 388.46; *m/z* (observed) 389.8.

3-(1-(10-((benzyloxy)carbonyl)decyl)-1H-1,2,3-triazol-4-yl)-2-aminopropanoic acid **(2d)**

Yield: 90% (70 mg)

MS (ESI^+): Mass (calculated) $C_{23}H_{35}N_4O_4$ 430.54; *m/z* (observed) 431.9; [M-Benzyl] 341.

3-(1-(14-((benzyloxy)carbonyl)tetradecyl)-1H-1,2,3-triazol-4-yl)-2-aminopropanoic acid **(3d)**

Yield: 95% (83 mg)

MS (ESI^+): Mass (calculated) $C_{27}H_{43}N_4O_4$ 486.65; *m/z* (observed) 487.9; [M-Benzyl] 397.9.

ω-(amino propanoic acid) triazole fatty acid **(1e-3e)**

Compound **1d-3d** (0.15 mmol) was added to a stirred suspension of 10% Pd-C (20 mg) in absolute ethanol (10 mL). H_2 gas was bubbled through the above reaction mixture and reducing environment maintained overnight with continued stirring at room temperature. Upon completion of reaction (Cf. TLC), the reaction mixture was filtered and ethanol removed under vacuum to yield compound **1e-3e**.

8-(4-(2-amino-2-carboxyethyl)-1h-1,2,3-triazol-1-yl)octanoic acid **(1e)**

Yield: quantitative (47 mg)

MS (ESI^+): Mass (calculated) $C_{13}H_{23}N_4O_4$ 298.34; *m/z* (observed) 320.8 [M+Na-H].

11-(4-(2-amino-2-carboxyethyl)-1H-1,2,3-triazol-1-yl)undecanoic acid **(2e)**

Yield: quantitative (51 mg)

MS (ESI^+): Mass (calculated) $C_{16}H_{29}N_4O_4$ 340.42; *m/z* (observed) 341.5.

15-(4-(2-amino-2-carboxyethyl)-1H-1,2,3-triazol-1-yl)pentadecanoic acid **(3e)**

Yield: quantitative (60 mg)

MS (ESI^+): Mass (calculated) $C_{20}H_{37}N_4O_4$ 396.52; *m/z* (observed) 396.9.

Radiolabeling

The intermediate $[^{99m}Tc(CO)_3]^+$ synthon was produced on addition of freshly eluted sodium pertechnetate ($Na^{99m}TcO_4$) (1 mL, 740 MBq) to a lyophilized kit containing potassium boranocarbonate (1 mg) and heating the contents in boiling water bath for 30 minutes. The formation of intermediate was characterized using TLC [MeOH: HCl (95:5 v/v); R_f ~ 0.4] and HPLC.

To the freshly prepared intermediate (0.5 mL, 370 MBq), fatty acid derivative (**1e/2e/3e)** (3-5 mg) in MeOH (0.5 mL) was added and contents heated under boiling water conditions for another 30 min to yield the respective complexes **1f, 2f** and **3f**.

Quality control

HPLC

Formation of ^{99m}Tc-fatty acid complexes (**1f-3f**) was characterized by reversed phase HPLC. Gradient elution program was followed using water (solvent A) and acetonitrile (solvent B) as the mobile phase (0 min 90% A, 30min 10% A, 40 min 0% A) each containing 0.1% TFA. Flow rate of the solvent was maintained at 1 mL/min. Test solution (20 µL) was injected into the column using a micro-syringe and elution was monitored by observing the radioactivity profile. The same C18 reversed phase analytical column was used for the purification of the radiolabeled complexes **1f**, **2f** and **3f** using the same gradient elution with same mobile phase but without 0.1%TFA. Peaks corresponding to labeled complexes were collected, dried and reconstituted in 10% ethanol. Around 7.4 MBq of pure radiolabeled complex was obtained by this method which was subsequently used for performing *in vitro* and biodistribution studies.

