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Research Article

Synthesis of 2-[(2-chloro-2′- [^{18}F]fluoroethyl)amino]-2H-1,3,2-oxazaphosphorinane-2-oxide (^{18}F-fluorocyclophosphamide), a potential tracer for breast tumor prognostic imaging with PET

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Summary

A fluorine-18 labeled analog of the widely used chemotherapeutic agent cyclophosphamide was synthesized as a tracer for prognostic imaging with positron emission tomography. 2-[(2-Chloro-2′-[^{18}F]fluoroethyl)amino]-2H-1,3,2-oxazaphosphorinane-2-oxide (^{18}F-fluorocyclophosphamide), was prepared by direct halogen exchange reaction from the parent cyclophosphamide. In small-scale syntheses, radiochemical yields of up to 4.9% and specific activities of 960 Ci/mmol were achieved in a total synthesis time of 60–75 min. The [^{18}F]-labeled cyclophosphamide analog with radioactive purity >99% and chemical purity >96% was suitable for *in vivo* (microPET imaging) and *ex vivo* studies of a murine model of human breast tumors. Copyright © 2005 John Wiley & Sons, Ltd.

Key Words: fluorine-18; cyclophosphamide; PET prognostic imaging; breast tumor therapy

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Introduction

Cyclophosphamide (CP) (2-[bis(2-chloroethyl)amino]-2*H*-1,3,2-oxazaphosphorinane 2-oxide) **7** is one of the most widely used agents in cancer chemotherapy. The report of its synthesis[1,2] marked the beginning of rational design and development of chemotherapeutic drugs.[3] It was the first synthetic compound with a profound anticancer activity in humans. CP itself is a pro-drug, which undergoes metabolic activation by hepatic cytochrome P450 enzymes to generate alkylating species.[4] Phosphoramidic mustard that is formed in a series of enzymatic and non-enzymatic transformations is the active species that crosslinks strands of DNA preventing DNA replication and promoting cell death. Various by-products of CP biotransformations (e.g. acrolein, and chloroacetaldehyde) are neurotoxic, urotoxic and cardiotoxic, increasing and complicating the overall toxicity of chemotherapy.

Cyclophosphamide has been a drug of choice for the treatment of breast cancer. However, it has been realized that individual patient differences in the biodistribution, pharmacokinetics, and metabolism of cyclophosphamide and its analogs (e.g. ifosfamide) can influence the clinical outcome of therapy.[5,6] Due to its toxicity, it is particularly worthwhile to assess the likelihood of benefit from treatment with CP prior to administration of the drug to the patient. It has been shown that cancer cell lines *in vitro* can quickly develop resistance to CP.[7–9] In addition, prior exposure to alkylating agents increases the likelihood of resistance to CP.[7–11] Both genetic and physiologic alterations can occur to render the cells resistant to CP.[12–14] An inverse correlation has been shown between the cellular sensitivity to CP and the cellular content of at least two aldehyde dehydrogenases, ALDH1A1 and ALDH3A1[15] that catalyze the detoxification of CP and its metabolites.[16] Glutathione S-transferase activity, has also been implicated in cellular resistance to CP.[8] Information about the biodistribution and kinetics of metabolism of cytotoxic drugs and CP in particular in individual patients could help to further improve the selection of appropriate chemotherapeutic regimens.

Positron emission tomography (PET)[17–19] and single photon emission computed tomography (SPECT)[20] have been recently used for the assessment of the potential response to chemotherapy in patients with breast cancer. Nuclear medicine imaging modalities allow for the pre-therapy studies of pharmacokinetic parameters (tissue concentration, times of transit and retention) *in vivo* with tracer levels of chemotherapeutics, potentially enabling a better individualized approach to the administration of pharmacologic doses of chemotherapy compounds.

