Bioorganic & Medicinal Chemistry Letters 26 (2016) 5472–5475

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

A two-step strategy to radiolabel choline phospholipids with ^{99m}Tc in S180 cell membranes via strain-promoted cyclooctyne–azide cycloaddition reaction

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ARTICLE INFO

Article history:
Received 18 July 2016
Revised 6 October 2016
Accepted 11 October 2016
Available online 12 October 2016

Keywords:
Tumor imaging
AECho
ADIBO-BPA
^{99m}Tc(CO)$_3$-labeling
Pretargeting
SPAAC

ABSTRACT

As tumor markers, the radiolabeling of choline (Cho)-containing phospholipids in cellular membranes with ^{99m}Tc is a challenge. The conventional strategy to combine the metallic radionuclide with Cho by large ligand damages the bioactivity of Cho, resulting in low tumor-to-nontumor ratios. Pretargeting strategy based on strain-promoted cyclooctyne–azide cycloaddition (SPAAC) reaction was applied to solve this general problem. Functional click synthons were synthesized as pretargeting components: azidoethyl-choline (AECho) serves as tumor marker and azadibenzocyclooctyne (ADIBO) conjugated to bis(2-pieolyl) amine (BPA) ligand (ADIBO-BPA) as ^{99m}Tc(CO)$_3$-labeling and azido-binding group. Both in vitro cell experiment and in vivo biodistribution experiment indicate that it is versatile to radiolabel Cho in cellular membranes via this two-step pretargeting strategy. We believe that this pretargeting strategy can indeed enhance the target-specificity and also reduce background signals to optimize imaging quality.

Phospholipids are important components of cellular membranes. Choline (Cho) is a common precursor of phospholipid via intrinsic biosynthesis route.[1] Cho-containing phospholipids are important structural components of membranes and play critical roles in cell signaling, either as signaling molecules in their own right or as precursors of secondary messengers.

Because tumor proliferates more rapidly than normal tissues, the uptake of choline in the former is obviously higher than that in the latter.[2] So far, Cho derivatives have already been widely researched as tumor markers in magnetic resonance imaging[3] and radionuclide imaging of tumor.[4,5] However, modifications of the Cho molecule will probably decrease its bioactivity[6], restraining its applications in nuclear medical imaging. In the case of ^{99m}Tc nuclide, the defect is especially obvious, as it requires large ligands linked with Cho to coordinate with the ^{99m}Tc core. Therefore, the pretargeting strategy is potentially able to overcome this general problem.[7,8]

The pretargeting strategy is a multi-step process that utilizes the high affinity of biomolecules, avoiding the drawbacks associated with decreasing bioactivity and slow clearance.[8] For example, the first step of a pretargeting strategy in Cho-containing phospholipids involves the administration of a tagged unlabeled choline and subsequent tumor targeting. The second step follows with injection of a radiolabeled molecule with a high specificity and bioorthogonal reactivity for the tag on the tumor localized Cho-containing phospholipids. This strategy has been increasingly used in radioimmunodetection and radio-immunotherapy of tumors.[9–11] The pretargeting strategy consists of two indispensable components: click synthons used as reporter/effector and click reaction as the link.[12–14] Among all the click reactions, the strain-promoted cyclooctyne–azide cycloaddition (SPAAC) represents a rapid, efficient, and catalyst-free in vivo click reaction with high specificity and versatility under mild conditions.[15] SPAAC reaction is being increasingly applied in microscopic optical visualization of macromolecules such as glycoproteins[16], proteins[17], DNA[18] and RNA[19], as well as in radiochemistry for molecular imaging.[20–23]

Labeling of Cho-containing phospholipid in vitro with pretargeting strategy has been tried[24–31]; however, the in vivo trial especially with radiochemical method has been rarely reported. In this study, we attempted to use this strategy to radiolabel Cho-containing phospholipid with the SPAAC reaction.

Next, we investigated Cho and cyclooctyne derivatives suitable as reporter/effector in the pretargeting strategy.

