DOI: 10.1002/ange.201405924

Bioconjugation

Site-Selective Labeling of a Lysine Residue in Human Serum Albumin**

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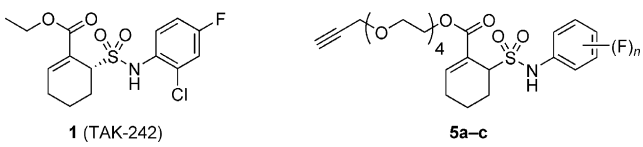
Abstract: *Conjugation to human serum albumin (HSA) has emerged as a powerful approach for extending the in vivo half-life of many small molecule and peptide/protein drugs. Current HSA conjugation strategies, however, can often yield heterogeneous mixtures with inadequate pharmacokinetics, low efficacies, and variable safety profiles. Here, we designed and synthesized analogues of TAK-242, a small molecule inhibitor of Toll-like receptor 4, that primarily reacted with a single lysine residue of HSA. These TAK-242-based cyclohexene compounds demonstrated robust reactivity, and Lys64 was identified as the primary conjugation site. A bivalent HSA conjugate was also prepared in a site-specific manner. Additionally, HSA-cyclohexene conjugates maintained higher levels of stability both in human plasma and in mice than the corresponding maleimide conjugates. This new conjugation strategy promises to broadly enhance the performance of HSA conjugates for numerous applications.*

Protein conjugates to therapeutic moieties have been reported and evaluated in clinical trials.[1] Compared to the parental small molecule, protein–drug conjugates offer several advantages, including half-life extension, localization to a target tissue, avoidance of drug–drug interactions, and reduction of toxicity. Human serum albumin (HSA) has proven to be a valuable protein for the conjugation of small molecules, peptides, and proteins. For example, the half-lives of the small molecules DOXO-EMCH (INNO-206)[2] and AWO54[3] have been dramatically extended by conjugation to HSA, facilitating their progression into clinical trials for the treatment of cancer and rheumatoid arthritis, respectively.

Drug conjugation to target proteins is commonly achieved through maleimide–cysteine chemistry or labeling of lysine with N-hydroxysuccinimide ester. However, these methods have several disadvantages, including the formation of heterogeneous mixtures of protein conjugates[4] that have varied in vivo efficacies, pharmacokinetics, and toxicities.[5] To overcome this obstacle, we previously reported a serum-stable alternative to maleimide-based protein conjugation that allows site-specific labeling of cysteine residues.[6] Additionally, several compounds, such as β-lactam-based antibiotics, have been reported to react with lysine residues present on the surface of HSA.[7] To date, however, none of these compounds have displayed site specificity for lysine.

Recently, TAK-242 (**1**, Figure 1), a potent Toll-like receptor 4 (TLR4) inhibitor,[8] was shown to form a covalent lysine adduct with both rat and human albumin in plasma after intravenous dosing.[9] TAK-242 inhibits protein–protein interactions between TLR4 and its adapter proteins by associating with a single cysteine residue within an intracellular domain of TLR4.[10] A covalent binding mechanism based on Michael addition with cysteine or lysine, and subsequent elimination of the sulfonamide moiety through allylic rearrangement has been proposed.[9] Based on this potential mechanism, we hypothesized that linkers derived from TAK-242 could react with lysine on HSA to enable the formation of stable conjugates. Thus, we prepared polyethylene glycol (PEG)-modified TAK-242 derivatives using fluorobenzene sulfonamide as a leaving group to 1) maintain labeling activity for HSA and 2) reduce TLR4 inhibitory activity (Figure 1).



1 (TAK-242) **5a–c**

Figure 1. Structures of TAK-242 (**1**) and PEG-modified TAK-242 derivatives used for HSA conjugation.

We evaluated the HSA reactivity of the TAK-242 analogues **5a–c** and compared labeling to that of maleimide compound **10** (Schemes 1 and 2). Recombinant HSA was incubated with one or two equivalents of **5a–c** or **10** at 37 °C for two hours, then a click reaction was performed with the azide–rhodamine compound **12a** to give a fluorescent conjugate. Products were resolved by SDS-PAGE, and the reaction rates were calculated by measuring the fluorescence intensities of HSA-conjugate bands (Figures 2A and S1). Additionally, we performed conjugation reactions between HSA and compounds **5a–c** or **10** (50 μM) in the presence of human plasma (Figures 2B and S2). The PEG-modified TAK-242 analogues reacted with recombinant HSA at rates similar to the maleimide compound. Compound **5b**, which contains a 2,4-difluorophenyl sulfonamide, had the highest reactivity. Following conjugation of **5b** with HSA, the by-