Here, we describe the synthesis of a [^{18}F]-labeled analog of cyclophosphamide (**8**) as a potential tracer for the study of tumor chemosensitivity *in vivo*. Since the multistep synthesis of fluorocyclophosphamide[21,22] (**6**) was not compatible with the rapid time-frame required for a radiosynthesis using a

short-lived isotope such as ^{18}F, we approached labeling through a direct halogen exchange reaction, starting with a commercially available precursor.[23]

Results and discussion

In order to identify unequivocally product of the radiosynthetic fluorination, the desired cold standard (**6**), a fluorine analogue of cyclophosphamide, was prepared (Figure 1). The preparation of **6** was based on the modified reported synthetic procedures.[21,22] The multi-step synthesis yielded a racemic **6**, an oil which, unlike cyclophosphamide, did not form crystalline hydrate. Previous biological testing of this compound has shown its cytotoxicity in HeLa (KB) cell cultures.[22] A ^{31}P NMR study[3] of apparent half-lives at pseudo-equilibrium of **6** and its acyclic metabolite in 1 M lutidine at pH 7.4, 37°C has shown that **6** has a longer average apparent half-life compared to cyclophosphamide (48 ± 1 min vs 38 ± 2 min). At the same time, the relative percentage ratio of metabolites (cyclic: acyclic) in both cases remains very similar (for **6** it is 84:16 and for cyclophosphamide it is 86:14).

Various synthetic strategies for the halogen exchange reaction were tested in order to optimize conditions for the [^{18}F]F labeling of **7**. For example, fluorination of **7** with KF/tetrabutylammonium bromide in acetonitrile did not yield fluoroethyl functionality. However, Kryptofix®222-catalyzed KF fluorination of anhydrous **7**[24] in acetonitrile[25] gave the desired product **6** (Figure 2), whose identity was confirmed by spectroscopic and chromatographic analyses. Chemical shifts and the splitting patterns with the corresponding proton–fluorine spin–spin coupling values that are characteristic of the fluoroethyl functionality[26] were identified in the ^{19}F and ^{1}H NMR spectra of the obtained **6**. Among the multiple products in this reaction, no other substances containing fluoroethyl group(s) were identified. Fluorination with KF/Kryptofix®222 in DMSO at higher temperatures (120–150°C) gave a mixture of products without formation of **6**. Similarly, the attempt to synthesize the monotosyl congener of cyclophosphamide as a potential

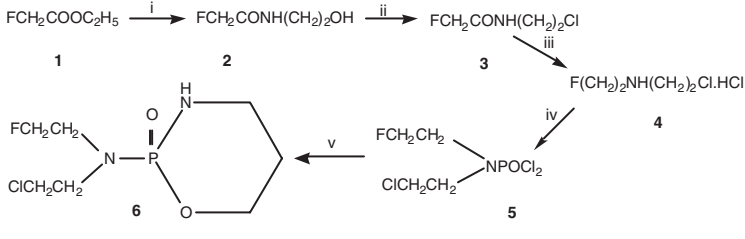

Figure 1. Synthesis of 2-[(2-chloro-2′-fluorodiethyl)amino]-2H-1,3,2-oxazaphosphorinane 2-oxide (6): (i) ethanolamine; (ii) $SOCl_2/CHCl_3$; (iii) diborane/THF; (iv) $POCl_3$; (v) 3-aminopropanol, triethylamine/dioxane

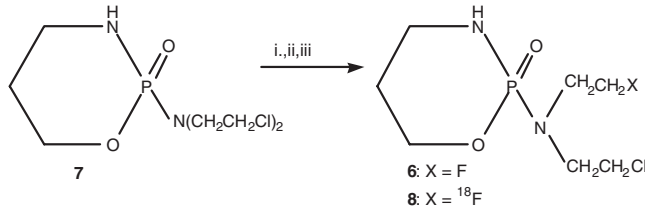

Figure 2. Direct halogen exchange synthesis of 6 and radiochemical synthesis of 2-[(2-chloro-2′-[^{18}F] fluoroethyl)amino]-2H-1,3,2-oxazaphosphorinane-2-oxide (8): (i) KF or $^{18}F^{-}/K_2CO_3$/acetonitrile; (ii) Kryptofix®222 110°C/10 min; (iii) chromatography

substrate for [^{18}F] fluorination, via **7**/silver tosylate reaction[27–29] failed to produce the desired product.

