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Design and synthesis of biotinylated cardiac glycosides for probing Nur77 protein inducting pathway

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ABSTRACT

The orphan nuclear receptor Nur77 (also known as TR3 or nerve growth factor-induced clone B NGFI-B) functions as a nuclear transcription factor in the regulation of target gene expression and plays a critical role in the regulation of differentiation, proliferation, apoptosis, and survival of many different cell types. Recent studies demonstrate that Nur77 also involves many important physiological and pathological processes including cancer, inflammation and immunity, cardiovascular diseases, and bone diseases. Our previous studies showed that cardiac glycosides could induce the expression of Nur77 protein and its translocation from the nucleus to the cytoplasm and subsequent targeting to mitochondria, leading to apoptosis of cancer cells. In order to probe the Nur77 protein inducting pathway, we designed and synthesized a series of novel biotinylated cardiac glycosides from β-Antiarin and α-Antiarin, two typical cardiac glycosides from the plant of *Antiaris toxicaria*. The induction of Nur77 protein expression of these biotinylated cardiac glycosides and their inhibitory effects on NIH-H460 cancer cell proliferation were evaluated. Results displayed that some biotinylated cardiac glycosides could significantly induce the expression of Nur77 protein comparable with their parent compounds β-Antiarin and α-Antiarin. Also, their streptavidin binding activities were evaluated. Among them, biotinylated cardiac glycosides **P4b** and **P5a** exhibited significant effect on the induction of Nur77 expression along with high binding capacity with streptavidin, suggesting that they can be used as probes for probing Nur77 protein inducting pathway.

Nur77, also called TR3, NGFIB, and NR4A1, is an orphan member of the nuclear receptor superfamily and an immediate-early response gene that functions as a nuclear transcription factor in the regulation of target gene expression. Nur77 plays a critical role in a plethora of cellular processes including cell differentiation, proliferation, apoptosis, and survival, etc.[1–3] Recent studies demonstrated that Nur77 also involves many important physiological and pathological processes including cancer,[4] inflammation and immunity,[3,5,6] cardiovascular diseases,[2,7,8] and bone diseases.[9] For example, upon stimulation by apoptosis-inducing agents, Nur77 is usually induced and the induced Nur77 protein subsequently translocate from the nucleus to mitochondria to induce cytochrome *c* release and apoptosis.[10] Further study revealed that Nur77 interacts with the N-terminal loop region of Bcl-2 and induces the conformational change of Bcl-2, resulting in conversion of Bcl-2 from a protector to a killer.[11] Nur77 also participates in the pathogenesis of inflammation. *In vivo* study demonstrated that Nur77 deficiency in mice increases their susceptibility to systemic inflammation. Liebmann et al.[6] reported that Nur77 is a key regulator of T cell immunometabolism, controlling oxidative phosphorylation and aerobic glycolysis during T cell activation that elevates the threshold for T cell activation and confers protection in different T cell-mediated inflammatory diseases. Recently, Hu et al.[3] unraveled a mechanism of Nur77-dependent clearance of inflamed mitochondria by mitophagy to alleviate inflammation. The pivotal role of Nur77 in cardiovascular diseases also attracts much attention of researchers. Zhang et al.[2] reported that Nur77 play a role in the regulation of hepatic cholesterol metabolism and reduces the cholesterol level in hepatocytes. Qin et al.[7] reported that Nur77 is a novel negative regulator

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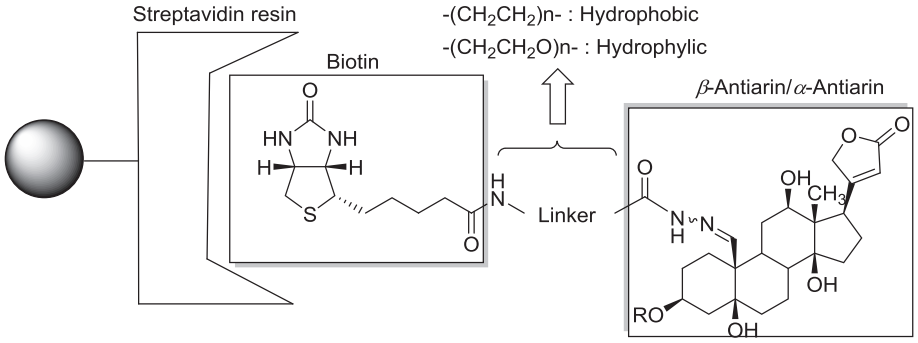