Partition coefficient **(Log $P_{o/w}$)**

The purified complex (**1f/2f/3f)** (0.1 mL, ~185 kBq) was mixed with double distilled water (0.9 mL) and n-octanol (1 mL) and vortexed for 3 min. The mixture was then centrifuged at 3500 g for 3 min to effect clear separation of the two layers. Equal aliquots from the two layers were withdrawn in triplicates and measured for associated radioactivity. The readings thus obtained were used to calculate Log $P_{o/w}$ value (octanol-water partition) of the complexes.

Serum stability

The purified fatty acid complex (**1f/2f/3f**) (50 µL, ~ 370 KBq) was incubated in human serum (450 µL) at 37°C for 30 min. Thereafter, serum proteins were precipitated by addition of ethanol (500 µL). The solution was centrifuged and the supernatant was analyzed by HPLC to ascertain the stability of the complex in serum.

Biodistribution studies

All procedures performed herein were in accordance with the national laws pertaining to the conduct of animal experiments. Normal female Swiss mice (20-25 g body weight) were used for the *in vivo* distribution studies. The mice were fasted for 4-5 h prior to the experiment. The HPLC purified ^{99m}Tc-labelled fatty acid complex (**1f/2f/3f**) reconstituted in 6% human serum albumin (HSA) was then administered (100 µL, ~370 KBq) intravenously through the tail vein of each mouse. Individual sets of animals (n = 3) were utilized for studying distribution at four different time points (2 min, 5 min, 10 min and 30 min). At the end of each time point, respective set of animals were sacrificed and the relevant organs and tissue were excised for the measurement of associated activity. The organs were weighed and the activity associated with each organ was measured in a flat-bed type NaI(Tl) counter with suitable energy window for ^{99m}Tc (140 keV ± 10%). The activity associated with each organ/tissue was expressed as a percent injected dose per gram (% ID per g).

Results

Three fatty acids of variable chain lengths 8, 11 and 15 carbon were selected for screening the efficacy of new ^{99m}Tc fatty acid complexes designed via 'click chemistry' approach for myocardial imaging. All the three fatty acids bearing ω-bromo group were modified at bromo terminal with triazole glycine chelate in a five step synthetic procedure (**Scheme 1**). The first step is an esterification reaction where the carboxylic acid group was converted to a benzyl ester in presence of EDC and DMAP activator. The introduction of benzyl chromophore favored unambiguous monitoring and product isolation during all the intermediary steps of reaction scheme. The second step involved direct substitution of bromo group with an azide functionality via S_N2 reaction mechanism. IR stretching frequency at 2095 cm^{-1} confirmed azide formation, but NMR could not differentiate azide derivative from step 1 bromo compound. NMR δ values of **1a-3a** and **1b-3b** were identical probably because of similar electron pull from both bromine and azide groups. Third step of the reaction is a 'click reaction' where the cyclic triazole ring gets formed via a cycloaddition reaction between azide group of **1b-3b** with the terminal alkyne group of *N*-Boc-propargyl glycine in presence of in-situ generated Cu(I) catalyst. Subsequent steps involved deprotection of Boc with TFA, followed by benzyl group removal with 10% Pd-C catalytic hydrogenation. IR, NMR and ESI-MS spectroscopic analyses confirmed intermediates and final product formation during different steps of the reaction.

The radiolabeling procedure (**Scheme 2**) involved prior generation of intermediate synthon $[^{99m}Tc(CO)_3(H2O)_3]^+$ through a lyophilized kit containing CO donor, potassium boranocarbonate ($K_2[H_3B(CO)_2]$). The formation yield of intermediate synthon as characterized using TLC and HPLC was >95%. Subsequently labeled ^{99m}Tc fatty acid complexes (**1f-3f**) were obtained on reaction of intermediate synthon with respective fatty acid conjugates (**1e-3e**). HPLC characterization of the three complexes **1f-3f** showed

retention times of 21, 23.5 and 28 min respectively **[Figure 1]** whereas the intermediate synthon eluted out as broad peak at 15 min. Radiolabeling yield calculated from the percentage peak area under the observed complex peak in HPLC spectrum was observed to be >80% for all three complexes. The increase in HPLC retention times of the respective fatty acid complexes was consistent with the increasing chain length (lipophilicity). The latter factor was established on ascertaining the Log $P_{o/w}$ of the HPLC purified ^{99m}Tc labeled fatty acid complexes (**1f-3f**) between n-octanol and water (**Table 1).**The complexes exhibited serum stability for complete period of study upto 30 minutes.