The [^{18}F] analogue of **6** was prepared in a single step by a halogen exchange reaction with no-carrier-added [^{18}F]F^{-}/Kryptofix®222 in anhydrous acetonitrile with a commercially available cyclophosphamide monohydrate (**7**) (Figure 2). Anhydrous cyclophosphamide that was used in the analogous synthesis of **6** is poorly soluble in cold acetonitrile. An additional evaporation step upon the addition of cyclophosphamide monohydrate in acetonitrile to the dried no-carrier-added [^{18}F]F^{-}/Kryptofix®222 mixture did not improve radiochemical yield and was omitted in the radiosyntheses. Normal phase semi-preparative HPLC (Econosil silica column, dichloromethane:methanol, 98:2) was initially tested for the separation and purification of **8**, but was found to require additional filtration and evaporation steps which was determined to be impractical. A significant difference in the retention times between the product and the precursor in the reversed phase HPLC, allowed for their baseline separation. The reversed phase HPLC separation in combination with a Sep-Pak purification step was used for the work-up of the reaction mixture. This procedure lends itself to automation in higher activity syntheses. In small-scale syntheses, starting with 20 mCi $^{18}F^{-}$ and a total synthesis time of 60–75 min, labeling yields (recalculated to the end of bombardment (EOB)) of up to 4.9% (1.1–4.9%; $n = 16$) were achieved. Typically, the activity of injectable product thus obtained (0.35–1.6 mCi) was sufficient to be used in microPET studies with rodents.

The chromatographic (HPLC, thin-layer chromatography) behavior of the labeled product **8** was shown to be identical to the unlabeled standard (**6**) synthesized in a multi-step procedure and the product obtained in a cold synthesis with KF/Kryptofix®222. The radiochemical purity of product **8** determined by analytical HPLC was greater than 99% and chemical purity greater than 96%, with specific activities up to 960 Ci/mmol (54–960 Ci/mmol).

Preliminary *in vivo* experiments (microPET) performed with tumor-bearing nude mice have shown potential of **8** as a tracer for chemosensitivity study in a murine model of human breast cancer.

Experimental

Chemicals for the syntheses were purchased from commercial sources (Sigma-Aldrich, Fisher Scientific) and were used without further purification. All solvents were of the highest grade commercially available. Tetrahydrofuran (THF) (Aldrich) was distilled over $LiAlH_4$ under argon. Melting points were determined with a Thomas–Hoover capillary melting point apparatus and are uncorrected, as are the reported boiling points. Proton (^{1}H) and ^{19}F spectra were recorded with a Bruker AM-360 WB spectrometer operating at 360.14 MHz and 338.87 MHz, respectively. Chemical shifts (δ, ppm) were referenced to tetramethylsilane (TMS) for ^{1}H and $CFCl_3$ for ^{19}F as standards. Coupling constants (J) are reported in Hz. High resolution electron impact (EI HRMS) mass spectral data were collected on a VG Analytical AutoSpec mass spectrometer.

Reversed phase analytical high performance liquid chromatography (HPLC) (Alltech Econosil C18 250×4.6 mm, 5 μm column) and semi-preparative HPLC (Alltech Econosil C18, 250×10 mm, 5 μm column) were performed with 0.025 M KH_2PO_4: acetonitrile (pH 3.0), 75:25 mixture as an eluent at a flow rate of 1.5 ml/min and 5 ml/min, respectively. The effluent in semi-preparative HPLC was monitored with an ultraviolet detector at 200 nm coupled with a gamma-radioactivity PIN diode detector (Carrol and Ramsey Associates, Berkeley, CA; Model 105-S). For analytical HPLC an UV detector was coupled with a NaI(Tl)/PMT gamma detector (Bioscan Flowcount, Model FC-3200). Semi-preparative normal phase HPLC was performed on Econosil silica 250×10 mm column (Alltech) with either chloroform:methanol (95:5) or dichloromethane:methanol (98:2). Specific activity of the product **8** was determined by analytical reversed phase HPLC with an UV detector at 200 nm, 0.02 AUF. The linear-response standard curve for the determination of mass of the carrier product was plotted for the concentrations of **6** between 0.001 and 0.01 mg/ml.

Analytical thin-layer chromatography (TLC) of cyclophosphamide and fluorinated analogue was carried out on aluminum backed Silica gel 60 (0.25 mm) plates (EM Science) with ethyl acetate:dichloromethane:methanol (5:3:1). Separated components were visualized by exposure to iodine vapor or by spraying with 0.2% ninhydrin solution containing 0.15% acetic acid and heating.[30] Flash chromatography was carried out with Silica gel 60, 0.04–0.063 mm (EM Science).