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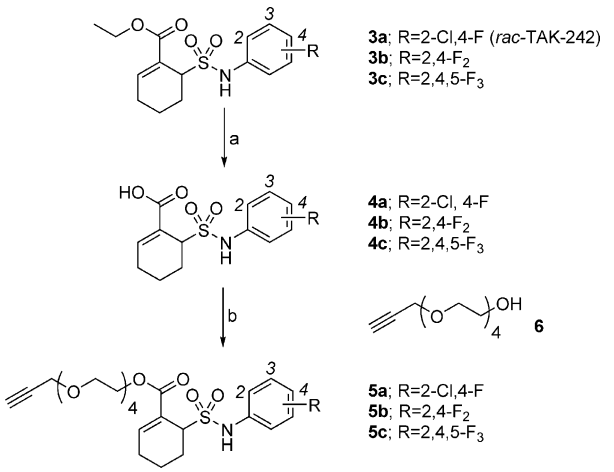
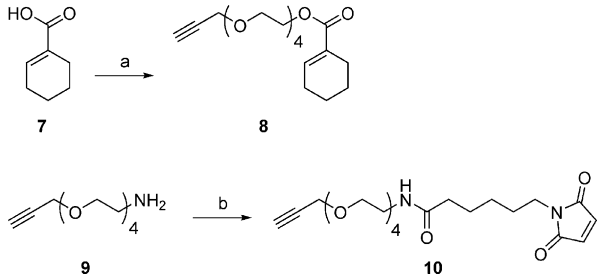
[**] This study was supported by The Skaggs Institute for Chemical Biology. We thank Linh Truc Hoang for performing LC-MS/MS analysis and Diane M. Kubitz for animal work. We also thank Yoshihiro Ishihara for comments on the manuscript.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201405924.



Scheme 1. Synthesis of PEG-linked TAK-242 analogues. Reagents and conditions: a) $Ba(OH)_2$ octahydrate, H_2O, CH_3CN, 60 °C, 1 h, 70 % **4a**, 83 % **4b**, 64 % **4c**; b) **6**, DEAD, PPh_3, THF, 0 °C → RT, overnight, 32 % **5a**, 44 % **5b**, 42 % **5c**. DEAD = diethyl azodicarboxylate.



Scheme 2. Synthesis of comparative compounds for HSA labeling. Reagents and conditions: a) **6**, DEAD, PPh_3, THF, 0 °C → RT, overnight, 28 %; b) triethylamine, EMCS, DMF, RT, overnight, 46 %. DMF = *N*,*N*-dimethylformamide; EMCS = N-(ε-maleimidocaproyloxy)succinimide ester.

product 2,4-difluoroaniline was detected in solution by HPLC (Figure S3), suggesting that the mechanism of the reaction for compounds **5a–c** with HSA is similar to that of the reaction with TAK-242 (Scheme S5).

In order to assess specificity for HSA, we incubated our TAK-242 derivatives in human plasma depleted of HSA (Figures 2C and S4). No proteins in the depleted serum reacted with the TAK-242 compounds **5a–c** (Figure 2C, red arrow); however, maleimide (**10**) did react with several proteins (Figure 2C, yellow arrows). Moreover, inclusion of saturating amounts of myristic acid had no impact on recombinant HSA labeling, thereby supporting the use of cyclohexene compounds for in vivo bioconjugation (Figure S5). Evaluation of TAK-242 analogue stability indicated that **5b** was sufficiently stable for labeling applications (Figure S6). Furthermore, structure–activity relationship analyses showed that TLR4 inhibition requires the alkyl group of the TAK-242 ester moiety and the hydrophilic chain.[8] As expected, none of the cyclohexene compounds inhibited TLR4 activity (Figure S7).

The simple cyclohexene derivative compound **8** was incubated with recombinant HSA to confirm the impact of the sulfonamide group on conjugation (Scheme 2). No labeling was observed with this compound (Figure S8), thus

