Bioorganic & Medicinal Chemistry Letters 19 (2009) 4764–4767

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of radiolabeled cytarabine conjugates

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ARTICLE INFO

Article history:
Received 8 April 2009
Revised 8 June 2009
Accepted 15 June 2009
Available online 17 June 2009

Keywords:
Ara-C
Nucleoside transporters
Technetium-99m
Cancer

ABSTRACT

N4-Modified, novel Ara-C conjugate capable of radiolabeling with gamma ray-emitting (^{99m}Tc) as well as positron emitting (^{18}F) radionuclides, that is, *N*4-hydrazine derivative was synthesized. The radiolabeling of *N*4-(hydrazinonicotinyl)-1-β-arabinofuranosyl cytosine (HAra-C) with ^{99m}Tc was performed with over 95% labeling yield. To label HAra-C with ^{18}F, 4-fluoro(^{18}F)-benzaldehyde was synthesized from 4-formyl-*N*,*N*,*N*-trimethylanilinium triflate in 30% radiochemical yield; it quantitatively formed hydrazone derivative with HAra-C within 45 min. The radiolabeled conjugates were analyzed by radio-UV-RP-HPLC. The cold precursors were characterized by ^{1}H, ^{13}C NMR. Additionally, HAra-C was evaluated for cytotoxicity in lung adenocarcinoma (H441) cells and found to be comparable in cell killing efficiency to that of Ara-C. Uptake of ^{99m}Tc-HAra-C in cultures of H441 cells and sensitive pancreatic cancer cells (MIAPaCa-2) was inhibited by nucleoside transporter inhibitor nitrobenzylthioinosine. The results suggest that ^{99m}Tc-labeled HAra-C is a substrate for the membrane nucleoside transporters, and that it may be used in molecular imaging of nucleoside transporter expression for the verification of potential anticancer efficacy of nucleoside drugs, such as Ara-C and gemcitabine.

Nucleoside analogs are an important family of compounds which are of great interest in view of their pharmacological and pharmaceutical applications.[1] Cytarabine or cytosine arabinoside (Ara-C) is a pyrimidine nucleoside analogue used for the treatment of acute myeloid leukemia, meningial leukemia and lymphoma.[2] The clinical utility of Ara-C is limited to blood cancers, because of its unfavorable pharmacokinetics, inactivation by deamination, and acquisition of resistance to membrane transport. Besides inactivation by deamination to arabino uracil, it is also rapidly cleared with a half life of 1–3 h, limiting its efficacy in the treatment of solid tumors.[3,4] It has been demonstrated that derivatization of *N*4 amine of cytosine may increase its stability towards deaminase-mediated inactivation. As such, protection of Ara-C by encapsulating the drug inside liposomes, or DepoCyt, or by preparing derivatives of Ara-C, such as *N*4-acyl polyethylene glycol derivatives have shown promise.[5,6]

Ara-C enters the cells by such a carrier-mediated process, mainly involving human equilibrative nucleoside transporter 1 (hENT1). Once inside, it is activated by a sequence of phosphorylations to produce cytosine arabinoside triphosphate (Ara-CTP) which is a potent inhibitor of DNA polymerase, and results in DNA chain termination. One of the mechanisms of acquired resistance to the nucleoside drugs is the downregulation of nucleoside transporters.[7] A vast majority of work has been conducted in vitro, but a technology that can assess expression of nucleoside transporters in vivo remains to be developed. Our overall objective has been to radiolabel Ara-C with imaging radionuclides, and at the same time, impart resistance to deamination. Here we describe a modification of amine group *N*4 amine of Ara-C that can add the capability of radiolabeling Ara-C with imaging radionuclides. We also demonstrate the potential of ^{99m}Tc-labeled HAra-C in imaging expression of nucleoside transporters by testing the labeled conjugate in vitro.