No-carrier-added [^{18}F]fluoride ion was produced with CTI/Siemens RDS 112 negative ion cyclotron (Knoxville, TN) by the $^{18}O(p,n)^{18}F$ reaction

with 11 MeV protons in a silver target using [^{18}O]H_2O (97% isotopic enrichment).

N-(2-hydroxyethyl)fluoroacetamide (**2**)

The title product **2** was obtained as an oil (b.p. 98–103°C; 0.05 mmHg; 89% yield) (Lit[21] b.p. 110–115°C; 0.1–0.2 mmHg). ^{1}H NMR ($CDCl_3$): 3.13 (bs, 1H, OH); 3.51 (t, 2H, OCH_2, $J_{H,H} = 5.2$); 3.75 (t, 2H, NCH_2, $J_{H,H} = 5.2$); 4.82 (d, 2H, FCH_2, $J_{F,H} = 47.3$); 6.96 (bs, 1H, NH). ^{19}F NMR ($CDCl_3$): −226.3 (t, $J_{H,F} = 47.0$).

N-2-chloroethylfluoroacetamide (**3**)

The product **3** was synthesized according to the published procedure[31] (m.p. 65–66°C; yield 91%) (Lit[31] m.p. 63–65°C). ^{1}H NMR ($CDCl_3$): 3.70 (m, 4H, CH_2CH_2); 4.85 (d, 2H, FCH_2, $J_{F,H} = 47.0$); 6.75 (bs, 1H, NH). ^{19}F NMR ($CDCl_3$): −226.5 (t, $J_{H,F} = 47.0$).

N-(2-chloroethyl)-N-(2-fluoroethyl)amine hydrochloride (**4**)

Diborane reduction of amide **3**[21] yielded after recrystallization from anhydrous acetone a crystalline **4** (m.p. 193–194°C; yield 78%) (Lit[21] m.p. 193–195°C). ^{1}H NMR (D_2O): 3.49 (t, 2H, NCH_2-(CH_2F) $J_{H,H} = 4.6$); 3.57 (m, 2H, CH_2Cl); 3.94 (t, 2H, NCH_2-(CH_2Cl), $J_{H,H} = 5.6$); 4.8 (dt, 2H, CH_2F, $J_{H,H} = 4.6$, $J_{F,H} = 46.7$). ^{19}F NMR ($CDCl_3$): −223.5 ($^2J_{H,F} = 46.7$, $^3J_{H,F} = 29.3$).

N-(2-chloroethyl-N-(2-fluoroethyl)phosphoramidic dichloride (**5**)

The product **5** was obtained[22] as a colorless oil (b.p. 97–103°C; 0.5 mmHg; yield 47%) (Lit.[22] b.p. 97–99°C at 0.4–0.5 mmHg). ^{1}H NMR ($CDCl_3$): 3.66 (m, 6H, $3CH_2$); 4.66 (dt, 2H, FCH_2, $J_{F,H} = 47.3.0$ Hz, $J_{H,H} = 4.6$ Hz). ^{19}F NMR ($CDCl_3$): −222.6 ($^2J_{H,F} = 47.3$ Hz, $^3J_{H,F} = 29.3$ Hz).

2-[(2-Chloro-2′-fluorodiethyl)amino]-2H-1,3,2-oxazaphosphorinane-2-oxide (**6**)

The condensation of **5** and 3-aminopropanol afforded **6** as a colorless viscous oil[22] (yield 84%). A portion of the product (740 mg) was dissolved in dichloromethane and flash-chromatographed on a silica gel column with dichloromethane:methanol, 18:1. Fractions were examined with TLC, and ninhydrin positive fractions with $R_f \sim 0.3$ were pooled and evaporated under reduced pressure. Colorless oil was obtained (612 mg, 83%). ^{1}H NMR ($CDCl_3$): 1.86 (m, 2H, $^5CH_2$); 2.63 (bs, 1H, NH); 3.44 (m, 6H, N-CH_2-(F); N-CH_2-(Cl), $^4CH_2$); 3.65 (t, 2H, CH_2Cl, $J_{H,H} = 7.0$); 4.36 (m, 2H,$^6CH_2$); 4.55 (dt, 2H, FCH_2, $J_{F,H} = 47.3.0$, $J_{H,H} = 4.9$). ^{19}F NMR ($CDCl_3$): −222.6

($^2J_{H,F} = 47.3$, $^3J_{H,F} = 29.3$); EI HRMS $M^+ + H$ calculated for $C_7H_{16}N_2O_2FPCl$: 245.0622, found: 245.0618.