Table 2 summarizes the results of biodistribution studies carried in normal swiss mice. **Figure 2** gives a comparison between the myocardial uptake values of the three ^{99m}Tc labeled complexes **1f-3f** and the reference compound ^{125}I-IPPA. All the complexes showed an increased uptake in myocardium at 2 min post injection (p.i.) with **1f** showing the highest uptake of 8.70 ±1.41 %ID/g, which was close to uptake shown by ^{125}I-IPPA [9.51 ± 1.61 %ID/g]. This high initial uptake gradually decreased with time but significant activity (~1 %ID/g) was found retained even at 30 min p.i. for all the complexes. The absolute retention values observed were much below than that for ^{125}I-IPPA [7.10 ± 1.79 %ID/g] at 30 min p.i. Varying the fatty acid carbon backbone from 8 to 15 carbons resulted in no significant improvement in the myocardial uptake and washout kinetics.

Heart/blood ratio for the complex **3f** was initially superior (0.8 vs. 0.3) to the other two complexes up to 10 min p.i., which decreased to identical ratio (~ 0.4) as observed for **1f** and **2f** at 30 min p.i. Significant activity was found associated with the liver owing to the lipophilic nature of the complexes but rapid movement of activity from liver to GI was observed for all three complexes with time. Similar washout behavior of the complexes was observed from other non-target organs *viz.* blood and lungs, as well. This was a definite improvement over ^{125}I-IPPA[13] which had 30.40±6.82 %ID/g and 11.25±1.18 %ID/g activity

associated with liver and lung respectively, at 30 min p.i. Unlike ^{125}I-IPPA, all the three complexes showed high activity accumulation in kidneys, followed by rapid decline indicating renal pathway to be the competing excretion route. The excretion percentage of the ^{99m}Tc complexes via renal decreased with increase in fatty acid chain length (lipophilicity).

Discussion

There has been prolonged search for a ^{99m}Tc labeled fatty acid complex that would possess the advantages of high myocardial uptake, optimum retention and rapid clearance of activity from blood pool and surrounding non-target organs like liver and lung. Efforts have been put forward in this direction and several fatty acid analogs have been reported. In all such cases, as the metal chelate is introduced into the fatty acid chain there is significant alteration in the properties of the fatty acid molecule, particularly its molar volume and lipophilicity/hydrophilicity as compared to the parent molecule, which in turn plays an important role in governing the *in vivo* properties. Hence, a ^{99m}Tc core with small size but high stability and lipophilicity is required to counter the effects of introduction of the radiometal. These attributes can be found in the $[^{99m}Tc(CO)_3]^+$ core and work with fatty acid analogs having different tridentate donor moiety have been reported before using this core. Also complexes based on [^{99m}Tc(CO)$_3$-cyclopentadienyl] core, such as ^{99m}Tc-CpTT-Pentadecanoic acid and ^{99m}Tc-CpTT-16-oxo-Hexadecanoic acid,[18-20] could be found in the literature but no such literature where the fatty acid chain has been modified through Huisgen cycloaddition to introduce a tridentate donor set for labeling with the said core is available to the best of our knowledge.