Synthesis of Ara-C-SBHN conjugate: Our goal of chemically modifying Ara-C was to protect it against N4 deamination, and at the same time, render the new molecule amenable to radiolabeling with ^{99m}Tc and ^{18}F. The dual purpose was accomplished by conjugation of succinimidyl-6-boc-hydrazinonicotinate (SBHN) at the N4 position of Ara-C. SBHN (**4**) was synthesized according to Scheme 1 (Fig. 1) from commercially available 6-chloronicotinic acid.[8,9] SBHN was conjugated to Ara-C (**5**) to yield *N*4-(6-Boc-hydrazinonicotinyl)-1-β-arabinofuranosyl cytosine (**6**) as shown in scheme 2 (Fig. 2). Several reaction conditions (Table 1) were tested in order to optimize the yield. Our initial attempts of Ara-C (**5**) (0.04 mmol) reaction with SBHN (**4**), in the presence of *N*,*N*-dimethyl formamide and diisopropylethyl amine at 50 °C, afforded **6** in poor yields (29%) and the reaction was not reproducible. Next, we coupled Ara-C (**5**) with hydrazinonicotinic acid (**2**) via DCC (dicyclohexylcarbodiimide). This reaction was also unsatisfactory where the starting material remained unreacted even after 5 days. The best yields of the conjugate **6** were obtained in presence of pyridine at 70 °C. The conjugate **6**; was characterized with ^{1}H NMR by the appearance of aromatic protons at δ 8.80–6.50, H-1′ at δ 6.10, and tertiary butyl protons of Boc at δ 1.44.

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doi:10.1016/j.bmcl.2009.06.056

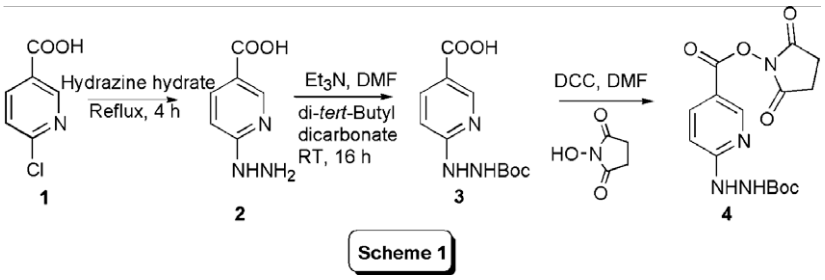

Figure 1. Scheme 1, Synthesis of succinimidyl-Boc-hydrazinonicotinate (SBHN).

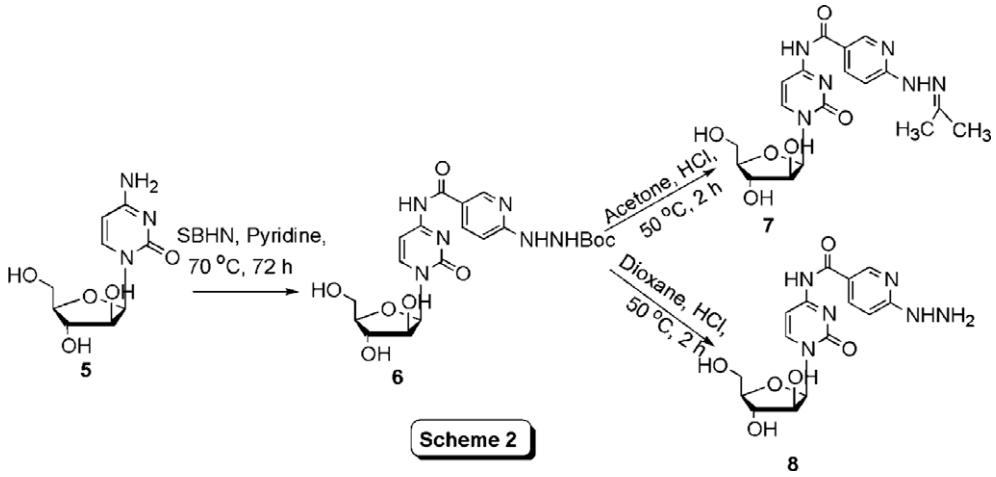

Figure 2. Scheme 2 showing the synthesis of *N*4-(6-*tert*-boc-hydrazinonicotinyl)-1-β-arabinofuranosyl Cytosine or HAra-C.