Fig. 1. Representative structure of cardiac glycoside probes.

of endothelin-1 (ET-1) expression in vascular endothelial cells (ECs) through an inhibitory interaction with the c-Jun/AP-1 pathway. Yan et al.[8] reported that Nur77 is a novel negative regulator for the *β*-adrenergic receptor (*β*-AR)-induced cardiac hypertrophy through inhibiting the NFATc3 and GATA4 transcriptional pathways. To sum up, Nur77 mediates many important physiological and pathological processes of humankind diseases.

Cardiac glycosides have been used for the treatment of congestive heart failure (CHF) for centuries and their known cellular target is the alpha subunit of the sodium (Na^+)/potassium (K^+)-ATPase.[12,13] Our previous studies showed that treatment of cancer cells with cardiac glycosides significantly resulted in the expression of Nur77 protein at a concentration of 20 nM. Immunofluorescence assay displayed that the induced Nur77 protein subsequently translocated from the nucleus to the cytoplasm and subsequent targeting to mitochondria, leading to apoptosis of cancer cells, which may be independent on Na^+/K^+-ATPase.[14,15] However, it's still unclear that how does cardiac glycosides affect the Nur77 signaling pathway.

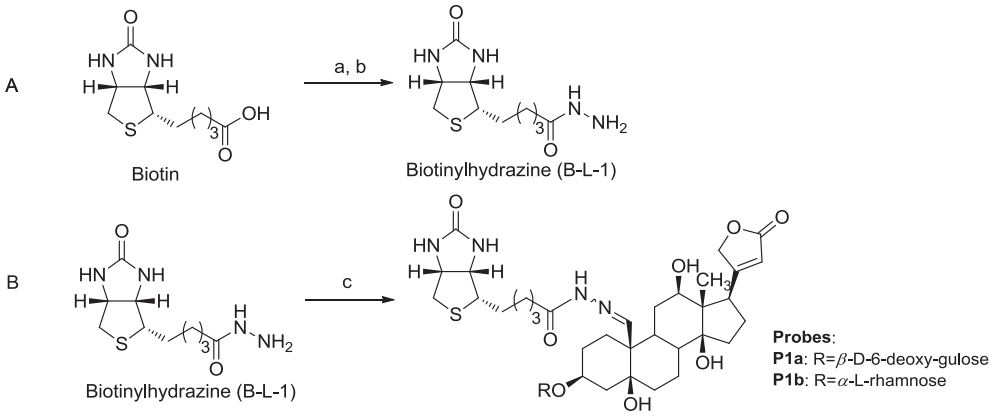
Compound-centric chemical proteomics (CCCP) is a very powerful technique to unveil the specific molecular targets of bioactive compounds,[16] which has been successfully applied for identifying the cellular targets of NSC751382,[17] Orlistat,[18] *β*-lactones[19] and a variety of natural products.[20] CCCP combines the affinity chromatography method and advanced mass spectrometry technique whereby the small molecule compound is covalently immobilized to a solid support, and incubated with a protein lysate to pull down the interacting proteins which will be identified using LC–MS/MS subsequently. Biotin is an optimal tag for labeling the bioactive molecules due to its strongest non-covalent biological interaction with streptavidin, which made it feasible for the enrichment and purification of biomolecule(s) binding to the biotinylated chemical probes. Here, in order to probe the Nur77 protein inducting pathway, we designed and synthesized a series of novel biotinylated cardiac glycosides from β-Antiarin and α-Antiarin for fishing the molecular target (s) binding to cardiac glycosides.