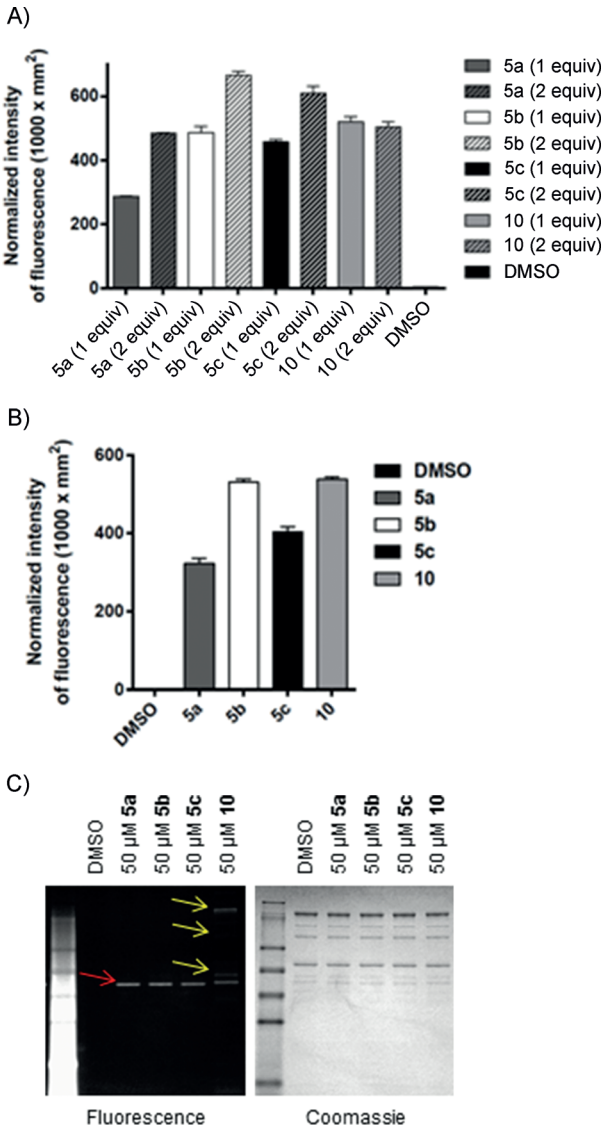
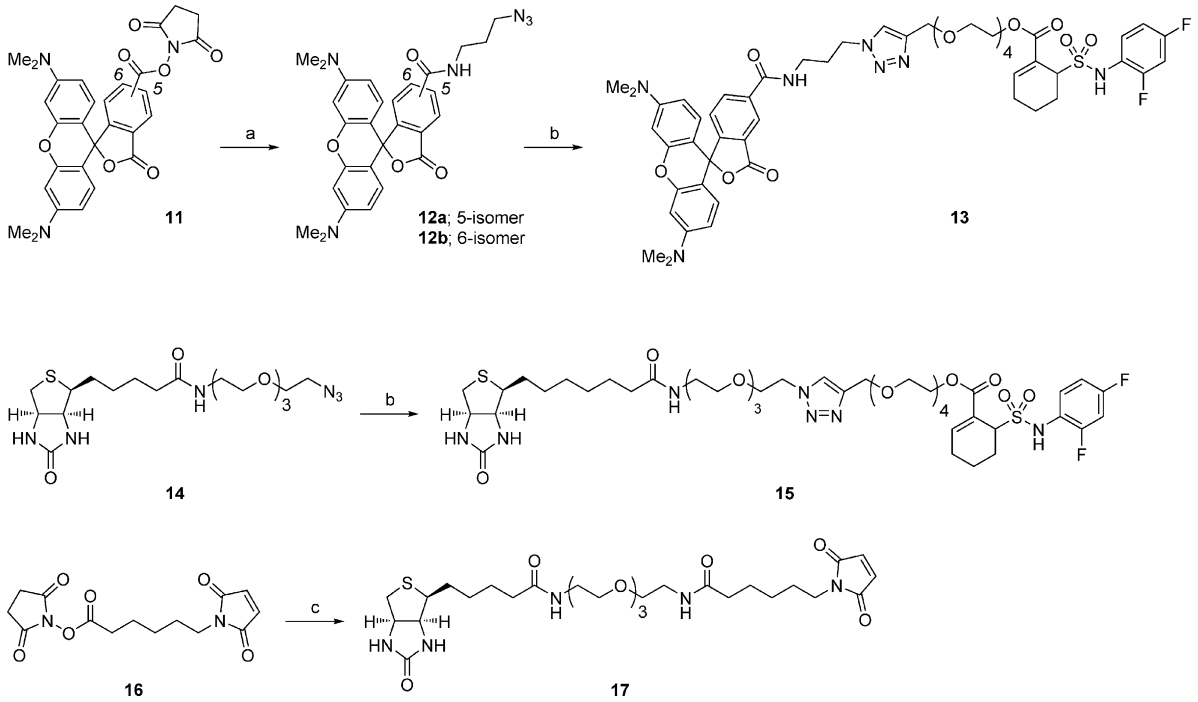


Figure 2. Conjugation reactions of HSA with cyclohexene compounds (**5a–c**) and with the maleimide compound (**10**). A) Normalized intensities of conjugation reaction with recombinant HSA. B) Normalized intensities of conjugation reaction with HSA in human plasma. C) SDS-PAGE of conjugation reaction in HSA-depleted human plasma (red arrow: HSA conjugate; yellow arrow: other protein conjugates).

indicating that the sulfonamide group of **5b** is an important trigger for conjugation. Compound **13**, which was modified to contain rhodamine, was incubated with recombinant HSA in various PBS solutions to evaluate dependence on the pH value (Scheme 3). Conjugation was positively correlated with the basicity of the solution, as the reaction rate at pH 9 was approximately two times higher than at pH 7.4 (Figure S9), likely as a result of an increase in amino acid nucleophilicity.