2-[(2-Chloro-2′-fluorodiethyl)amino]-2H-1,3,2-oxazaphosphorinane-2-oxide (**6**) *by direct fluorination of* (**7**)

Anhydrous cyclophosphamide[24] **7** (20 mg, 0.08 mmol) was heated at reflux in anhydrous acetonitrile (2.5 ml) (oil bath temperature 95–100°C) in the presence of Kryptofix®222 (78 mg, 0.21 mmol) and anhydrous potassium fluoride (11 mg, 0.19 mmol) for up to 3 h. Thin layer chromatography of the reaction mixture showed the appearance of multiple products. ^{19}F NMR ($CDCl_3$) of the reaction mixture indicated the presence of a signal at −221 ppm that is characteristic of the fluoro-alkyl functionality. The reaction mixture was evaporated under reduced pressure and dissolved in dichloromethane. Chromatography on a silica gel column with dichloromethane:methanol (18:1) afforded the product **6** (2 mg; 10.7%) whose ^{19}F and ^{1}H NMR spectra were identical with the standard synthesized by the above described methods. The product obtained by the KF/Kryptofix®222 fluorination was co-chromatographed on analytical reversed phase HPLC with the standard **6** (R_t **6** = 4.5 min; R_t **7** = 7.5 min).

2-[(2-Chloro-2′-[^{18}F]fluorodiethyl)amino]-2H-1,3,2-oxazaphosphorinane-2-oxide (*^{18}F-fluorocyclophosphamide*)(**8**)

[^{18}F]-fluorocyclophosphamide (**8**) was prepared by a single-step halogen exchange as shown in Figure 2. Typically, [^{18}F]F^- recovered in water (300 µl) was added to a 0.1 M solution of potassium carbonate (30 µl) and Kryptofix®222 (2.25 mg, 6 µmol) in 50 µl acetonitrile) and evaporated to dryness at 110°C in a stream of argon. Acetonitrile was added (3 × 300 µl) and evaporated to dryness. The solution of cyclophosphamide monohydrate (6 mg, 21 µmol)) in acetonitrile (600 µl) was then added and the reaction mixture heated at 110°C in a closed vial for 10 min. The reaction mixture was, after brief (3 min) cooling to room temperature in an ice bath, directly injected onto a semi-preparative reversed phase HPLC column eluted with a flow rate of 5 ml/min. The radioactive fraction at 7.7–8.7 min was collected (R_t **8** = 8.2 min; R_t cyclophosphamide (**7**) = 12.8 min). pH of the collected eluate was adjusted to 7.8 with 0.025 M NaH_2PO_4 and diluted tenfold with water (total volume ~50 ml). The diluted eluate was passed through a preconditioned C18 Sep-Pak column (Waters, C18 Sep-Pak, 300 mg), activated with methanol (5 ml) and water (10 ml). The column was then washed with water (25 ml). The radioactive product **8** was eluted with acetonitrile (1 ml). The acetonitrile was evaporated under reduced pressure, the residue was dissolved in ethanol (1 ml) and then evaporated to dryness ($T < 35$°C). The injectable radioactive tracer was obtained after reconstitution in 0.9% saline and filter-sterilization

(0.22 μm filter, Millex®GP, PES membrane, Millipore). Radiochemical and chemical purities were determined by analytical HPLC and were found to be >99% and >96%, respectively.

Conclusion

We have synthesized an analog of cyclophosphamide that contains a single [^{18}F]-fluoroethyl group ('fluorocyclophosphamide') by direct halogen exchange reaction from a commercially available precursor. The resulting radioactive tracer, of high radiochemical and chemical purity, is suitable for *in vivo* (microPET) studies of pharmacokinetic parameters relevant to individualized pre-therapeutic assessment of therapy regimens.

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