Design of biotinylated cardiac glycosides for probing Nur77 protein inducting pathway. Generally, chemical probe is consisted of three parts including a tag easy to detect or to purify, a flexible linker, and the bioactive compound that reacts with the target(s).[21] Here, biotin was chosen as the tag. The selection of the bioactive compound determines the efficiency of the chemical probes. What's more, it is better that biotinylation of the bioactive compound has no or less effect on its bioactivity. Structure-activity relationship (SAR) studies demonstrated that the sugar moiety in C-3 of cardiac glycosides is necessary for its induction of Nur77 expression. Meanwhile, the substituents at C-10 of cardiac glycosides showed significant effects on their bioactivities, with methyl (–CH_3), hydroxymethyl (–CH_2OH), or formyl group (–CHO) substituents at C-10 exhibiting comparable effects on induction of Nur77 expression.[14,15,22] Based on the SAR analysis and the relatively conservative structures of cardiac glycosides, we decided to biotinylate cardiac glycosides at the formyl group (–CHO) of C-10, which is highly reactive chemically. Finally, β-Antiarin and α-Antiarin, two typical cardiac glycosides from the plant of *Antiaris toxicaria*, were selected to prepare the chemical probes due to their significant difference in induction Nur77 expression bioactivities (β-Antiarin is better than α-Antiarin). A linker between the bioactive compound and the tag (biotin) should be flexible and have appropriate length in order to provide enough space for biotin to interact with streptavidin.[21] Meanwhile, the amphiphilicity of the linkers influences the penetrability, and then the bioactivity of the chemical probes.[21] Initially, the hydrophobic diamine [NH_2–(CH_2)*n*-NH_2] and hydrophilic PEG-bis(amine) with different chain length were used as the linkers. We proposed that one primary amine of the linker reacted with the carboxylic acid of the biotin to form amide bond and the other primary amine of the linker reacted with formyl group of C-10 of β-Antiarin (α-Antiarin) to form imine bond. However, the products were obtained in low yields and were unstable due to the instability of imine bond. Then, an alternative synthetic route was designed by introduction of hydrazine between linkers and the formyl group (–CHO) of cardiac glycosides. Two kinds of linker, [NH_2–(CH_2)*n*-COOH and NH_2–(CH_2–CH_2–O)*n*-CH_2–COOH], were introduced to formyl group at C-10 of β-Antiarin (α-Antiarin) after reacting with hydrazine (Fig. 1). The synthetic probes were obtained in high yield and were stable under neutral conditions. To our knowledge, this is the first time for systematic synthesis of biotinylated cardiac glycosides for fishing their target proteins.

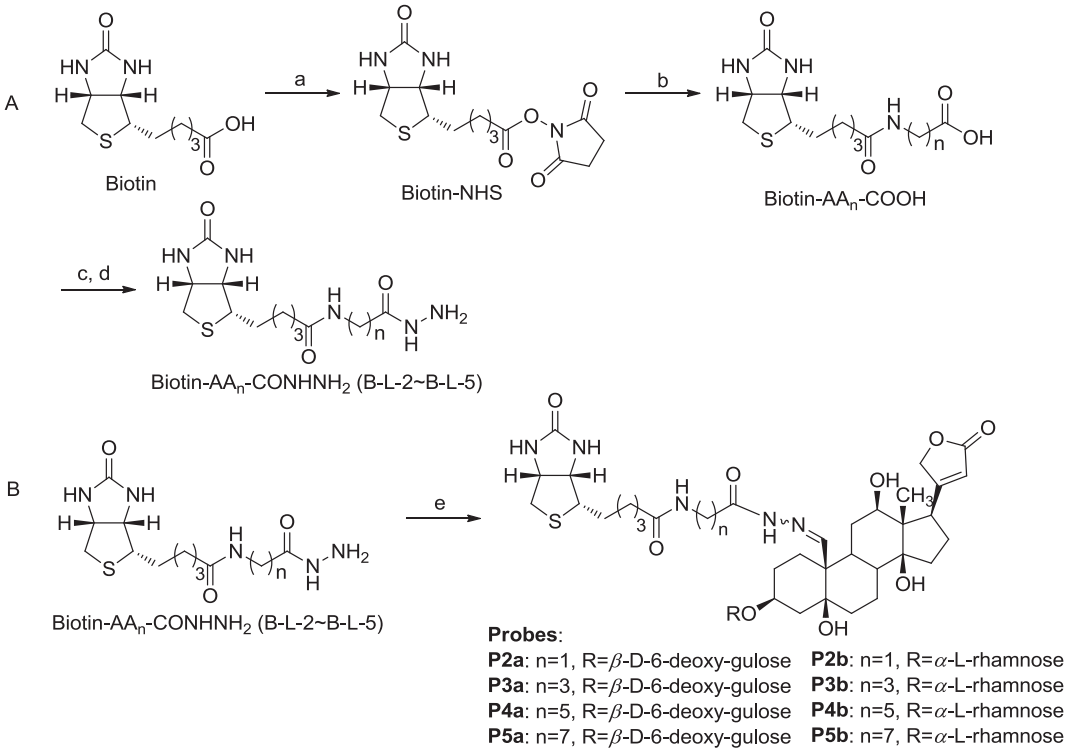
*Synthesis of biotinylated cardiac glycosides **P1a** and **P1b** from Biotinylhydrazine (B-L-1) and β-Antiarin (α-Antiarin)*. The synthesis of biotinylated cardiac glycosides **P1a** and **P1b** started from the synthesis of Biotinylhydrazine (B-L-1) which was prepared according the literature.[23] With the biotinylhydrazine (B-L-1) and cardiac glycosides (β-Antiarin and α-Antiarin) in hands, probes **P1a** and **P1b** were synthesized by the hydrazone formation under the catalysis of 1% AcOH.[24] The crude products were obtained by concentrated under reduced pressure. The residues were purified by semipreparative RP-HPLC (35% MeOH-H_2O) to obtain **P1a** (50.8% yield) and **P1b** (41.8% yield) (Scheme 1). The products **P1a** and **P1b** were both identified as mixtures of trans and cis epimers due to the presence of imine bond at C-19. The ratios of the trans and cis epimers of **P1a** and **P1b** were both about 3:1, which were identified by the analysis of ^{1}H NMR spectra.