In 2013, Huang et al.[25] biosynthesized and incorporated choline (Cho) analogs such as azidoethyl-choline (AECho) and

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http://dx.doi.org/10.1016/j.bmcl.2016.10.026
0960-894X/

azidopropyl-choline (APCho) into the phospholipids of cells and enveloped virus. The results show that both AECho and APCho can be incorporated into phospholipids via the intrinsic biosynthesis route, indicating that the azido group has little destruction on the Cho bioactivity. In addition, the incorporation efficiency of AECho is higher than APCho, owing to a higher similarity with natural Cho. In contrast, Gordon and co-workers[32] studied the effect of ring strain and electronic effect on the SPAAC reaction kinetics. The results illustrate that azadibenzocyclooctyne has a superior reaction kinetics compared to that of other cyclooctyne derivatives.

Therefore, it was reasonable to choose AECho as the reporter to target tumor tissues and azadibenzocyclooctyne conjugated to bis (2-pieolyl) amine (BPA) ligand for $^{99m}Tc(CO)_3$-labeling as the effector. After a suitable time interval, in vivo SPAAC reaction occurs between the effector and the prelocalized reporter. This strategy was used in this study, anticipating to protect the bioactivity of Cho, resulting in superior tumor targeting.

Herein, ADIBO-BPA and AECho were synthesized according to the published procedures (the Supplementary data, Schemes S1 and S2).

$^{99m}Tc(CO)_3$-labeled compounds were then prepared following literature procedure (the Supplementary data, S4). The radiochemical purities of all the $^{99m}Tc(CO)_3$-labeled complexes were analyzed by radio-HPLC. The radio-HPLC chromatograms are shown in Figure 1. The retention time of $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$ is slightly shorter than that of $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^{+}$, indicating that the former has greater polarity. It's worth mentioning that $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$ was successfully prepared both in water and serum media, and the detailed procedure is mentioned in the Supplementary data. Inspiringly, in both the media, $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^{+}$ could be completely transformed into $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$ via in vitro SPAAC reaction even at a very low AECho concentration of 1.0 mM, providing further evidence for its applicability in vivo.

The partition coefficient, which is a typical physicochemical parameter and a relevant indication of pharmacokinetic behavior for the radiopharmaceutical, was determined by distributing the radiolabeled complex in a mixture of 1-octanol and deionized water. The results are listed in Table 1. The log $P_{(O/W)}$ indicates that $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^{+}$ is more lipophilic than $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$, thus might result in a slower blood clearance rate than the latter.

The $^{99m}Tc(CO)_3$-labeled complexes are stable in both phosphate buffer (pH 7.4, 0.1 M) and fetal bovine serum media, and show no detectable decomposition at room temperature during 7 h

Table 1
Stability, PBPP, and partition coefficients of $^{99m}Tc(CO)_3$-labeled complexes

Complex	Stability in plasma[a] (%)	PBPP[b] (%)	Log P[c]
$[^{99m}Tc(CO)_3$-BPA-ADIBO$]^{+}$	>99	15.1 ± 0.4	0.77 ± 0.04
$[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$	>99	9.4 ± 0.2	0.22 ± 0.02

[a] Radiochemical purities after 7 h at room temperature.
[b] PBPP, percentage of binding to plasmatic proteins.
[c] log P = Radioactivity (1-octanol)/Radioactivity (deionized water).