In the present work, by making use of this reaction, ω-terminal of three fatty acids having 8, 11 and 15 carbons respectively has been modified to introduce a tridentate moiety consisting of an amine nitrogen atom, a carboxylic oxygen atom and the nitrogen atom of the triazole ring to facilitate ^{99m}Tc labeling through the carbonyl core. Fatty acids with varying chain

lengths from 8-15 were selected to screen the effect of varying lipophilicity on myocardial uptake and its *in vivo* pharmacokinetic behavior. The present 'click approach' introduces a heterocyclic triazole which may mimic the phenyl group present in IPPA. All the ligands were synthesized with moderate yields and characterized by spectroscopic techniques confirming their structure. Radiolabelling was carried out following the method described in the experimental section and the radiolabeled complexes were purified from the excess ligand mass by HPLC before *in vivo* studies. The complexes synthesized in the present study are lipophilic in nature and the longer chain fatty acid complexes of 11 and 15 carbon elute out broadly in the HPLC spectrum. This may be due to improper elution of the complexes from the C18 column in the present choice of mobile phase used for characterization. The click labeling approach do not add radiochemical impurities, as the starting raw materials for triazole formation *viz.* fatty acid azide and propargyl glycine, do not independently complex with $^{99m}Tc(CO)_3$ core and the complexation is feasible only with clicked adducts. The scheme 1 followed here for the synthesis of clicked adducts (**1e-3e**) is via de-protection of clicked benzyl protected adducts (**1d-3d**), hence there can be a possibility of benzyl protected derivative participating in the complexation. But the NMR spectral data confirmed the absence of such derivatives (**2d** and **3d**) in the final ligands **2e** and **3e** respectively. However, to confirm the above proposition a representative complexation of **1d** with intermediate $^{99m}Tc(CO)_3$ core was carried and the complex eluted beyond 30 min (data not shown) indicating significant difference in the HPLC retention times between benzyl protected and de-protected clicked derivatives. The radiochemical purity observed for the complexes is > 80% which is not sufficient for new radiotracer preparation. The lower labeling efficiency observed may again be due to lipophilic nature of ligands which has affected the solubility of ligands in water:methanol mixture and hence the final complexation yield. The authors in the present study have not attempted to optimize the labeling conditions so as to improve the

labeling efficiency as the focus of the present study was to ascertain the biological efficacy of synthesized clicked complexes for myocardial imaging in comparison to ^{125}I-IPPA. Hence complexes have been HPLC purified before conducting the biological experiments.

All the complexes exhibited stability in human serum which ensured kinetic inertness of the complexes *in vivo*. Biodistribution studies of ^{99m}Tc fatty acid complexes (**1f-3f**) in normal swiss mice showed prominent heart uptake and significant retention up to 30 min p.i. which is important from SPECT imaging perspective. Another important aspect in myocardial imaging is the clearance of activity from the blood pool and the surrounding non-target organs like liver and lungs to obtain high contrast images. Although activity declined rapidly for this design from non-target organs but heart/blood ratios for all the three complexes were significantly lower compared to the standard agent. Favorable feature observed for this 'click' approach of labeled complexes was their excretion via both hepatobiliary and renal pathways. Initial uptake in liver was observed to clear rapidly with time. These factors highlight the significance of the present design, where early improved heart/liver ratios are expected favoring increased image resolution during metabolic cardiac imaging.

Several approaches for labeling long chain fatty acids with ^{99m}Tc have been explored in past. The results obtained in the present work cannot be directly compared with many of reported data due to difference in species such as rats utilized for biodistribution experiments. Our group has evaluated before ^{99m}Tc fatty acid utilizing two different approaches.[13,21] All three designs including the present evaluation exhibited high serum stability but suffered from reduced myocardial activity retention, albeit HYNIC complex **2e** [21] which showed moderately high retention values up to 30 min p.i. Among the three labeling approaches, the present click design revealed highest myocardial uptake at initial time points close to ^{125}I-IPPA. Unlike pharmacokinetics of previous two designs where complexes cleared mainly via the hepatic route, the clicked complexes excreted via both hepatic and renal pathways.

Further, the movement of residual activity from liver towards intestines was rapid in comparison to previous approaches thereby favoring improved resolution for metabolic cardiac imaging.