*Synthesis of biotinylated cardiac glycosides **P2a**-**P5a** and **P2b**-**P5b** from Biotin–AA_n–$CONHNH_2$ (B-L-2–B-L-5) and β-Antiarin (α-Antiarin)*. Biotin–AA_n–$CONHNH_2$ (n = 1, 3, 5, and 7, B-L-2–B-L-5) were prepared firstly. Biotin was activated by *N*-hydroxysuccinimide (NHS) in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) to obtain Biotin–NHS as reported methods.[25] Then, Biotin–NHS was reacted with hydrophobic linkers [glycine, γ-aminobutyric acid, 6-aminocaproic acid and 8-aminooctanoic acid, AA_n (n = 1, 3, 5 and 7)] to achieve biotin-linker products (Biotin–AA_n–COOH).[25,26] Finally, Biotin–AA_n–$CONHNH_2$ (n = 1, 3, 5, and 7, B-L-2–B-L-5) were prepared by the reaction of B-AAn-COOH with $SOCl_2$ under excess MeOH and subsequent hydrazinolysis with hydrazine hydrate in MeOH.[23]

The prepared Biotin–AA_n–$CONHNH_2$ (B-L-2–B-L-5) were reacted with β-Antiarin and α-Antiarin under the catalysis of 1% AcOH in



Scheme 1. Synthetic route of biotinylated cardiac glycosides **P1a** and **P1b**. (A) Preparation of Biotinylhydrazine (B-L-1) from biotin. (B) Preparation of probes **P1a** and **P1b** from Biotinylhydrazine (B-L-1) and β-Antiarin (α-Antiarin). Reagents and conditions: a. $SOCl_2$, MeOH, r.t., overnight; b. NH_2NH_2, r.t., 17 h; c. β-Antiarin (α-Antiarin), 1% AcOH, MeOH, 60 °C, 10 h.