Table 1
Coupling of Ara-C with SBHN

S. No	Precursors	Condition	Temp	Time	Yield of **6** based on TLC
1	Ara-C and SBHN	DIPEA (5 equiv), DMF	rt-1 day, 50 °C-1 day	2 days	29% of **6** formed, but not reproducible
2	Ara-C and SBHN	DMSO	rt	7 days	10%
3	Ara-C and SBHN	Pyridine	rt	7 days	No reaction
4	Ara-C and 6-hydrazino nicotinic acid	DMF (DCC)	rt-1 day, 70 °C-5 days	6 days	10–15%
5	Ara-C and SBHN	Pyridine	70 °C	3 days	50–60%

Generally, Boc-deprotection of amines is carried out with trifluoroacetic acid (TFA).[10] We used HCl to eliminate the possibility of the formation of trifluoroacetyl derivatives as reported elsewhere.[11] When HCl-mediated Boc-deprotection of HAra-C (**6**) was performed in acetone, we obtained a hydrazone derivative, **7**. Formation of **7** was confirmed by the appearance of molecular ion peak at 419.07 in mass spectrometry; ^{1}H NMR showed CH_3 peaks at δ 2.15 integrating for 6 protons. However, deprotection of **6** with HCl in dioxane at 50 °C (Fig. 2, Scheme 2) provided us the required product *N*4-(6-hydrazinonicotinyl)-1-β-arabinofuranosyl cytosine (**8**). The compound **8** was confirmed by the disappearance of tertiary butyl protons at δ 1.40 in ^{1}H NMR.

Techenetium-99m labeling of HAra-C: ^{99m}Tc radiolabeling of sugar part of thymidine has been previously reported,[12] but this is the first report of ^{99m}Tc radiolabeling of the base part of Ara-C by which it may also be protected from deamination. We performed ^{99m}Tc labeling of conjugate **8** by following a standard method using stannous chloride as a reductant and tricine as a co-ligand.[13,14] Quantitative radiolabeling (>95%) was obtained within 15 min of incubation at room temperature (Fig. 3, Scheme 3). The labeling was followed by Whatman paper chromatography in acetone where free pertechnetate had an R_f of 0.8 and the labeled compound had an R_f of 0.1. Upon radio-RP-HPLC, ^{99m}Tc-labeled HAra-C eluted at 5.3 min without any contamination with free pertechnetate (retention time 11 min).

Flourine-18 labeling of HAra-C: Our next goal was to label HAra-C with positron-emitting radionuclide ^{18}F for Positron Emission Tomography or PET. We synthesized 4-fluoro(^{18}F)-benzaldehyde (**11**) as a radiolabeled synthon using a krytpofix-mediated nucleophilic substitution of 4-formyl-*N*,*N*,*N*-trimethylanilinium triflate (Fig. 4, Scheme 4, >30% radiochemical yield).[15] For labeling HAra-C, an ethanolic solution of **11** was reacted with aqueous solution of HAra-C at 70 °C for 45 min. The hydrazone (**12**) formation was confirmed by comparing with authentic hydrazone of 4-fluorobenzaldehyde and HAra-C which eluted at 3.5 min in RP-HPLC (Reverse Phase-High Pressure Liquid Chromatography) and showed a molecular ion peak at 484.09 in the ESI MS (Electron Spray Ionization Mass) spectrum. During radiolabeling, the hydrazone was monitored by following an increasing UV-radio peak at 3.50 min and vanishing 4-fluoro(^{18}F)-benzaldehyde peak at 8.53 min.

