Published in final edited form as:

Bioorg Med Chem Lett. 2015 April 15; 25(8): 1684–1687. doi:10.1016/j.bmcl.2015.03.011.

N-Sulfonyl- β -lactam hapten as an effective labeling reagent for aldolase mAb

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Abstract

Utilization of chemically programmed antibodies (cpAbs) is regarded to be one of the most efficient methods for the development of therapeutic systems. cpAbs can extend the half-life of programming reagents, activate immune systems via the Fc region of antibodies and achieve universal vaccination by attaching varieties of small, programmed molecules. In the current study, we aimed to develop a novel labeling reagent for the preparation of cpAbs and found that *N*-sulfonyl- β -lactams (NSBLs) were optimal. NSBL can be synthesized from readily available 4-(bromomethyl)benzenesulfonyl chloride via few simple manipulations and can label the aldolase monoclonal antibody (mAb) 84G3, which could not be labeled effectively by the conventional labeling reagent, *N*-acyl- β -lactam (NABL). We also demonstrated that the conjugate, which consists of mAb 84G3 and an NSBL bearing a biotin moiety, maintained strong binding activity to streptavidin. In addition, the stability assay of NSBL revealed that NSBLs can tolerate aqueous media without significant decomposition over 24 hours.

Graphical abstract

Keywords

Bioconjugation; Chemically programmed antibody; Aldolase antibody; *N*-sulfonyl- β -lactam

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Bioconjugation is an essential tool to attach valuable functional units onto proteins, such as antibodies,^{1,2} nucleic acids^{3,4} and carrier proteins,⁵ by assembling proteins and biologically active small molecules.^{6,7} Among them, conjugates of antibodies and small molecules, which are called “chemically programmed antibodies (cpAbs),” have proved to be one of the most useful devices for the development of therapeutic systems (Figure 1).^{8,9} If the antibody could be labeled properly by certain small-molecule compounds, the resulting conjugate would possess an extended serum half-life relative to that of the parent small molecule due to a significant increase in molecular weight. In addition, the Fc domain of the antibody would activate effector function systems such as T-cell or complement systems, and show several preferable activities such as antibody-dependent cell cytotoxic activity (ADCC) or complement-dependent cell cytotoxic activity (CDC).¹⁰ By using various kinds of therapeutic agents such as anti-HIV,¹¹ anti-cancer¹² or anti-flu¹³ compounds as the programming molecules, one could potentially target a wide range of diseases via universal vaccination. For the preparation of cpAbs, several procedures for labeling antibodies by small molecules have been reported.¹⁴⁻¹⁶ We reported an effective labeling system of the aldolase monoclonal antibody (mAb) 38C2¹⁷⁻²¹, which was generated by conjugating 1,3-diketone **1** (see Figure 2 for the structure of **1**) and carrier protein KLH via *N*-acyl β -lactam (NABL)-mediated amidation (Scheme 1).^{13,22-26} In this case, the NABLs react selectively and irreversibly with the two amino groups of the lysine residues to form stable covalent bonds with mAb 38C2.

For effective generation of catalytic antibodies by immunization with small molecules, it is essential to use a small molecule that has a structure that is close to the transition state of the target reaction. For this purpose, we also reported that hapten **2**, which mimics the tetrahedral transition state of the C–C bond-forming step of an aldol reaction, induced seven out of seventeen antibodies showing aldolase activity, while only two out of twenty possessed catalytic activity in the case of the immunization by hapten **1**.^{27,28} Although the availability of the catalytic antibody series generated by the sulfonyldiketone hapten **2** is superior to those generated by the diketone hapten **1**, labeling studies using these compounds have yet to be accomplished. Therefore, we surmised that if we could develop the methodology of effectively labeling the aldolase antibody series that are derived from the immunization by **2**, it would be valuable for the preparation of cpAbs due to the availability of the catalytic antibodies. Herein, we report the labeling study of 84G3, which is one of the aldolase antibodies induced by the immunization with hapten **2**, by using a newly designed labeling reagent.