Comparing the present *in vivo* distribution results with most promising [99mTc]MAMA-HDA,[11] carrying N_2S_2 framework and evaluated in the same specie, the present evaluated complexes showed improved initial myocardial uptake and heart/liver ratios. Heart/blood ratio for clicked complex **3f** was observed to be similar to [99mTc]MAMA-HDA (~ 0.82) at 5 min p.i. Thus the present design shows reasonable myocardial uptake and favorable *in vivo* pharmacokinetics, which merits high potential for metabolic cardiac imaging.

Conclusion

Three fatty acids with 8, 11 and 15 carbon chains were modified at the terminal carbon through Huisgen 1,3-dipolar cycloaddition reaction to give clicked adducts in high purity and moderate yields. Radiolabelling of clicked fatty acid ligands with technetium tricarbonyl core yielded complexes with purity > 80%. Biodistribution studies of these complexes in normal Swiss mice revealed affinity towards myocardium with significant retention up to 30 min p.i. The complexes demonstrated improved *in vivo* pharmacokinetics with rapid washout of activity from non-target organs via hepatic and renal pathways. All these data provide conclusive evidence in support of the promising nature of this chemistry but lower retention of tracers in the myocardium in comparison to ^{125}I-IPPA limit the potential of these tracers as myocardial metabolic markers.

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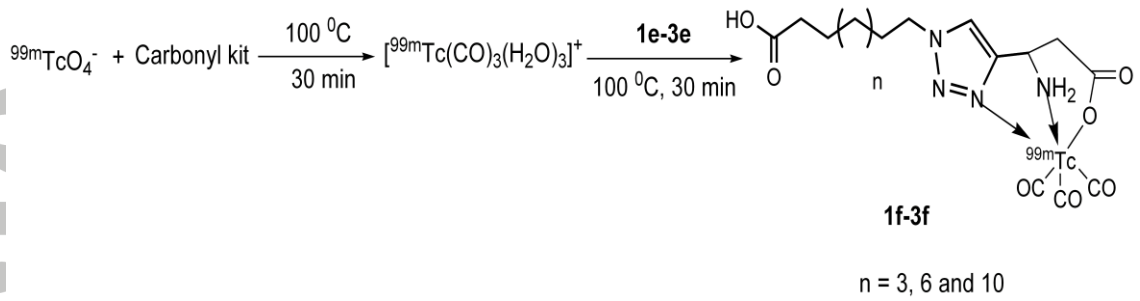
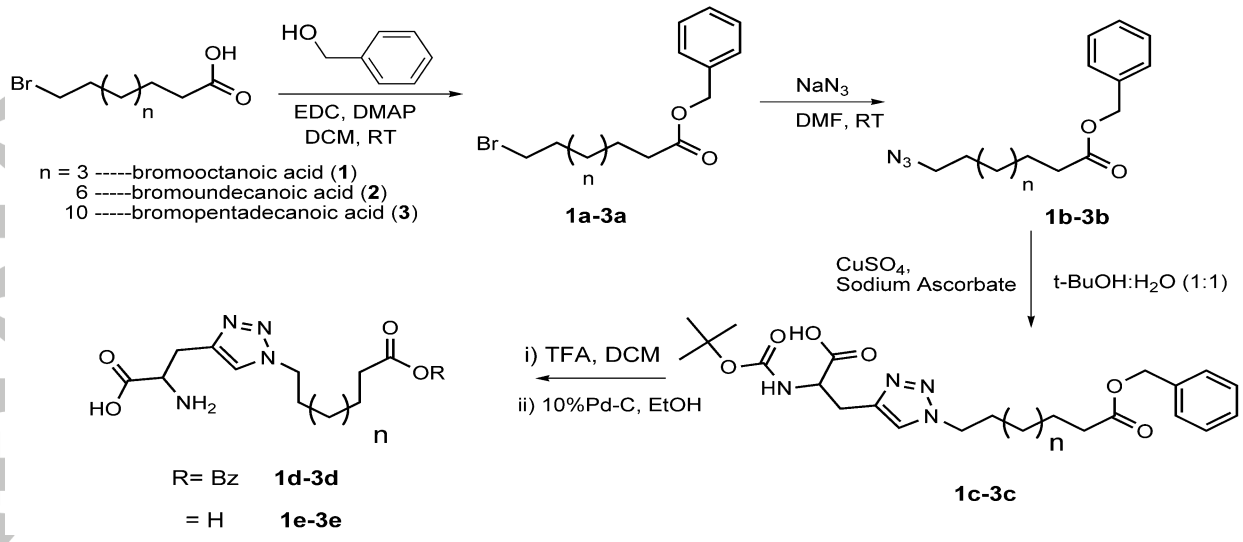