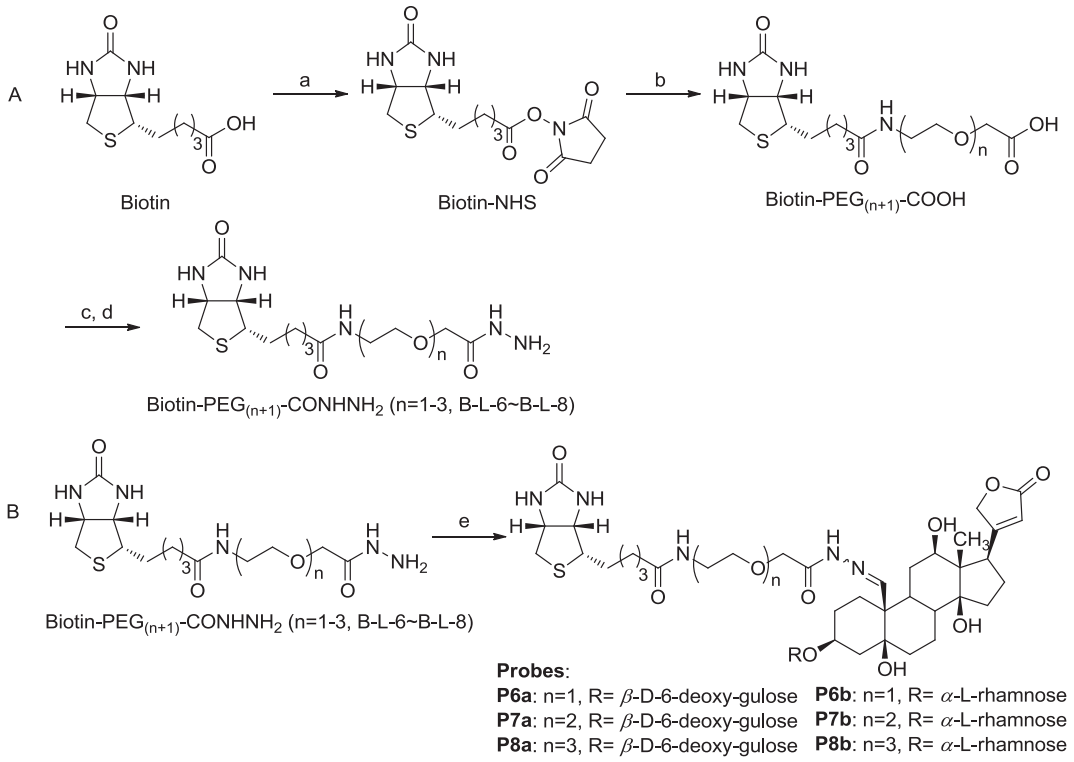
Scheme 2. Synthetic route of biotinylated cardiac glycosides **P2a**-**P5a** and **P2b**-**P5b**. (A) Preparation of Biotin-AA_n-$CONHNH_2$ (n = 1, 3, 5 and 7, B-L-2–B-L-5) from biotin. (B) Preparation of probes **P2a**-**P5a** and **P2b**-**P5b** from Biotin-AA_n-$CONHNH_2$ (n = 1, 3, 5 and 7, B-L-2–B-L-5) and β-Antiarin (α-Antiarin). Reagents and conditions: a. DCC, NHS, DMF, r.t., 8 h; b. Glycine (γ-aminobutyric acid, 6-Aminocaproic acid and 8-Aminooctanoic acid), H_2O, acetone, r.t., 12 h; c. $SOCl_2$, MeOH, r.t., overnight; d. NH_2NH_2, r.t., 17 h; e. β-Antiarin (α-Antiarin), 1% AcOH, MeOH, 60 °C, 10 h.

MeOH at 60 °C for 10 h, respectively. The crude products were obtained by concentrated under reduced pressure. The residues were purified by semipreparative RP-HPLC (17% ACN-H_2O) to obtain biotinylated cardiac glycosides **P2a–P5a** and **P2b–P5b** with around 30.1–44.8% yield (Scheme 2).[24] The ratios of the trans and cis epimers of probes **P2a–P5a** and **P2b–P5b** were about 3:1 from analysis of the ^{1}H NMR spectra.

*Synthesis of biotinylated cardiac glycosides **P6a–P8a** and **P6b–P8b** from Biotin–$PEG_{(n+1)}$–$CONHNH_2$ (B-L-6–B-L-8) and β-Antiarin (α-Antiarin).* The hydrophilic linkers [NH_2-$PEG_{(n+1)}$–COOH, n = 1, 2, 3] were designed and synthesized from $(PEG)_n$ (diethylene glycol, triethylene glycol and tetraethylene glycol) according to the literatures due to commercial unavailability.[26–28] Then, biotin was reacted with NH_2-$PEG_{(n+1)}$–COOH (n = 1, 2 and 3) followed by hydrazine to prepare Biotin–$PEG_{(n+1)}$–$CONHNH_2$ (n = 1, 2 and 3, B-L-6–B-L-8) according to the procedure preparing Biotin–AA_n–$CONHNH_2$ mentioned above.