Inspired by the structure of hapten **2**, which has an additional sulfonyl group at the carbon between the two carbonyl groups of the 1,3-diketone moiety, we speculated that the *N*-sulfonyl β -lactam (NSBL) would label 84G3 more effectively than the NABL hapten that was reported previously.^{13,22-24} Therefore, we synthesized *N*-sulfonyl- β -lactam **3a** (Scheme 2). At first, the commercially available 4-(bromomethyl)benzene-1-sulfonyl chloride (**4**) was converted to the corresponding methylsulfonate **5**²⁹ by methanolysis in the presence of Et₃N. Methylsulfonate **5** was etherified with alcohol **6**,³⁰ which is derived from tetraethyleneglycol, to produce **7**. In an effort to convert the methylsulfonate moiety of **7** to a sulfonyl chloride, we found that it can be realized by nucleophilic demethylation with LiCl

followed by chlorination with phosgene in one-pot. The resulting sulfonyl chloride **8** was coupled with 2-azetidinone to afford the desired NSBL **3a**. We also prepared the corresponding *N*-sulfonyl γ -lactam (NSGL) **3b** in an analogous manner to evaluate the influence of the size of the lactam moiety.

With the novel *N*-sulfonyl lactam haptens in hand, we evaluated the efficacies of the labeling of mAb 84G3 by these haptens (Scheme 3). The labeling was performed by incubating the mixture of 84G3 and 2.2 equivalents of each hapten in neutral PBS solution at room temperature for 60 min. NABL **3c**, which is the effective labeling reagent for mAb 38C2, was also tested for labeling of 84G3 to compare the labeling efficacy with those of *N*-sulfonyl lactams **3a** and **3b**. We evaluated the efficacy of this conjugation process by monitoring the catalytic activity of the *retro*-aldol reaction of methodol **10** to form the fluorescent compound **11** as reported previously (Figure 3).²⁰ As we expected, NSBL **3a** reacted smoothly and the catalytic activity of the resulting antibody **9a** completely disappeared within 1 h. In contrast, NSGL **3b** did not affect the aldolase activity of the antibody presumably due to its stable and less reactive five-membered ring structure. We also found that the existing NABL hapten **3c** did not label the catalytic site of 84G3 as well. These results imply that both the highly strained, less hindered four-membered lactam moiety and *N*-sulfonyl functional group are necessary for effective labeling of 84G3 selectively at the catalytic lysine site. The ESI-MS analysis of the conjugate of **9a** and 84G3 indicated that the two molecules of **3a** labeled 84G3 (Figures S1 and S2).

One of the goals for the development of cpAbs is to obtain biologically active novel conjugates that are anticipated to have the three benefits outlined in Figure 1. To achieve this purpose, it is necessary to maintain the function of the small molecule after conjugation. To test whether the conjugation affects the function of the small molecule, we installed a biotin moiety as a model function to 84G3 by using the NSBL labeling system (Scheme 4). NSBL **3a**, which has a terminal alkyne moiety, was coupled with a biotin bearing an azide group (**12**)³¹ by Cu-mediated Huisgen reaction³² to obtain an NSBL possessing the biotin moiety (**13**).

Labeling of 84G3 by **13** was performed under the same conditions as described above and we successfully obtained a cpAb bearing the biotin moiety (**9d**). The labeling efficacy of the conjugation was confirmed by methodol assay and ESI-MS analysis (Figures S3 and S4).

To confirm the binding activity of 84G3-biotin **9d** against streptavidin, we performed ELISA using a plate coated with streptavidin (Figure 4). When **9d** was used as the primary antibody, we observed strong binding to streptavidin. In contrast, both the mAb 84G3 itself and cpAb **9a**, which do not bear a biotin moiety, did not show any binding signal toward streptavidin. From these results, it can be concluded that the cpAb of 84G3 and small programming reagents maintain the binding activity of the small compound to the target protein. This labeling system would be useful to prepare novel biologically active cpAbs.