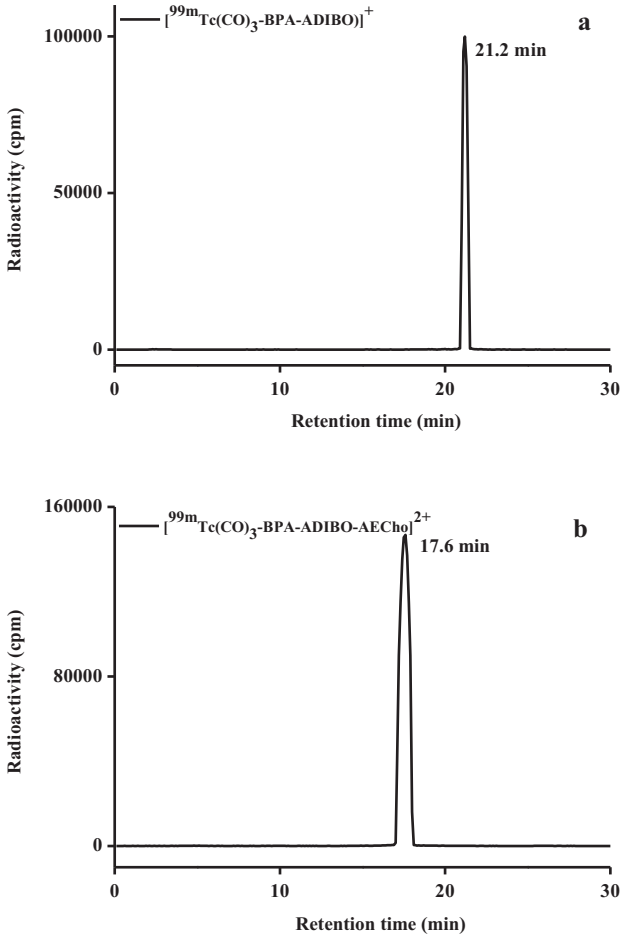

Figure 1. HPLC analysis of $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^{+}$ (a) and $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$ (b).

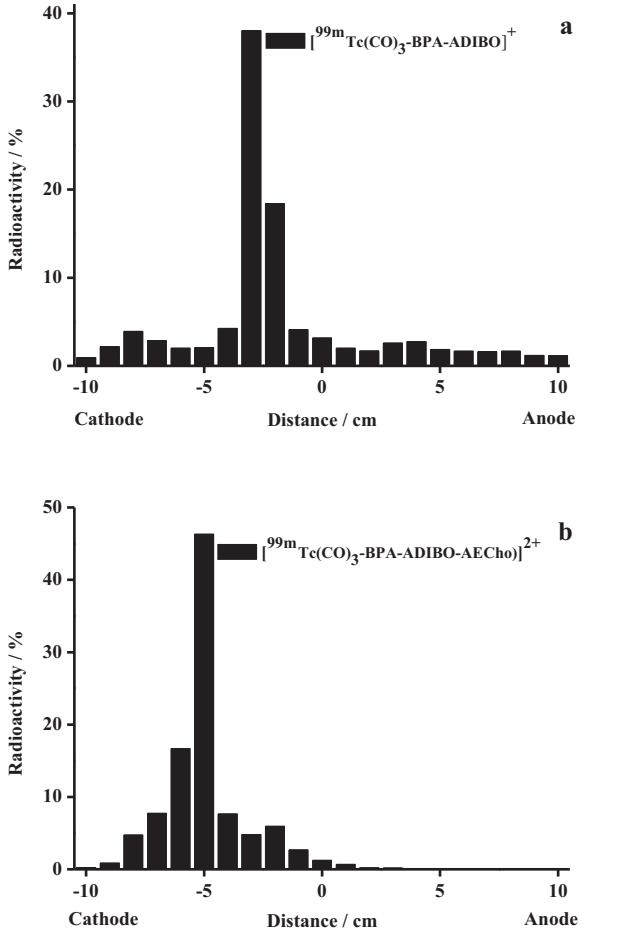

Figure 2. Paper electrophoresis patterns of $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^{+}$ (a) and $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$ (b).

(Table 1), which is a guarantee for them to be used in vivo. In plasma protein binding experiment, lower protein binding rate of $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$ was observed (Table 1), probably attributed to its lower log*P*.

In order to determine the charges of $^{99m}Tc(CO)_3$-labeled complexes, paper electrophoresis was performed in phosphate buffer (0.05 M, pH 7.4). Pertechnetate ($^{99m}TcO_4^-$) was used as the reference under the same electrophoretic conditions. The paper electrophoresis patterns of the complexes are shown in Figure 2. Pertechnetate shows a trend of migration toward the anode, while the radioactivity of $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ and $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$ shows the opposite trend under the same electrophoretic conditions, indicating that both $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ and $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$ have positive charges.

The incorporation of AECho through intrinsic metabolic route and SPAAC reaction between $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ and AECho were used to radiolabel the Cho-containing phospholipids in S180 cell membranes in vitro (Fig. 3a). The result in Figure 3b demonstrates that without AECho, cellular uptake of $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ with time fluctuates in a narrow scope. The final ~14% non-specific uptake might be ascribed to its excellent ability to cross cell membranes. However, with the addition of AECho, the cellular uptake of $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ gradually increases. Moreover, with increasing concentration of AECho, the cellular uptake of $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ increases simultaneously.