The intermediate Biotin–$PEG_{(n+1)}$–$CONHNH_2$ (B-L-6–B-L-8) were reacted with β-Antiarin and α-Antiarin under the catalysis of 1% AcOH in MeOH at 60 °C for 10 h. The crude products were obtained by concentrated under reduced pressure. Then, the residues were purified by semipreparative RP-HPLC (17% ACN-H_2O) to obtain probes **P6a–P8a** and **P6b–P8b** with around 12.8–23.2% yield (Scheme 3).[24] The ratios of the trans and cis epimers of probes were around 1:1 ratio by analysis of the ^{1}H NMR spectra.

Induction of Nur77 expression. The induction of Nur77 expression of the synthesized biotinylated cardiac glycosides (**P1a–P8a** and **P1b–P8b**) were evaluated on NIH-H460 cancer cells by western blot (Fig. 2). DMSO and the derivatized linkers, including Biotinylhydrazine (B-L-1), Biotin–AA_5–$CONHNH_2$ (B-L-4), and Biotin–PEG_2–$CONHNH_2$ (B-L-6), were used as blank and negative controls. β-Antiarin and α-Antiarin were used as positive control. As a result, the biotinylated cardiac glycosides **P1b–P5b**, **P7b** and **P8b**, especially **P4b**, could significantly induce the expression of Nur77 protein comparable with the parent compound β-Antiarin, while biotinylated cardiac glycosides **P3a–P6a**, especially **P5a**, exhibited stronger effects on the induction of



Scheme 3. The synthetic route of biotinylated cardiac glycosides **P6a**-**P8a** and **P6b**-**P8b**. (A) Preparation of Biotin-$PEG_{(n+1)}$–$CONHNH_2$ (n = 1–3, B-L-6–B-L-8) from biotin. (B) Preparation of probes **P6a**-**P8a** and **P6b**-**P8b** from Biotin-$PEG_{(n+1)}$–$CONHNH_2$ (n = 1–3, B-L-6–B-L-8) and β-Antiarin (α-Antiarin). Reagents and conditions: a. DCC, NHS, DMF, r.t., 8 h; b. NH_2-$PEG_{(n+1)}$–COOH (n = 1, 2, 3), H_2O, acetone, r.t., 12 h; c. $SOCl_2$, MeOH, r.t., overnight; d. NH_2NH_2, r.t., 17 h; e. β-Antiarin (α-Antiarin), 1% AcOH, MeOH, 60 °C, 10 h.

Nur77 expression, compared with their parent compound α-Antiarin, at a concentration of 20 nM. SAR analysis showed that the chain length and amphiphilicity of the linkers have less effect on the induction of Nur77 expression for β-Antiarin derived biotinylated cardiac glycosides. Nevertheless, the induction of Nur77 expression of **P5a** is stronger than that of **P2a–P4a**, indicating that the long hydrophobic linker is well tolerated than short hydrophobic linker for α-Antiarin derived biotinylated cardiac glycosides; while it was opposite for hydrophilic linker since probe **P6a** exhibited stronger effect than that of **P7a** and **P8a**. In addition, all biotinylated cardiac glycosides did not show obvious inhibitory effects on the proliferation of NIH-H460 cancer cells at a concentration of 50 nM by MTT assay (viability rate > 90%) (Fig. 3).