Scheme1 Scheme for the synthesis of fatty acid triazole conjugates



Scheme 2 Radiolabeling of fatty acid triazole conjugates with technetium tricarbonyl core

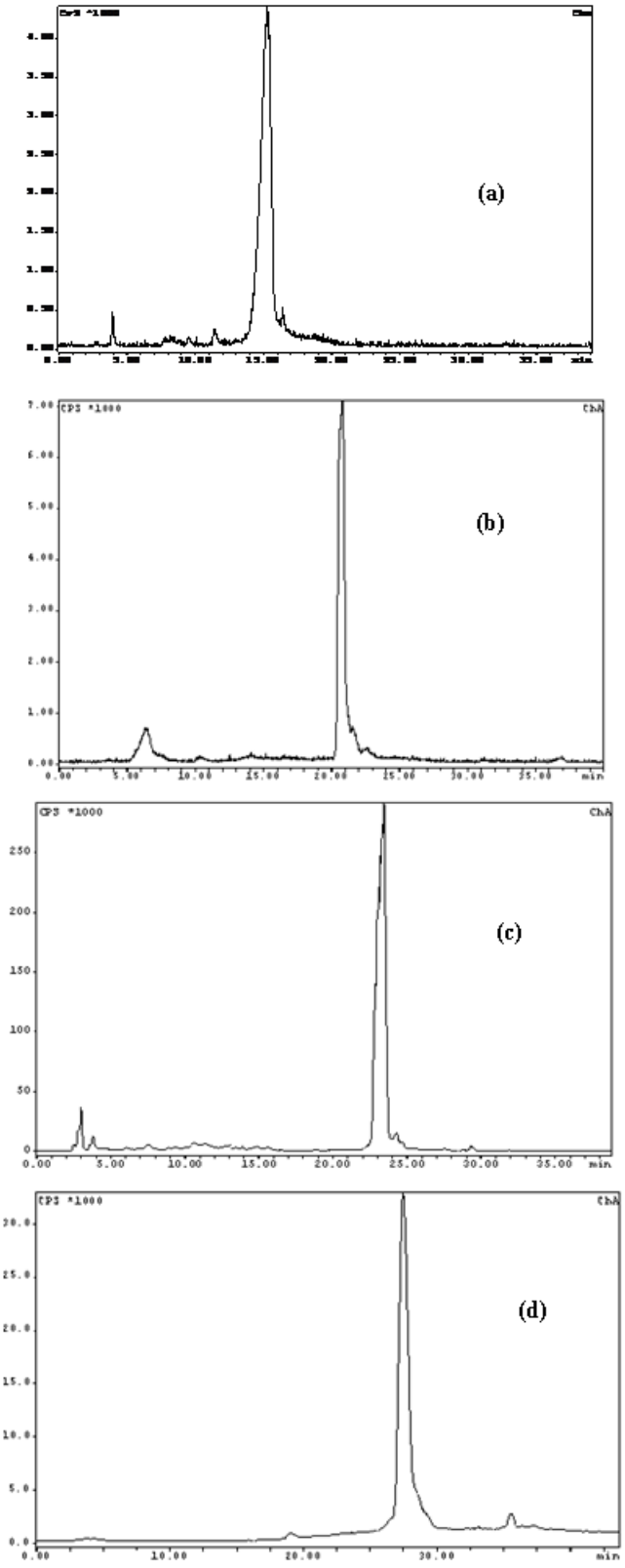


Figure 1 HPLC profile of (a) $^{99m}Tc(CO)_3$ intermediate synthon (b) Complex **1f** (c) Complex **2f** and (d) Complex **3f**