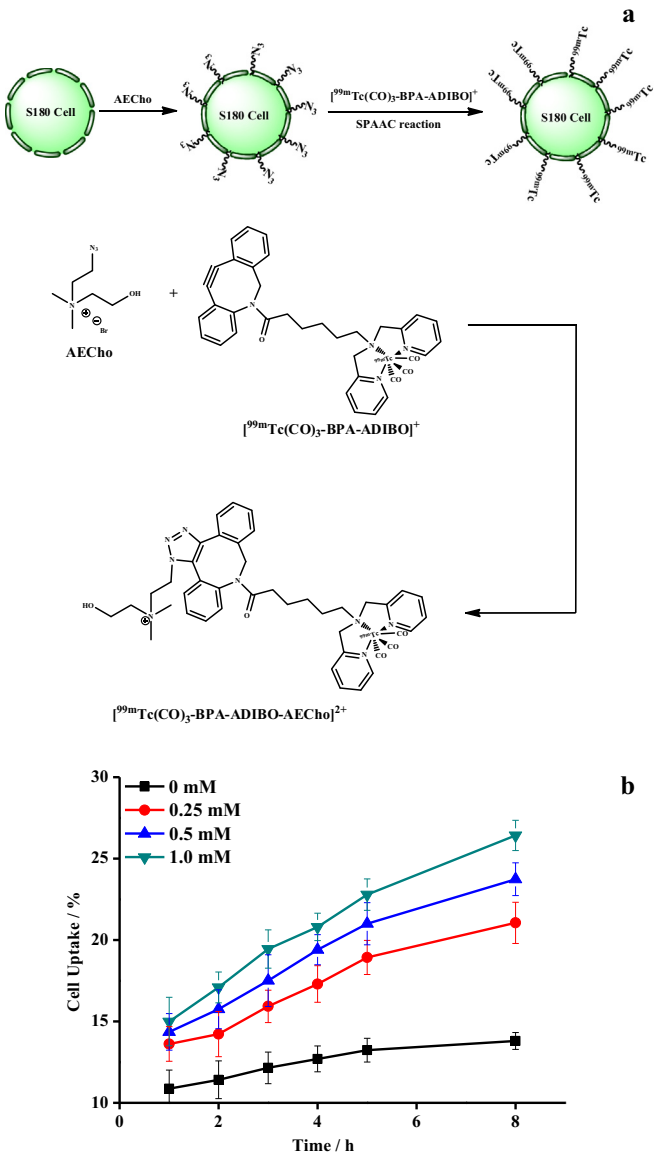

Figure 3. Schematic representation of metabolic incorporation of AECho in S180 cells and bioorthogonal labeling (a). Cellular uptake of $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ at different concentrations of AECho (b).