*Streptavidin binding capacity of biotinylated cardiac glycosides (**P4b***

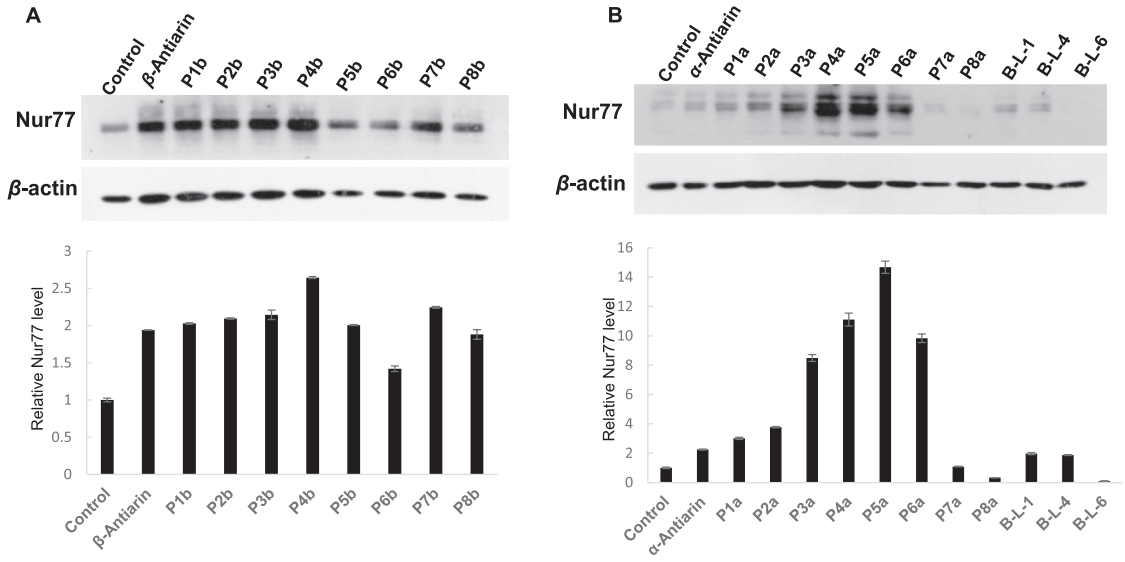


Fig. 2. Induction of Nur77 expression by biotinylated cardiac glycosides on NIH-H460 cancer cells. (A) Induction of Nur77 expression by β-Antiarin and **P1b**-**P8b**. (B) Induction of Nur77 expression by α-Antiarin, **P1a**-**P8a**, B-L-1 (Biotinylhydrazine), B-L-4 (Biotin-AA_5-$CONHNH_2$), and B-L-6 (Biotin-PEG_2-$CONHNH_2$). NIH-H460 cells were incubated for 3 h with probes (20 nM), and Nur77 expression was determined by western blotting using anti-Nur77 antibody.

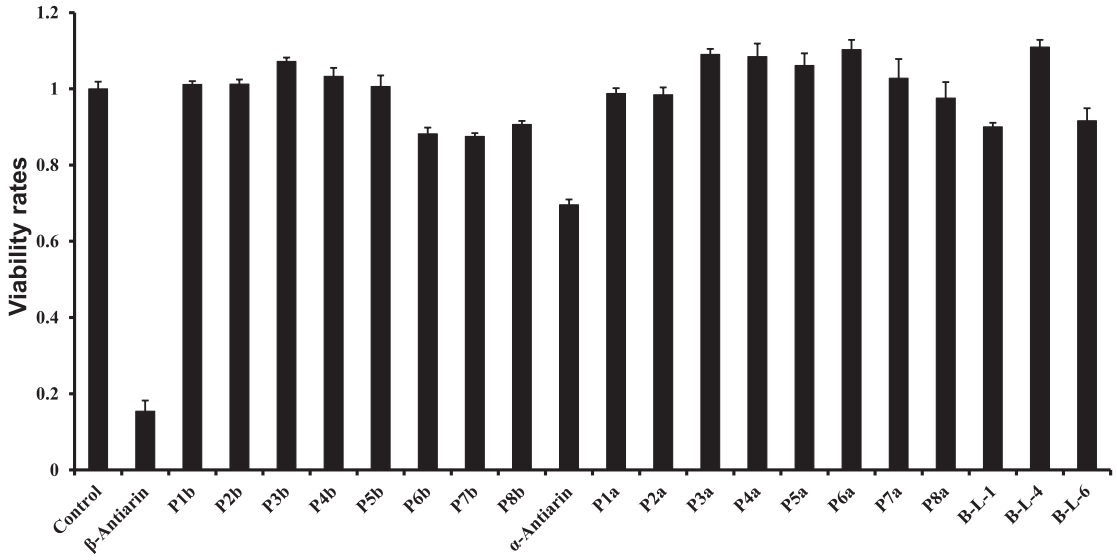


Fig. 3. Viability rates of β-Antiarin, α-Antiarin, biotinylated cardiac glycosides **P1b**-**P8b** and **P1a**-**P8a**, and negative controls B-L-1 (Biotinylhydrazine), B-L-4 (Biotin-AA_5-$CONHNH_2$), and B-L-6 (Biotin-PEG_2-$CONHNH_2$). NIH-H460 cells were incubated for 48 h with probes (50 nM) and cell viability was assayed by MTT method.