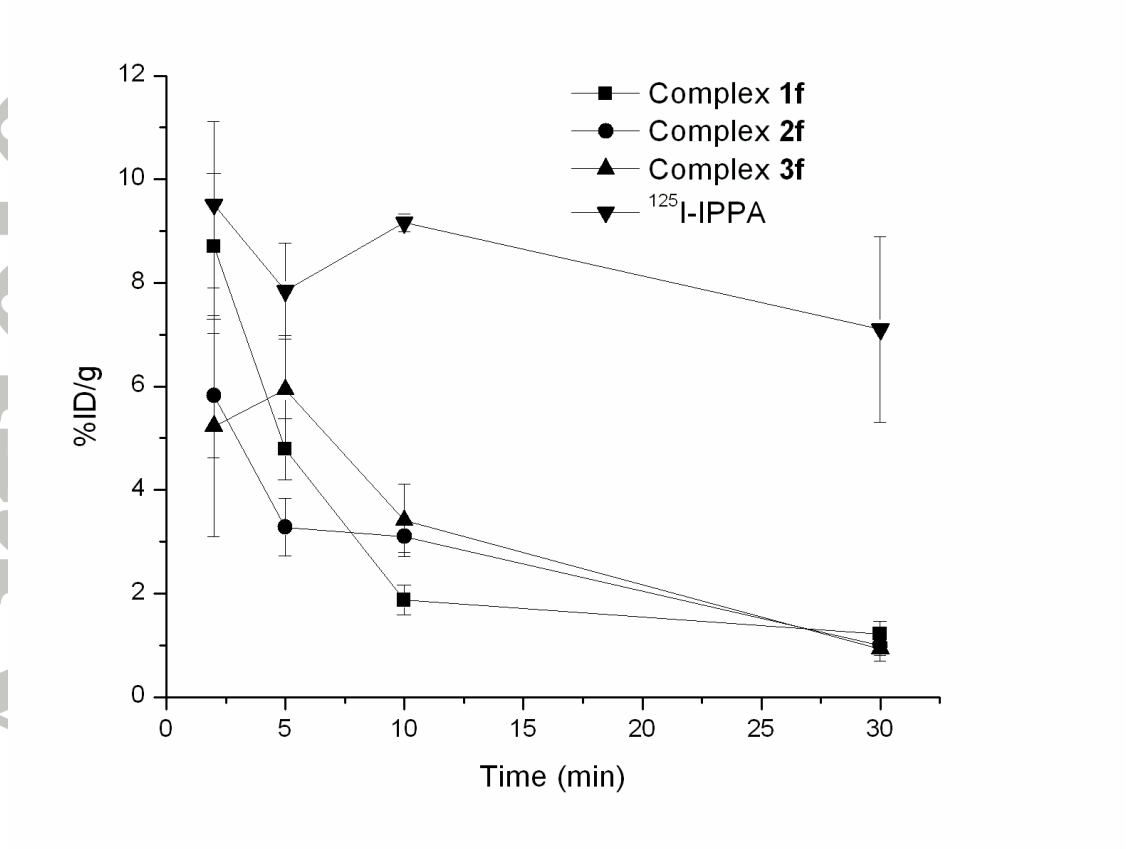


Figure 2 Myocardial uptake and washout kinetics profile of $^{99m}Tc(CO)_3$- fatty acid complexes (**1f-3f**) in Swiss mice

Table1 Partition coefficient (Log $P_{o/w}$) of ^{99m}Tc labeled fatty acid complexes **1f-3f**

Complex	Log $P_{o/w}$
1f	0.10
2f	0.38
3f	0.89

Table 2: Biodistribution studies of ^{99m}Tc fatty acid complexes **1f-3f** (values expressed in %ID/g) in Swiss mice