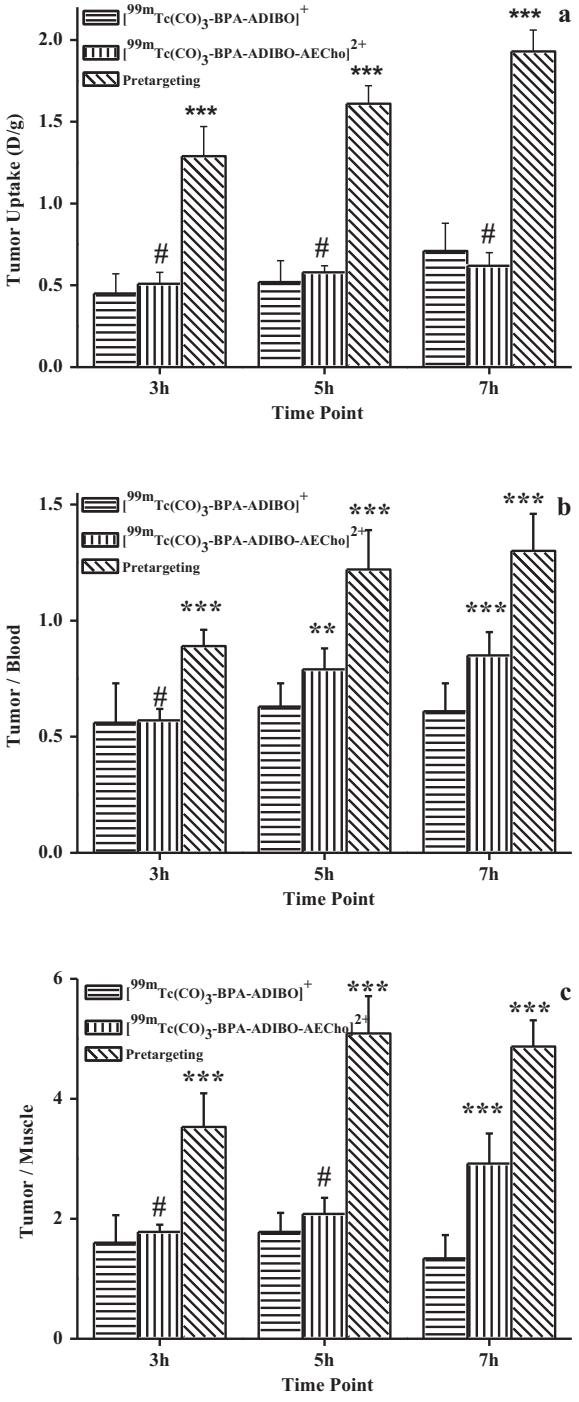

Figure 4. Comparison of biodistribution (tumor uptake (a), tumor/blood (b), tumor/muscle (c)) among blank control ($[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$), conventional control ($[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$), and pretargeting strategy (each mouse was injected daily for three days with Azido-Choline, and then injected with $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ on the fourth day). Data are presented as mean ± SD (n = 5) and analyzed by *T*-test. $^{\#}P$ >0.10, 0.01< $^{**}P$ <0.05, $^{***}P$ <0.01.

According to the result, it's reasonable to speculate that AECho can be effectively incorporated into S180 cell membranes and successfully labeled with the SPAAC reaction in vitro.

The in vivo biodistribution experiment was divided into three parts: $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ and $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$ were used as the blank control and potential conventional tumor imaging agent control, respectively, whereas the in vivo SPPAC reaction between $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ and AECho was used as the tumor pretargeting strategy[7]. As shown in Figure 4 and Table S4, $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$ shows a similar tumor uptake efficiency with $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$, but slightly higher tumor/blood ratios after 5 h and tumor/muscle ratios at 7 h because of former's faster blood clearance rate and lower muscle uptake efficiency. In contrast, the pretargeting strategy apparently shows higher tumor uptake efficiency, tumor/blood and tumor/muscle ratios than the other two at each time point, indicating that AECho effectively targets tumor cells in vivo, and its SPAAC reaction with $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ in tumor tissues promotes the latter's tumor uptake efficiency immensely.

In conclusion, functional click synthons were synthesized as the pretargeting components: AECho and ADIBO-BPA with $^{99m}Tc(CO)_3$ labeling serve as the tumor marker and azido-binding group, respectively. ADIBO-BPA was radiolabeled with $^{99m}Tc(CO)_3$, and $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$ was obtained by the in vitro click reaction between $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ and AECho. In vitro cell experiment and in vivo biodistribution experiment were studied with S180 cell line. The in vitro cell experiment illustrates that AECho can be effectively incorporated into S180 cell membranes and successfully radiolabeled with the SPAAC reaction. In the biodistribution experiment, $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ and $[^{99m}Tc(CO)_3$-BPA-ADIBO-AECho$]^{2+}$ were used as the blank control and potential conventional tumor imaging agent control, respectively. Compared to the control groups, the pretargeting group exhibits higher tumor uptake efficiency, tumor/blood and tumor/muscle ratios than the other two at each time point. To some extent, it can be concluded that the pretargeting strategy protects the bioactivity of Cho and therefore provides an innovative approach for the development of tumor SPECT (single photon emission computed tomography) imaging agents.

The biodistribution experiment also shows slow blood clearance rate of $[^{99m}Tc(CO)_3$-BPA-ADIBO$]^+$ because of its lipophilicity. This defect results in high background interferences; therefore, further studies are required to improve the hydrophilicity of ADIBO-BPA. Even so, our trial convincingly demonstrates that the two-step pretargeting strategy to SPECT imaging can indeed enhance the target-specificity and also reduce background signals to optimize imaging quality.

Acknowledgement

This study was supported by the National Natural Science Foundation of China (Grant Nos. 21371017 and 81371592).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.10.026.

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