Since the NSBL has a highly electrophilic β -lactam moiety in its structure, it might be problematic in the labeling step if the NSBL has a more complex structure and the labeling step is slow. Therefore, we evaluated the stability of NSBL **13** in aqueous media by HPLC

analysis. When 100 μ L of 1 mg/ml CH₃CN solution of NSBL **13** was mixed with 100 μ L of PBS (100 mM, pH 7.4), HPLC showed only one signal corresponding to **13** (Figure S5-A). Even after incubating this mixture at room temperature for 24 h, we could not observe any other signals and we concluded that **13** is stable enough in neutral aqueous conditions. We also conducted the same test by using PBS (100 mM, pH 8.5) to check the stability in basic media. In this case, a new signal appeared as time progressed, and we found that the new signal corresponds to the ring-opened product **14** by LC-MS analysis (Figure 5, S5-B and S7). Furthermore, in the presence of *n*-BuNH₂ as a model primary amine, NSBL **13** immediately reacted to form the corresponding ring-opened product **15** in neutral pH media, as was demonstrated by HPLC and LC-MS analysis (Figure 5, S5-C and S8). When the same conditions were applied in basic media (pH 8.5), hydrolyzed product **14** was not observed and the NSBL was converted into the ring-opening amide **15**. This result implies that NSBL reacts with a primary amine more quickly than H₂O, and aqueous conditions do not pose a problem for the desired amidation with highly reactive primary amines of the catalytic lysine residue in the antibody.

In conclusion, a new NSBL (compound **3a**) was designed as a labeling reagent for mAb 84G3 and synthesized from readily available compounds in a few simple steps. Evaluation of the efficacies of the labeling of mAb 84G3 by this programming reagent revealed that this new hapten can be used as a highly efficient labeling reagent for mAb 84G3, while the conventional NABL or five-membered derivative NSGL could not be used for the labeling of the same antibody. The binding activity of the small molecule to the target protein was maintained in cpAbs, therefore it is expected to be used for the synthesis of biologically active cpAbs. In addition, the small NSBL compound is stable under the labeling reaction conditions. Further studies for the development of cpAbs for the treatment of significant diseases, including the evaluation of the three features of cpAbs, [(1) prolonged half-life, (2) effector activation via Fc domain and (3) universal vaccination] are currently underway and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by National Institutes of Health grants Pioneer Award DP1 CA174426, RO1 AI095038, and The Skaggs Institute for Chemical Biology. TI thanks The Uehara Memorial Foundation for a research fellowship for research abroad. The authors are indebted to Dr. Yoshihiro Ishihara for assistance in the preparation of the manuscript. TI dedicates this work to the memory of Carlos F. Barbas III, an outstanding mentor and friend.

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Figure 1.
Concept and expected benefits of cpAbs.

Figure 2.
Small-molecule haptens that induce aldolase antibodies.

Figure 3. Labeling study of mAb 84G3 with lactam haptens **3a**–**3c**. Reagents and conditions: (a) cpAb (0.3 mol %), PBS (pH 7.4), rt.

Figure 4.
ELISA of the cp84G3s.

Figure 5.
Chemical structure of **14** and **15**.

Scheme 1.
Labeling of mAb 38C2 by NABL.

Scheme 2.

Synthesis of the *N*-sulfonyl lactam haptens **3a** and **3b**. Reagents and conditions: (a) Et₃N, MeOH, rt, 30 min (52%). (b) NaH, **6**, THF, 0 °C to rt, 3 h (33%). (c) LiCl, acetone, 55 °C, 2 h then phosgene, DMF, CH₂Cl₂, 0 °C to rt, 4 h (74%). (d) NaHMDS, 2-azetidinone, THF, -78 °C, 2 h or *n*-BuLi, 2-pyrrolidinone, -78 °C, 1 h (16% for **3a**, 12% for **3b**).

Scheme 3.

Labling of 84G3 with lactam haptens. Reagents and conditions: (a) **3a** or **3b** or **3c**, PBS (pH 7.4), rt, 60 min.

Scheme 4.

Preparation of 84G3-biotin **9d**. Reagents and conditions: (a) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, Na-(L)-ascorbate, **12**, tBuOH, H_2O , rt, 1 h (60%). (b) mAb 84G3, PBS (pH 7.4), rt, 60 min.