To measure the equivalence and time dependence of the labeling reaction, we conjugated HSA with the fluorescent TAK-242 derivative **13**. We calculated the dye/protein molar ratios from the absorbance values of both the protein and dye, which demonstrated that use of ten equivalents of compound **13** led to the highest rates of labeling, giving a dye/protein molar ratio of higher than 1:1 after five hours (Figure S10).



Scheme 3. Synthesis of functionalized compounds for HSA labeling. Reagents and conditions: a) NEt_3, 3-azidopropan-1-amine, CH_3CN, RT, overnight, 51% **12a**, 31% **12b**; b) **5b**, $CuSO_4$, THPTA, sodium ascorbate, *t*BuOH, H_2O, RT, 3 h, 34% **13**; 84% **15**; c) *N*-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethoxy)biotinamide, DMF, RT, 3 h, 46%. THPTA = tris(3-hydroxypropyltriazolylmethyl)amine.

This result suggests that the TAK-242 analogue may react selectively with one amino acid residue of HSA, but that large excesses of the electrophile can lead to labeling at secondary positions. Our data also indicates that determining the appropriate number of compound equivalents and reaction kinetics is critical for sufficiently driving the reaction while maintaining selectivity. Nonetheless, the cyclohexene linker is highly site-selective, particularly with regard to the finding that non-enzymatic glycation of lysine in HSA results in modification at 21 sites.[11]

Next, we blocked the lysine residues of HSA with NHS-biotin or the cysteine residues with iodoacetamide (IAA), and then incubated each mixture with **5b** to further establish that TAK-242 reacts specifically with lysine and not cysteine. After blocking with IAA, no change in labeling was observed with **5b**; however, the rate of conjugation with maleimide compound **10** was greatly reduced (Figure S11). By contrast, reaction of **5b** with HSA was significantly reduced after blocking with NHS–biotin (Figure S12). Furthermore, monomeric amino acids Lys, Cys, Ser, Thr, His, and Trp did not react with **5b** (data not shown), which suggests that the tertiary structure of HSA is an important factor for conjugation.

To determine the labeling site, we subjected the HSA–**5b** conjugate to proteolysis and analyzed the digested fragments by nano LC-MS/MS. One equivalent of **5b** was used for the preparation of the HSA conjugate to limit secondary labeling (Figure S10). Forty-four unique peptides were identified, and 68% sequence coverage was obtained (Figure S13). The peptide containing amino acids 61 to 82 [NCDKSLHTLFGDKLCTVATLRE] had a 339 Da modification at Lys64 in the sample reacted with **5b** (Figure S14). These findings were confirmed through the presence of b and y fragment ions in the MS/MS spectrum. Given the stoichiometry of HSA labeling and LC-MS/MS analysis, this data indicates that Lys64 is the primary site of labeling.

We recently reported a phenyloxadiazole (ODA) compound that efficiently labeled Cys34 of HSA and demonstrated high stability in human plasma.[6] To test whether conjugation at both Lys64 and Cys34 is feasible, recombinant HSA was labeled with rhodamine-linked cyclohexene compound **13** or fluorescein-linked ODA compound, and then sequentially labeled at the second site with the respective compound. The increase in fluorescence for the HSA conjugates treated with both compounds relative to labeling with **13** or ODA alone confirmed that each site was conjugated (Figure 3A). To further validate that HSA was modified at both sites, the fluorescence emission spectra were collected for HSA conjugated with **13**, ODA, or **13** + ODA (Figure 3B). As anticipated, rhodamine fluorescence was detected for HSA conjugated with **13** but not with ODA, and fluorescein signal was observed for the ODA conjugate but not with **13**. Fluorescence for the rhodamine and fluorescein labels was detected with **13** + ODA, demonstrating that HSA can be simultaneously conjugated in a site-specific manner.