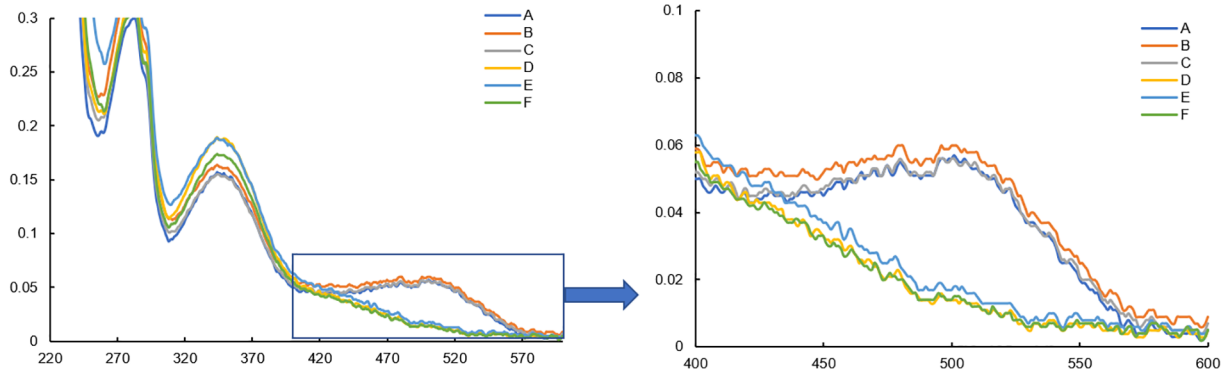


Fig. 4. Monitoring of streptavidin–biotin-probes (**P4b**/**P5a**) interaction by UV–vis spectroscopy: (A) streptavidin + HABA; (B) streptavidin + *α*-Antiarin + HABA; (C) streptavidin + *β*-Antiarin + HABA; (D) streptavidin + biotin + HABA; (E) streptavidin + **P4b** + HABA; (F) streptavidin + **P5a** + HABA.

and P5a). The streptavidin-HABA (4′-hydroxyazo-benzene-2-carboxylic acid) assay was performed to evaluate the specific streptavidin binding activity of the biotin chain-end probes **P4b** and **P5a** which showed remarkable induction effect for Nur77 expression.[29] HABA (λ_{max} 350 nm) changes color from yellow to red (λ_{max} 500 nm) upon binding to free streptavidin (Fig. 4, trial A). The same color changes were observed when HABA was added to a solution of streptavidin with non-biotin-containing cardiac glycosides (α-Antiarin and β-Antiarin) as monitored with UV spectroscopy (Fig. 4, trials B and C). However, when HABA was added to a solution of streptavidin saturated with free biotin or biotin probes **P4b** and **P5a**, a red shift was not observed (Fig. 4, trials D, E, and F). These results demonstrate specific streptavidin binding capacity of biotin probes **P4b** and **P5a**, indicating its future application for fishing the cardiac glycoside targets.

In conclusion, we have successfully designed and synthesized 16 novel biotinylated cardiac glycoside chemical probes with a mixture of trans and cis epimers. Western blot result displayed that some biotinylated cardiac glycosides could significantly induce the expression of Nur77 protein comparable with their parent compounds β-Antiarin and α-Antiarin. Meanwhile, the biotinylated cardiac glycosides didn't show obvious cytotoxicity toward cancer cells. Among them, biotinylated cardiac glycosides **P4b** and **P5a** exhibited specific high affinity binding capacity with streptavidin along with significant effect on induction of Nur77 expression, suggesting that they can be used as probes for probing Nur77 protein inducting pathway. The potential applications of the biotinylated cardiac glycosides as probes for probing Nur77 protein inducting pathway will be investigated in our immediate study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.01.015.

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