Cytotoxicity of HAra-C and uptake mechanism: After successfully labeling HAra-C with ^{99m}Tc, we investigated the effects of HAra-C

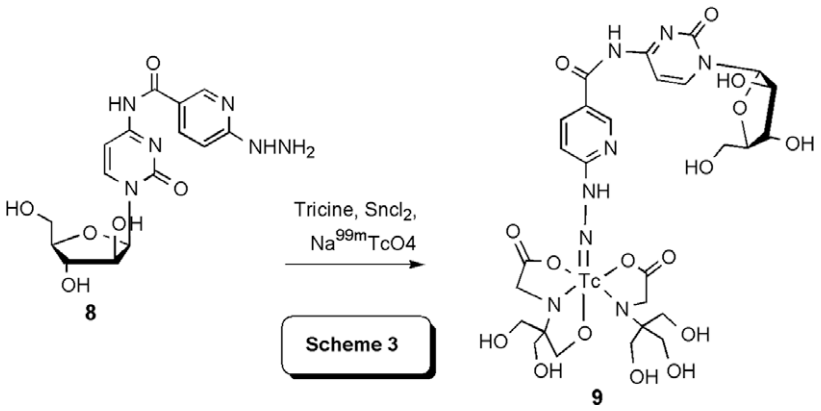

Figure 3. Scheme 3 showing ^{99m}Tc labeling of HAra-C.

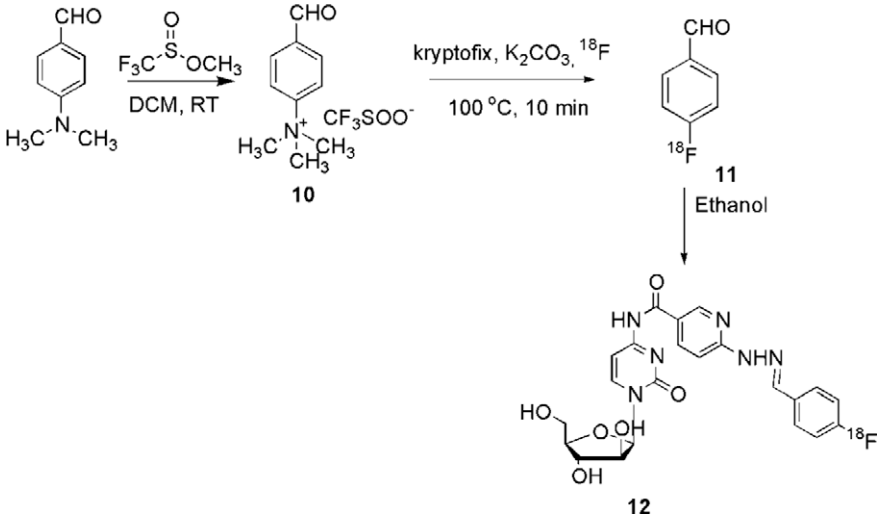

Figure 4. Scheme 4 showing synthesis of 4-fluoro(^{18}F)benzaldehyde and its hydrazone formation with HAra-C.

and Ara-C on human lung adenocarcinoma cell line H441. The total number of cells after 72 h of treatment were counted using trypan blue cell exclusion assay. It was observed that there is a dose dependent reduction in cell viability; at 2 μM dose both drugs showed comparable viability of around 55% (Fig. 5). The results were confirmed by serum lactate dehydrogenase and cellular glucosaminidase assays (not shown). No attempt was made to test the cytotoxicity of ^{99m}Tc-labeled HAra-C, because it is meant to be used in tracer doses for imaging studies.