Finally, we determined the stability of HSA conjugates. As a preliminary in vitro evaluation, we prepared HSA–cyclohexene and HSA–maleimide fluorescent conjugates based on compounds **13** and our previously described fluorescein-linked maleimide linker,[6] respectively, and incubated the conjugates in human plasma at 37 °C for one month (Figure S15). The stability of the HSA–cyclohexene conju-

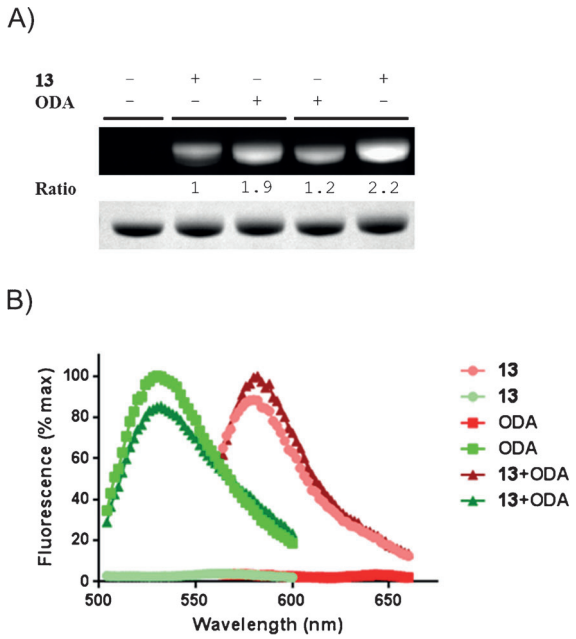


Figure 3. Site-specific labeling of recombinant HSA at Lys64 and Cys34. Conjugation reactions were performed using rhodamine-linked **13** or fluorescein-linked ODA, then the respective second labeling site was sequentially conjugated. A) SDS-PAGE of HSA dual labeling reactions. Ratios for normalized fluorescence intensity values relative to labeling with **13** alone are shown. B) Fluorescence emissions spectra for HSA conjugates. Fluorescein (shaded green) and rhodamine (shaded red) spectra were collected for each conjugate.

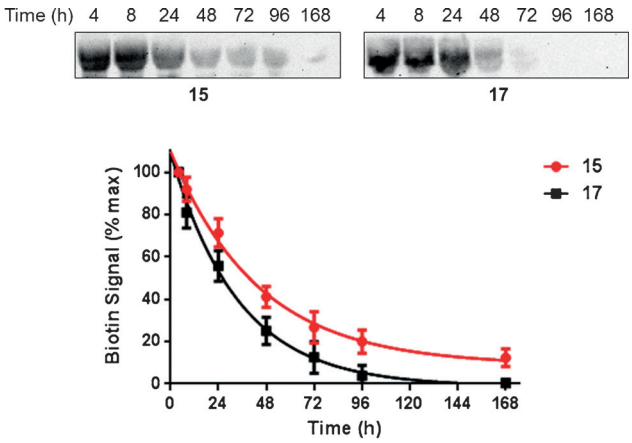


Figure 4. Stability of HSA conjugate in mice. Top: representative Western blot for cyclohexene (**15**) and maleimide (**17**) biotinylated HSA conjugates. Bottom: averaged remaining biotin conjugate values.

gate ($t_{1/2} > 28$ d) proved to be much greater than that of the corresponding HSA–maleimide conjugate ($t_{1/2} = 7.2 \pm 0.4$ d). We next evaluated the in vivo stability of HSA conjugates prepared with biotinylated compounds **15** and **17** (Scheme 3 and Figure 4). Biotinylated compounds were administered to BALB/c mice at a single subcutaneous dose of 20 mg kg^{-1} ($n = 3$). Blood was collected at 4, 8, 24, 48, 72, 96, and 168 hours, and samples were analyzed by Western blot. Assuming the linker did not influence clearance, the biotinylated cyclohexene–HSA conjugate ($t_{1/2} = 39.9 \pm 4.4$ h) was more stable than the maleimide–HSA conjugate ($t_{1/2} = 25.0 \pm 2.8$ h). Thus, the application of cyclohexene compounds for site-selective lysine conjugation of HSA appears to be a viable means for improving the therapeutic half-life of drug moieties.

In conclusion, we designed and synthesized TAK-242 derivatives to overcome the limitations of maleimide conjugation in preparing HSA conjugates. TAK-242 analogues possessed high reactivity and specificity for HSA, with Lys64 being identified as the primary labeling site. Additionally, the HSA conjugates of cyclohexene compounds **13** and **15** showed excellent stability in human plasma and in mice, respectively. This conjugation framework should find great utility for the generation of HSA conjugates with improved serum stability.

Received: June 4, 2014
Published online: September 4, 2014

Keywords: chemoselectivity · drug conjugates · human serum albumin · lysine labeling · site-specific conjugation

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