	2 min	5 min	10 min	30 min
Complex 1f (n=3)				
Liver	47.06 (5.59)	36.20 (3.80)	20.74 (5.71)	13.08 (1.35)
Int + GB	3.94 (0.60)	4.45 (1.95)	9.58 (2.39)	15.34 (0.33)
Stomach	4.92 (1.07)	5.75 (2.46)	1.80 (0.43)	2.86 (0.14)
Kidney	75.24 (8.41)	77.22 (13.45)	39.62 (5.89)	19.03 (1.81)
Heart	8.70 (1.41)	4.79 (0.59)	1.87 (0.29)	1.21 (0.25)
Lungs	17.22 (3.10)	9.01 (1.32)	3.64 (0.58)	2.31 (0.90)
Muscle	3.52 (0.58)	1.98 (0.14)	0.86 (0.11)	0.44 (0.05)
Blood	23.62 (1.40)	14.06 (1.27)	5.65 (0.19)	3.18 (0.50)
Heart/Blood	0.36 (0.07)	0.35 (0.11)	0.33 (0.4)	0.40 (0.18)
Heart/Liver	0.17 (0.02)	0.13 (0.02)	0.09 (0.01)	0.09 (0.03)
Heart/Lung	0.54 (0.22)	0.55 (0.12)	0.52 (0.03)	0.60 (0.29)
Complex 2f (n=3)				
Liver	46.02 (14.52)	48.60 (13.86)	36.95 (10.07)	12.03 (2.54)
Int + GB	1.94 (1.22)	2.43 (1.30)	4.53 (1.39)	8.77 (2.56)
Stomach	2.24 (0.93)	5.39 (2.77)	6.00 (2.51)	2.53 (0.24)
Kidney	18.62 (8.23)	47.46 (22.60)	55.27 (15.97)	17.19 (5.74)
Heart	5.82 (1.20)	3.28 (0.56)	3.10 (0.31)	1.00 (0.31)
Lungs	10.26 (2.39)	5.82 (2.38)	5.30 (1.72)	1.55 (0.49)
Muscle	0.89 (0.25)	1.18 (0.35)	1.40 (0.21)	0.61 (0.33)
Blood	21.41 (5.60)	10.44 (4.60)	6.83 (1.35)	2.38 (0.69)
Heart/Blood	0.28 (0.06)	0.35(0.14)	0.43 (0.11)	0.42 (0.01)
Heart/Liver	0.13 (0.03)	0.07 (0.02)	0.08 (0.03)	0.08 (0.01)
Heart/Lung	0.58 (0.11)	0.61(0.19)	0.57 (0.15)	0.65 (0.13)
Complex 3f (n=3)				
Liver	31.88 (3.94)	40.10 (10.68)	21.17(3.07)	5.29 (0.23)
Int + GB	0.98 (0.18)	1.89 (1.09)	5.31 (0.27)	6.08 (1.17)
Stomach	1.51 (0.49)	1.44 (0.30)	2.35 (0.66)	2.69 (1.04)
Kidney	7.86 (1.62)	14.17 (8.54)	22.01 (5.86)	6.73 (0.72)
Heart	5.23 (2.13)	5.94 (1.04)	3.41 (0.70)	0.93 (0.13)
Lungs	8.41 (1.90)	6.82 (1.41)	3.24 (0.71)	1.68 (0.30)
Muscle	1.07 (0.80)	1.19 (0.15)	1.69 (0.68)	1.24 (0.48)
Blood	19.61 (0.85)	6.20 (3.55)	4.51 (0.88)	2.10 (0.2)
Heart/Blood	0.28 (0.06)	0.82 (0.11)	0.76 (0.20)	0.44 (0.15)
Heart/Liver	0.21 (0.11)	0.14 (0.05)	0.20 (0.05)	0.18 (0.06)
Heart/Lung	0.58 (0.11)	0.94 (0.55)	1.11 (0.49)	0.55 (0.29)