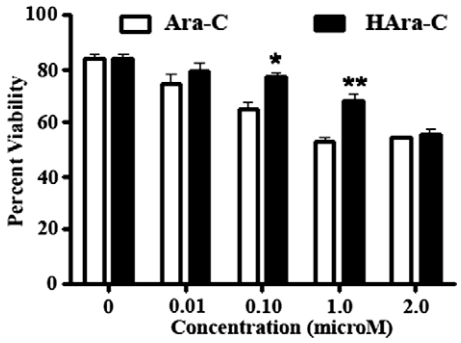

Figure 5. Cytotoxicity of Ara-C and HAra-C in lung adenocarcinoma H441 cells in culture. Cell viability was studied by trypan blue exclusion. The * and ** indicate significance in difference between Ara-C and HAra-C analyzed by analysis of variance Bonferroni post-test.

Among the purported mechanisms of resistance to pyrimidine analogs is the downregulation of nucleoside transporters in cancer cells.[16,17] A close analog of Ara-C is gemcitabine which is pharmacologically similar to Ara-C.[7,18] Expression of hENT1 has been shown to be associated with the chemosensitivity of gemcitabine in human pancreatic adenocarcinoma and biliary tract carcinoma cells.[19] In order to design a molecular imaging agent for quantitation of hENT1 expression, we investigated the effect the nucleoside transporter inhibition on the uptake of tracer dose of ^{99m}Tc-HAra-C in H441 cells as well as in two pancreatic cancer cell lines MIAPaCa-2 and PANC-1. Nucleoside transport is specifically inhibited by nitrobenzylmercaptopurine ribonucleoside (NBTI).[20] It is clear from Figure 6 that the specific ENT inhibitor NBTI reduces ^{99m}Tc-HAra-C uptake in H441 and MIAPaCa-2 cells, but not in PANC-1 cells. In separate experiments we have observed that MIAPaCa-2 cells are more sensitive to gemcitabine than PANC-1 cells (data not shown). Resistance of PANC-1 cells to gemcitabine has also been reported.[21] Recently, researchers have shown that the adenoviral-mediated overexpression of hENT1 enhances gemcitabine response in human pancreatic adenocarcinoma. Our results confirm these published reports, and provide a novel method for potential molecular imaging of nucleoside transporters in vivo.

In summary, we synthesized N4-conjugate of Ara-C (HAra-C) whose cytotoxicity was comparable to that of Ara-C in H441 cells.

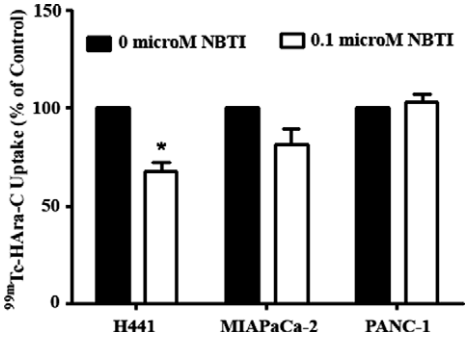

Figure 6. ^{99m}Tc-labeled HAra-C uptake in H441 lung adenocarcinoma cells in presence or absence of nucleoside transport inhibitor (NBTI). The * indicates significance in difference in uptake of HAra-C between cells treated with 0 μM and 0.1 μM NBTI.

Based on our preliminary in vitro cytotoxicity investigations, it is anticipated that the modification retains cell killing potential of Ara-C in addition to the nucleoside transporter-mediated intracellular uptake. The amenability to radiolabel HAra-C can be used to follow its biological disposition in vivo by both SPECT as well as PET imaging. The non-invasive imaging of the expression of hENT1 in cancer cells may influence clinical interventions dependent on pyrimidine nucleosides. For instance, gemcitabine or Ara-C therapy in cancers with no ENT expression may be futile. We are further exploring the utility of this concept in vivo in animal models of lung and pancreatic cancers.

Acknowledgements

The generous supply of ^{99m}Tc-pertechnetate by the OUHSC-Nuclear Pharmacy and ^{18}F-water supply from Midwest Medical Isotopes, Oklahoma City is acknowledged. Authors also thank Dr. Shrikant Anant for providing pancreatic cancer cell lines PANC-1 and MIAPaCa-2.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.06.056.

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